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# Replication of Vesicular Stomatitis Virus Facilitated in Nonpermissive Cells by Early Functions of Shope Fibroma Virus (40026)

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Padgett and Walker reported several years ago (1) that replication of vesicular stomatitis virus (VSV), an RNA virus, is markedly facilitated in serially cultured domestic rabbit kidney ($DRK_3$) cells (2) persistently infected with a noncytocidal strain of Shope fibroma virus (SFV), a DNA virus. They observed that superinfection of $DRK_3$ cultures with VSV 3–5 days after treatment with infectious SFV resulted in yields of VSV 10,000-fold greater than in parallel cultures challenged by VSV alone. Recently, we reported (3) that this dramatic facilitation of VSV also occurs when $DRK_3$ cultures are inoculated simultaneously with both viruses, suggesting that contemporaneous replication of SFV, if it occurs in these cells, does not interfere with the replication of VSV. In the same study we found that ultraviolet irradiation of SFV abolishes its ability to induce facilitation, indicating that a functional SFV genome is necessary. It was therefore of interest in the present study to determine (i) whether SFV does in fact replicate in the presence of VSV and (ii) whether the postulated viral gene functions required for facilitation are expressed early or late in the replication of SFV, i.e., before or after DNA synthesis. In addition, since VSV by itself appears unable to replicate in $DRK_3$ cells (1, 3), cultures challenged with VSV in the absence of SFV were examined for the induction of viral cytopathic effects (CPE) to determine whether the cells are completely nonpermissive or whether they become abortively infected.

*Materials and methods.* Cloned $DRK_3$ cells originally isolated by Dr. H. C. Hinze at the University of Wisconsin, Madison, were grown as monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 0.23% sodium bicarbonate, 100 units/ml of penicillin, and 100 $\mu$g/ml of streptomycin. Vero cells provided by W. Wong, State Hygienic Laboratory, University of Iowa, Iowa City, were grown in monolayers in the same medium with only 5% serum. For maintenance of both kinds of cells following viral infection, the concentration of serum was reduced to 2.5%. Experimental cultures were prepared by seeding screw-capped Leighton tubes (11 × 38-mm window) with $1 \times 10^5$ $DRK_3$ or Vero cells in 1 ml of the appropriate growth MEM. After incubation at 37° in 5% $CO_2$ for 2 days, the cultures routinely contained $2\text{–}4 \times 10^5$ actively growing cells and were used for experimentation.

The Patuxent strain of SFV, noncytolytic in DRK cultures (4), was also obtained from Dr. Hinze. Stocks of cloned virus were grown at 34° and harvested as described previously (2). The Indiana strain of VSV was provided by Dr. J. E. Rodriguez, University of Iowa, Iowa City. Stocks of cloned VSV were prepared by inoculation of Vero cultures with about $1 \times 10^{-5}$ infectious units/cell. This low multiplicity of infection was used to avoid production of defective T forms of the virus (5). When VSV-specific cytopathic effects (CPE) characterized by rounded, detached cells were apparent in 100% of the cells, generally 36 hr after incubation at 37°, the culture fluids were clarified by centrifugation at 800*g*, pooled, and stored at −55°.

Infectious SFV and VSV present together in doubly infected $DRK_3$ cultures were quantitated by a differential assay that exploited a marked difference in the host range of the two viruses. Whereas diluted SFV caused foci of aggregated cells in $DRK_3$ monolayers, as previously described (6), diluted VSV had no demonstrable affect. In contrast, VSV rapidly produced cytolytic plaques in monolayers of Vero cells, while SFV was unable to form either plaques or foci. Cultures to be assayed for both viruses



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were frozen and thawed to detach the cells and then subjected to sonification in a Branson Sonifer (Cole-Parmer Instruments & Equipment Co.) for cellular disruption. After serial dilution of the resultant culture fluids in cold Tris-buffered saline (pH 7.4), 0.1-ml amounts were inoculated onto confluent monolayers of both $DRK_3$ and Vero cells in 35-mm plastic petri dishes. Following viral adsorption for 2 hr at 37°, 2 ml of MEM containing 0.5% methylcellulose (Matheson, Coleman, and Bell), 5% heat-inactivated fetal bovine serum, and the usual concentrations of sodium bicarbonate and antibiotics was added to each monolayer. The Vero cultures were then incubated at 37° and the $DRK_3$ cultures at 34°. After 24 hr the inoculated Vero monolayers were treated with 10% formalin containing 0.05% methylene blue; the $DRK_3$ monolayers were similarily treated after incubation for 5 days. With the aid of a binocular dissecting microscope, after the fixed and stained cultures were rinsed and air-dried, foci of aggregated SFV-infected $DRK_3$ cells and plaques of cytopathically affected VSV-infected Vero cells were readily counted. Based on experiments in which known amounts of SFV and VSV were mixed, diluted, and assayed in this manner, the presence of each virus had no demonstrable affect on quantitation of the other in its respective permissive host.

To quantitate amounts of DNA synthesized, the material in cultures labeled with 10 $\mu$Ci of [$^3$H]thymidine ([*methyl*-$^3$H]thymidine; 58 Ci/mmol; Schwarz/Mann) was first digested and then subjected to either equilibrium centrifugation in cesium chloride or direct acid-precipitation. Each culture to be assayed was thawed and treated for 3 hr at 37° with a mixture containing a final concentration of 0.5% Sarkosyl NL30 (Geigy Chemical Corp.), 0.02 *M* disodium (ethylenedinitrilo)tetraacetate (EDTA; pH 7.0), and 0.1% Pronase (Calbiochem, B grade). Sarkosyl and Pronase were prepared in 0.015 *M* sodium chloride and 0.0015 *M* sodium citrate (0.1× SSC, pH 7.3). For equilibrium centrifugation, 200 $\mu$l samples from each digested culture were mixed in separate nitrocellulose tubes (0.5 × 2.5 in.) with 3.8 ml of 0.1× SSC-buffered cesium chloride (Kerr-McGee Chemical Corp., $\rho$ = 1.733 g/cm$^3$) and centrifuged at 30,000 rpm for 60 hr in a refrigerated Beckman L centrifuge using a Type 40 fixed-angle rotor. Ten-drop fractions were dripped from the bottom of the gradients and collected on filter paper disks (Whatman 3M, 25 mm). After collection, the filters were placed in 5% trichloroacetic acid at room temperature to precipitate acid-insoluble material. The filters were then dehydrated in ethanol and acetone, air-dried, and placed in 10 ml of Omnifluor (New England Nuclear Corp.) to measure $^3$H by liquid scintillation. Gradient densities were determined by measuring the refractive indexes of periodic fractions in a Bausch and Lomb refractometer. For direct precipitation, a 0.5-ml sample from each digested culture was transferred to a glass tube containing 0.5 ml of 10% trichloroacetic acid. Precipitates were allowed to form at room temperature for 1 hr. The entire content of each tube was then transferred quantitatively to separate glass-fiber disks (Whatman GF/C, 24 mm) positioned in a Millipore filtration manifold. After washing the precipitates with 5% trichloroacetic acid and 95% ethanol, the disks were removed, air-dried, placed in 10 ml of Omnifluor, and analyzed by liquid scintillation for total $^3$H counts per minute.

*Results.* To determine whether SFV replicates in the presence of VSV, actively growing $DRK_3$ cells were inoculated with 0.2 ml of a mixture containing SFV plus VSV diluted in MEM without serum to give respective multiplicities of 10 and 20 infectious units/cell. Control cultures were inoculated with each virus separately at similar multiplicities. Following viral adsorption at 37° for 2 hr, the inocula were removed, each culture was rinsed once with 5 ml of Tris-buffered saline, and 1 ml of maintenance MEM was then added. At 3 and 30 hr after inoculation, replicate cultures were placed at −55° and assayed later for infectious SFV and VSV by the differential method described above.

Data in Table I show that both viruses replicated in the dual-inoculated cultures. At 3 hr, the amount of residual input virus was routinely only $10^3$–$10^4$ SFV focus-form-

TABLE I. REPLICATION OF SFV AND VSV IN DUALLY INOCULATED $DRK_3$ CELLS.

| Inoculum[a] | Yields of infectious virus[b] | |
|---|---|---|
| | SFV FFU/ culture | VSV PFU/ culture |
| SFV + VSV | $1.2 \times 10^6$ | $2.5 \times 10^8$ |
| SFV | $1.3 \times 10^6$ | |
| VSV | | $6.4 \times 10^3$ |

[a] Cultures were inoculated with a mixture of SFV and VSV or with each virus separately at multiplicities of 10 SFV FFU/cell and 20 VSV PFU/cell.

[b] Infectious virus was assayed 30 hr after infection.

ing units (FFU)/culture and $10^4$–$10^5$ VSV plaque-forming units (PFU)/culture (data not shown). Since the amount of infectious SFV produced by 30 hr in the doubly infected cultures was essentially the same as in the cultures infected with SFV alone, the contemporaneous replication of VSV caused no interference or enhancement of SFV. In contrast, while production of SFV was independent of VSV, replication of VSV appeared to be completely dependent on the presence of SFV.

To determine whether facilitation of VSV in these dually infected $DRK_3$ cultures requires early or late functions of the SFV genome, replication of VSV was examined in the presence of increased concentrations of hydroxyurea to inhibit DNA synthesis. Early poxviral genes are expressed before synthesis of the viral DNA (7). Cultures of actively growing cells were treated with different amounts of the inhibitor at 37° for 4 hr and then inoculated with virus. For inoculation, immediately after removal of the medium 0.1 ml of 2× concentrated hydroxyurea appropriately diluted in MEM without serum was added to each culture. To this was added 0.1 ml of a mixture containing both SFV and VSV diluted in the same MEM to give the usual final multiplicities of infection. Control cultures were inoculated in the same manner with either SFV or VSV alone. After viral adsorption the cultures were washed and nourished with MEM as described in the previous experiment. Appropriate concentrations of hydroxyurea were included in the medium. The cultures were then divided into two groups and given an additional 0.1 ml of medium plus or minus 10 μCi of [$^3$H]thymidine. Incubation was continued at 37°. At 3 and 30 hr after infection, cultures not treated with radioisotope were placed at −55° to be assayed later for infectious SFV and VSV. Cultures labeled continuously with [$^3$H]thymidine until 30 hr after infection were also frozen at −55° and then assayed later by equilibrium centrifugation.

Results in Table II show that SFV was able to facilitate VSV replication in the presence of hydroxyurea. At all of the concentrations of inhibitor tested, by 30 hr after infection, yields of VSV in the dually infected cultures were more than 10,000-fold greater than in controls inoculated with VSV alone. These yields were comparable to those produced in the absence of the drug. Interestingly, in repeated experiments, yields of VSV were somewhat lower at 100 m*M* hydroxyurea than at either 10 or 50 m*M*. Because the presence of hydroxyurea did not permit VSV to replicate in cultures inoculated with VSV alone, the facilitation of VSV observed in the doubly infected cultures was caused by SFV rather than by some effect induced by the drug. When these various cultures were assayed for infectious SFV, including those inoculated with SFV alone, production of virus was found to have been inhibited by greater than 99% at all three concentrations of hydroxyurea (data not shown).

The extent of inhibition of DNA synthesis by hydroxyurea in the dually infected cultures is presented in Fig. 1. Rather than for successive short pulses, [$^3$H]thymidine was

TABLE II. FACILITATION OF VSV IN $DRK_3$ CELLS BY SFV IN THE PRESENCE OF HYDROXYUREA.

| Inoculum[a] | Hydroxyurea[b] (m*M*) | Yield of VSV[c] (PFU/culture) |
|---|---|---|
| SFV + VSV | 0 | $7.5 \times 10^8$ |
| SFV + VSV | 10 | $3.3 \times 10^9$ |
| SFV + VSV | 50 | $3.8 \times 10^9$ |
| SFV + VSV | 100 | $1.0 \times 10^8$ |
| VSV | 0 | $7.8 \times 10^3$ |
| VSV | 100 | $1.0 \times 10^3$ |

[a] Cultures were inoculated with a mixture of SFV and VSV or with VSV alone at multiplicities of 10 SFV FFU/cell and 20 VSV PFU/cell.

[b] The drug was present 4 hr before, as well as throughout the entire 30 hr period of infection.

[c] Infectious VSV was assayed 30 hr after infection.

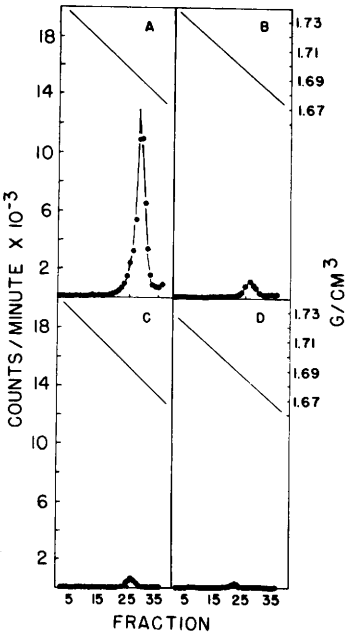


FIG. 1. Analysis of DNA synthesis in cultures of $DRK_3$ cells inoculated simultaneously with SFV and VSV in the absence (A) or presence of hydroxyurea at concentrations of 10 m*M* (B), 50 m*M* (C), or 100 m*M* (D). Nonconfluent $DRK_3$ cultures were treated with the various concentrations of hydroxyurea for 4 hr at 37° and then inoculated with a mixture containing 10 SFV FFU/cell, 20 VSV PFU/cell, and the appropriate amounts of the inhibitor. After 2 hr at 37°, 10 $\mu$Ci of [$^3$H]thymidine was added and remained continuously until 30 hr postinfection. Samples were then digested with Pronase and Sarkosyl in the presence of EDTA, subjected to isopycnic centrifugation in cesium chloride, and assayed for acid-precipitable radioactivity.

present continuously for an extended period of time to detect low levels of DNA synthesis. Because the guanidine–cytosine content of SFV DNA (8) is similar to that of rabbit cell DNA (9), the assay method used was not expected to distinguish between viral and cellular DNA. Equilibrium centrifugation was used as a means to identify the $^3$H-labeled material as DNA. In the absence of hydroxyurea there was a single sharp peak of $^3$H-labeled DNA at the characteristic buoyant density of 1.69 g/cm$^3$. Despite inhibition of infectious SFV production by greater than 99%, continuous labeling for 28 hr revealed the synthesis of small amounts of DNA at all of the concentrations of hydroxyurea tested. Calculations based on the total counts per minute in each peak showed inhibitions of 89, 95, and 98% at respective drug concentrations of 10, 50, and 100 m*M*. Similar results were found in repeated experiments. Although these small amounts of DNA were synthesized, it is unlikely that they were necessary for SFV to facilitate VSV. As shown in Table II, except for the somewhat decreased yield of infectious VSV at 100 m*M* hydroxyurea, probably caused by drug cytotoxicity, which was apparent in parallel noninfected control cultures, the marked inhibition of DNA synthesis by hydroxyurea did not affect the extent of facilitation. In fact, inhibitor concentrations of 10 and 50 m*M* seemed to enhance replication of the VSV.

To determine whether $DRK_3$ cells are nonpermissive for VSV alone, or whether they are abortively infected, cultures treated with the virus were examined for (i) inhibition of cellular DNA synthesis and (ii) induction of viral CPE. As a positive control for this experiment, permissive Vero cells were examined in parallel with the $DRK_3$ cells. Actively growing cultures containing comparable numbers of each kind of cell were inoculated with 0.2 ml of VSV diluted in maintenance MEM to give a multiplicity of 20 PFU/cell. Control cultures were mock-infected with the MEM only. After 2 hr at 37°, 1 ml of the same medium was added to each culture without removal of the inoculum. At various times after inoculation replicate cultures were pulse-labeled for 2 hr by the addition of 10 $\mu$Ci of [$^3$H]thymidine. Following each pulse, the cultures were frozen at −55° until assayed for the amount of [$^3$H]thymidine incorporated into acid-precipitable material, as described above. Total $^3$H counts per minute present in the virus-treated cultures were then compared with those present in the mock-infected controls.

As shown in Fig. 2A, in $DRK_3$ cultures VSV was unable to inhibit incorporation of [$^3$H]thymidine into material presumed to be cellular DNA. Even after 24 hr (data not shown) no inhibition was observed. Contrastingly, synthesis of cellular DNA was markedly inhibited by VSV in the permissive Vero cultures. By 10 hr after inoculation, incorporation of label into the virus-treated cultures was about 95% less

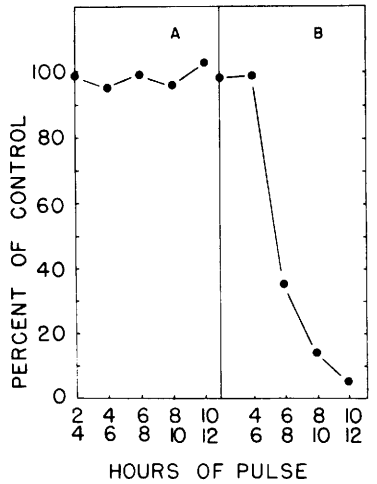


FIG. 2. Comparison of cellular DNA synthesis in VSV-treated and untreated $DRK_3$ cells (A) and Vero cells (B). Nonconfluent cultures of both kinds were inoculated with maintenance MEM or the same medium containing 20 VSV PFU/cell. At the indicated times, 10 $\mu$Ci of [$^3$H]thymidine was added to replicate cultures for a pulse period of 2 hr at 37°. Each point represents the total amount of acid-precipitable $^3$H-labeled material in the virus-treated cultures relative to that present in the mock-treated controls pulsed at the same time.

than in the controls. Microscopic examination of the various cultures showed similar differences. At 24 hr, whereas no detectable VSV CPE was seen in the virus-treated $DRK_3$ cultures, 100% of the Vero cells manifested the rounded appearance characteristically induced by VSV infection. In permissive cells inoculated with VSV alone, inhibition of cell macromolecular synthesis appears to be associated with the development of CPE (10, 11). Since induction of CPE at low input multiplicities requires synthesis of VSV messenger RNA (12, 13) transcribed by the virion-associated transcriptase (14), failure of VSV to inhibit DNA synthesis or induce CPE in $DRK_3$ cells indicates that these cells are completely nonpermissive for this virus.

*Discussion.* The results of this investigation demonstrate that some as yet unknown event early in the replicative cycle of SFV, prior to synthesis of its DNA, facilitates the replication of VSV in $DRK_3$ cells. This finding is similar to that of Thacore and Youngner (15), who reported that replication of VSV in rabbit cornea (RC-60) cells is markedly facilitated by vaccinia virus, a cytolytic poxvirus related to SFV, in the absence of viral DNA synthesis. Thus, it appears that facilitation of VSV in certain restrictive cells is a property common to different poxviruses.

In addition to supporting the contention that poxviruses characteristically facilitate VSV in some cells, the present study also shows that contemporaneous replication of VSV in such cells has no affect on replication of the facilitating poxvirus. In $DRK_3$ cells, at least, SFV replicated to the same extent whether or not VSV was replicating at the same time. Apparently, there is little or no competition between the two viruses during synthesis and maturation of progeny virions.

While similarities exist between facilitation of VSV by poxviruses in $DRK_3$ and RC-60 cells, both require early poxviral functions, a significant difference is also apparent. Replication of VSV alone in RC-60 cells reportedly is abortive; at multiplicities of 5–10 PFU/cell, viral CPE occurs, some viral RNA is synthesized, and small amounts of viral cores are detectable (15). In contrast, the present study indicates that $DRK_3$ cells are nonpermissive for VSV. At a comparable multiplicity of 20 PFU/cell, no virus-induced inhibition of cellular DNA synthesis occurred, and viral CPE did not develop. Thus, interaction between VSV and SFV in these cells appears to be unique. Since in permissive cells infected by VSV alone at these low input multiplicities induction of CPE seems to require primary transcription of the viral genome (12), the failure of VSV to cause CPE in $DRK_3$ cells suggests that viral replication in these cells, unlike RC-60 cells, is blocked before this initial synthetic event occurs. One very early step in VSV replication that may be blocked is viral adsorption. Since it is known that SFV alters the plasma membrane of infected $DRK_3$ cells (16), it is conceivable that SFV modifies the cell surface to provide receptors which then allow VSV to be adsorbed. This may explain why the presence of VSV does not affect focus formation by SFV in our differential assay of the two viruses. If VSV in a mixed inoculum is unable to attach to monolayers of $DRK_3$ cells during

the period for viral adsorption, addition of the overlay medium would likely prevent it from later infecting the foci of SFV-infected, permissive cells. Whether infection by SFV does in fact facilitate VSV adsorption to these cells, however, will require further investigation, especially since in their original study Padgett and Walker (1) concluded that VSV is adsorbed in the absence of SFV.

*Summary.* In serially cultured domestic rabbit kidney ($DRK_3$) cells, vesicular stomatitis virus (VSV) alone was unable to replicate, inhibit synthesis of cellular DNA, or induce CPE. When these cells were inoculated simultaneously with VSV plus Shope fibroma virus (SFV), both viruses replicated without apparent interference. This faciliation of VSV by SFV also occurred when these dually inoculated cells were treated with hydroxyurea at concentrations which inhibited production of infectious SFV by greater than 99% and synthesis of viral and cellular DNA with a buoyant density in cesium chloride of 1.69 g/cm$^3$ by greater than 90%. These results indicate that an early event in the replication of SFV, before viral DNA synthesis, converts $DRK_3$ cells from a nonpermissive state, in which VSV alone is not even able to induce CPE, to a permissive state that allows complete VSV replication.

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