Further Studies of the Physical and Metabolic Properties of Foot-and-Mouth Disease Virus Temperature-Sensitive Mutants

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Three temperature-sensitive (ts) mutants of foot-and-mouth disease virus were classified as ribonucleic acid negative and as belonging to the same complementation group when measured by virus yields and [3H]uridine incorporation in paired, mixed infections at the nonpermissive temperature (38.5 C). Mutant ts-22, the only mutant able to produce plaques at 38.5 C, was more sensitive to acid than were the parental wild-type or other mutant viruses. Diethylaminoethyl-dextran did not enhance the plaque-forming ability of the mutant viruses at 38.5 C. All of the viruses inhibited host cell protein synthesis at both permissive (33 C) and nonpermissive (38.5 C) temperatures.

Temperature-sensitive (ts) mutants of animal viruses are used in the detailed analysis of viral genomes and provide a basis for formulating attenuated live-virus vaccines (4, 14). In a number of reports, the production and attenuation of FMD virus mutants, including genetic complementation and recombination studies, are described (5, 7, 8).

Recently, three ts mutants of type A24 FMD virus were isolated and characterized by their growth in tissue culture and several animal species (12). Mutant ts-22 was considered "leaky" because of its limited growth at the nonpermissive temperature (38.5 C), whereas ts-24 and ts-42 were unable to produce plaques under agar at this temperature. Both ts-22 and ts-24 had greatly reduced pathogenicity for infant mice, although ts-42 retained its virulence. These observations suggested that the cistrons governing mouse pathogenicity and growth of the virus at elevated temperatures are different.

The experiments reported here were designed to determine other phenotypes expressed by these mutants and to gain a better understanding of the FMD viral genome.

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MATERIALS AND METHODS

Tissue cultures. Primary bovine kidney (BK') monolayer cultures were prepared by the Research Services section of this Center and propagated in Hanks balanced salts solution supplemented with 0.5% lactalbumin hydrolysate (HLH). Growth medium contained 6% bovine serum and maintenance medium contained 2%. We prepared secondary cultures (BK') in 16- by 150-mm test tubes by seeding with 1 ml of trypinized BK' cells (3 × 10⁶ cells/ml of HLH growth medium). The cultures, maintained at 37 C, were used 2 to 3 days after seeding; the medium was changed daily. Unless stated otherwise, cultures were washed with HLH medium before infection.

Viruses. Type A, subtype 24, FMD virus (wild type [w]) was mutagenized with 5-fluorouracil as described previously (12). Mutants were initially selected that produced plaques well at 33 C (permissive temperature) but poorly, or not at all, at 38.5 C (nonpermissive temperature). We prepared stock viruses by infecting BK' monolayers grown in Fovitsky bottles, incubating at 37 C for 1.5 h, and overlaying with HLH medium containing 80 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) and 2% bovine serum. When the cytopathic effect was maximum after incubation at 33 C for 22 to 26 h, the infectious culture fluid was clarified by centrifugation and dispensed into 2-ml ampoules, frozen, and stored at −56 C. Viruses were also concentrated from the clarified supernatants by two cycles of precipitation with 8% polyethylene glycol (17) and frozen.

Virus studies. Infectivity of samples was determined by plaque assays under agar overlay (2) at various temperatures in BK' cultures grown in 4-oz. (ca. 0.12-liter) prescription bottles. The cultures were generally incubated for 2 days at 38.5 C and for 3 days at 33 C to allow 2- to 4-mm plaques to develop. Diethylaminoethyl (DEAE)-dextran (1 mg/ml final concentration) was included in the agar overlay for some experiments (9). Air incubators were used to incubate cultures at 33 C and during virus adsorp-
tion (37 C); all other temperatures were maintained by immersing the culture vessels in the appropriate water baths.

When BK2 cultures were used, the cells were washed with 0.16 M Tris buffer, drained, and infected at an input multiplicity of 10 plaque-forming units (PFU)/cell. In complementation experiments, tube cultures were doubly infected with pairs of mutants premixed to give a multiplicity of infection of 10. The cultures were incubated at 37 C for 15 min, and the cells were then treated with pH 5.7 acetate buffer for 1 min (16) to remove residual virus. Maintenance medium was added after multiple washings of the cultures with Tris buffer, and the cultures were incubated at 38.5 C for 6 h. At the time of harvest, sodium dodecyl sulfate (0.5% final concentration) was added to each culture to lyse the cells, and the tubes were frozen at -56 C.

We determined the comparative sensitivity of the mutant and parental viruses to acid pH by diluting the viruses in appropriate phosphate buffers as described elsewhere (7).

Isotope incorporation. Viral ribonucleic acid (RNA) synthesis was measured by adding 1 ml of maintenance medium containing 1 μCi of [3H]-uridine and 5 μg of actinomycin D 15 min after infection. We harvested cultures at hourly intervals by treating them with sodium dodecyl sulfate and adding cold 5% trichloroacetic acid to precipitate the RNA. The precipitates were collected on 25-mm nitrocellulose disks, washed repeatedly with trichloroacetic acid, and transferred to liquid scintillation counting vials. One milliliter of 1 M NH4OH and 10 ml of counting fluid were added, and the samples were counted in a Beckman model L335 scintillation counter.

Cell protein synthesis was measured by pulsing for 20 min with 2 μCi of tritiated reconstituted protein hydrolysate in 0.25 ml of a Tris-buffered Hanks solution containing 0.1% glucose (10). After adding 0.5% sodium dodecyl sulfate and 10 ml of 5% trichloroacetic acid, the precipitate was heated for 15 min at 90 C, cooled, collected on nitrocellulose filters, and counted.

RESULTS

Comparative growth cycles at 33 and 38.5 C. Single-cycle replication of wt and ts mutants in BK2 cells after high multiplicity of infection was studied. None of the mutant viruses showed an increase in titer at 38.5 C during the 6 h after infection with 10 PFU/cell (Fig. 1). The wt virus reached maximum titer in 3 h at the nonpermissive temperature (38.5 C); growth of all viruses was slower at the permissive temperature (33 C).

Plaque production at various temperatures. BK1 monolayers were infected with 50 PFU and incubated at various temperatures. With this inoculum, none of the mutant viruses produced plaques at 38.5 C. At 37.5 C, ts-22, ts-24, and ts-42 produced 10, 20, and 30%, respectively, the number of plaques observed with the same inoculum when the cultures were incubated at 33 C. Mutant ts-22 can produce some plaques at 38.5 C (12) after inoculation with large quantities of virus. Therefore, a wide range of virus dilutions was used to ascertain the cut-off temperatures for these viruses. Data in Table 1 indicate that the three mutants are inhibited in their plaque-producing abilities at temperatures above 37 C. Although the number of plaques formed by mutant ts-22 was inhibited 95% at 37.5 C, it was the only mutant able to produce plaques at 38.5 C.

Effect of DEAE-dextran on plaque development. Polyanions found in agar may inhibit the development of plaques by some viruses. DEAE-dextran added to this overlay apparently blocks this inhibition (9, 13). To determine whether plaque production by the ts mutants was affected by such inhibitors, we infected BK1 monolayer cultures as follows: cultures incubated at 33 C were infected with 20 PFU; cultures incubated at 38.5 C were infected with 20 PFU of wt or 2 × 104 PFU of each mutant. After 1-h adsorption at 37 C, the cultures were overlaided with agar or agar containing 1 mg of DEAE-dextran per ml and incubated at 33 or 38.5 C. The results of one such experiment are presented in Table 2. At 33 C no differences in numbers of plaques were observed with the two overlays. All of the plaques were 2 to 4 mm in diameter. At 38.5 C the wt virus produced a similar number of slightly larger (3- to 4-mm) plaques. The only mutant that produced plaques

![Fig. 1. Comparative growth of FMD A14 wt virus and three ts mutants in secondary BK tube cultures at 33 C (●) and 38.5 C (○).](http://iai.asm.org/)

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Infections. These mutants are apparently in the same complementation group and can be considered to be defective in similar functions.

In the second procedure, viral RNA synthesis at 38.5°C was measured by [3H]uridine incorporation in the presence of actinomycin D at 1 and 6 h postinfection. A comparison of the net incorporation in mixed infections (Table 5) indicates no restoration of *wt* RNA synthesizing capabilities.

**Inhibition of host cell protein synthesis.** To determine the effect of mutant infection on BK<sup>+</sup> protein synthesis, the infected cells were pulse-labeled with tritiated protein hydrolysates. All viruses tested inhibited protein synthesis to a similar extent at both the permissive and nonpermissive temperatures (Table 6).

**DISCUSSION**

The isolation and characterization of mutants provide one means of developing information related to the genomic organization of viruses.
Establishing linkage groups, developing genetic "maps," and using such information to select specific recombinants all are useful applications for these data. Moreover, certain virus-host cell interactions can be explored when viruses containing specific markers are used.

FMD viruses are sensitive to acid pH (1). Mutants are considered significantly acid labile when their survival is less than 10% of that of the parental virus (7). By this criterion, ts-22 is acid labile at pH 6.8 (Table 3). Mutant ts-42 appears to be more resistant than the parental virus to acid conditions. Pringle (11) has suggested that resistance to low pH and high thermal conditions may be linked, but other authors (7) have questioned this relationship.

Certain virus mutants are apparently affected by the sulfated polyanions contained in agar used to prepare the solid-matrix overlay (6, 15). Wallis and Melnick feel that either the virus is bound to this agar-contained inhibitor, forming a noninfectious complex, or that the sulfated polymers enter the culture cell and interfere directly with virus replication (18). Adding polycations such as DEAE-dextran to the agar overlay allows multiplication of viruses that previously were apparently inhibited by these agar factors (9, 13). DEAE-dextran was used in the present experiments to confirm that these conditional lethal mutants were indeed inhibited in their replication at 38.5 C by virtue of the elevated temperature and not of an increased sensitivity to these agar-contained polyanions. Plaque development was not enhanced at either 33 or 38.5 C (Table 2); in fact, DEAE-dextran may have inhibited wt virus replication somewhat at 38.5 C.

Because free virus and cell-bound virus are inactivated by pH 5.7 treatment (16), the growth curves observed after infecting BK' cells with 10 PFU/cell (Fig. 1) reflect events only associated with intracellular virus. Although none of the mutants showed an increase in titer during incubation for 6 h at 38.5 C, these viruses remained viable after entering the cells.

Indeed, one of the early events after FMD virus infection of cells is the inhibition of host cell protein synthesis before virus replication (10). Each of the mutants behaved like the wt virus in this respect (Table 6); that is, the mutants were able to infect the cells and initiate these events necessary to shut down the further production of cellular proteins at both 33 and 38.5 C growth temperatures.

Mutant ts-22 was reported to replicate to a limited extent during long-term growth in primary BK cultures at 38.5 C and was therefore considered "leaky" (12). "Leaky" viruses generally multiply better under liquid medium than under solid overlay (7). Although ts-22 was more inhibited in its ability to produce plaques at 37.5 C than ts-24 or ts-42 (Table 1), ts-22 was the only mutant that produced even very small, irregular plaques at 38.5 C. To produce plaques at 38.5 C, 10²-fold more infectious particles were needed to infect BK cells than were required at 33 C. Thus, with some combinations of host cell, multiplicity of infection, and overlay, ts-22 can exhibit limited growth at the nonpermissive temperature.

The mutants were unable to complement

**Table 4. Complementation measured by virus yields at 38.5 C after mixed infections**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Single</th>
<th>Mixed with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ts-22</td>
</tr>
<tr>
<td>ts-22</td>
<td>$0.5 \times 10^7$</td>
<td>$2.3 \times 10^4$</td>
</tr>
<tr>
<td>ts-24</td>
<td>$6.6 \times 10^4$</td>
<td>$2.3 \times 10^4$</td>
</tr>
<tr>
<td>ts-42</td>
<td>$8.5 \times 10^6$</td>
<td>$1.239$</td>
</tr>
</tbody>
</table>

* a Complementation level = (virus yield, mixed infection at 38.5 C)/(sum of single infection yields at 38.5 C) (3).

**Table 5. Complementation measured by net $[^3H]$uridine incorporation after mixed infections at 38.5 C**

<table>
<thead>
<tr>
<th>Virus</th>
<th>$\Delta$ Counts/min*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ts-22</td>
<td>1,445</td>
</tr>
<tr>
<td>ts-24</td>
<td>1,144</td>
</tr>
<tr>
<td>ts-42</td>
<td>1,239</td>
</tr>
<tr>
<td>wt</td>
<td>12,290</td>
</tr>
</tbody>
</table>

* $\Delta$ Counts/min = counts/min at 6 h - counts/min at 1 h.

**Table 6. Inhibition of host-cell protein synthesis by FMD viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>33 C</th>
<th>38.5 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts/min</td>
<td>% Inhibition</td>
</tr>
<tr>
<td>ts-22</td>
<td>636</td>
<td>73.8</td>
</tr>
<tr>
<td>ts-24</td>
<td>729</td>
<td>70.0</td>
</tr>
<tr>
<td>ts-42</td>
<td>573</td>
<td>72.3</td>
</tr>
<tr>
<td>wt</td>
<td>896</td>
<td>63.1</td>
</tr>
<tr>
<td>Uninfected</td>
<td>2,424</td>
<td>2,326</td>
</tr>
</tbody>
</table>

- Adding polycations such as DEAE-dextran to the agar overlay allows multiplication of viruses that previously were apparently inhibited by these agar factors (9, 13). DEAE-dextran was used in the present experiments to confirm that these conditional lethal mutants were indeed inhibited in their replication at 38.5 C by virtue of the elevated temperature and not of an increased sensitivity to these agar-contained polyanions. Plaque development was not enhanced at either 33 or 38.5 C (Table 2); in fact, DEAE-dextran may have inhibited wt virus replication somewhat at 38.5 C.
- Because free virus and cell-bound virus are inactivated by pH 5.7 treatment (16), the growth curves observed after infecting BK' cells with 10 PFU/cell (Fig. 1) reflect events only associated with intracellular virus. Although none of the mutants showed an increase in titer during incubation for 6 h at 38.5 C, these viruses remained viable after entering the cells.
- Indeed, one of the early events after FMD virus infection of cells is the inhibition of host cell protein synthesis before virus replication (10). Each of the mutants behaved like the wt virus in this respect (Table 6); that is, the mutants were able to infect the cells and initiate these events necessary to shut down the further production of cellular proteins at both 33 and 38.5 C growth temperatures.
- Mutant ts-22 was reported to replicate to a limited extent during long-term growth in primary BK cultures at 38.5 C and was therefore considered "leaky" (12). "Leaky" viruses generally multiply better under liquid medium than under solid overlay (7). Although ts-22 was more inhibited in its ability to produce plaques at 37.5 C than ts-24 or ts-42 (Table 1), ts-22 was the only mutant that produced even very small, irregular plaques at 38.5 C. To produce plaques at 38.5 C, 10²-fold more infectious particles were needed to infect BK cells than were required at 33 C. Thus, with some combinations of host cell, multiplicity of infection, and overlay, ts-22 can exhibit limited growth at the nonpermissive temperature.
- The mutants were unable to complement
each other in mixed infections at 38.5 C as measured by virus yield (Table 4) or uridine incorporation (Table 5) and can therefore be considered defective in the same function (3, 14). This assay for RNA synthesis in mixed infections at the nonpermissive temperature may prove to be a more critical and simpler method to screen for complementation between RNA- mutants. Mutant ts-24 may contain defects both in the genes controlling mouse pathogenicity and in those related to ability to replicate at 38.5 C (12). Thus, several apparently diverse functional events may be controlled by genes within the same complementation group.

These ts mutants were initially selected because of their reduced ability to replicate at 38.5 C. This reduced ability reflects a deficiency in at least one gene product necessary for development of infectious virus at the elevated temperature (12). This defect must be in a gene product that is normally active after host cell protein synthesis has shut down, but before the synthesis of infectious viral RNA. Efforts to identify the defective protein(s) are under way.

ACKNOWLEDGMENTS

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LITERATURE CITED


