Temperature-Sensitive Host Range Mutants of Herpes Simplex Virus Type 2

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Herpesviruses are capable of several types of infection of a host cell. To investigate the early events which ultimately determine the nature of the virus-host cell interaction, a system was established utilizing temperature-sensitive mutants of herpes simplex virus type 2. Four mutants have been isolated which fail to induce cytopathic effects and do not replicate at 39°C in hamster embryo fibroblast cells. At least one mutant is virus DNA negative. Since intracellular complementation is detectable between pairs of mutants, a virus function is known to be temperature sensitive. However, all four mutants induce cytopathic effects and replicate to parental virus levels in rabbit kidney cells at 39°C. This suggests that a host cell function, lacking or nonfunctional in HEF cells but present in rabbit kidney cells at 39°C, is required for the replication of these mutants in hamster embryo fibroblast cells at 39°C. Therefore, we conclude that these mutants are both temperature sensitive and exhibit host range properties.

A characteristic of herpes simplex virus infection of a host cell is the diversity of relationships which may be established within that cell. The most common effect is the production of the cytopathic cycle which has been described by many investigators for many cell systems (7, 24). Virus latency has long been known to be a property of the herperviruses (13). This property of herpes simplex virus type 1 (HSV-1) has recently been characterized in both animal (3, 27-29) and human (1, 2) systems. Recurrent infections due to herpes simplex virus type 2 (HSV-2) (18) are well known. A recent report by Walz et al. (31) has given further evidence of such HSV-2 latency in experimental systems. The scope of herpesvirus latency has recently been reviewed (22).

The suspected transforming and oncogenic potential of the herpesviruses, with all its important implications, has been demonstrated in many laboratories (In E. Kurstak and K. Maromorosch (ed.), Viruses, evolution, and cancer, in press). Although these three manifestations of transformation, latency, and cytopathic infection by herpes simplex viruses are under investigation, little is known of the control mechanisms regulating events leading to different interactions. To delineate such control mechanisms would greatly facilitate research efforts into virus latency and cell transformation. Since both virus latency and cell transformation require a more subtle interaction than the complete destruction of an infected cell, we sought to establish a virus-cell system utilizing mutants of HSV-2 which did not induce cytopathology under nonpermissive conditions.

In this report we describe the isolation and preliminary characterization of four mutants of HSV-2 strain 333 which are temperature sensitive in hamster embryo fibroblast (HEF) cells but show a surprising host range property.

MATERIALS AND METHODS

Cell cultures. HEF cells were prepared from 12-day-old LSH strain (Lakeview Hamster Colony) hamster embryos. The cells were grown at 37°C in 8-ounce (0.473 liter) glass prescription bottles in medium 199 supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, and 0.075% sodium bicarbonate. One hundred units of penicillin per milliliter and 100 μg of streptomycin/ml were used in all cell culture media. At confluency, cell monolayers were trypsinized and reseeded into plastic microwell trays (Linbro Diaposo Trays, Linbro Chemical Co., New Haven, Conn.) and the growth medium containing increased sodium bicarbonate (0.23%) was replaced. These cultures were grown to confluency at 37°C in a 5% CO₂ atmosphere.

Primary rabbit kidney (RK) cell cultures were prepared from the kidneys of 3-week-old New Zealand white rabbits. These cells were similarly grown at 37°C in 8-ounce (0.473 liter) glass prescription bottles and passaged at confluency into plastic microwell trays. Confluent monolayers in plastic dishes (Falcon...
Plastics) (60 by 15 mm) were used for infectious virus assay described below. All RK cells were grown in Eagle minimal essential medium supplemented with 10% calf serum, 0.075% or 0.23% sodium bicarbonate, and antibiotics. After virus inoculation, all cultures were maintained in Eagle medium supplemented with 2% fetal calf serum, 0.075% sodium bicarbonate, and antibiotics.

**Virus.** HSV-2 strain 333 was used as the parental virus from which mutants were derived, and is referred to as the parental virus. This strain was originally obtained from William Rawls (Baylor College of Medicine, Houston, Tex.) and had been passaged in our laboratory four times in human embryo kidney cells. A fifth passage was harvested, quick frozen, and stored at -70°C for these studies.

**Inoculation of cells.** Confluent monolayers consisting of 10⁴ to 2 x 10⁵ cells per microwell were drained of medium and exposed to 0.1 ml of virus. For complementation studies, 0.05 ml of each two mutants was inoculated. Multiplicity of infection for all experiments was 2 PFU per cell. After adsorption at room temperature for 1 h, cultures were washed twice with 1.0-ml volumes of 0.025 M Tris-buffered saline (pH 7.4). One milliliter of Eagle medium containing 2% fetal calf serum, 0.23% sodium bicarbonate, and antibiotics was added to each microwell. Microwell trays were incubated at either 33, 37, or 39°C in a 5% CO₂ atmosphere. The time of virus inoculation was regarded as zero time for all experiments.

**Plaque assay.** Cultures were frozen and thawed, replicate cultures were pooled, sonically treated for 60 s each in a Branson Sonifier (Cole-Palmer Instrument and Equipment Co.), and cell debris was removed by centrifugation at 200 x g. Serial 10-fold dilutions of each supernatant were made in Tris-buffered saline (pH 7.4) and 0.1 ml of each dilution of culture material was inoculated into two plastic petri dishes (60 by 15 mm) containing confluent monolayers of RK cells. After 1 h of adsorption at room temperature all dishes were overlayed with 0.5% methylcellulose (Matheson, Coleman, and Bell) in Eagle medium supplemented with 5% fetal calf serum, 0.23% sodium bicarbonate, and antibiotics. Infectious assays were incubated at 33°C in 5% CO₂ for 4 days, neutral red (diluted 1:7,500 in Tris-buffered saline) was added for 3 h at 37°C, and plaques were counted.

**Mutagenesis by nitrosoouaridine (NTC).** Parental virus (3.0 x 10⁴ PFU) in 0.1 ml was added to 0.9 ml of 50 μg/ml of N-methyl-N-nitro-N-NTG (K and K Laboratories, Plainview, N.Y.) in medium. At 0.5, 1, 3, 5, 10, 20, and 60 min, a 0.1-ml sample was removed from the incubation mixture and triturated directly in RK cells at 37°C as described above. At 4 days, plaques were counted. Progeny from plaques of 1 mm in diameter or less in size were picked and plaque purified at 37°C an additional two times.

**Mutagenesis by UV irradiation.** Parental virus was diluted 1:4 in Tris-buffered saline and the virus suspension was sonically treated for 30 s to disrupt virus aggregates. One milliliter of this virus suspension was placed into each of seven sterile plastic dishes (60 by 15 mm) and exposed to 4,000 ergs/s per cm² of UV irradiation. At 15-, 20-, 30-, 45-, 60-, 90-, and 120-s exposure, one dish was removed and the contents were titrated in RK cells at 37°C. Plaques of 1 mm diameter or less were picked and purified at 37°C an additional two times.

**Mutagenesis by BUdR.** Concentrations of 5, 10, and 20 μg of 5-bromodeoxyuridine (BUdR)/ml were prepared in Eagle medium containing 10% dialyzed fetal calf serum, 0.23% sodium bicarbonate, and antibiotics. Parental virus was inoculated, at a multiplicity of 3 PFU/cell, onto RK cells in dishes (60 by 15 mm) and allowed to adsorb at room temperature for 1 h. After adsorption, 5.0 ml of medium containing BUdR was added to each culture and all were incubated at 37°C in 5% CO₂ atmosphere. All cultures were harvested at 24 h at which time virus cytopathic effect (CPE) was complete at all drug concentrations. Cultures were quick frozen and thawed, sonically treated for 45 s, and cell debris was removed by low-speed centrifugation. Supernatants were held at -70°C as mutagenized stocks.

**DNA assay.** Confluent monolayers of HFF cells in 1-ounce (ca. 0.0591 liter) bottles (∼5.0 x 10⁴ total cells) were inoculated, virus was allowed to adsorb, and 2.0 ml of maintenance medium per bottle was added. At this time, 10 μCi of 3'-methyl-tritium thiadmine (specific activity 14.1 Ci per mmol; Schwarz Mann Co.) in 0.1-ml volume was added. Cultures were incubated at 33 and 39°C for an additional 23 h, at which time CPE was observed, and all labeled cultures were harvested by freezing at -4°C. Cultures were thawed, and lysates were combined at 37°C with 0.1% Sarkosyl NL 30 (Geigy Chemical Corp.), 0.02 M ethylenediaminetetraacetic acid, and 0.1% heat-inactivated Pronase (Calbiochem, B grade). Pronase and Sarkosyl were prepared in 0.1 × SSC (0.1 × SSC: 0.015 M sodium chloride and 0.0015 M sodium citrate, pH 7.3). The digestion mixture was allowed to incubate at 37°C for a minimum of 4 h, after which 0.2 ml of each sample was mixed with 3.8 ml of cesium chloride in 0.1 × SSC (density equals 1.745 g/cc). These were centrifuged at 30,000 rpm for 60 h at 20°C in a Beckman L2-65B ultracentrifuge with a 40.3 fixed-angle rotor. Eight drop fractions were collected by bottom puncture of centrifuge tubes onto Whatman filter paper disks. Acid-insoluble material was precipitated by three washes with 5% trichloroacetic acid at room temperature. Filters were dehydrated by one washing with 95% ethanol and one washing with acetone. After drying, filters were placed in 10 ml of toluene containing 0.25% Omnifluor (New England Nuclear Corp.) and counted in a Beckman LS-250 liquid scintillation counter. Every tenth fraction was collected for density determination utilizing a Bausch and Lomb refractometer.

**RESULTS**

Selection by plaque characteristic. The parental strain of HSV-2 333 was known to produce in RK cells a heterogeneous mixture of plaque sizes ranging in diameter from 1 to 3 mm, large plaques (≥ 2 mm) being the predominant type. After treatment of parental viruses...
with mutagens, an increase in the number of small plaques (<1 mm) was observed. Although this was not quantitated at the time, it occurred regardless of the mutagen used. This finding is consistent with other reports indicating that various mutagens can induce an increased number of small plaques within the mutagenized virus population (5, 14, 25, 30).

To obtain a readily detectable genetic marker, only those plaques ≤1 mm in diameter were picked and purified by further titration and selection in RK cells. Figure 1 demonstrates this difference in plaque size between predominantly large plaque-forming parental virus and homogeneous small plaque-forming mutants. The stability of small plaque size has been reported for nonmutagenized isolates of HSV-1 (20) and HSV-2 (16).

**Isolation of mutants.** In three separate experiments the parental virus was subjected to treatment with one form of mutagenic agent: UV irradiation, BUdR, or NTG. After mutagenesis, the virus was titrated by 10-fold serial dilution as described in Materials and Methods, and small plaques (<1 mm) were picked, and then suspended in 1.0 ml of Eagle growth medium. There was a greater than 1,000-fold decrease in infectious virus detectable after 120 s of UV irradiation (Fig. 2). Mutant 69 was derived from a single plaque picked from the titration in RK cells of parental virus exposed to 90 s of UV irradiation. This dosage represents a 0.1% survival rate. Figure 3 indicates that at concentrations of 5 to 20 μg per ml of BUdR, infectivity of parental virus 333 is inhibited by 100-fold. Mutant 74 was obtained from a single plaque formed upon titration in RK cells of parental virus 333 previously replicated in the

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**Fig. 1.** Plaques in RK cells of parental HSV-2 strain 333 and mutants.

**Fig. 2.** Inactivation of parental HSV-2 strain 333 after exposure to UV irradiation.

**Fig. 3.** Inactivation of parental HSV-2 strain 333 by replication in the presence of 5'-BUdR.
presence of 20 μg of BUdR per ml of nutrient medium.

Exposure of parental virus to NTG for 60 min resulted in only a four-fold decrease in initial virus titer. It was at this point that mutant 46 was isolated from a single plaque. Mutant 41 was isolated similarly from a single plaque picked from the titration in RK cells of parental virus 333 exposed to 10 min of NTG. Since the parental virus was not plaque purified before mutagenesis, the possibility that a spontaneously arising variant has been selected cannot be ruled out.

All plaques were purified by repeated titration and selection. Small plaque-purified isolates were then screened at 33 and 39 C in RK and HEF cells for their ability to induce CPE at the permissive but not at the nonpermissive temperature. Parental virus will induce complete CPE at both temperatures with equal efficiency.

**Cytopathology and infectivity in HEF cells.** Stocks of four mutants which yielded no CPE at 39 C in HEF cells were grown in RK cells at the permissive temperature and stored in aliquots at -70 C. Table 1 demonstrates that these small plaque mutants induce complete CPE by 48 h in HEF cells at 33 C. This cytopathology correlates with a 1,000 to 10,000-fold increase in infectious virus between 4 and 24 h (Fig. 4 and 5), and is similar for all mutants tested.

Conversely, at 39 C in HEF cells, only the parental virus produces complete cytopathology by 24 h representing a 1,000-fold increase in infectious virus from the 4-h point (Fig. 4 and 5). Although there are slight amounts of leakiness, no mutant has progressed further than ±CPE at 24 h or 1+ at 48 h. Less subjective are the actual titers representing infectious virus which demonstrate only a decrease in residual input virus. Table 1 further indicates that these mutants are capable of inducing complete CPE but are less efficient in replicating at 37 than at 33 C.

**DNA results.** Mutant infected HEF cultures were labeled with 3'-methyl tritiated-thymidine for 24 h at which time cells were harvested and cell lysates were analyzed by isopycnic banding in cesium chloride gradients. By this method, virus DNA and host cell DNA can be separated based on their characteristic densities. Figure 6 demonstrates that HEF cells inoculated with parental virus 333 synthesize virus DNA at both 33 and 39 C. However, mutant 74-inoculated HEF cells show little or no virus DNA formation at 39 C. Interestingly, at 33 C, mutant 74 appears to depress host cell DNA synthesis (41%) but this characteristic is less marked at 39 C (7%).

Mutant 69 (Fig. 7) induces only small amounts of virus DNA in HEF cells at 33 C over a 24-h continuous label period. Moreover, some depression of host cell DNA synthesis is evident (21%). At 39 C, little or no virus DNA is made and there is a suggestion that host cell DNA synthesis is stimulated. Initial experiments also indicate that both mutants 46 and 41 (Fig. 7) are capable of inducing reduced amounts of virus DNA in HEF cells at 39 C and depression of host cell DNA synthesis occurs at both 33 and 39 C.

**Cytopathology and infectivity of doubly infected HEF cells.** To determine if the four noncytopathic mutants derived from parental virus 333 were mutant in different cistrons, complementation studies were performed. HEF cells were mixedly infected with pairs of mutants, the virus was allowed to adsorb for 1 h at room temperature, the cell sheets were washed twice with Tris-buffered saline, maintenance medium was added, and the cultures were incubated at 33 or 39 C. In all complementation experiments, singly infected HEF cells were included for calculation of complementation levels.

Two parameters were utilized for the determination of complementation: (i) formation of cytopathology in mixedly infected cells and (ii)

<table>
<thead>
<tr>
<th>Virus</th>
<th>33 C</th>
<th>37 C</th>
<th>39 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum CPE</td>
<td>Maximum titer</td>
<td>Maximum CPE</td>
</tr>
<tr>
<td>Parental 333</td>
<td>4+</td>
<td>1.0 x 10^4</td>
<td>4+</td>
</tr>
<tr>
<td>Mutant 69</td>
<td>4+</td>
<td>1.2 x 10^4</td>
<td>4+</td>
</tr>
<tr>
<td>Mutant 74</td>
<td>4+</td>
<td>2.3 x 10^4</td>
<td>4+</td>
</tr>
<tr>
<td>Mutant 46</td>
<td>4+</td>
<td>6.0 x 10^4</td>
<td>4+</td>
</tr>
<tr>
<td>Mutant 41</td>
<td>4+</td>
<td>5.1 x 10^4</td>
<td>4+</td>
</tr>
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</table>

*4+, 100% cytopathology; ±, questionable cytopathology.
* Maximum titers in PFU per milliliter (checked at 24 and 48 h post-inoculation).
Fig. 4. Replication of parental HSV-2 strain 333 in HEF cells.

Fig. 5. Replication of mutant 69 in HEF cells.

Fig. 6. DNA profiles of uninfected HEF cells and of cells infected with parental HSV-2 strain 333 and mutant 74.
replication of viruses mixedly infecting HEF cells at 39°C as compared to singly infected cells. To quantitate this latter parameter, the method of Burge and Pfefferkorn (4) was used. In this method, the 24-h infectious virus yields were used. Complementation levels were derived by dividing infectious virus yields of mixedly infected cells by the sum of the yields of each mutant under identical conditions in singly infected cells. A complementation level greater than one indicates complementation. However, as suggested by Burge and Pfefferkorn (4), we considered only those values greater than two as indicative of complementation. Noncomplementing pairs of mutants produce complementation levels of one or less. Values less than one may indicate interference of one mutant with the replication of the other. This means of determining complementation values for temperature-sensitive mutants of HSV-1 has also recently been used by Schaffer et al. (26).

By this method, two complementation groups, designated A and B, have been defined (Table 2). The combination of mutant 74 with any other mutant induced complete cytopathology, and replication approaching parental virus yields under nonpermissive conditions. Any two combinations of mutants 41, 46, and 69 did not induce cytopathology nor replication under nonpermissive conditions. In addition to demonstrating that at least two different mutant genotypes exist, intracellular complementation also indicates that these mutants are capable of adsorbing, penetrating, and uncoating within the HEF cell, thereby making available virus genetic information for interaction with the host genome.

Table 2. Complementation of HSV-2 mutants at 39°C in HEF cells

<table>
<thead>
<tr>
<th>Mutant combination</th>
<th>Complementation level</th>
<th>Complementation group</th>
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</thead>
<tbody>
<tr>
<td>74 × 69</td>
<td>6.25</td>
<td>A 74</td>
</tr>
<tr>
<td>74 × 46</td>
<td>19.23</td>
<td>A 69</td>
</tr>
<tr>
<td>74 × 41</td>
<td>2.31</td>
<td>B 46</td>
</tr>
<tr>
<td>46 × 41</td>
<td>0.69</td>
<td>B 41</td>
</tr>
<tr>
<td>46 × 69</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>69 × 41</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7. DNA profiles of uninfected HEF cells and of cells infected with HSV-2 mutants 69, 46, and 41.
Regulation of replication by host cell type.

To this point, the mutants described have been considered as to their virus temperature-sensitive nature in HEF cells; the permissive temperature was 33°C and the nonpermissive temperature 39°C. It has been demonstrated that selected pairs of mutants are capable of complementation in mixedly infected HEF cells at 39°C, further supporting the conclusion that a temperature-sensitive virus function is involved.

The first suggestion that cell type is a determinant of permissiveness was observed when small plaque-purified isolates were screened at 33 and 39°C in RK and HEF cells for their ability to induce cytopathology at 39°C. It was noted that while little or no cytopathology occurred in HEF cells, complete cytopathology eventually occurred in RK cells at 39°C. In returning to further investigate this unusual behavior of the temperature-sensitive mutants, it was found that mutants of parental virus 333, in addition to inducing complete cytopathology, could also replicate in RK cells to levels approaching that of parental virus under identical conditions (Table 3). This implies a host regulatory mechanism as well as a temperature-sensitive virus function is involved.

**DISCUSSION**

The purpose of this study was to establish a system to study interactions between herpes simplex virus and host cells. The approach taken was to isolate conditional lethal mutants of HSV-2 defective, under nonpermissive conditions, in their ability to induce cytopathology.

Before the development of this mutant virus system, studies on herpes virus latency in cell culture have been confined to observation of infected cultures to which the DNA inhibitor cytosine arabinoside has been added (12, 19). Preliminary studies on virus latency have been attempted by infecting HEF cells with noncytopathic mutants and maintaining cultures at 39°C. Cultures have been maintained as long as 6 weeks post-inoculation. When cultures were shifted down to permissive temperature (33°C), complete cytopathology was observed. The recovered virus had mutant properties (Koment and Rapp, unpublished data) and may therefore be useful in the study of virus latency in cell culture.

The unique observation that the mutants replicated in RK cells at 39°C indicates that there may be a specific host component required for their replication. W. Munyon (personal communication) has made a similar observation with mutants of HSV-2. The extent of the host range property is under investigation.

The ability of herpes simplex viruses to induce heritable changes within cell populations has been demonstrated in several laboratories (6, 8, 9, 15, 17, 21, 23). This has been made possible in most cases by lethal treatment of virus by physical (UV irradiation) or chemical (neutral red) means before inoculation of cells to circumvent the deleterious effects of cytopathology. Few transformation events have been reported using intact virus. Darai and Munk (6) exposed human embryo lung cells to HSV-2, shifted cultures to 42°C and recovered transformants. Garfinkle and McAuslan (10, 11) “transformed” cells carrying Rous sarcoma virus with HSV type 1 or 2 and observed the expression of some virus functions. Utilizing mutants described in this study, transformation studies in HEF cells at 39°C could conceivably identify a high frequency transforming mutant if the temperature-sensitive block occurred after transcription and translation of a “transforming” cistron (5). A variety of different mutants, such as those isolated, could be valuable in defining the virus information required for a given transformational event.

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**LITERATURE CITED**


