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Effect of Sindbis Virus Infection on Induction of Heat Shock Proteins in *Aedes albopictus* Cells

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When *Aedes albopictus* cells (clone C7) were infected with Sindbis virus, the production of cytopathic effect CPE depended largely on the conditions under which the cells were cultured. We observed marked inhibition of cellular RNA and protein synthesis, as well as a loss of the ability to induce heat shock proteins, e.g., hsp70, under conditions which led to cytopathic effect. Infected cells in which heat shock proteins could no longer be induced contained much lower amounts of hsp70 mRNA after heat shock than did mock-infected cells which were similarily treated. It is suggested that this decreased level of hsp70 mRNA is due to a failure of these cells to synthesize hsp70 mRNA after heat shock.

Considerable work has been done studying the replication of Sindbis virus (SV) (family *Togaviridae*, genus *Alphavirus*) in cultured mosquito cells. Although it was first reported that SV or Semliki Forest virus (another alphavirus) grew to high titer in mosquito cells without producing any obvious cell damage (1, 13), we and others subsequently showed that cell clones isolated from the parental population varied considerably in their response to virus infection (10, 17). Thus, we identified clones which showed marked cytopathic effect (CPE) after infection with Sindbis virus (CPE^+) and clones which showed no CPE (CPE^-). The 24-h yields of virus from CPE^+ and CPE^- cells were, in our experiments, not significantly different (10). More recently, we demonstrated with hybrid cells formed by fusion of CPE^+ and CPE^- cells that the CPE^+ phenotype was dominant (16).

In experiments with vesicular stomatitis virus (VSV) in *Aedes albopictus* cells (clone C7, a clone which is CPE^+ with SV), we showed that the production of CPE and the inhibition of protein synthesis depended on the conditions under which the infected cells were maintained (5). Thus, to produce maximal CPE and inhibition of protein synthesis it was necessary to incubate cells at 34.5°C instead of the usual 28°C (uninfected *A. albopictus* cells grow well at both 28 and 34.5°C) and in the presence of serum. Infected cells incubated at 28°C in 10% serum, or at 34.5°C without serum in the medium, showed little CPE or inhibition of protein synthesis.

To probe more deeply into how infection with SV affects mosquito cells, we wished to examine the effect of virus infection on a specific gene system, rather than simply on overall protein or RNA synthesis. The system we chose was the heat shock system and specifically the expression of hsp70, a prominent heat shock protein with a molecular weight of about 70,000 (2, 15). This system had the following advantages. (i) Since hsp70 (and other heat shock genes) is strongly conserved through evolution, we anticipated that we would be able to use a *Drosophila* hsp70 DNA probe to assess the expression of the gene in mosquito cells. (ii) Because the synthesis of hsp70 is inducible and little or no hsp70 is made in the uninduced state, there is a very low background of hsp70 or of hsp70 mRNA. (iii) The induction of the heat shock proteins involves both the transcription of the gene and the translation of the newly synthesized mRNA. Conceivably, then, the effect of virus infection could be examined both at the level of transcription and at the level of translation (for a review, see reference 7).

Effect of culture conditions on the development of CPE and inhibition of macromolecular synthesis in SV-infected *A. albopictus* cells. *A. albopictus* C7-10 cells were infected (10) with SV and were incubated under different conditions. As with VSV, the SV-infected cells incubated at 34.5°C in medium containing 10% serum (E-10) developed marked CPE, whereas the cultures maintained at 28°C or in serum-free medium (E-0) developed only minimal or no CPE (Table 1). The lack of CPE at 28°C or in E-0 medium was not due to a failure to produce virus; on the contrary, virus yields were actually higher in the cultures which showed no CPE, probably because these cells were able to produce virus for a longer period of time.

To examine the effect of SV infection on cellular RNA synthesis, RNA was extracted from [^{3}H]uridine-labeled cells and was analyzed by polyacrylamide gel electrophoresis. Our results (not shown) demonstrated the following: (i) that RNA synthesis in infected C7-10 cells maintained at 34°C in E-10 medium, relative to that in a control culture, began to decline about 12 h after infection and by 16 h was 25% of the control level, and (ii) that this severe decline in RNA synthesis occurred only in cells maintained at 34°C in the presence of serum. RNA synthesis in SV-infected cells maintained at 28°C with serum or at 34°C without serum was

TABLE 1. Effect of culture conditions on virus titer and CPE in SV-infected *A. albopictus* C7-10 cells[a]

Temp (°C)	Medium	CPE[b]	Titer (PFU/ml)
28	E-10	1+	1.1×10^9
34.5	E-10	4+	2.3×10^8
34.5	E-0	0	1.8×10^9

[a] The C7-10 clone has been described elsewhere (16). The SV was a cloned derivative of the HR strain of Burge and Pfefferkorn (14). Monolayer cultures were infected at an input multiplicity of 100 PFU per cell, as described elsewhere (10). Samples of medium were harvested 24 h after infection and were assayed for infectious virus (11).

[b] The degree of CPE (evaluated at 24 h) was rated on a scale of 0 (no CPE) to 4+ (90 to 100% destruction of the cell monolayer).

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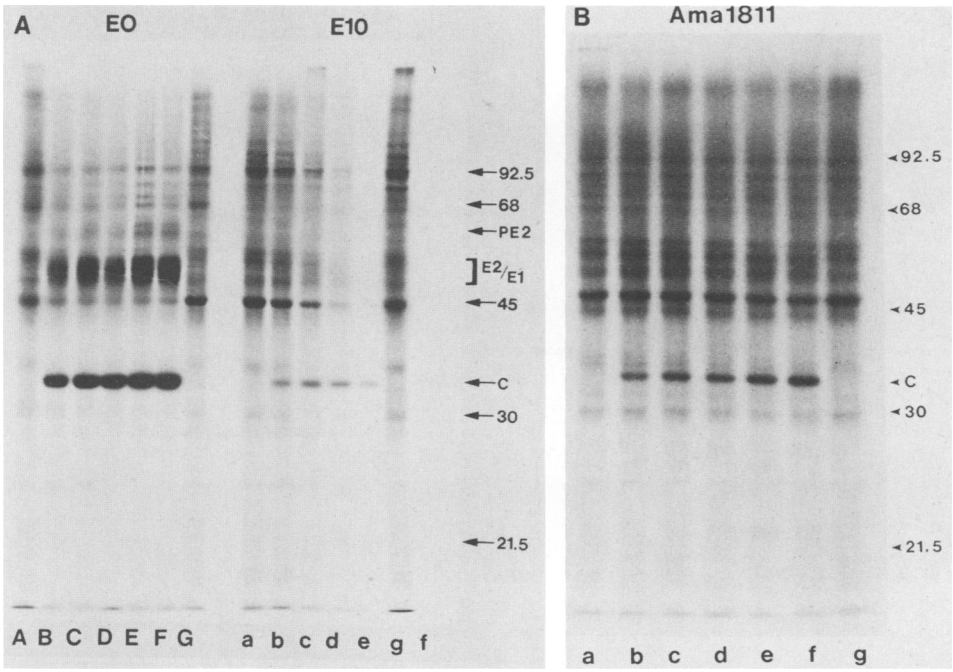
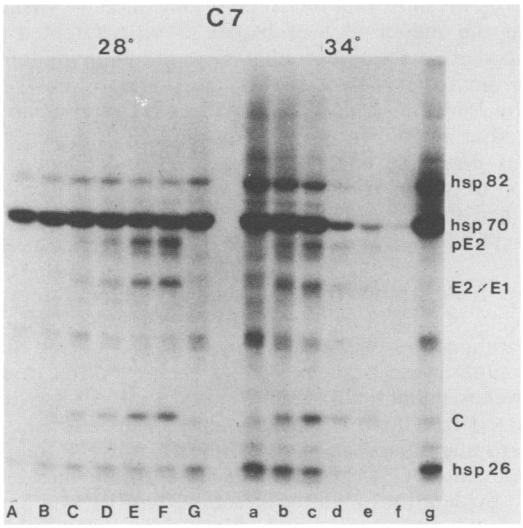


FIG. 1. Protein synthesis in SV-infected *A. albopictus* cells in the presence and absence of serum. (A) Monolayer cultures of C7 cells in 35-mm plates (2×10^6 cells per plate) were infected with SV and maintained at 34°C in E-0 or E-10 medium. Cultures were labeled with [^{35}S]methionine (1,125 Ci/mmol; 10 μCi/ml of medium) for 60 min at 10 (lanes B and b), 12 (lanes C and c), 14 (lanes D and d), 16 (lanes E and e), and 18 (lanes F and f) h after infection. Control cultures were similarly labeled at 10 (lanes A and a) and 18 (lanes G and g) h after mock infection. Labeling was terminated by lysing the cell monolayers in 1.0 ml of lysis buffer (0.15 M KCl–0.02 M Tris hydrochloride [pH 7.4]–2.5 mM magnesium acetate–0.5% Triton X-100–2 mM β-mercaptoethanol). After the nuclei were pelleted, a sample of the supernatant equivalent to 8×10^5 cells was transferred to a fresh tube and brought to a final volume of 500 μl with lysis buffer. The protein was precipitated by adding 500 μl of cold 20% trichloroacetic acid and chilling the mixture on ice for at least 10 min. After the precipitate was collected by centrifugation, the protein was soaked in 1.0 ml of acetone-water solution (9:1) for 5 min on ice, vortexed, pelleted, and then air dried. The protein precipitates were then dissolved in 40 μl of gel sample buffer (0.13 M Tris hydrochloride [pH 8.8]–1.25% sodium dodecyl sulfate–1.25% mercaptoethanol–12.5% glycerol–0.001% bromophenol blue) and were heated to 100°C for 2 min, after which 10 μl of 0.49 M HCl was added to bring the pH to 6.8. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) by using the discontinuous buffer system of Laemmli (6). Molecular weight standards, as well as the SV proteins C, E1/E2, and pE2, are indicated. (B) Monolayer cultures of Ama1811 cells were treated as described for the C7 cells except that they were maintained only in E-10 medium. Infected cultures were labeled at 10, 12, 14, 16, and 18 h after infection (lanes b, c, d, e, and f), and control cultures were labeled at 10 and 18 h after mock infection (lanes a and g).



not very different from that observed in the control mock-infected cultures. As expected, SV infection had no effect on host RNA synthesis in Ama1811 cells, a CPE$^-$ clone, even when maintained at 34°C with serum.

Figure 1 shows the effect of SV infection on overall protein synthesis in the infected cells. In the experiment shown in Fig. 1A, the two sets of C7-10 cell cultures were

FIG. 2. Induction of heat shock proteins in SV-infected C7 cells maintained at different temperatures. Monolayer cultures of C7 cells in 35-mm dishes were infected with SV and maintained in E-10 medium at 28 or 34°C. The medium was removed and replaced with leucine-deficient E-10 medium 1 h before heat shock. At 10 (lanes B and b), 12 (lanes C and c), 14 (lanes D and d), 16 (lanes E and e), and 18 (lanes F and f) h after infection, the cultures were heat shocked for 30 min at 40°C and were then labeled for 15 min at 28°C with [^{3}H]leucine (120 Ci/mmol; 15 μCi/ml of medium) in E-10 medium lacking leucine. Uninfected cultures were treated similarly at 10 (lanes A and a) and 18 (lanes G and g) h after mock infection. The labeled proteins were analyzed by gel electrophoresis as described in the legend to Fig. 1. The positions of the heat shock proteins hsp82, hsp70, and hsp26 and the SV proteins C, E2/E1, and pE2 are indicated.

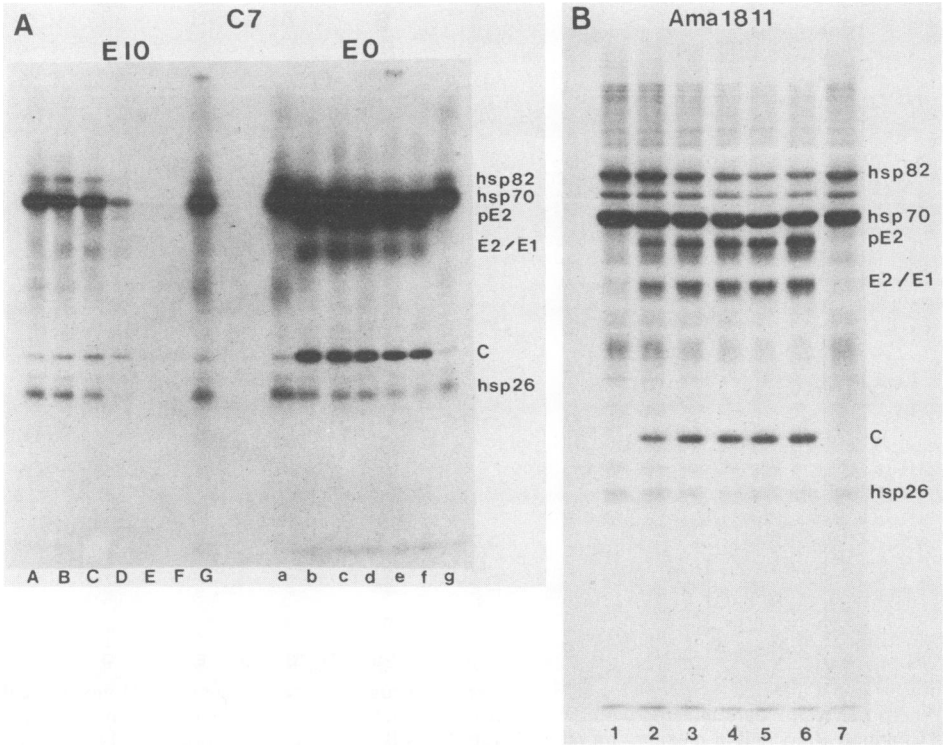


FIG. 3. Induction of hsp70 in *A. albopictus* cells infected with SV and maintained with or without serum in the medium. (A) Monolayer cultures of C7 cells were infected with SV and were incubated at 34.5°C in E-0 or E-10 medium. Cells were heat shocked and labeled with leucine as described in the legend to Fig. 2. Heat shock treatments were at 10 (lanes B and b), 12 (lanes C and c), 14 (lanes D and d), 16 (lanes E and e), and 18 h (lanes F and f) after infection. Control cultures were heat shocked at 10 (lanes A and a) or 18 (lanes G and g) h after mock infection. The labeled proteins were analyzed by gel electrophoresis. The positions of the heat shock proteins hsp82, hsp70, and hsp26 and the SV proteins C, E2/E1, and pE2 are indicated. (B) Monolayer cultures of Ama1811 cells were infected with SV and maintained at 34.5°C in E-10 medium. Otherwise they were treated as the C7 cells in panel A. Cultures were heat shocked at 10, 12, 14, 16, and 18 h (lanes 2 to 6, respectively) after infection. Control cultures were heat shocked at 10 and 18 h (lanes 1 and 7, respectively) after infection.

maintained at 34°C but in one case in E-0 and in the other in E-10 medium. In the E-10 medium, beginning at 12 h after infection (lane c), there was a clear and progressive decrease in the levels of both host and viral protein synthesis. By 18 h (lane f), very little protein synthesis was observed. Interestingly, synthesis of the viral capsid protein persisted at a time when no other proteins, cellular or viral, were being made. In contrast, infected cells in E-0 medium showed little decrease in overall protein synthesis, and viral proteins continued to be made at high levels up to 18 h after infection. When the α-amanitin-resistant clone Ama1811 was infected with SV and maintained at 34°C in E-10 medium, overall protein synthesis continued at control levels over the 18-h time interval examined (Fig. 1B).

It is important to emphasize that the inhibition of protein synthesis which we observed in C7-10 cells maintained at 34°C in E-10 medium affected viral as well as cellular protein synthesis. This also appears to be the case with VSV-infected cells (for example, see references 3 and 5).

Effect of virus infection on expression of the hsp genes. In preliminary experiments, we observed that when C7-10 cells which are normally grown at 28°C were incubated at 34, 37, or 40°C, three new proteins with estimated molecular weights of 82,000, 68,000, and 28,000 were made. These proteins likely correspond to the heat shock proteins hsp82, hsp70, and hsp26, respectively, which are induced in *Drosophila* cells; we shall therefore use these designations in referring to the mosquito cell proteins. As in other systems, hsp70 was made in substantially greater amounts than the other heat shock proteins. Also, as seen below, although hsp70 was made in equivalent amounts at 34, 37, and 40°C, the level of normal polypeptide synthesis decreased as the temperature was elevated; thus, at 40°C virtually the only proteins made were the heat shock proteins. The heat response of the Ama1811 cells to heat shock was similar to that of the C7-10 cells.

Since transiently heating cells at 34.5°C induced heat shock proteins in *A. albopictus* cells and since we wished to examine the inducibility of hsp70 in virus-infected cells maintained at 34.5°C, we used 40°C as the inducing temperature in our experiments. It was necessary to know, however, how long the synthesis of hsp70 would continue in cells incubated at 34.5°C. In brief, we found that although hsp70 synthesis began to decline between 30 and 60 min after elevation to 34.5°C it was still detectable at 2 h. By 4 h at 34.5°C, hsp70 synthesis was no longer detectable. However, when such cells were shifted to 40°C, hsp70 was again induced.

Figure 2 illustrates the effect of SV infection on the inducibility of hsp70. Cells (C7-10) were infected with SV, were maintained in E-10 medium at 28 or 34.5°C, and then at various times were heat shocked by being shifted to 40°C. For the cells maintained at 28°C, inducibility of hsp70 was not affected even up to 18 h after infection. In contrast, when cells were maintained at 34°C, synthesis of hsp70 following heat shock at 40°C was profoundly diminished. The decline was first evident by 14 h after infection (lane d), and by 18 h (lane f) only a small amount of hsp70 was made following

shift-up to 40°C. Thus, the ability to synthesize hsp70 following heat shock was lost at approximately the same time that effects on overall protein synthesis were seen.

In another experiment (Fig. 3A), we examined the effect of serum on the inducibility of hsp70. SV-infected cells were maintained at 34.5°C either in E-0 or in E-10 medium. In the latter case, by 14 h after infection (lane D) the inducibility of the heat shock proteins was greatly diminished, and by 16 or 18 h (lanes E and F) no heat shock protein synthesis could be detected following shift-up to 40°C. In contrast, when infected cells were maintained in E-0 medium, although there was some decrease in the amount of heat shock proteins made (especially of hsp26 and hsp82) after shift-up, it was clear that the ability to make the heat shock proteins was maintained in these cells, along with the ability to synthesize viral proteins.

Consistent with observations already presented, the inducibility of heat shock proteins in Ama1811 cells maintained at 34.5°C in E-10 medium was not affected by infection with SV (Fig. 3B).

hsp70 mRNA in SV-infected cells. To determine whether hsp70 mRNA could be made in SV-infected cells, we used a plasmid containing the *Drosophila* hsp70 gene to quantitate by the dot blot hybridization method hsp70 mRNA sequences in SV-infected *A. albopictus* cells.

RNA was extracted from cells at 14 h after infection, serially diluted in twofold steps, dotted onto a nitrocellulose membrane, and then hybridized with the nick-translated plasmid containing the *Drosophila* hsp70 gene. In this experiment, all cultures were maintained at 34.5°C prior to heat shock.

Heat shock of mock-infected C7 or Ama1811 cells led in each case to a significant increase in the amount of RNA containing hsp70 nucleotide sequences (Fig. 4). More important, when Ama1811 cells in E-10 medium or C7 cells in E-0 medium were infected with SV, there was no effect on the ability to induce the synthesis of hsp70 mRNA. In contrast, when C7 cells were incubated in E-10 medium, the concentration of hsp70 mRNA after heat shock was between 8- and 16-fold less in the SV-infected cells than in the mock-infected cells. We conclude from these results that the ability to transcribe the hsp70 gene is impaired in SV-infected C7 cells maintained at 34.5°C in E-10 medium and that the failure to transcribe the hsp70 gene under these conditions is sufficient to explain the decreased synthesis of hsp70 after heat shock. Our results, however, do not rule out the possibility of an additional defect in these cells affecting translation of preexisting mRNAs.

There are certain similarities between what we have found in SV-infected *A. albopictus* cells and what we had earlier described for VSV-infected mosquito cells (5). For instance, in both cases severe CPE was observed at 34°C but not at 28°C and only in the presence of serum. Also, with both viruses the inhibition of macromolecular synthesis involved virus-directed RNA and protein synthesis, as well as that directed by the host cell.

It is also of interest that although the translation of most host cell mRNAs was drastically reduced when C7-10 cells in E-0 medium were shifted to 40°C, the translation of SV mRNA was relatively unaffected (Fig. 3A). Thus, the viral mRNA and the heat shock mRNAs may share certain features which permit their translation under conditions not favorable for the translation of most cellular mRNAs. It should be noted, however, that although hsp70 is a stress protein, and it has been reported that stress proteins are induced after infection with certain viruses, including SV (4),

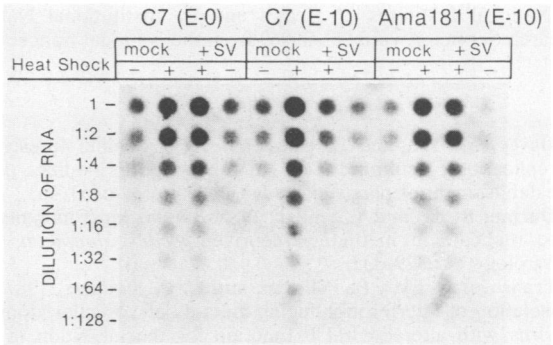


FIG. 4. Dot blot analysis to quantitate hsp70 mRNA in SV infected heat-shocked *A. albopictus* cells. C7 or Ama1811 cells were infected with SV or were mock infected and then incubated at 34.5°C in E-0 or E-10 medium. At 14 h after infection, cultures were heat shocked (+) or mock infected (−) as indicated for 30 min at 40°C, after which the total RNA was extracted with phenol and chloroform-isoamyl alcohol (24:1) and was then ethanol precipitated. We used a pSP65 plasmid (9) into which the *Drosophila* hsp70 gene had been inserted as a probe for the hsp70 mRNA (the plasmid was a generous gift from Marilyn Sanders). The plasmid DNA was labeled by nick translation with [α-^{32}P]dATP basically by the procedure described by Maniatis et al. (8). A specific activity of 1.1×10^8 cpm/μg of DNA was obtained. For the dot blot hybridization, 40 μg of whole-cell RNA (200 μl) was heat denatured (5 min, 65°C) in an equal volume of 12× SSPE (1× SSPE is 0.15 M NaCl–10 mM $NaPO_4$ [pH 7.7]–1 mM EDTA)–15% formaldehyde and was then diluted in twofold steps in ice-cold 12× SSPE. A 150-μl sample of each RNA dilution was applied to a nitrocellulose filter (0.45-μm pore size) through a minifold apparatus (Schleicher & Schuell, Inc.). The filter was air dried and then baked in a vacuum oven (80°C for 60 min). The filter was prehybridized at 42°C for 24 h in 10 ml of a solution containing 50% formamide, 5× SSPE, 0.04% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin, and 250 μg of denatured salmon sperm DNA per ml. ^{32}P-labeled probe DNA (1×10^6 cpm) was then added to the 10 ml of buffer in the plastic bag and was allowed to hybridize to the filter for 24 h at 42°C. After hybridization, the filter was washed four times (each time for 5 min at room temperature) in 2× SSPE buffer containing 0.1% sodium dodecyl sulfate and twice (each time for 20 min at 50°C) in 0.1× SSPE containing 0.1% sodium dodecyl sulfate. The filter was then dried and exposed to Kodak XAR-5 film at −70°C with two intensifying screens. The RNA dots which represent the undiluted samples (top row) contain 15 μg of total RNA.

the ability to induce hsp70 in our experiments was not spared under conditions in which SV infection led to a general inhibition of macromolecular synthesis.

One of the features which makes the SV (*A. albopictus*) cell system especially interesting for studying how virus infection causes injury to the host cell is that the balance between a cytopathic and a noncytopathic infection is so readily altered. Specifically, we have shown that by the choice of the host cell, the temperature of incubation, and the composition of the medium we can tip the balance either towards a cytopathic infection or towards a noncytopathic infection and that we can do this without appreciably affecting the yield of virus. It would not be surprising, finally, if SV mutants could be obtained which varied in their ability to affect this delicate balance, i.e., mutants which were less or more cytopathic than our standard virus. Such a mutant has been described, in fact, for VSV, i.e., the P^- mutant initially described elsewhere (12) which is less cytopathic than the standard VSV.

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