Involvement of an Early Human Cytomegalovirus Function in Reactivation of Quiescent Herpes Simplex Virus Type 2

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We have previously described an *in vitro* system in which the function lacking for herpes simplex virus type 2 (HSV-2) replication can be induced by human cytomegalovirus (HCMV). The mechanism of this reactivation of quiescent HSV-2 by HCMV has been further defined. The HCMV function(s) responsible for HSV-2 stimulation was examined temporally, and the fraction of cells in quiescent cultures producing HSV-2 after superinfection was determined. Using independent biological, genetic and molecular techniques we have made the following observations. (i) As early as 12 h after HCMV superinfection, HSV-2 RNA was expressed in latently infected cells. (ii) At 24 h after HCMV superinfection, a time when newly synthesized HCMV was not yet apparent, infectious HSV-2 was produced by reactivated cultures. (iii) Four HCMV temperature-sensitive mutants, which are DNA-negative at nonpermissive temperature and represent four different complementation groups, induced reactivation of HSV-2 at 39.5°C. (iv) Early after HCMV superinfection, 1.6% of quiescent cells could be induced to transcribe HSV-2 information. (v) Early after HCMV superinfection, 0.3% of cells in the quiescent cultures could be induced to yield infectious HSV-2. The finding that a significant interaction can occur between HCMV and quiescent HSV-2 in an *in vitro* model is noteworthy in light of the knowledge that both of these herpesviruses often reside simultaneously in the human host.

In the absence of overt clinical infection, herpes simplex virus (HSV) is maintained in a latent state in the neural tissues of animals (22, 23) and humans (1-3, 21, 26). Development of *in vitro* HSV latency systems has allowed study of persistence (8-10, 12, 13, 27) and detailed examination of cell-virus interactions, such as reactivation and the state of the virus genome during latency. We have developed an *in vitro* HSV type 2 (HSV-2) latency system (5, 6, 18, 19) in which HSV-2-infected human embryonic lung (HEL) cells, when treated with cytosine arabinoside (ara-C; 19) or phosphonoacetic acid (5), do not produce infectious virus for several days after drug removal. The inapparent period of HSV-2 can be extended by incubation of infected cultures at 39.5°C after drug removal (5, 6, 18). Examination of these quiescent cultures when HSV-2 was undetectable at 39.5°C yielded no evidence of infectious HSV-2 in total cell lysates by plaque formation (5, 6, 18). Furthermore, very few quiescent cells formed HSV infectious centers (8, 19) and, within the detection limits of our assay system, cells positive for HSV-2 RNA were not found. Infectious HSV-2 production was reinitiated either by alteration of incubation

temperature (5, 18) or by superinfection of quiescent cultures at 39.5°C with a heterologous herpesvirus, human cytomegalovirus (HCMV; 6). The time of HSV-2 reappearance varied; infectious HSV-2 reappeared 11 days after temperature shift of quiescent cultures (18) or within 24 h after HCMV superinfection.

We chose to examine the mechanism of HSV-2 reactivation in this *in vitro* system because it eliminates the complexities of animal studies, provides a simple, reproducible method to examine inapparent HSV-2 infection, and also allows the reactivation of HSV-2 after specific manipulation of quiescent cultures. HCMV-dependent stimulation of HSV-2 replication may result from interactions between the two viruses or between HCMV and the HSV-2-containing host cell. In either event, since HSV-2 was reproducibly induced within 24 h after HCMV superinfection, this procedure was used to determine indirectly the number of cells capable of expressing HSV-2 RNA.

The studies presented in this paper were designed to determine: (i) whether the HCMV function(s) involved in this stimulation was an early or a late event; and (ii) what fraction of the cells contained HSV-2 in quiescent cultures and produced HSV-2 after superinfection.

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MATERIALS AND METHODS

Cells and viruses. Confluent monolayers of HEL cells were grown in Dulbecco medium supplemented with 10% fetal calf serum, 0.03% glutamine, penicillin (25 U/ml), streptomycin (25 µg/ml), kanamycin (100 µg/ml), and 0.075% sodium bicarbonate. Maintenance medium contained 2% fetal calf serum, 0.15% sodium bicarbonate, and supplements similar to those added to growth medium. Flow 5000 cells (Flow Laboratories, Rockville, Md.) and primary rabbit kidney (PRK) cells were grown to confluency in modified Eagle minimum essential medium supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, penicillin (100 U/ml), streptomycin (100 µg/ml), and 0.225% sodium bicarbonate. For latency experiments, HEL cells were grown in T-25 flasks or in 60-mm petri dishes. Titration and infectious center assays were carried out on confluent HEL, PRK, or Flow 5000 cells in 60-mm petri dishes.

HSV-2 (186) and HCMV (AD169) were prepared and stored as previously described (6). HCMV temperature-sensitive (*ts*) mutants (28, 29) were grown in Flow 5000 cells in a manner similar to that for HSV-2.

Virus titrations. The presence of infectious HSV-2 and HCMV was determined as previously described (6).

Infectious center assays. Cultures were treated as previously described (18). Briefly, infected cultures were washed twice with phosphate-buffered saline and dispersed using 0.5% trypsin. Cells to be assayed for HSV infectious centers were then treated with 5% rabbit anti-HSV-2 serum for 15 min at room temperature and diluted serially. The number of viable cells in each sample was determined by exclusion of 0.1% trypan blue. After adsorption of the diluted cells to PRK cell monolayers for 2 to 3 h at 37°C, monolayers were overlaid with 0.5% methylcellulose nutrient medium. The cells were then incubated at 37°C for 2 to 3 days, fixed with 5% Formalin, and stained with 1% crystal violet. The infectious center titer was determined using the formula: Infectious center titer = (number of infectious centers × dilution)/total number of viable cells.

For HCMV infectious centers, the following modifications were used: cells were treated with 20% human anti-HCMV serum and 5% rabbit anti-HSV-2 serum, adsorbed to HEL cells, and overlaid with 0.5% agarose containing 5% rabbit anti-HSV-2 serum. The cells were incubated at 37°C for 2 weeks before fixation and stained with 0.02% methylene blue. The rabbit anti-HSV-2 serum and human anti-HCMV serum used were as previously described (6).

Latency experiments. Confluent HEL monolayers were treated as previously described (6, 18). Maintenance medium containing 25 µg of ara-C per ml (Sigma Chemical Co., St. Louis, Mo.) was placed on HEL cells 5 h before infection with low levels (0.2 PFU/cell) of HSV-2 (186). Cultures were exposed to ara-C-containing medium and incubated at 37°C after adsorption. During the 7-day period postinfection (p.i.), maintenance medium containing ara-C was changed daily. At 7 days p.i., cultures were washed twice with Tris-buffered saline (pH 7.4), fed medium without inhibitor, and incubated at 39.5°C.

Superinfection of quiescent cultures. Cultures previously infected with HSV-2, treated with ara-C, and shifted to 39.5°C were superinfected with HCMV (AD169) at a multiplicity of infection (MOI) of 2, 0.2, or 0.02 PFU/cell. HCMV *ts* mutants (*ts*13, *ts*9, *ts*153, and *ts*155) were used at the same MOI as the parental virus. Irradiation of HCMV with UV light was carried out as previously described (6).

Purification and in vitro labeling of HSV-2 DNA. HEL cells were infected with HSV-2 (186). To obtain HSV-2 DNA, virions from infected cells and supernatant were separated from cell debris by banding on discontinuous CsCl gradients (1.2 and 1.4 g/ml). Virions were digested with 2 mg of proteinase K (EM Biochemicals, Elmsford, N.Y.) per ml in 0.5% sodium dodecyl sulfate for 2 h at 37°C, placed in CsCl (initial average density of 1.70 g/ml), and centrifuged to equilibrium at 87,600 × *g* for 65 h in a Beckman 50.3 rotor. The gradients were collected by bottom puncture, and fractions containing HSV-2 DNA, as determined by buoyant density in CsCl, were pooled and dialyzed against 10 mM Tris-1 mM EDTA buffer (pH 7.8).

HSV-2 DNA was labeled in vitro with [³H]dTTP (81.5 Ci/mmol, New England Nuclear Corp., Boston, Mass.) and [³H]dCTP (21.8 Ci/mmol, New England Nuclear) with *Escherichia coli* DNA polymerase I (Boehringer Mannheim Corp., Indianapolis, Ind.) as described by Maniatis and co-workers (15). HSV-2 [³H]DNA had a specific activity of 1.2×10^7 to 2.2×10^7 cpm per µg. The size of the nick-translated HSV-2 probe, as determined by sedimentation in isokinetic alkaline sucrose gradients (16), ranged from 94 to 230 nucleotides with an average size of 177 nucleotides (data not shown).

In situ hybridization. Cells grown on cover slips (15 mm) and treated as described above were hybridized with HSV-2 [³H]DNA as described by Copple and McDougall (7) with some modifications of Brahic and Haase (4). Before hybridization, the cells were treated with 1 µg proteinase K per ml in 20 mM Tris-hydrochloride (pH 7.4) and 2 mM CaCl₂ for 15 min at 37°C and then dehydrated. The hybridization mixture consisted of 50,000 cpm of HSV-2 [³H]DNA, 50% deionized formamide, 10 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 6× standard saline citrate (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), and 100 µg of calf thymus DNA per ml. Hybridizations were carried out at 47°C for 48 h. The slides were then washed extensively with 2× SSC, dipped in 50% nuclear track emulsion (NTB-2, Eastman Kodak Co., Rochester, N.Y.) in 600 mM ammonium acetate, exposed at 4°C for 1 to 3 weeks, and developed using a 1:200 dilution of Rodinal (Agfa-Gevaert, Teterboro, N.J.). Developed slides were stained with 4% Giemsa in 10 mM potassium phosphate (pH 7.4) before examination by light microscopy. Background levels of hybridization showed 2 to 7 grains per cell; cells were considered positive when greater than 20 grains per cell were present.

RESULTS

Fraction of cell population capable of synthesizing HSV-2 in quiescent HSV-2-infected HEL cells. These studies employed a

system of HSV-2-infected human cells in which infectious HSV-2 remains undetectable for extended periods. Infectious center assay was used to establish the number of cells containing HSV-2 genetic information. When cultures were assayed immediately after HSV-2 infection (MOI of 0.2 PFU per cell), 2 to 4% of the cells were infected as determined by infectious center assay (Table 1). After temperature shift but before superinfection, the infectious center titer ranged from $<2 \times 10^{-6}$ to 2×10^{-4} , a value similar to that previously reported ($<1.5 \times 10^{-5}$ to 5×10^{-4} ; 18). The variation was random and did not correlate with time after temperature shift (Table 1). Increasing the MOI during initial HSV-2 infection in the presence of ara-C resulted in destruction of the monolayer. Using the [3 H]-DNA probe prepared for this study, we were unable to detect expression of HSV-2 RNA during the quiescent state.

Kinetics of HSV-2 reactivation by HCMV superinfection. To determine temporally which HCMV function(s) was responsible for HSV-2 stimulation, we examined the reappearance of infectious HSV-2 early after superinfection. HSV-2 production observed within 24 h after HCMV superinfection (0.2 or 0.02 PFU per cell; Fig. 1) showed HSV-2 titers increasing to maximal levels within 36 h. Quiescent cultures treated with either UV-irradiated HCMV or mock-infecting fluid failed to yield HSV-2. Subculturing quiescent cultures 1:2 also failed to yield HSV-2. To further determine specificity of the HCMV-dependent stimulation, a second virus known to stimulate host cell macromolecular synthesis, simian virus 40 (SV40), was used to superinfect quiescent cultures. Superinfection of quiescent cultures with SV40 at multiplicities of

100, 10, or 1 PFU per cell did not yield infectious HSV-2 (data not shown). At an MOI of 100 PFU of SV40 per cell 48 h post-superinfection, 10% of the SV40-superinfected quiescent cultures expressed SV40 nuclear T antigen, as determined by indirect immunofluorescence staining (data not shown).

It is interesting to note the apparent independence of the amount of HSV-2 produced relative to the initial MOI of HCMV used for superinfection. Quiescent cultures superinfected with 0.2 or 0.02 PFU of HCMV per cell produced 10^2 PFU of HSV-2 per culture within 24 h and $\sim 10^4$ PFU per culture within 36 h. This lack of multiplicity dependence was not simply a reflection of the number of HCMV-infected cells, since the number of quiescent cells that produced HCMV (as determined by infectious center assay) was dependent on the initial MOI of HCMV (Table 2).

The presence of infectious HCMV in HCMV-superinfected cultures was also examined (Fig. 2). Low levels of infectious HCMV did not replicate to detectable levels within 72 h p.i. (Fig. 2). Since HSV-2 appeared within 24 h and HCMV did not replicate during the initial 72 h p.i., these studies suggest that an early HCMV function is involved in HSV-2 stimulation.

Reactivation of HSV-2 by superinfection with HCMV *ts* mutants. To examine more fully the early HCMV function involved, we carried out studies with HCMV *ts* mutants. HCMV *ts* mutants from four different complementation groups (29) and unable to synthesize virus DNA at 39.5°C were used to superinfect

TABLE 1. Infectious center assays with latently infected HEL cells

Treatment	Assay temp	Infectious center titer ^a	
4 h p.i. (+ara-C) ^b	37°C	4×10^{-2}	
4 h p.i. (-ara-C)	37°C	2×10^{-2}	
4 h p.i. (+ara-C)	39.5°C	2×10^{-2}	
4 h p.i. (-ara-C)	39.5°C	1×10^{-2}	
13 days p.i. ^c	39.5°C	2×10^{-4}	4×10^{-6}
14 days p.i.	39.5°C	1×10^{-5}	$<2 \times 10^{-6}$
19 days p.i.	39.5°C	4×10^{-6}	4×10^{-6}
22 days p.i.	39.5°C	4×10^{-6}	3×10^{-5}

^a Calculated by the formula: infectious center titer = (number of infectious centers $\times$ dilution)/total number of viable cells.

^b Cultures were infected with HSV-2 at 37°C in the presence (+ara-C) or absence (-ara-C) of ara-C treatment and assayed 4 h p.i. for infectious center formation at 37 or 39.5°C.

^c A culture which was 13 days p.i. was infected and treated for 7 days with ara-C and shifted to 39.5° for 6 days before assay.

FIG. 1. Kinetics of HSV-2 appearance early after superinfection of quiescent cultures with HCMV. HSV-2-infected cells treated with ara-C were shifted to 39.5°C after drug removal. At 12 days p.i., cultures were superinfected with HCMV at 0.2 (○) or 0.02 (●) PFU of HCMV per cell. Control cultures were superinfected with UV-irradiated HCMV (×) or mock-infected (□).

quiescent cultures. Superinfection with HCMV *ts9* (0.2 or 0.02 PFU per cell) stimulated HSV-2 production (Fig. 3). Studies showed that HCMV *ts155* also stimulated HSV-2 production (Fig. 4). The higher MOI (2 PFU of HCMV per cell) not used previously in kinetic experiments was examined in this study. Superinfection with multiplicities greater than 2 PFU per cell caused cytopathology; latently infected cultures did not survive and produce HSV-2. Although cultures superinfected with wild-type HCMV (2, 0.2, or 0.02 PFU per cell) yielded infectious HSV-2, a marked decrease in HSV-2 production was repeatedly observed at the highest MOI used. Superinfection with HCMV *ts155* induced HSV-2 at all the multiplicities studied. Superinfection at 39.5°C with two other DNA-negative HCMV *ts* mutants (*ts13* and *ts153*) produced very similar results, i.e., stimulation of HSV-2 production to levels comparable to those produced after superinfection with wild-type HCMV (data not shown). We conclude from these studies that HCMV DNA synthesis is not required for reactivation of HSV-2.

Fraction of latently infected cells induced to yield HSV-2 by HCMV superinfection. In previous studies (6) the yield of reactivated HSV-2 after HCMV superinfection was determined. To establish the number of latently infected cells that produced HSV-2 after HCMV superinfection, we superinfected HSV-2-infected cultures with HCMV and examined them by

TABLE 2. Fraction of quiescent cell population synthesizing HCMV after superinfection

Treatment ^a		
Infection	HCMV superinfection (PFU/cell)	HCMV infectious center titer ^b
Mock	0.02	5×10^{-3}
	0.2	6×10^{-2}
	2.0	1.2×10^{-1}
HSV-2	0.02	3×10^{-3}
	0.2	2×10^{-2}
	2.0	1.6×10^{-1}

^a Confluent monolayers of HEL cells were infected with HSV-2 and treated as described in the text. Control cultures were mock infected. At 11 or 19 days p.i. with HSV-2, cultures were superinfected with 2, 0.2, or 0.02 PFU of HCMV per cell. After adsorption, cells were fed medium containing 5% rabbit anti-HSV-2 serum. At 16 h after superinfection, cultures were harvested and assayed for infectious center formation in HEL cells.

^b Calculated by the formula: infectious center titer = (number of infectious centers $\times$ dilution)/total number of viable cells. Numbers represent the mean of values obtained in two separate experiments.

FIG. 2. Lack of HCMV replication at early times after HCMV superinfection. Cultures were treated as described in Fig. 1. Aliquots superinfected with 0.2 (○) or 0.02 (●) PFU of HCMV per cell were treated with rabbit anti-HSV-2 serum and assayed for the presence of infectious HCMV.

FIG. 3. Reactivation of HSV-2 after superinfection of quiescent cultures at 39.5°C with HCMV *ts9*. HSV-2-infected HEL cells treated with ara-C and shifted to 39.5°C upon ara-C removal were superinfected 10 days p.i. with HCMV *ts9* at 0.2 (○—○) or 0.02 (●—●) PFU per cell. Parallel cultures were superinfected with wild-type HCMV at 0.02 (○...○) PFU per cell. Control cultures were mock infected (□—□). The curve (---) represents the establishment of the quiescent state.

infectious center assay before virus spread. Upon HCMV superinfection (0.2 or 0.02 PFU per cell), HSV-2-infected cultures showed a dramatic increase in the number of cells that produced HSV-2, as measured by infectious center assays on PRK cells (Fig. 5). At early times after superinfection, 0.03 to 0.3% of the cell population yielded HSV-2. This value represents a minimum estimate of cells containing the HSV-2 genome before superinfection. At times post-su-

FIG. 4. Reactivation of HSV-2 after HCMV *ts155* superinfection of quiescent cultures at 39.5°C. HSV-2-infected HEL cells treated with ara-C and shifted to 39.5°C after ara-C removal were superinfected 13 days p.i. with (A) parental HCMV at 2 (●), 0.2 (○) or 0.02 (●) PFU per cell; and (B) parallel cultures were infected with 2 (●), 0.2 (○) or 0.02 (●) PFU of HCMV *ts155* per cell. Control cultures were mock infected (□).

perinfection when reactivated HSV-2 was replicating in the cultures, the fraction of HSV-2 infectious centers increased to 7 to 10% of the cells. HSV-2 infectious center titers for time points immediately after superinfection were of most interest; however, the increase in infectious center titer at 24 and 48 h, although due to the spread of newly replicated virus, was also of interest because it demonstrated that other cells in the population were not resistant to HSV-2 superinfection. Cultures treated with UV-irradiated HCMV or mock-infecting fluid produced 10^2 to 10^4 fewer infectious centers than did HCMV-superinfected cultures.

As discussed above, the number of quiescent cells that produced infectious HCMV was determined by infectious center assay (Table 2). Quiescent HSV-2-infected cultures incubated at 39.5°C were superinfected with HCMV at 11 or 19 days (two separate experiments) p.i. Sixteen hours after HCMV superinfection, the cells were assayed for HCMV infectious center formation. To avoid falsely high estimates of infected cells in the infectious center assay, a harvest time (16 h post-superinfection) before infectious HCMV production and release was chosen. Although the number of quiescent cells that produced HCMV infectious centers was dependent on the MOI, the number of HCMV infectious centers was lower than calculated for the MOIs used; the number of cells infected with HCMV was 3×10^{-3} at the lowest MOI (0.02 PFU/cell) and 1.6×10^{-1} at the highest (2 PFU/cell). Failure of ara-C-treated and latently infected HEL cells to support HCMV infection, quantitatively the same as an untreated HEL monolayer, was most

likely due to cell damage produced during ara-C treatment and virus infection.

HSV-2 RNA in HCMV-superinfected cultures. In situ hybridization was carried out to determine the time course of HSV-2 RNA appearance after HCMV superinfection and the number of cells that transcribed HSV-2 information. To establish the specificity and the sensitivity of the HSV-2 [3 H]DNA probe, we examined HSV-2- and mock-infected HEL cell control cultures. The percentage of cells positive for HSV-2 RNA was dependent upon the MOI (Table 3) and it was possible to detect HSV-2 RNA in HEL cells when very low MOIs of HSV-2 were used; at 0.01 or 0.001 PFU per cell, 1.5 and 0.5% of the cells, respectively, were positive for HSV-2 RNA. Failure of the HSV-2 [3 H]DNA probe to hybridize to mock-infected cells demonstrated the specificity of the hybridizations.

Cells positive for HSV-2 RNA were not found in latently infected cultures, indicating that either transcription did not occur in latently infected cells or the probe was not sufficiently sensitive. At 6 h after HCMV superinfection, cells containing HSV-2 RNA were not detected (Table 4). However, by 12 h a very low percentage (0.02%) of positive cells hybridized strongly with the HSV-2 [3 H]DNA probe (Fig. 6). Within 24 h after superinfection the percentage of cells positive for HSV-2 RNA increased to 6.6% (Table 4). The percentage of cells positive for HSV-

FIG. 5. Fraction of cells in HCMV-superinfected cultures that produced HSV-2 as determined by infectious center assays in PRK cells. HSV-2-infected HEL cells treated with ara-C and shifted to 39.5°C upon drug removal were superinfected 12 days p.i. with HCMV at 0.2 (○) or 0.02 (●) PFU per cell. Parallel cultures were infected with UV-irradiated HCMV (×). Control cultures were mock infected (□). At various times after superinfection, infectious center assays were performed using PRK cells as indicator cells.

2 RNA increased to 17.5% by 36 h and to 26.2% by 48 h after superinfection. Control HSV-2-infected cells treated with mock-infecting fluid at the time of superinfection did not hybridize with the HSV-2 [³H]DNA probe at the times examined. Mock-infected cultures treated with HCMV or mock-infecting fluid also showed no hybridization. It is important to note in analyzing this time course that although at 12 h after superinfection with HCMV HSV-2 was measurable by infectious center assay (Fig. 5), HSV-2 infectious virus was not detectable in titrations of lysed cultures and culture fluid (Fig. 1). The percentage of cells that expressed HSV-2 RNA 12 h after HCMV superinfection most likely represents only latently infected cells that con-

tained HSV-2 DNA before HCMV superinfection. In contrast, at later times, cells expressing HSV-2 RNA included those infected by reactivated HSV-2 as well as latently infected cells containing HSV-2 DNA before HCMV superinfection.

To estimate the percentage of cells transcribing HSV-2 information after superinfection in the absence of extracellular virus spread, we used specific antiserum. HSV-2- and mock-infected cultures treated with ara-C and shifted to 39.5°C at the time of drug removal were superinfected with HCMV or mock-infecting fluid 15 days p.i. After adsorption, medium containing 5% rabbit anti-HSV-2 serum was placed on the cells. At various times after superinfection, cells positive for HSV-2 RNA were assayed by in situ hybridization (Table 4). As in the previous findings (Table 4), a low percentage of cells (0.01%) positive for HSV-2 RNA appeared within 12 h after HCMV superinfection (Table 4; Fig. 7). The increase to 22.3% at 48 h after HCMV superinfection represents intercellular spread of HSV-2, as determined by clustering of cells positive for HSV-2 RNA. In the absence of extracellular spread and before intercellular spread of HSV-2, 1.6% of the cells demonstrated HSV-2 RNA. Once again, control cultures did not hybridize with the HSV-2 [³H]DNA probe.

The appearance of HSV-2 RNA 12 h after HCMV superinfection is a further indication that an early HCMV function induced HSV-2 reactivation. Also as indicated by in situ hybridization, the small fraction (1.6%) of the cell population that transcribed HSV-2 RNA early after

TABLE 3. *Percentage of cells positive for HSV-2 RNA in productively infected HEL cells as determined by in situ hybridization*

Treatment ^a	MOI (PFU/cell)	% Positive cells ^b
Mock		0
HSV-2	1	92.8
	0.1	8.4
	0.01	1.5
	0.001	0.5

^a Confluent monolayers of HEL cells were infected with various multiplicities of HSV-2 and harvested 8 h p.i. for in situ hybridization with HSV-2 [³H]DNA. Control cells were mock infected and assayed in parallel.

^b Results represent the average of two independently determined counts. At least 1,000 cells were counted.

TABLE 4. *Percentage of cells positive for HSV-2 RNA in latently infected cells superinfected with HCMV*

Treatment			% Positive cells ^a				
Infection	Superinfection	Antibody	6 h ^b	12 h	24 h	36 h	48 h
Mock	Mock	No antibody ^a	— ^c	—	—	—	—
	HCMV		—	—	—	—	—
HSV-2	Mock	+ Rabbit ^d anti-HSV-2 serum	—	—	—	—	—
	HCMV		—	0.02	6.6	17.5	26.2
Mock	Mock		—	—	—	ND ^e	—
	HCMV		—	—	—	ND	—
HSV-2	Mock		—	—	—	ND	—
	HCMV		—	0.01	1.6	ND	22.3

^a Confluent monolayers of HEL cells were infected with HSV-2 and treated as described in the text. Control cultures were mock infected. At 16 days p.i. with HSV-2, cultures were superinfected with 0.2 PFU of HCMV per cell or mock infected and harvested at various times after superinfection for assay by in situ hybridization with HSV-2 [³H]DNA.

^b Time after HCMV superinfection.

^c —, Negative.

^d Confluent monolayers of HEL cells were infected with HSV-2 and treated as described in the text. Control cultures were mock infected. At 15 days p.i. with HSV-2, cultures were superinfected with 0.2 PFU of HCMV per cell or mock infected. After adsorption, cells were fed medium containing 5% rabbit anti-HSV-2 serum. At various times after superinfection, cultures were harvested and assayed by in situ hybridization with HSV-2 [³H]DNA.

^e ND, Not determined.

FIG. 6. *In situ* hybridization of HSV-2 [^3H]DNA to HCMV-superinfected quiescent cultures at 12 h post-superinfection. Confluent monolayers of HEL cells were infected with HSV-2 or mock infected and treated as described in Table 2. Autoradiographs show hybridization of HSV-2 [^3H]DNA to (A) mock-infected cultures superinfected with mock-infecting fluid, (B) mock-infected cultures superinfected with HCMV, (C) HSV-2-infected cultures superinfected with mock-infecting fluid, and (D) HSV-2-infected cultures superinfected with HCMV. $\times 650$.

HCMV superinfection is an indirect estimate of the number of latently infected cells capable of being induced to replicate HSV-2. It is, of course, possible that a larger fraction of cells contained the HSV-2 genome but expressed little or no HSV-2 information.

DISCUSSION

Superinfecting with HCMV overcame the block in HSV-2 replication. It is significant that when quiescent monolayers were superinfected with HCMV (0.2 PFU/cell), 1.6% of the cells expressed HSV-2 RNA. Although this finding would indicate that almost every HCMV-infected cell can be induced to express HSV-2 RNA, such a conclusion is not plausible since only 3% of the cells in the monolayer were

initially infected with HSV-2. There are at least two alternative explanations: (i) that defective HCMV particles can induce HSV-2; and (ii) that during the establishment of the quiescent state, a larger percentage of cells obtains HSV-2 genetic information than would be expected from the initial infection. The small percentage of quiescent cells induced to transcribe HSV-2 RNA in this system was similar to values reported for neurons that transcribe HSV RNA in human sensory ganglia (0.4 to 8%; 11). It is also noteworthy that whereas 1 to 2% of quiescent cells expressed HSV-2 RNA after HCMV superinfection (0.2 PFU/cell), only 0.3% of the cells produced HSV-2 as measured by infectious center assay. That is, a higher percentage of cells can be induced to express HSV-2 information

than can be induced to replicate infectious HSV-2 particles.

The function lacking for HSV-2 replication in quiescent cultures was induced by HCMV infection,

FIG. 7. *In situ* hybridization of HSV-2 [^3H]DNA to HCMV-superinfected quiescent cultures. Confluent monolayers of HEL cells were infected with HSV-2 and treated as described in Table 4. Autoradiographs show hybridization of HSV-2 [^3H]DNA to HCMV-superinfected cells treated with rabbit anti-HSV-2 serum and harvested (A) 12 h after HCMV superinfection, (B) 24 h after HCMV superinfection, and (C) 48 h after HCMV superinfection. $\times 370$.

and the data suggest that the required HCMV function was an early or immediate early event. Approximately 50 HCMV-specific polypeptides have been identified (24). Of these, the immediate early proteins are of particular interest. Using gel electrophoresis of extract from cycloheximide-treated HCMV-infected cells, Stinski (25) has identified three immediate early polypeptides, ranging from 68,000 to 75,000 in molecular weight. Immunoprecipitation studies have established the presence of two HCMV-specific polypeptides (76,000 and 82,000 in molecular weight) early after HCMV infection (17).

Several mechanisms could account for the HCMV-dependent stimulation of HSV-2 replication. (i) HCMV infection may stimulate a cellular function required for HSV-2 replication that is lacking in the quiescent state. (ii) HCMV function(s) may stimulate HSV-2 transcription directly. (iii) Finally, HCMV function(s) may provide a function or a product necessary for HSV-2 replication. HCMV DNA shares little or no base sequence homology with HSV DNA (14); thus, HCMV-dependent stimulation of HSV-2 production at 39.5°C would be difficult to explain simply as a result of complementation between these two viruses. Nevertheless, an HCMV protein(s) which shares functional roles with an HSV-2-coded protein(s) may result in complementation.

Under natural conditions, the human host is frequently a reservoir for numerous viruses. Both HSV and HCMV are ubiquitous human pathogens. HSV-1 and HSV-2 infect 50 to 75% and 20 to 40% of the human population, respectively, and neutralizing antibody titers to HCMV have been found in 80 to 100% of adults (20). The simultaneous presence of both herpesviruses in the human host, coupled with findings described here, suggest that interaction between latent HSV-2 and HCMV could occur *in vivo*.

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LITERATURE CITED

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