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Temperature-Sensitive Virus from *Aedes albopictus* Cells Chronically Infected with Sindbis Virus

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Cultures of *Aedes albopictus* cells persistently infected with wild-type Sindbis virus (SV-W) give rise to small plaque-forming mutants which are also temperature sensitive. These mutants, designated SV-C, are neutralized by antiserum produced against SV-W. Mutant ts clones were isolated from SV-C by plaque purification. After serial undiluted passage in BHK or mosquito cells, each of the clones gave rise to ts^+ revertants which, however, remained mutant with respect to plaque morphology. Nineteen of 20 clones derived from SV-C were RNA^+, and one was RNA^- (SV-C-2). The RNA synthesizing activity, once induced in infected cells by SV-C-2, was stable at the nonpermissive temperature (39.5 C). All clones derived from SV-C were inactivated at 60 C much more quickly than was SV-W. It was not possible to demonstrate complementation between any of the SV-C clones.

When mosquitoes are infected with viruses of the togavirus group, they become persistently infected, shedding progeny virus for the rest of their lives. In several instances involving group A togaviruses, it has been shown that, although the virus stock used to infect the mosquitoes contained predominantly large plaque variants, after several weeks the progeny virus consisted largely of small plaque variants (6, 11). Similarly, small plaque variants became predominant in cultures of *Aedes aegypti* cells chronically infected with Semliki forest virus (12) and in *Aedes albopictus* cells chronically infected with wild-type Sindbis virus (SV-W) (18). After several weeks, all of the virus recovered from such chronically infected *Aedes albopictus* cultures (SV-C) produced small, irregularly shaped plaques measuring 1 to 2 mm in their longest dimension, whereas the original infecting virus (SV-W) produced large, round plaques averaging 8 mm in diameter.

This report demonstrates that the small plaque variants of SV obtained from chronically infected *A. albopictus* cultures are temperature sensitive. We have also obtained temperature-sensitive variants of SV by serial, undiluted passage of the virus in vertebrate cells at low temperature. This suggests that the low temperature at which the mosquito cell cultures are maintained may favor the accumulation of the ts mutants.

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Plaque-purified derivatives of an SV-C stock were isolated and characterized. Complementation could not be demonstrated between any of the SV-C clones.

MATERIALS AND METHODS

Cells, media, and viruses. The primary chicken embryo fibroblasts, the *A. albopictus* and BHK-21 cell cultures, and the media in which they were maintained have all been described (19). The plaque-purified derivatives of SV (SV-W) and Eastern equine encephalitis virus (EEEV) have been described previously (18). Uncloned virus from chronically infected mosquito cell cultures was designated SV-C and in these experiments was from cultures initially infected with SV-W 2 to 3 months previously. Virus clones obtained from the SV-C stocks were designated SV-C-1, SV-C-2, etc. Cloned virus was obtained from well-isolated plaques, a portion of which was then grown to a stock. Both the initial plaque purification and the growth of the stock were performed in baby hamster kidney (BHK)-21 cells at 34 C.

The chemically induced SV mutants ts 4 and 10 (1, 2) were obtained from Elmer Pfefferkorn. The virus stocks of these mutants were prepared in primary chicken embryo cells.

Plaque assays. SV and EEEV were assayed on monolayers of BHK-21 cells. After a 60-min adsorption period, the inoculum was removed, and the monolayers were overlaid with nutrient agar consisting of the HT medium of Rouse et al. (1966) and containing 0.015% DEAE-dextran and 0.9% Noble agar (Difco Laboratories). After incubation for 48 to 72 h at the appropriate temperature, the cultures were stained with neutral red, and the plaques were counted.

Incubation at 39.5 ± 0.2 C was in an incubator

(Wedco, Inc.) modified so that the temperature was controlled by a mercury thermoregulator (Bronwill Scientific, Inc.). Incubations at other temperatures were in unmodified incubators (Wedco, Inc. and National, Inc.), and the temperature varied approximately ± 0.5 C.

Neutralization and hemagglutination assays. Antiserum to SV-W was obtained by immunization of white rabbits with concentrated SV-W (15). Preimmune serum was also obtained from these rabbits. The test virus was diluted to an estimated 600 PFU/ml, mixed with an equal volume of appropriate dilutions of antiserum, and incubated at room temperature for 45 min. The surviving infectivity was determined by plaque assay and was expressed as the ratio of the PFU remaining after treatment with immune serum to the PFU present after treatment with the same dilution of preimmune serum.

Hemagglutination assays were performed with goose erythrocytes by the method of Clarke and Casals (3) at pH 5.8. When virus yields were to be assayed by hemagglutination, serum was replaced by 0.4% bovine serum albumin in the culture medium. This did not affect the yield of infectious SV at 24 h.

Assay of viral RNA synthesis. Viral RNA synthesis was assayed in the presence of actinomycin D (5 μg/ml) in monolayers of BHK-21 cells grown in 4.708-g glass vials. Assays were performed in a water bath, the temperature of which varied approximately ± 0.1 C, and which was controlled by a mercury thermoregulator. Incorporation of radioactive precursors was terminated by washing the cell monolayer with 5 ml of phosphate-buffered saline (PBS) and then dissolving it in 1% sodium dodecyl sulfate. Portions were then assayed for trichloroacetic acid-precipitable radioactivity.

Complementation experiments. Complementation experiments were carried out in BHK-21 cell cultures grown in 15-ml glass vials. Before the start of the experiment, the BHK-21 cultures and virus inocula were warmed to 39.5 C. Cultures were then infected with SV-C clones or ts 4 or 10. After a 1-h adsorption period, the cultures were washed three times with prewarmed PBS, and medium was added to the cultures. At 4 h after infection, the cultures were again washed three times with PBS to remove any reversibly adsorbed virus which had eluted and which might obscure complementation (2). Samples (20 μliter) were taken at 4 and 10 h after infection for plaque assay in BHK-21 cells at 34 C (28 C in the case of ts 4 × ts 10). The complementation level was calculated as described by Burge and Pfefferkorn (2) and was equal to the yield from mixed infections divided by the sum of the yields of each variant grown separately.

Chemicals and radioisotopes. Actinomycin D was a gift from Merck, Sharpe and Dohme Research Laboratories. Cycloheximide was purchased from Sigma Chemical Co., and [5-^{3}H]uridine (26.5 Ci/mmol) from New England Nuclear Corp.

RESULTS

SV-C is antigenically related to SV-W. In order to demonstrate that the small plaque variants were indeed SV and not some unrelated virus harbored by the mosquito cells, antiserum produced against SV-W was tested for its ability to neutralize SV-C. Both SV-W and SV-C were effectively neutralized by the serum, whereas EEEV was not (Fig. 1). The 50% plaque reduction titer was about 25-fold lower with SV-C than with SV-W. It has not yet been determined whether the reduced sensitivity to neutralization was due to reversible antigenic alterations resulting from growth in mosquito cells (17) or to mutation.

SV-C is temperature sensitive. When SV-C was assayed on BHK-21 cell monolayers at 28 C, the temperature at which the chronically infected mosquito cultures were maintained, or at 34 C, the maximum number of plaques was produced (Table 1). However, there was a 50-fold reduction in the number of plaques formed at 37 C, and no plaques were detected on monolayers inoculated with undiluted SV-C

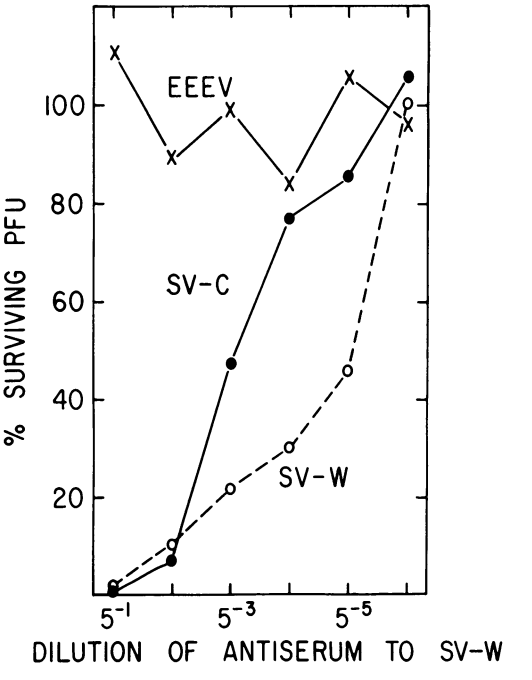

FIG. 1. *Neutralization of SV-C, SV-W, and Eastern equine encephalitis virus (EEEV) with antiserum produced against SV-W. Neutralization was carried out as described in Materials and Methods. Percentage of surviving PFU is the ratio of the PFU remaining after treatment with immune serum to the PFU present after treatment with the same dilution of preimmune serum. One hundred percent survival at a 5 to 6 dilution of serum was 438 PFU for SV-W, 169 PFU for SV-C, and 166 PFU for EEEV.* ○, *SV-W;* ●, *SV-C;* ×, *EEEV.*

TABLE 1. *Temperature-sensitive plaque formation on BHK cell monolayers by Sindbis virus from chronically infected cultures of A. albopictus*[a]

Incubation temp (C)	SV-C (PFU/ml)	EOP[b]	SV-W[b] (PFU/ml)	EOP[b]
28	4.1×10^6	1.0	1.6×10^8	1.0
34	4.4×10^6	1.1	1.7×10^8	1.1
37	8.2×10^4	2.0×10^{-2}	1.7×10^8	1.1
39.5	<10	$<2.4 \times 10^{-6}$	1.6×10^8	1.0

[a] Adsorption was at 28 C, after which the cultures were incubated at the temperatures indicated for 48 h.
[b] Values represent the titer at the indicated temperature divided by the titer at 28 C.

and then incubated at 39.5 C for 48 h. SV-W, in contrast, produced a similar number of plaques at all temperatures.

The growth kinetics of SV-W and SV-C in chicken embryo cells at permissive and nonpermissive temperatures were compared (Fig. 2). SV-W replicated well at all temperatures tested. It reached a maximum titer sometime between 8 and 18 h after infection, when cells were incubated at 39.5, 37, or 34 C, but its growth rate was slightly slower at 28 C. In SV-C-infected chicken cells, the rate of virus production at 28 and 34 C was significantly slower than in cells infected with SV-W, and the yields at 48 h after infection were about 10-fold lower than the maximum titers of SV-W. The rate of SV-C growth was further decreased in cultures incubated at 37 C, and no increase in virus titer occurred at 39.5 C. Similar results were obtained in experiments on BHK-21 cells.

In order to rule out the possibility that a normal yield of SV-C was produced at 39.5 C, but that it was masked by rapid inactivation at this temperature, the inactivation kinetics of SV-W and SV-C at 39.5 C were compared. The small difference in the rate of inactivation of SV-W and SV-C at this temperature could not account for the difference in virus yields (Fig. 3).

It was also possible that noninfectious progeny virions were produced in SV-C-infected cultures at 39.5 C. As Table 2 indicates, equivalent amounts of hemagglutinin were produced by SV-W at 34 or 39.5 C, but no detectable hemagglutinin appeared in SV-C infected cultures incubated at 39.5 C (Table 2). We have not, however, ruled out the production of particles which were neither infectious nor hemagglutinating.

Isolation of viral clones from SV-C. Cloned derivatives of SV-C were prepared by plaque isolation and growth of stocks in BHK-21 cells at 34 C. All of the clones isolated from SV-C were temperature sensitive. The fact that these isolates remained temperature sensitive after several passages in BHK-21 cells ruled out the possibility that the ts phenotype was due to a

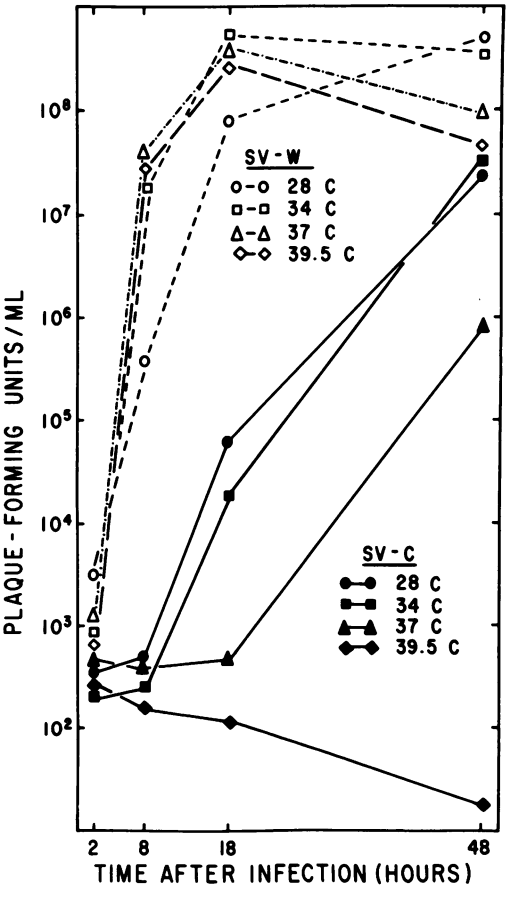

FIG. 2. *Growth curves of SV-W and SV-C in chicken cells incubated at 28, 34, 37, and 39.5 C. Prior to infection, phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.2 mM Na_2HPO_4) and viral inocula were heated to 42 C. The virus inocula were warmed only for 2 min to prevent inactivation. The chicken monolayers were washed once with PBS and then were infected with either SV-C or SV-W at a multiplicity of infection of 1 PFU/cell. After adsorption for 1 h at 39.5 C, the inocula were removed, the plates were washed three times with prewarmed PBS, and prewarmed medium was added. Plates then were incubated at the appropriate temperatures, and samples for plaque assay were taken at the times indicated. SV-W: ○, 28 C; □, 34 C; △, 37 C; ◇, 39.5 C. SV-C: ●, 28 C; ■, 34 C; ▲, 37 C; ◆, 39.5 C.*

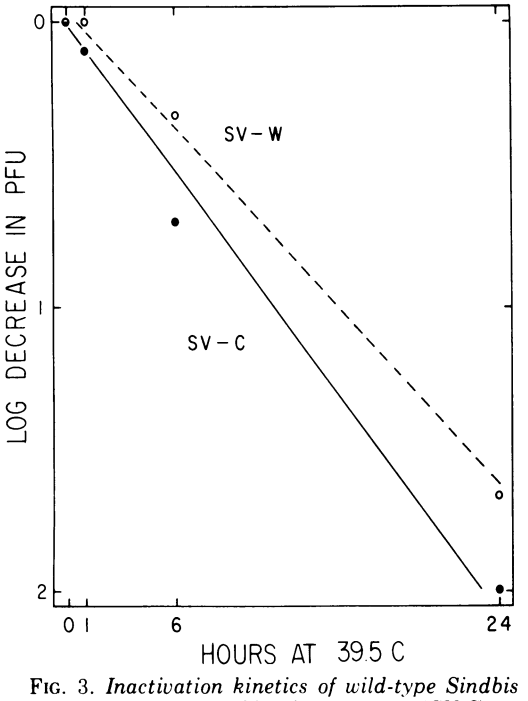

FIG. 3. *Inactivation kinetics of wild-type Sindbis virus (SV-W) and Sindbis virus mutant (SV-C) at 39.5 C. Undiluted, 1-ml. portions of SV-W (1.7 × 10⁸ PFU/ml) and of SV-C (4.4 × 10⁶ PFU/ml) were heated in a 39.5 C water bath, and samples were withdrawn, rapidly chilled, and assayed for surviving PFU at the indicated times.* ○, *SV-W;* ●, *SV-C.*

TABLE 2. *Absence of hemagglutinin production by BHK-21 cells infected with SV-C and maintained at 39.5 C*

Infecting virus[a]	Incubation temp (C)	HAU/ml[b]
SV-W	34	1280
SV-W	39.5	1280
SV-C	34	640
SV-C	39.5	0

[a] Multiplicity of infection = 1 PFU/cell.

[b] HAU, Hemagglutination units per milliliter at 24 h. The assay was performed as described in Materials and Methods.

host-specified modification acquired during growth of the virus in mosquito cells.

Stocks of six representative cloned mutants were tested for ts⁺ revertants by direct plaque assays at 34 and 39.5 C. Efficiencies of plating (EOP) at 39.5 C relative to those at 34 C of 6.7×10^{-5} and 3×10^{-6}, respectively, could be detected for two of the clones (Table 3). In both cases, the plaques produced at 39.5 C were of the small or mutant type. No ts⁺ revertants were found by this method in the remaining four clones.

Reversion to ts⁺ phenotype. The observed low frequency or absence of ts⁺ particles in the uncloned SV-C stock or in the clones derived from it might be more apparent than real for several reasons. First, selective pressure during the growth of stock populations at permissive temperature could have operated in favor of ts mutants through interference. Although the SV-C clones did not interfere when they were present in only a 10-fold excess, a 10^5-fold multiplicity advantage, such as might exist in a chronically infected culture, did result in marked interference by SV-C clones with the replication of SV-W. A similar interference therefore might be expected with the replication of ts⁺ revertants in chronically infected cultures. Because the experiment was carried out in the presence of actinomycin D, it is unlikely that this interference was due to interferon (4). Alternatively, rare wild-type progeny or ts⁺ revertants present in SV-C stocks might not have expressed themselves in plaque assays at the nonpermissive temperature because of interference or some other effects induced by the majority ts particles. This suggestion was based on two considerations. (i) Infection of BHK-21 cells with clones SV-C-4, -16, and -19 at multiplicities greater than 1 PFU/cell led to cell death at 39.5 C, and this circumstance would have precluded recognition of plaques. No cell killing at this temperature was observed with clone SV-C-2, even at an input multiplicity of 35 PFU/cell. (ii) Plaque production by SV-W has been shown to be inhibited in presence of high multiplicities of ts mutants at the nonpermissive temperature (1, 2), and it seemed possible that ts⁺ revertants might be similarly inhib-

TABLE 3. *Frequency of revertants in stocks of Sindbis virus mutant clones*[a]

SV-C clone	Frequency of revertants[b]
SV-C-2	$<10^{-7}$
SV-C-4	$<10^{-5}$
SV-C-8	6.7×10^{-5}
SV-C-13	3×10^{-6}
SV-C-16	$<10^{-6}$
SV-C-19	$<10^{-6}$

[a] Cloned virus was obtained from well-isolated plaques, and a portion of this virus then was grown to a stock. Both the initial plaque purification and the growth of the stock were in BHK-21 cells at 34 C.

[b] Frequency of ts⁺ revertants is the ratio of the titer measured at 39.5 C to that measured at 34 C.

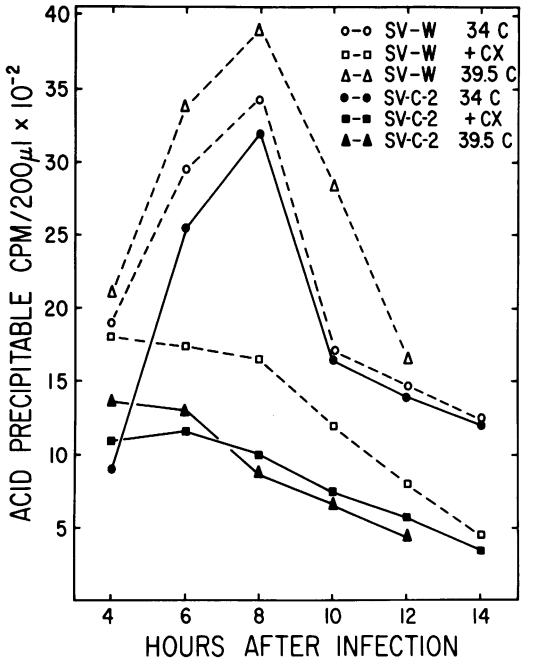

FIG. 4. *Viral RNA synthesis directed by wild-type Sindbis virus (SV-W) and Sindbis virus mutant 2 (SV-C-2): effect of temperature shift up to 39.5 C and effect of cycloheximide. Cultures of BHK-21 cells grown in 15-ml glass vials were infected with SV-W (open symbols) or SV-C-2 (closed symbols) at a multiplicity of 5 PFU/cell. After a 1-h adsorption period, 0.8 ml of medium containing 4 μg of actinomycin D was added to each culture. After 3.8 h of incubation at 34 C, the cultures were divided into three groups. One group was shifted up to 39.5 C (triangles), another was left at 34 C, but received cycloheximide (300 μg/ml) (squares), and the third group was maintained at 34 C without further treatment (circles). At the times indicated, 20-min pulse labels were initiated by using [5-³H]uridine (20 μCi/culture). Cultures were harvested and acid-precipitable counts per minute were determined as described in Materials and Methods.*

ited in stocks containing vast excesses of ts virus. Accordingly, reconstruction experiments were carried out by examining the plating efficiency at 39.5 C of SV-W in cell monolayers co-infected with clones SV-C-4, -16, and -19 at nonkilling concentrations (multiplicity of infection [MOI] < 1 PFU/cell). No reduction in plaque number was observed. Clone SV-C-2 prevented plaque formation by SV-W only when it was used undiluted (MOI of 35 PFU/cell).

Thus frequencies of ts⁺ revertants lower than the maxima given in Table 3 could not have been detected because of either cell killing (SV-C-4, -16, and -19) or interference (SV-C-2).

That clones, such as SV-C-8 and -13, could indeed yield ts⁺ revertants was demonstrated by passaging them serially through BHK-21 cells at 34 C without dilution of the seed inocula. Under these conditions, as shown in Table 4, each of the cloned stocks yielded ts⁺ revertant progeny with surprising frequencies. Similar results were obtained when the ts clones were subjected to serial undiluted passages at 28 C in mosquito cells.

SV-C stocks contain predominantly RNA⁺ mutants. Twenty SV-C clones were tested for their ability to synthesize viral RNA at the nonpermissive temperature. Apart from SV-C-2, the remaining 19 clones tested synthesized viral RNA at 39.5 C, suggesting a strong selection for RNA⁺ phenotypes. Typical results are shown in Table 5.

SV-C-2-directed RNA polymerase activity once formed is stable at 39.5 C. The temperature-sensitive lesion in RNA⁻ variant SV-C-2 was examined further. Cultures of BHK-21 cells were infected with SV-W or SV-C-2 at a MOI of 5 PFU/cell and incubated at the permissive temperature for 3.8 h. At this time one group of cultures was shifted up to the nonpermissive temperature (39.5 C), a second group received

TABLE 4. *Accumulation of revertants by serial undiluted passage in BHK cells at 34 C*

SV-C clone	Frequency of revertants[a]	
	Passage 2	Passage 4
SV-C-2	4.4×10^{-6}	1.6×10^{-4}
SV-C-4	5.0×10^{-3}	5.9×10^{-2}
SV-C-8	7.5×10^{-4}	6.3×10^{-2}
SV-C-13	8.5×10^{-4}	1.0×10^{-3}
SV-C-16	1.9×10^{-4}	7.7×10^{-2}
SV-C-19	5.6×10^{-4}	8.5×10^{-2}

[a] Frequency of revertants is as described in the legend to Table 3.

TABLE 5. *RNA synthesis phenotype of Sindbis virus variants*[a]

Sindbis virus	SA at 39.5 C: SA at 34 C	RNA synthesis phenotype
SV-W	0.84	+
SV-C-2	<0.01	−
SV-C-4	0.34	+
SV-C-8	0.39	+
SV-C-13	0.52	+
SV-C-16	0.53	+
SV-C-19	1.03	+

[a] Specific activity (SA) is expressed as counts per minute per absorbancy at 260 nm. The experimental details are described in Materials and Methods.

cycloheximide (300 μg/ml), a third group was left at the permissive temperature with no further treatment. Cultures were then pulse labeled for 20-min periods beginning at 4 h after infection and every 2 h thereafter. At 34 C both SV-W and SV-C-2 infected cells synthesized RNA at increasing rates until approximately 8 h after infection, after which time the rates of synthesis sharply declined. After shift-up to 39.5 C, cells infected with SV-W synthesized RNA with kinetics similar to those kept at 34 C. SV-C-2 infected cells, however, showed no further increase in the rate of RNA synthesis after the shift-up to 39.5 C. Rather, the rate slowly declined with a half-life of 6 to 8 h. After cycloheximide treatment, cells infected with both SV-W and SV-C-2 showed a similar slow decline in RNA synthesizing activity with a half-life of 6 to 8 h. Control experiments demonstrated that the concentration of cycloheximide used in these experiments inhibited the incorporation of labeled amino acids into acid-precipitable material by more than 99%. We conclude that in the case of the RNA$^-$ mutant, SV-C-2, the RNA synthesizing system, once formed, is stable even at the nonpermissive temperature.

SV-C clones are heat labile. All of the SV-C clones tested were much more quickly inactivated by heating to 60 C than was SV-W, a plaque-purified derivative of the HR strain of Burge and Pfefferkorn (1; Fig. 5). Burge and Pfefferkorn (1) found a strong correlation between the RNA synthesis and heat inactivation phenotypes of mutagen-induced, temperature-sensitive SV variants. In general, RNA$^+$ variants were much more heat labile than RNA$^-$ variants, which were, in turn, only slightly less stable than the parent virus. In this case, the fact that the RNA$^-$ variant, SV-C-2, was inactivated at a rate similar to the RNA$^+$ variants suggests that SV-C-2 contains at least two lesions, one affecting RNA synthesizing activity and another affecting a structural protein.

SV-C clones fail to complement each other. In the case of the mutagen-induced variants, Burge and Pfefferkorn (2) found that RNA$^-$ variants always complemented RNA$^+$ variants. However, we failed to find any complementation between SV-C-2, the RNA$^-$ variant, and any of the RNA$^+$ clones (Table 6). Furthermore, no combinations of the RNA$^+$ variants tested complemented each other. Control crosses between ts 4 (RNA$^-$) and ts 10 (RNA$^+$) (2) did result in complementation, indicating that the experimental conditions were appropriate for complementation to occur.

The lack of complementation again suggests that, at least in the case of SV-C-2, the viral genome contains more than one mutation. We have not ruled out the possibility that our failure to detect complementation is due to interference between the pairs of variants tested.

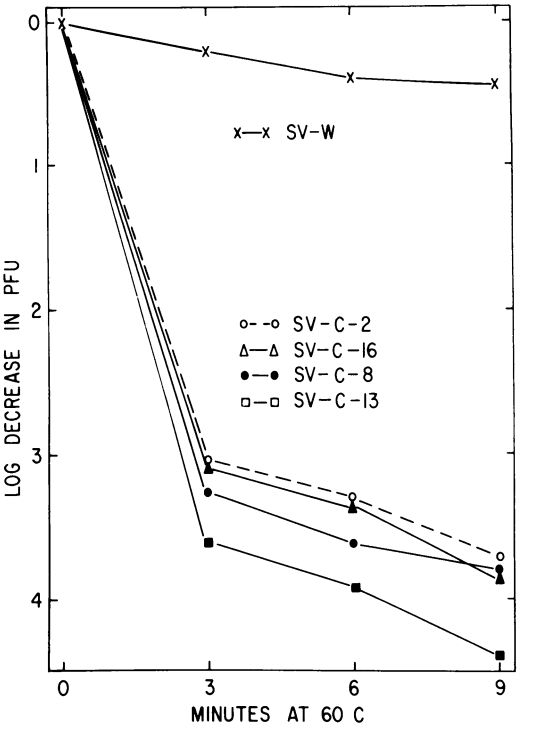

FIG. 5. *Inactivation kinetics of wild-type Sindbis virus (SV-W) and Sindbis virus mutant (SV-C) clones at 60 C. Undiluted, 0.6-ml portions of SV-W and selected SV-C clones were heated in a 60 C water bath. Samples were withdrawn at the indicated times, rapidly chilled, and assayed for surviving PFU.*

Serial undiluted passage of SV-W in BHK cells at low temperature produces ts mutants. The accumulation of ts mutants in chronically infected cultures is not novel to mosquito or invertebrate cell cultures. For example, virus from cultures of L cells chronically infected with Newcastle disease virus (NDV) also has been shown to be temperature sensitive (13).

There have been two recent reports of vertebrate cells chronically infected with SV (7, 14). Nevertheless, because of the destructiveness of SV for vertebrate cells, such systems are more difficult to establish than a similar mosquito cell system.

Another approach was used to look for ts mutants in vertebrate cell culture, and this was the technique of serial undiluted passage at low

TABLE 6. *Lack of complementation between Sindbis virus mutant clones*[a]

Virus	Yield from single infection	Yield in mixed infection with					
		SV-C-4	SV-C-8	SV-C-13	SV-C-16	SV-C-19	ts-10
SV-C-2	2.2×10^6	2.2×10^6 (0.5)	3.2×10^6 (0.8)	1.3×10^6 (0.5)	2.3×10^6 (0.5)	4.9×10^6 (1.1)	
SV-C-4	2.2×10^6		1.1×10^6 (0.3)	8.0×10^5 (0.3)	1.5×10^6 (0.3)	1.4×10^6 (0.3)	
SV-C-8	1.8×10^6			1.1×10^6 (0.5)	3.1×10^6 (0.7)	3.3×10^6 (0.8)	
SV-C-13	5.6×10^5				1.2×10^6 (0.4)	1.2×10^6 (0.4)	
SV-C-16	2.4×10^6					4.0×10^6 (0.8)	
SV-C-19	2.4×10^6						
ts-4	2.0×10^6						7.1×10^7 (28.4)
ts-10	5.2×10^5						

[a] Complementation was carried out as described in Materials and Methods at 39.5 C in BHK-21 cells. Cells were infected with each mutant at an approximate multiplicity of 10 PFU/cell. The complementation level (Burge and Pfefferkorn, ref. 2) for each cross is given in parentheses below the yield in mixed infections and is defined as the yield from mixed infections divided by the sum of the yields of each mutant grown separately. Ts 4 and 10 are from Burge and Pfefferkorn, ref. 2.

temperatures. SV-W thus was serially passaged through BHK-21 cells at 34 C, both undiluted (MOI > 10 PFU/cell) and diluted (MOI 0.01 to 0.8 PFU/cell).

In the undiluted passage series, there was a gradual drop in the EOP (39.5 C: 34 C) reaching 9×10^{-2} after 25 passages (Table 7). In contrast, in the diluted passage series, there was very little, if any, drop in the EOP. Presumably, in this case, any ts mutants which might have arisen during each passage were diluted to ineffective levels before the next passage was initiated.

When serial undiluted passage of SV-W was done at 37 C in either BHK or chicken cells, the EOP dropped only to 0.5.

To confirm that SV was not the only RNA virus to produce ts variants as a result of serial, undiluted passage, type-1 poliovirus was serially passaged through Vero cells at 34 C. Although the EOP 39.5 C: 34 C was close to 1.0 in the initial stock, the EOP had dropped to 0.1 after 18 passages. This is consistent with the reports of Dubes and Chapin (5) and Lwoff (8) demonstrating that cold-adapted strains of poliovirus could no longer replicate at higher temperatures.

These results with both SV and poliovirus indicate that enrichment for ts mutants occurs not only in chronically infected cultures, but also during serial passage at high multiplicity if such passage is done at a low temperature.

It should be noted, however, that after high-multiplicity passage through the BHK cells, the EOP (39.5 C: 34 C) dropped only to 9×10^{-2} as compared with $< 2 \times 10^{-6}$ in the case of SV-C (Table 1). Also, since we are presumably describing a mixed population of ts mutants, until

TABLE 7. *Temperature sensitivity of wild-type Sindbis virus serially passaged at 34 C through BHK-21 cells*[a]

Passage	Titer at 34 C	No. of plaques counted		EOP 39.5 C: 34 C
		34 C	39.5 C	
Undiluted				
0	1.7	309	296	0.96
5	7.2	435	487	1.12
9	2.5	321	146	0.45
13	1.3	1,176	365	0.31
17	0.9	523	132	0.25
21	3.4	582	109	0.18
25	1.5	1,226	117	0.09
Diluted				
5	8.1	623	645	1.03
9	46.0	344	220	0.64
13	40.1	541	399	0.72
17	61.0	372	435	1.17
25	130.0	798	746	0.93

[a] The SV-W was first passaged once through BHK-21 cells (MOI = 0.1 PFU/cell) at 39.5 C. This is designated passage 0. For each subsequent passage, 1 ml of either undiluted virus or virus diluted 1:1000 from the previous passage was used to infect 10^7 BHK-21 cells. The virus from all passages was harvested at 16 hours after infection. Titer is expressed as PFU per milliliter $\times 10^{-8}$. EOP, Efficiency of plating.

plaque purified derivatives are studied, little more can be said about this virus.

DISCUSSION

This report has demonstrated that, several months after the initial infection of a culture of *A. albopictus* cells with SV-W, the progeny virus in the chronically infected cultures (SV-C) is temperature sensitive. This result has been obtained in three independently established, chronically infected mosquito cell cultures each infected initially with SV-W.

The evolution of temperature-sensitive mutants, however, may be a general correlate of persistent infections involving a variety of host cells and viruses. This is suggested by the reports of Preble and Youngner with NDV in L cells (13) as well as by reports describing properties of Western Equine encephalitis virus (16) and Sendai virus (10) from persistently infected cultures.

Our experiments also suggest that, besides persistent infection, serial undiluted passage of virus at low temperature is an effective means of enriching for ts mutants. In this case, probably both the serial undiluted passage and the low temperature are necessary. It should be noted, however, that even after 25 serial passages, about 10% of the progeny retained ts^+ phenotype, whereas no ts^+ particles could be demonstrated in the uncloned SV-C population.

An interesting feature of the SV-C is that nearly all of the clones derived from it are RNA^+. This is contrary to the result reported by Preble and Youngner, who found that ts mutants of NDV from chronically infected L cell cultures were all RNA^-.

The inability to demonstrate complementation between different RNA^+ mutants could be explained by postulating that (i) the mutations all involve the same cistron or (ii) each of these mutants contains two or more mutations. In the case of the RNA^- clone SV-C-2, the evidence indicates that there are at least two mutations: first, SV-C-2 is RNA^- and does not complement any of the RNA^+ clones; second, SV-C-2 is inactivated at 60 C as quickly as any of the RNA^+ clones, implying that it also has at least one mutation affecting structural proteins. Another point arguing in favor of multiple mutations in the viral clones is the observation that most ts^+ revertants remained mutant with respect to plaque morphology. A similar observation was made in the NDV L cell system (13).

All of the SV-C ts mutants described here were derived from chronically infected cultures 2 to 3 months old. Possibly variants with single temperature-sensitive defects can be isolated from virus produced soon after the establishment of a chronically infected culture, and this possibility is presently under study.

In considering the defect of SV-C-2, it was noted that RNA synthesis did not occur at 39.5 C, but that in shift-up experiments RNA synthesizing activity, once formed, was stable at the nonpermissive temperature. If the viral RNA polymerase is an oligomeric molecule, it is possible that a temperature-sensitive lesion could be present in a virus-specific protomer which does not affect the enzymatic activity of the oligomer, but does prevent assembly of protomers into the functional oligomeric form at the nonpermissive temperature. Such a hypothesis has been previously suggested by Martin (9) as a possible explanation of the temperature-sensitivity of certain RNA^- Semliki Forest virus variants.

Alternatively, one can postulate that SV-C-2 directs the synthesis of an essential precursor protein which cannot be cleaved at the nonpermissive temperature, but that the cleavage products, once formed, are functional and stable. Evidence for such a lesion has been presented and discussed in the case of ts-11, a mutagen-induced ts mutant of SV (20).

A final point of interest is that, although ts^+ revertants were not detected in the original mixed SV-C population, such revertants were readily demonstrated after undiluted passage of mutant clones in either BHK or mosquito cells. Indeed serial undiluted passage through BHK cells generated ts mutants from SV-W and generated ts^+ revertants from SV-C clones. These observations suggest that the systems under study are complex and are influenced by a number of factors such as temperature, viral interference, relative multiplicity of different viral genotypes, and perhaps the host cell. Although little mention has been made of defective particles in the SV-C cultures, it is probable that such particles are also present in the chronically infected cultures. Perhaps, then, a slight change in the conditions of culture or passage may be sufficient to tip the balance either toward enrichment for mutants or for the selection of revertants.

Because togaviruses may replicate in mosquitoes, cold-blooded vertebrates, mammals, and birds (body temperature >39 C) the importance of such selective pressures for the natural transmission of these viruses and possibly for the determination of varying virulence is evident.

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