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

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Two small-plaque mutants of herpes simplex virus type 2 (HSV-2) (strain 333), whose growth at 39 C was blocked in certain cell types (cell-dependent temperature sensitivity), were compared with the parental virus in a number of biological assays. One mutant (no. 69) was found to produce a large number of morphologically normal, but noninfectious, particles; under nonpermissive conditions, these mutant particles were able to interfere with the replication of wild-type HSV-2. The other mutant (no. 74), which is known to belong to a different complementation group, appeared to direct little virus DNA synthesis, even at the permissive temperature. Progeny production and virus DNA synthesis in cells infected by mutant 74 were delayed in comparison with wild-type virus-infected cells. Both mutants were found to be more sensitive to UV irradiation than the parental virus; this was especially marked in the case of mutant 74. Moreover, this mutant was found to have a high transforming efficiency at much lower doses of irradiation than those needed to abolish the cytopathic effect of wildtype HSV-2.

Herpes simplex viruses (HSV) are capable of a diversity of interactions with a wide range of host cells. The consequences of infection include cell death (2, 16, 35) with or without virus progeny production, virus persistence in a latent, nonreplicative form (8, 32, 38), and cell transformation with very little expression of virus-specified functions (10–13, 26, 30, 40). There is, as yet, little information about the nature of host- or virus-specified control mechanisms that determine the response to infection. Indeed, despite intensive study (16–18), many of the crucial steps in HSV replication remain unclear. A particularly profitable means of studying virus functions is the use of conditional, lethal, usually temperature-sensitive (ts) virus mutants. Such mutants have been successfully employed in the construction of the genetic map of HSV (5, 14, 36, 37, 39), and recently detailed examination of biological lesions responsible for the temperature-sensitive phenotype has begun in several laboratories (1, 3, 15). The products of infection and the ability of ts mutants to transform cells at the nonpermissive temperature have been areas of most extensive effort (26, 40).

Recently, Koment and Rapp (22–24) reported the isolation of a group of temperature-sensitive mutants with rather different properties from those described above. The temperature-sensitive phenotype of these mutants was only apparent in certain types of cell, conferring "host range" characteristics upon the group. Virus sensitivity to high (39 C) temperature was demonstrated in hamster and mouse fibroblast cultures but was much less apparent in epithelioid cells or human fibroblasts.

Believing that these mutants might provide a tool in the elucidation of virus-cell interaction, their behavior in a number of biological tests was compared with that of the parental virus. The work described in this communication is an extension of earlier studies on two of the temperature-sensitive host range mutants, carried out to determine more specifically the nature of the defect in virus production under nonpermissive conditions and to compare other biological properties of mutant and wild-type viruses.

Of particular interest was the transforming ability of the mutants. It has been established in many laboratories that, if the cytopathic effect of HSV is destroyed, the virus has the capacity to transform cells from hamsters, rats, mice, and humans (10–13, 26, 40). It was thought that mutants whose capacity for productive growth was genetically reduced might more readily reveal their transforming potential, and this view is supported by transformation by ts mutants under nonpermissive condi-

tions, which has been described from other laboratories (26, 40, 41).

A quantitative assay for biochemical transformation has recently been developed in this laboratory using a selection system for cells bearing virus-induced thymidine kinase (TK) (19–21), based on cell growth in HAT medium (25, 30) (Rapp and Buss, Intervirology, in press). This technique was used to determine whether the mutants had abnormal transforming ability and whether transforming potential could be correlated with any other biological property.

MATERIALS AND METHODS

Cells. Hamster embryo fibroblast (HEF) cells were prepared from 12-day-old LSH strain hamster embryos (Lakeview Hamster Colony, New Field, N.J.), as described previously (9), and were suspended in medium 199 supplemented with 0.225% sodium bicarbonate solution and 10% fetal calf serum plus 100 U of penicillin and 100 μg of streptomycin per ml (complete medium 199). Primary HEF cells were added to plastic petri dishes (60 mm), plastic tissue culture flasks (25 cm^2), or glass prescription bottles (16 ounce [ca. 0.473 liter]) as appropriate. The cells were used when confluent monolayers had formed.

Rabbit kidney (RK) cells were prepared from the kidneys of 21- to 28-day-old rabbits by the technique previously described (11) and were seeded into plastic petri dishes (60 mm) in Eagle medium plus sodium bicarbonate, antibiotics, and fetal calf serum at the concentrations used with medium 199 (complete Eagle medium).

Human embryonic lung (HEL) cells were obtained from Flow Laboratories, Rockville, Md., and were maintained in complete Dulbecco medium (Dulbecco medium plus 0.225% sodium bicarbonate, 10% fetal calf serum, and antibiotics, as described). Also maintained in complete Dulbecco medium were BSC-1 African green monkey kidney cells and 3T3-4E TK^- mouse cells. The latter were phenotypically thymidine kinaseless (TK^-) 3T3 cells obtained from Howard Green (Massachusetts Institute of Technology, Boston, Mass.).

Virus. A strain of HSV-2 designated 333, isolated from a genital lesion by W. Rawls (when at Baylor College of Medicine, Houston, Tex.) and passaged only in human cells, was the parental wild-type HSV-2 used in this study. Two mutants of HSV-2 strain 333, whose isolation and preliminary characterization have been reported recently (22–24), were examined. One mutant (no. 69) was derived from a single small plaque produced in RK cells by a preparation of wild-type virus that had been subjected to 3.6×10^4 ergs of UV irradiation per cm^2. The second mutant (no. 74) was obtained from a single small plaque on RK cells produced by an HSV-2 strain 333 preparation that had been grown in medium containing 20 μg of bromodeoxyuridine per ml as a mutagen.

Both mutants and the wild-type virus were plaque-purified four times prior to use. Virus stocks were prepared from monolayers of HEL cells that had been infected, using an input multiplicity of 0.5 PFU/cell, and were maintained at 33 C until extensive cytopathic effects became apparent (36 to 40 h postinfection). At this point, the infected cells were harvested by a single cycle of freeze-thawing and were disrupted by ultrasonication, and the lysate was centrifuged at $200 \times g$ for 10 min. The supernatant was transferred to small vials and stored at −70 C as stock virus.

Virus assay. Virus was titrated in RK cells by a plaque assay that has previously been described in detail (31). Briefly, confluent monolayers of RK cells in plastic petri dishes (60 mm) were inoculated with 0.1 ml of virus serially diluted in Tris buffer. After an adsorption period of 1 h at room temperature, 5 ml of complete Eagle medium containing 0.5% methylcellulose was added to each plate. Cultures were incubated for 4 days at 33 C in a moist atmosphere of 5% CO_2 in air, at the end of which time the cells were fixed in 5% formalin and were stained using 0.03% methylene blue solution, and virus plaques were counted with the aid of a dissecting microscope. Plaque diameter was measured with the aid of a Nikon profile projector (model 6C) at ×10 magnification; at least 200 plaques from not less than four plates were measured for each virus-cell system. Plaque "diameter" was always taken at the widest point in a horizontal direction.

Assay for DNA synthesis. The method used for DNA analysis was similar to that described earlier by Crouch and Rapp (6). Confluent monolayers of HEL cells in plastic bottles (25 cm^2) were treated for 48 h prior to the experiment with complete medium 199, which had a reduced concentration of fetal calf serum (1%) to reduce cell DNA synthesis. The cells were then infected at an input multiplicity of approximately 5 PFU/cell in 0.1 ml of Tris buffer. After adsorption, the monolayers were rinsed with Tris buffer, and 5 ml of complete medium 199 containing only 2% fetal calf serum was added to each bottle.

The incorporation of [*methyl*-^{3}H]thymidine during 8-h pulses was assayed at different times after infection. [*Methyl*-^{3}H]thymidine at a concentration of 10 μCi/ml (45 Ci/mmol) was added directly to the nutrient medium and, at the end of the labeling period, medium was decanted from the cells, which were dislodged from the bottle by freeze-thawing. Cells that had become detached from the monolayer due to virus cytopathic effects were rescued from the culture medium by a single low-speed centrifugation and were added back to the appropriate bottle. Harvested cells were digested for a minimum of 4 h at 37 C in a mixture of 0.6 ml of Pronase (10 mg/ml); 0.3 ml of Sarkosyl (10% solution), and 0.3 ml of EDTA (0.2 M, pH 8.1) in 0.015 M sodium chloride and 0.0015 M sodium citrate (0.1× SSC), pH 7.3.

Mock-infected cultures were also used to provide a ^{14}C-labeled DNA marker in cesium chloride gradients. Such cultures were treated by the procedure described above, except that [*methyl*-^{14}C]thymidine at a concentration of 0.1 μCi/ml (54 μCi/mmol) replaced [*methyl*-^{3}H]thymidine, and the cultures were

incubated for 24 h prior to harvesting and digestion.

After digestion, 0.3 ml of [^{3}H]thymidine-labeled material and 0.1 ml of [^{14}C]thymidine-labeled material were added to 3.8 ml of cesium chloride solution in 0.1× SSC (density = 1.7936 g/cm^3) and centrifuged in a Beckman L3-50 ultracentrifuge at 35,000 rpm for 60 h using a no. 40-3 fixed-angle rotor. After centrifugation, 8-drop fractions were collected from the bottom of the gradient onto Whatman filter paper disks. The disks were washed three times in 5% trichloroacetic acid, once in 95% ethanol, and once in acetone; they were allowed to dry at room temperature and were placed into 10 ml of formula 949 toluene-based scintillation fluid (New England Nuclear Corp.). Acid-insoluble radioactivity was counted with a Beckman LS-250 liquid scintillation counter, which could distinguish the two radioactive labels. Counts in both channels were printed onto tape and the gradient profiles were plotted with the aid of a Hewlett-Packard 9820A calculator and 9862A graph plotter.

UV irradiation of virus. Virus (diluted in phosphate-buffered saline to a concentration of 10^7 PFU/ml) was irradiated in plastic dishes (60 mm) with a Thomas UV lamp with a GE-G8T5 bulb at a distance such that the incident dose was about 4,200 ergs/s per cm^2. Irradiated virus was titrated in RK cells, as described above.

Virus transformation assay. The ability of wild-type and mutant viruses to mediate "biochemical transformation" of 3T3-4E TK$^-$ cells to TK$^+$ cells was assayed by using selection for TK$^+$ cells in HAT medium (7, 25, 30). Mutant and wild-type virus stocks were inactivated by exposure to UV light for different periods of time. Trypsinized 3T3-4E TK$^-$ cells in suspension (4 × 10^6) were treated with 2 × 10^6 PFU of irradiated virus (calculated from preirradiation titer) in 4.0 ml of complete Dulbecco medium. The mixture was shaken at room temperature for 2 h, after which 0.3-ml aliquots were dispensed into each petri dish (12 by 60 mm) containing 4 ml of complete Dulbecco medium and were incubated at 37 C. Within 16 to 24 h after plating, the Dulbecco medium was replaced with 5 ml of complete Dulbecco containing aminopterin (0.4 μg/ml), glycine (10 μg/ml), hypoxanthine (15 μg/ml), and thymidine (10 μg/ml) (HAT medium). This medium was replaced every third day for 18 days and, on day 21, the plates were fixed using 5% formalin and were stained in 0.3% methylene blue for 20 min. After staining, colonies were counted and the mean number of colonies per plate was calculated.

Assay for defective interfering particles. (i) Confluent monolayers of HEF cells in bottles (16 ounce) were infected at different input multiplicities, with a constant amount of wild-type virus and increasing dilutions of mutant or wild-type "interfering virus." After adsorption at room temperature, the cells were incubated at 39 C in complete medium 199 for 30 h, at which time the cells were harvested and the virus was extracted and titrated, as described above.

(ii) A constant amount of wild-type virus and increasing dilutions of "interfering" wild-type or mutant virus were mixed in a 0.1-ml quantity, which was then added to confluent monolayers of HEF cells in plates (60 mm). After adsorption for 1 h at room temperature, the cells were incubated at 33 or 39 C for 4 days under complete Eagle medium containing 0.5% methylcellulose. After 4 days the cell sheet was fixed, stained, and examined for virus plaques, as described above.

Particle counts by electron microscopy. Virus suspensions in medium 199 were prepared for electron microscopy by the technique of Monroe and Brandt (29) and were examined at 75 kV in a Hitachi HU12 electron microscope. Virus particles were scored as enveloped or nonenveloped and cored or emptied of any electron-dense core material. All samples for electron microscopy were blind-coded prior to examination.

RESULTS

Host range, temperature sensitivity, and plaque morphology. The results of plating experiments in different cell types, several of which are in accord with previously published data (23, 24), are summarized in Table 1. Plaque size, although heterogeneous, fell within a single population for each virus in each cell type. Mutants 69 and 74, which produced smaller

TABLE 1. *Plating efficiencies and mean plaque size at 33 and 39 C of wild-type HSV-2 strain 333 and of mutants 69 and 74*[a]

Host cell	Wild-type 333			Mutant 69			Mutant 74		
	Plating efficiency 39 C/33 C (%)	Mean plaque size (mm)		Plating efficiency 39 C/33 C (%)	Mean plaque size (mm)		Plating efficiency 39 C/33 C (%)	Mean plaque size (mm)	
		33 C	39 C		33 C	39 C		33 C	39 C
HEF	10	3.3	1.3	3.3 × 10^{-4}	1.8		4.0 × 10^{-5}	2.0	1.7
RK	100	2.5	2.0	10	2.0	0.5	15	1.5	0.5
HEL	100	2.5	2.8	11	2.4	0.6	30	2.4	0.8
BSC-1 (African green monkey kidney line)	57	0.9	0.9	23	0.6	0.5	33	0.9	0.4
3T3-4E TK$^-$ (mouse cell line)	<10^{-3}	1.4		<10^{-2}	0.9		<10^{-3}	0.9	

[a] The plating efficiency of virus stocks (grown in HEL cells) was compared in different cell types, using the plaque assay technique.

plaques than parental 333 under all experimental conditions, were found to express a temperature-sensitive phenotype that was host cell dependent. Moreover, failure of the mutants to grow at 39 C was not merely due to a prolonged virus growth cycle since infected HEF cells could be maintained for up to 9 days at 39 C without a detectable increase in plaque number. Mutant virus growth at 39 C was due to leakage, rather than reversion to wild type. Very little wild-type or mutant virus growth was observed in 3T3-4E TK$^-$ cells at 39 C.

Figure 1 shows growth curves over the first 24 h after infection of wild-type 333, mutant 69, and mutant 74 in HEF cells. The results further demonstrate the temperature sensitivity of both mutants in HEF cells and also suggest that maturation of mutant 74 proceeds at a slower rate than that of wild-type virus, even at 33 C.

DNA studies. Because of their different densities, HSV-2 DNA (density = 1.73 g/cm^3) (35) and cellular DNA (density = 1.70 g/cm^3) can be differentiated by isopycnic banding in CsCl solution, as shown in Fig. 2. For each profile, the total amount of [^{3}H]thymidine incorporated into virus and cellular DNA was calculated by summing the radioactivity in the fractions comprising the virus and cell DNA peaks, respectively.

The pattern of virus DNA synthesis during the infectious cycle in HEF cells is shown in Fig. 3 for wild-type 333 and the two mutants. Wild-type virus was observed to synthesize most of its DNA during the first 9 h after infection, a finding that is consistent with earlier reports (2, 34, 35). This was the case both at 33 and 39 C, although at the higher temperature less virus DNA was made, supporting the slight temperature sensitivity of HSV-2 in hamster cells (6).

Virus DNA synthesis in HEF cells infected by mutant 69 occurred predominantly during the first 9 h postinfection but, even at 33 C, mutant 69 directed only half as much viral DNA synthesis as did the wild-type 333. At 39 C the discrepancy between wild-type and mutant 69 DNA synthesis is greater than twofold, but some mutant virus DNA synthesis was observed.

The results obtained suggest that mutant 74 directed relatively little virus DNA synthesis (compared with that directed by wild-type virus or mutant 69) for the first 16 h after infection of HEF cells, although some progeny production could be detected at this time. The peak of DNA synthesis occurred between 17 and 25 h postinfection, at both 33 and 39 C. Considerably more virus DNA was made at the lower temperature,

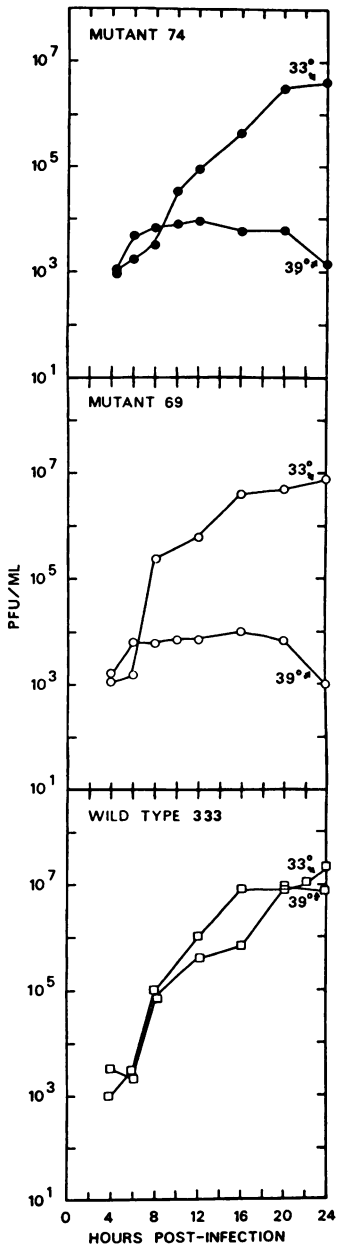

FIG. 1. *Growth curves of HSV-2 strain 333, mutant 69, and mutant 74 in HEF cells at 33 and 39 C. Cells were infected at an input multiplicity of 5.0 PFU/cell and were harvested, virus extracted, and titrated, as described.*

but this amounted to only about 30% of the amount of wild-type virus DNA made by HEF cells infected under similar conditions. The observation that mutant 74 makes progeny DNA "late" in the infectious cycle is consistent with the previous observation that the replication

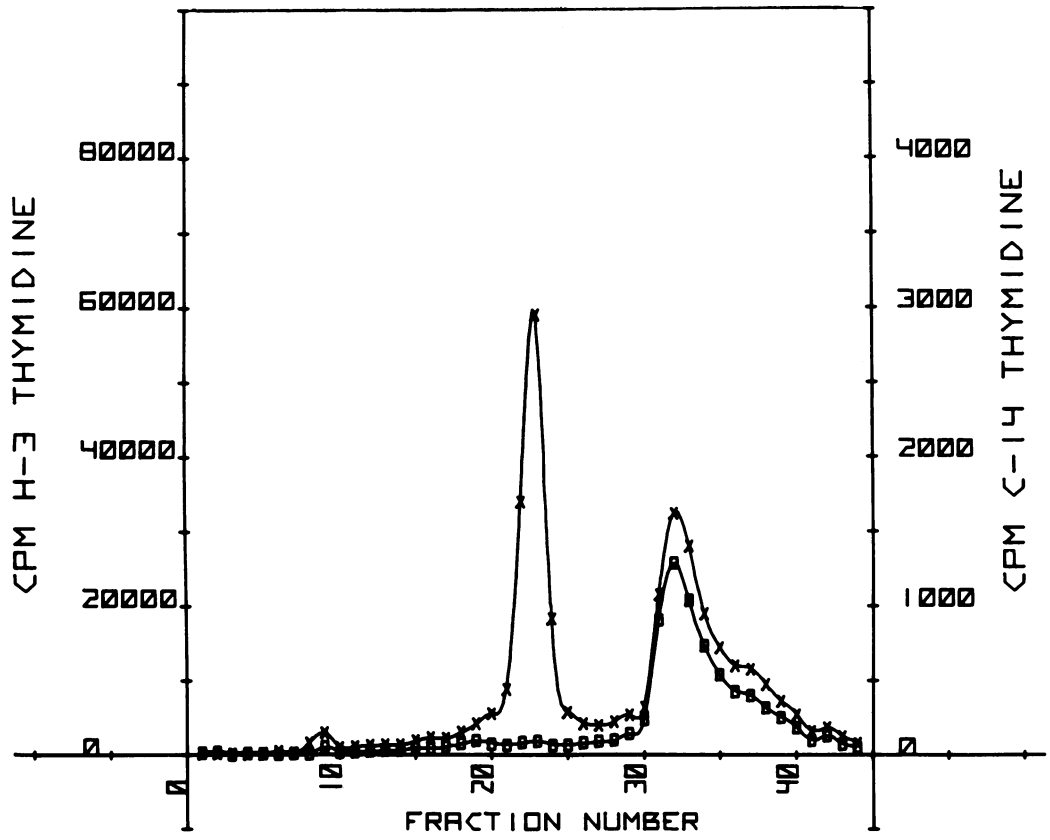

FIG. 2. *Separation of virus and cellular DNA by CsCl equilibrium centrifugation. This representative profile shows virus and cell DNA from a culture of HEF cells that had been infected with HSV-2 strain 333 17 h previously and incubated at 39 C. [^{3}H]TdR was added at 9 h postinfection, as described. Symbols: ×, radioactivity profile obtained from infected cells; □, radioactivity profile from mock-infected cell sample labeled with [^{14}C]TdR, which was cocentrifuged with material from infected cells.*

cycle of the virus is longer than that of mutant 69 or the parental virus at 33 C in HEF cells.

Electron microscope studies. The progeny of infection of HEF cells (at 33 and 39 C) by wild-type HSV-2 333 and by mutants 69 and 74 were harvested 24 h after infection and were examined in the electron microscope. Virus with a herpes-like morphology was seen in all preparations and was subdivided into particles with or without an electron-dense core (denoting the presence of virus DNA) and with or without a distinguishable envelope.

It soon became apparent, as shown in Table 2, which represents the results from a number of experiments, that even at the permissive temperature progeny of infection by mutant 69 had a very high ratio of particle to infectivity. It was earlier indicated (Fig. 1) that 24 h after infection at 33 C the progeny titers of infections by wild-type 333 and mutant 69 were not substantially different; the high ratio of particle to infectivity indicates that cells infected by mutant 69 produce a large number of virus particles, most of which are not infectious. Similar observations were made when mutant 69 was grown at 39 C in HEF cells, when ratios of particle to infectivity as high as a million to one were not unusual. (The infectivity of progeny virus was always assayed at 33 C in RK cells.)

The particles produced by mutant 69-infected cells were examined more closely to determine whether the vast number of defective particles was morphologically abnormal. No difference was found between the ratio of cored (probably DNA containing) to noncored particles in the progeny from wild-type virus-infected cells and in the progeny from mutant 69-infected cells. There was a slightly larger proportion of nonenveloped particles in the progeny of mutant 69 infections compared with those of wild-type infections, but this was not particularly striking and was insufficient to explain the defective

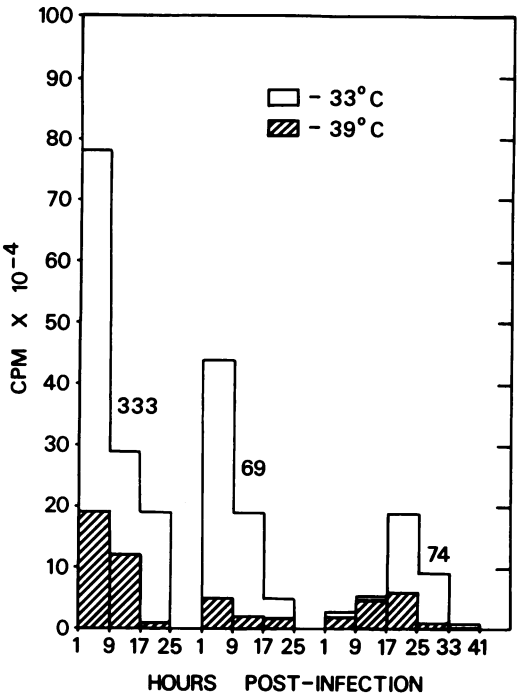

FIG. 3. *Pattern of virus DNA synthesis in HEF cells infected with HSV-2-333, mutant 69, or mutant 74 and maintained at 33 or 39 C. Columns represent total [³H]TdR incorporation in the virus peak (from CsCl equilibrium density gradient) during 8-h pulses of labeled material.*

TABLE 2. *Production of infectious and noninfectious progeny by HSV-2 strain 333 and mutants 69 and 74 in hamster cells at 33 and 39 C*[a]

Virus	Growth temp (C)	Particle/infectivity ratio	Nonenveloped/enveloped	No core/core
333	33	23–50	10–27	2–10
	39	50–100	16–30	8–20
74	33	27–70	88–200	30–70
	39	$1.5–9 \times 10^3$	100–300	50–100
69	33	$2.3–10 \times 10^3$	64–100	5–10
	39	$10^4–10^6$	40–200	10–50

[a] Virus prepared under different experimental conditions was titrated in RK cells at 33 C.

(i.e., morphologically normal but noninfectious) nature of the great majority of progeny produced by mutant 69.

Virus produced in HEF cells at 39 C showed a similar pattern. There was a slight increase in the proportion of nonenveloped particles compared with the progeny of wild-type virus grown at 39 C and no significant difference in the ratio of cored to noncored particles. Thus, there is no obvious morphological explanation for the defective nature of the bulk of the progeny virus produced by mutant 69.

Progeny produced by mutant 74 at 33 C appeared to have only a very slightly greater ratio of particle to infectivity than wild-type virus progeny, and both the proportion of nonenveloped and the proportion of empty particles were somewhat greater than in preparations of wild-type virus. At 39 C the ratio of particle to infectivity of the progeny was significantly higher than for wild-type virus, suggesting that mutant 74, like mutant 69, produces "defective" progeny at 39 C. Many of the noninfectious progeny of mutant 74 appeared to be nonenveloped, and there was an increase in the number of "empty" nucleocapsids produced at this temperature.

The propensity of mutant 69 for producing a large number of defective particles was also demonstrated in permissive cells. Virus stocks grown in HEL cells at 33 C had a higher ratio of particle to infectivity in the case of mutant 69 than for wild-type or mutant 74 virus. The degree of defectiveness of virus in stocks of 69 depended upon the host cell in which the virus was titrated (Table 3). The observations both at 33 and 39 C further support the "host range" properties of the two mutants.

Wild-type virus had essentially the same ratio of particle to infectivity (approximately 10 to 25 particles/PFU) when titrated in RK, HEL, or BSC-1 cells. In HEF cells a slightly higher ratio of particle to infectivity was observed at 39 C, reflecting an approximately 10-fold decrease in plating efficiency of HSV-2 at elevated temperatures in these cells. BSC-1 cells appeared to be permissive for all three viruses tested, at both temperatures, and the lowest ratios of particle to infectivity were observed for all viruses in these cells. RK cells, although more permissive than HEF cells for mutant virus growth, particularly at 39 C, were less permissive than HEL or BSC-1 cells. 3T3-4E TK⁻ cells supported little wild-type or mutant virus growth at 39 C.

Assay for defective interfering particles. Since stocks of mutant 74, and more particularly mutant 69, were known to contain many virus particles that were unable to replicate at 39 C, a phenomenon that was especially marked in HEF cells, experiments were designed to determine whether the presence of mutant virus would interfere with replication of wild-type HSV-2-333 at 39 C in HEF cells. Defective interfering particles have been previously reported in HSV stocks prepared by virus passage at high input multiplicities (4) and also between wild-type MPdk⁺ and MPdk⁻ strains of HSV growing in canine cells (33).

TABLE 3. *Relative plating efficiency of wild-type and mutant HSV-2 in different types of cells expressed as ratios of particle to infectivity*[a]

Cells	Plating efficiency of:					
	333 wild type		ts mutant 69		ts mutant 74	
	33 C	39 C	33 C	39 C	33 C	39 C
HEF	17-25	250-480	200-300	$7\text{-}9 \times 10^7$	40-110	9×10^5
Baby RK	10-18	15-30	100-200	1,500-1,900	20-50	60-100
HEL	17-25	17-25	80-100	750-900	20-50	50-80
BSC-1 cells	9-12	10-18	20-30	100-150	4-10	10-15
3T3-4E TK$^-$ cells	30-40	$>10^4$	5,000-8,000	$>10^6$	90-110	$>10^4$

[a] Virus infectivity was titrated using a plaque assay in which monolayers of cells were maintained in medium containing 0.5% methylcellulose for 4 days prior to the counting of virus plaques. Particle counting of the stock virus used in each experiment (grown in HEL cells at 33 C) was by electron microscope examination, as described in the text.

Such interference could be demonstrated (Table 4). Wild-type and both mutant viruses severely depressed the yield of HSV-2-333 when the "interfering" virus was added at an input multiplicity of 20 PFU/cell. Decreased concentration of interfering virus decreased the reduction in progeny synthesis, but this effect was least marked when mutant 69 was the interfering agent. It seems entirely reasonable to suppose that the large number of defective particles in preparations of this virus was responsible for interference with normal replication of HSV-2-333 at 39 C in HEF. Similar results to those shown in Table 4 were obtained using a constant input multiplicity of 0.2 PFU of HSV-2-333 per cell and similar multiplicities of "interfering virus."

Interference by mutant defective particles was also demonstrated using a plaque reduction technique (Table 5). At 33 C both large (presumably wild type) and small (presumably mutant) plaques were counted, and the total number of plaques was expressed. At 39 C, it was again observed that mutants 69 and 74 interfered with the development of wild-type plaques, and again this effect was more marked for preparations of mutant 69. Interference between viruses was not apparent at 33 C, possibly because at this temperature conditions are much more favorable for virus growth, which is hence favored over abortive infection and interference.

Biochemical transformation of 3T3-4E TK$^-$ cells by HSV-2 strain 333 and mutants 69 and 74. The effect of UV irradiation on the infectivity of mutant and wild-type HSV-2 is shown in Fig. 4. Wild-type strain 333 was found to be more resistant to UV irradiation than either mutant, with 74 the most sensitive, the same amount of irradiation causing a 1 to 2 $\log_{10}$ greater drop in titer of mutant 74 than of parental 333.

TABLE 4. *Assay for defective interfering particles in stocks of mutant virus, using virus yield as indicator*

"Interfering virus"		Input multiplicity of interfering virus (PFU/cell)		
		20	2	0.2
333 (Wild type)				
Challenge: PFU/cell[a]	2.0	2.0×10^5 (20)[b]	6.0×10^5 (60)	1.0×10^6 (100)
Challenge: PFU/cell[c]	0.2	2.0×10^5 (20)	3.0×10^5 (30)	1.0×10^6 (100)
Mutant 69				
Challenge: PFU/cell[a]	2.0	4.0×10^4 (4)	2.0×10^5 (20)	3.0×10^5 (30)
Challenge: PFU/cell[c]	0.2	3.4×10^4 (3.4)	5.0×10^5 (50)	8.0×10^5 (80)
Mutant 74				
Challenge: PFU/cell[a]	2.0	1.6×10^5 (16)	9.0×10^5 (90)	1.0×10^6 (100)
Challenge: PFU/cell[c]	0.2	1.4×10^5 (14)	7.0×10^5 (70)	2.0×10^6 (200)

[a] All cultures were challenged with HSV-2-333 wild-type virus at an input multiplicity of 2.0 PFU/cell. Control yield = 1.0×10^6 PFU/ml (20 PFU/cell). Titers represent the yield of HSV-2 (wild-type large plaques). Virus was titrated in RK cells as described in the text.

[b] Numbers in parentheses are percentages of control yield.

[c] All cultures were challenged with HSV-2-333 wild-type virus at an input multiplicity of 0.2 PFU/cell. Control yield = 10^6 PFU/ml (20 PFU/cell). Other conditions were similar to those described in footnote *a*.

3T3-4E TK$^-$ cells were exposed to virus that had been irradiated for 0 to 10 min and, after 21 days of incubation in selective HAT medium, the number of colonies that had formed on the 12 replicate plates for each virus sample was counted. In each experiment, the reversion frequency of 3T3-4E TK$^-$ cells to a TK$^+$ phenotype was estimated from the number of colonies forming on 12 replicate plates containing cells that had been exposed to UV-irradiated Tris buffer. An unfortunate feature of this transformation assay is that the reversion frequency of 3T3-4E TK$^-$ cells was found to be rather high

TABLE 5. *Assay for defective interfering particles in mutant stocks using plaque reduction as indicator*

Virus	Temp (C)	No. of plaques[a]					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
333	33	TNTC	TNTC	TNTC	162	113	96
	39	TNTC	TNTC	174	164	119	98
69	33	TNTC	TNTC	TNTC	136	118	112
	39	26	40	49	57	85	117
74	33	TNTC	TNTC	TNTC	142	137	116
	39	55	57	61	111	115	125

[a] A constant amount of wild-type 333 virus was plated which produced an expected number of plaques = 90 to 100/dish at both 33 and 39 C. TNTC, Too numerous to count.

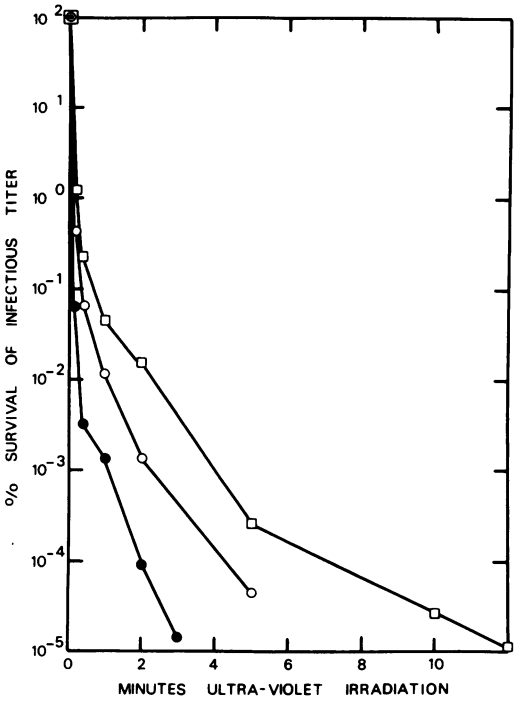

FIG. 4. *Survival of HSV-2 strain 333 and mutants 69 and 74 after exposure to increasing doses of UV light. Symbols: □, HSV-2 strain 333 (wild type); ○, mutant 69; ●, mutant 74.*

and variable; nevertheless, the results reported here are regularly reproducible. Reversion frequency was calculated for each experiment and subtracted from frequency of foci on virus-treated plates to give the "transformation frequency."

Unirradiated virus-induced cytopathology and no colonies were seen after exposure of cells to unirradiated wild-type virus or mutant 69. Cells infected with unirradiated mutant 74 did give rise to rare colonies, but these occurred at a much lower frequency than the reversion frequency of 3T3-4E TK$^-$ cells (approximately 10^{-5}) and their significance is obscure.

With increasing UV dose, as the infectivity of virus preparations decreased, the frequency with which transformed colonies were observed increased (Fig. 5). The data in Fig. 5 have all been corrected for the mean number of foci

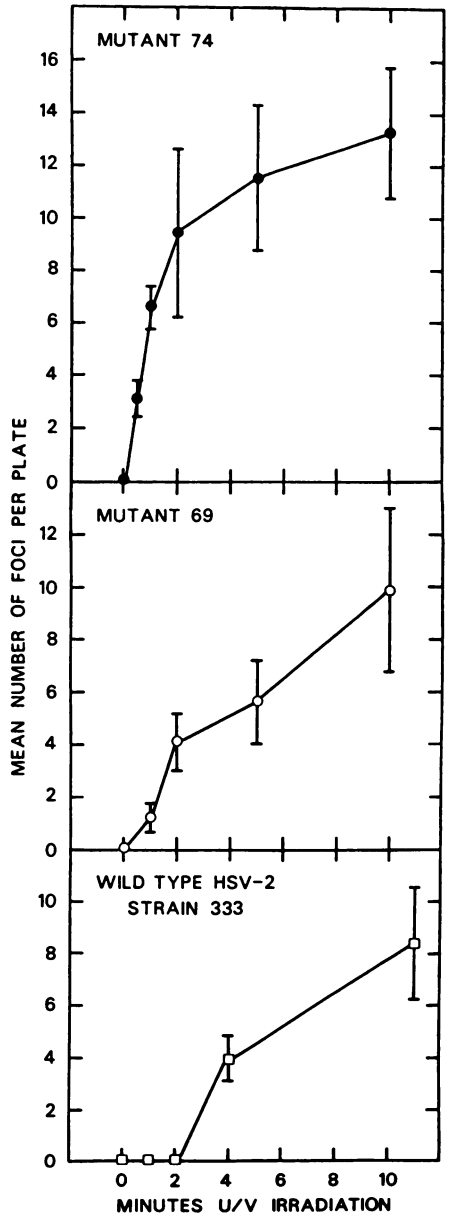

FIG. 5. *Biochemical transformation of 3T3-4E TK$^-$ by HSV-2-333 and mutants 69 and 74. Mean number of foci per plate was corrected for the mean number of foci on control (non-virus treated) plates. The results represent the mean of three experiments, and the bars give the standard error about the mean.*

observed on control plates. This varied from 0.17 to 2.5 in different experiments. Significant transformation was not observed with wild-type HSV-2-333 until the preparation had received at least 2 min of UV irradiation (4.8×10^5 ergs/cm^2); thereafter, the observed number of transformed foci increased. The maximum frequency of transformation events (i.e., numbers of foci corrected for mean foci on control plates) is approximately 3×10^{-4}.

Mutant 69, which was found to be more sensitive to UV irradiation than the parental virus, induced the formation of significant numbers of transformed foci at lower UV doses than wild-type 333, and a slightly higher maximum frequency of transformation was observed.

A more striking difference in transforming ability was shown by mutant 74. This virus, which was the most sensitive to UV irradiation, was able to transform significant numbers of cells at a low (12×10^4 ergs/cm^2) UV dose, and the frequency of transformation after exposure to this virus increased rapidly to a plateau level that was equal to, or slightly higher than, the maximum transformation frequency observed with wild-type virus or mutant 69. This plateau was maintained for UV doses from 2 to 10 min (4.8×10^5 to 2.4×10^6 ergs/cm^2). Virus infectivity dropped to undetectable levels after only 5×10^5 ergs of irradiation per cm^2.

The data presented in Fig. 5 represent the mean of three experiments. More recently, two further experiments, using virus that had been irradiated for as much as 15 min, have provided similar results.

Foci observed in transformation experiments consisted of homogeneous populations of cells, but at least two different morphological types were seen in different foci. One type was a flat, epithelioid cell that gave rise to large, apparently single-layered colonies. The second cell type was distinctly fibroblastoid and gave rise to multilayered foci. These observations are similar to those reported in an earlier study of HSV transformation of mouse 3T3 cells (13), in which the HAT selection system had not been employed.

DISCUSSION

In the present study, two mutants, previously reported to be defective for replication in HEF cells at 39 C, were compared with each other and with the parental virus for a number of biological properties. These mutants were chosen because they were found to be members of two distinct complementation groups (23) and were not as leaky as other temperature-sensitive host range mutants isolated (23).

Preliminary characterization of the mutants had demonstrated that their temperature sensitivity was not due to thermolability of the virion (R. W. Koment, unpublished data) or to a block in virus adsorption or penetration at 39 C (23). This was consistent with results reported in this communication, demonstrating that a number of virus-directed changes occurred in HEF cells infected at 39 C. Some virus DNA synthesis took place, host cell DNA synthesis was turned off (data not presented), and a few virus particles were produced, although these particles were mainly noninfectious. The observation that mutant 69 produces many morphologically normal particles of greatly reduced infectivity is similar to that made by Schaffer et al. (37), who reported a C group ts mutant of HSV-1 that produced apparently morphologically normal particles of greatly reduced infectivity.

Schaffer and co-workers (37) were also able to correlate the production of cored and empty particles with the DNA phenotype of the virus. To some extent, similar observations were made in this study using mutant 74. This mutant, even at permissive temperatures in HEF cells, had a delayed onset of DNA synthesis and at 39 C made very little virus DNA. Particles produced in cells infected by mutant 74 showed a preponderance of nonenveloped empty nucleocapsids at 39 C.

The "host range" properties of mutants 69 and 74 appear to form a gradual pattern from fully permissive cells (BSC-1) to extremely restrictive cells (HEF). This growth restriction in different cells is demonstrated at 33 C but, at 39 C, it is much more marked. It seems reasonable to conclude that the mutants have difficulty in replicating in certain cells and that the additional stress of high temperature (HSV-2 is known to grow poorly at elevated temperatures [6]) prevents virus growth almost completely. This conclusion could have important parallels in vivo, particularly with regard to latency. It may be that certain cells of the host are naturally resistant to HSV growth or even that there are certain naturally occurring HSV strains whose growth is restricted in some cells, e.g., of nervous tissue. Such strain differences would not be without precedent; Epstein-Barr virus for example, another herpesvirus of humans, appears to have at least two forms, one of which can replicate in lymphocytes, the other of which cannot replicate but can transform human cord blood lymphocytes (27, 28). It is even possible, of course, that such strain differences of Epstein-Barr virus are due to "defectives," like mutants 69 and 74.

The high transforming frequency observed

with mutant 74 after relatively low doses of UV irradiation was a particularly interesting result in this context. It had been thought possible that mutant 69, which produced a very large number of defective particles, might prove to be a particularly efficient transforming virus. This was not found to be the case, as extensive cytopathic effects were observed when this virus was added to cells after low UV doses, suggesting that, although unable to replicate, the majority of mutant 69 particles are able to induce cell death and, thus, preclude transformation. Mutant 74, although it did not produce a large number of defective particles, was abnormally sensitive to UV damage. It seems probable that this property, together with the naturally delayed growth cycle of the virus, tipped the balance toward cell transformation (rather than lysis). It should be mentioned again, however, that there are a number of shortcomings with the transformation system used in this study. The assay was based strictly on quantitative focus formation, and colonies were not examined for the presence of herpes-specific antigens. Considerable variation was seen in the number of foci produced in different experiments, and the reversion frequency of the 3T3-4E TK^- cells was high (1×10^5 to 5×10^{-5}). Similar transformation experiments using L(TK^-) cells (obtained from W. Munyon) gave transformation frequencies too low to be quantified, but again a greater transforming efficiency was observed using mutant 74 than mutant 69 or wild-type 333 virus. Taken together, the data on the transforming potential of the three viruses tested lead to the conclusion that great care must be exercised in the development of attenuated herpes simplex virus vaccines. Mutant 74 is attenuated, both for growth in cell culture and for virulence in mice (22), and on this criterion would be considered a suitable vaccine candidate. However, by selecting for growth attenuation, selection has also been made for a virus whose cytolytic properties are more than usually sensitive to exogenous damage, but whose transforming capabilities remain intact. This situation would clearly be most undesirable in a virus vaccine.

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LITERATURE CITED

1. **Aron, G. M., D. M. Purifoy, and P. A. Schaffer.** 1975. DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. 16:498–507.
2. **Ben-Porat, T., and A. S. Kaplan.** 1973. Replication-biochemical aspects, p. 163. *In* A. S. Kaplan (ed.), The herpesviruses. Academic Press Inc., New York.
3. **Benyesh-Melnick, M., P. A. Schaffer, R. J. Courtney, J. Esparza, and S. Kimura.** 1975. Viral gene functions expressed and detected by temperature sensitive mutants of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. 39:731–746.
4. **Bronson, D. L., G. R. Dreesman, N. Biswal, and M. Benyesh-Melnick.** 1973. Defective virions of herpes simplex viruses. Intervirology 1:141–153.
5. **Brown, S. M., D. A. Ritchie, and J. H. Subak-Sharpe.** 1973. Genetic studies of HSV-1. Isolation of ts mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. 18:329–346.
6. **Crouch, N. A., and F. Rapp.** 1972. Cell-dependent differences in the production of infectious herpes simplex virus at a supraoptimal temperature. J. Virol. 9:223–230.
7. **Davis, D. B., W. Munyon, R. Buchsbaum, and R. Chawda.** 1974. Virus type-specific thymidine kinase in cells biochemically transformed by herpes simplex virus types 1 and 2. J. Virol. 13:140–145.
8. **Docherty, J. J., and M. Chopan.** 1974. The latent herpes simplex virus. Bacteriol. Rev. 38:337–355.
9. **Duff, R., and F. Rapp.** 1970. Quantitative characteristics of the transformation of hamster cells by PARA(defective simian virus 40)-adenovirus 7. J. Virol. 5:568–577.
10. **Duff, R., and F. Rapp.** 1971. Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. Nature (London) New Biol. 233:48–50.
11. **Duff, R., and F. Rapp.** 1971. Properties of hamster embryo fibroblasts transformed in vitro after exposure to ultraviolet-irradiated herpes simplex virus type 2. J. Virol. 8:469–477.
12. **Duff, R., and F. Rapp.** 1973. Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type 1. J. Virol. 12:209–217.
13. **Duff, R., and F. Rapp.** 1975. Quantitative assay for transformation of 3T3 cells by herpes simplex virus type 2. J. Virol. 15:490–496.
14. **Esparza, J. D., J. M. Purifoy, P. A. Schaffer, and M. Benyesh-Melnick.** 1974. Isolation, complementation and preliminary phenotypic characterization of temperature-sensitive mutants of herpes simplex virus type 2. Virology 57:554–565.
15. **Halliburton, I. W., and M. C. Timbury.** 1973. Characterization of temperature-sensitive mutants of herpes simplex virus type 2. Growth and DNA synthesis. Virology 54:60–68.
16. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpesvirus polypeptides in the infected cell. J. Virol. 12:1347–1365.
17. **Honess, R. W., and B. Roizman.** 1974. Regulation of macromolecular synthesis of three groups of viral proteins. J. Virol. 14:8–19.
18. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. U.S.A. 72:1276–1280.
19. **Kit, S., and D. R. Dubbs.** 1963. Acquisition of thymidine kinase activity by herpes simplex infected mouse fibroblast cells. Biochem. Biophys. Res. Commun. 11:55–59.
20. **Kit, S., W. C. Leung, G. N. Jorgensen, D. Trkula, and D. R. Dubbs.** 1975. Thymidine kinase isozymes of normal and virus infected cells. Cold Spring Harbor

Symp. Quant. Biol. 39:703-715.
21. **Klemperer, H. G., G. R. Haynes, W. H. Shedden, and D. H. Watson.** 1957. A virus specific thymidine kinase in BHK-21 cells infected with herpes simplex virus. Virology 31:120-128.
22. **Koment, R. W., and F. Rapp.** 1975. *In vivo* characteristics of temperature-sensitive host range mutants of herpes simplex virus type 2. Intervirology 5:10-20.
23. **Koment, R. W., and F. Rapp.** 1975. Temperature-sensitive host range mutants of herpes simplex virus type 2. J. Virol. 15:812-819.
24. **Koment, R. W., and F. Rapp.** 1975. Variation in susceptibility of different cell types to temperature-sensitive host range mutants of herpes simplex virus type 2. Virology 64:164-169.
25. **Littlefield, J.** 1965. Studies on thymidine kinase in cultured mouse fibroblasts. Biochim. Biophys. Acta 95:14-22.
26. **Macnab, J. C. M.** 1974. Transformation of rat embryo cells by temperature sensitive mutants of herpes simplex virus. J. Gen. Virol. 24:143-153.
27. **Miller, G., J. Robinson, and L. Heston.** 1975. Immortalizing and non-immortalizing strains of Epstein-Barr virus. Cold Spring Harbor Symp. Quant. Biol. 39:773-781.
28. **Miller, G., J. Robinson, L. Heston, and M. Lipman.** 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection and interference. Proc. Natl. Acad. Sci. U.S.A. 71:4006-4010.
29. **Monroe, J. H., and P. M. Brandt.** 1970. Rapid semiquantitative method for screening large numbers of virus samples by negative staining electron microscopy. Appl. Microbiol. 20:259-262.
30. **Munyon, W., E. Kraiselburd, D. Davies, and J. Mann.** 1971. Transfer of thymidine kinase to thymidine kinaseless L cells by infection with ultraviolet-irradiated herpes simplex virus. J. Virol. 7:813-820.
31. **Rapp, F.** 1963. Variants of herpes simplex virus: isolation, characterization, and factors influencing plaque formation. J. Bacteriol. 86:985-991.
32. **Rapp, F., and M. A. Jerkofsky.** 1973. Persistent and latent infections, p. 271-289. *In* A. S. Kaplan (ed.), The herpesviruses. Academic Press Inc., New York.
33. **Roizman, B.** 1965. Abortive infection of canine cells by herpes simplex virus. III. The interference of conditional lethal virus with an extended host range mutant. Virology 27:113-117.
34. **Roizman, B., and P. R. Roane.** 1964. The multiplication of herpes simplex virus. II. The relationship between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology 22:262-269.
35. **Roizman, B., P. G. Spear, and E. D. Kieff.** 1973. Herpes simplex viruses 1 and 2: a biochemical definition. Perspect. Virol. 8:129-169.
36. **Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick.** 1973. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation and partial characterization. Virology 52:57-71.
37. **Schaffer, P. A., J. P. Brunschwig, R. M. McCombs, and M. Benyesh-Melnick.** 1974. Electron microscope studies of temperature sensitive mutants of herpes simplex virus type 1. Virology 62:442-457.
38. **Stevens, J. G., and M. L. Coor.** 1973. Latent herpes simplex in sensory ganglia. Perspect. Virol. 8:171-188.
39. **Subak-Sharpe, J. H., S. M. Brown, D. A. Ritchie, M. C. Timbury, J. C. M. Macnab, H. S. Marsden, and J. Hay.** 1975. Genetic and biochemical studies with herpesvirus. Cold Spring Harbor Symp. Quant. Biol. 39:717-730.
40. **Takahashi, M., and K. Yaminishi.** 1974. Transformation of hamster embryo and human embryo cells by temperature sensitive mutants of herpes simplex virus type 2. Virology 61:306-311.
41. **Wilkie, N. M., J. B. Clements, J. C. M. Macnab, and J. H. Subak-Sharpe.** 1975. The structure and biological properties of herpes simplex virus DNA. Cold Spring Harbor Symp. Quant. Biol. 39:657-666.