

Repression and activation of the genome of herpes simplex viruses in human cells

(herpesviruses/virus-virus interaction/antiviral selectivity/virus gene regulation)

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ABSTRACT We have described previously a cell culture system in which the herpes simplex virus (HSV) type 2 (HSV-2) genome is maintained in a repressed form after treatment of infected cells with 1- β -D-arabinofuranosylcytosine and increase of incubation temperature from 37°C to 39.5°C. Infectious HSV-2 production was activated by altering incubation temperature or by superinfecting with human cytomegalovirus. We now report the establishment of an analogous system utilizing HSV type 1 (HSV-1). Human embryo lung cells were infected with HSV-1 and treated with 1- β -D-arabinofuranosylcytosine (25 μ g/ml) for 7 days to minimize both synthesis of virus DNA and infectious virus while allowing expression of early virus genes. HSV-1 was maintained in an undetectable form for at least 72 days when the incubation temperature was raised from 37°C to 40.5°C after removal of the inhibitor. HSV-1 gene expression was then predictably turned on by superinfection with human cytomegalovirus or by reducing the incubation temperature. Virus replicated after activation was compared with the respective parental virus with regard to inhibition by the HSV-1-specific antiviral (E)-5-(2-bromovinyl)-2'-deoxyuridine and *Eco*RI, *Hind*III, and *Xba* I restriction endonuclease cleavage patterns. The results show activation of HSV gene expression in human cells by a human cytomegalovirus early gene function(s), followed by synthesis of parental-like HSV.

Model systems developed *in vitro* have enabled studies concerned with the maintenance and regulation of the genome of herpes simplex virus (HSV) (1, 2). Although there is evidence for persistence and expression of at least a portion of the virus genome in these cells (2), activation of infectious virus production was not demonstrated. We have described an infection of human cells with HSV type 2 (HSV-2) after treatment for a 7-day interval with 1- β -D-arabinofuranosylcytosine (Ara-C) (3) or phosphonoacetic acid (4), in which infectious virus was not detectable for several days after Ara-C or phosphonoacetic acid removal. Studies by O'Neill (5) extended the period in which HSV-2 remained undetectable by increasing the incubation temperature from 37°C to 39.5°C after Ara-C treatment. Predictable and consistent synthesis of infectious HSV-2 has been attained by decreasing the incubation temperature (4-6) or by superinfecting with another herpesvirus, human cytomegalovirus (HCMV) (6, 7). Superinfection with four HCMV temperature-sensitive (ts) mutants unable to synthesize virus DNA at nonpermissive temperatures and from four different complementation groups (8, 9) also turned on HSV-2 production (7). In addition, *in situ* hybridization studies demonstrated HSV-2-specific RNA 12 hr after HCMV superinfection, with infectious HSV-2 detected as early as 24 hr after superinfection. These results have implicated an early HCMV-specific function in the positive regulation of HSV-2 replication.

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We now report establishment of a similar system with HSV type 1 (HSV-1). As in the analogous HSV-2 system (3, 5-7), HCMV superinfection resulted in activation of HSV-1 replication. Activated viruses have been characterized with respect to sensitivity to inhibition by the HSV-1-specific antiviral agent, (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVdUrd) (10, 11) and genome structure, as determined by restriction endonuclease cleavage analysis. This *in vitro* system, utilizing Ara-C and increased incubation temperature to repress and HCMV superinfection to activate synthesis of infectious HSV-1 or HSV-2, may facilitate studies concerned with regulation of HSV gene expression in human cells.

MATERIALS AND METHODS

Cells. Monolayer cultures of human embryo lung (HEL), Flow 5000 (Flow Laboratories, McLean, VA), and primary rabbit kidney (PRK) cells were grown and maintained as described (3, 6, 7).

Viruses. HSV-1 (strain Patton or KOS) and HSV-2 (strain 186) stocks were obtained by replication in HEL cells as described (3, 6, 7). HCMV (strain Towne) provided by M. Stinski (University of Iowa, Iowa City, IA) and HCMV (strain AD169) purchased from the American Type Culture Collection were grown in Flow 5000 cells, and stocks were prepared as described (3, 6, 7). HEL and Flow 5000 mock infecting fluids were prepared from uninfected HEL and Flow 5000 cells.

Infectious Virus Determination. Infectious HSV and HCMV were quantitated by plaque assay on confluent PRK and Flow 5000 cell cultures, respectively (12, 13).

Plaque Reduction Determination. Confluent PRK cell cultures were infected with a predetermined quantity of virus. The virus was allowed to adsorb for 1 hr; the cultures were then overlaid with a 0.5% methylcellulose nutrient medium supplemented with BVdUrd (provided by E. De Clercq, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium). Infected cells were incubated at 37°C, and plaques were enumerated as described (6, 7, 12).

Establishment and Maintenance of HSV in Repressed Form. The establishment and maintenance of HSV-2 in HEL cells in an undetectable form were carried out as described (6, 7). Infection of HEL cell cultures with HSV-1 (strain Patton or KOS) was accomplished in a manner similar to that described for HSV-2 (6, 7). Unless otherwise indicated, confluent HEL monolayer cell cultures were pretreated with 25 μ g of Ara-C (Sigma) per ml for 5 hr, infected with 0.05-0.1 plaque-forming

Abbreviations: HSV, herpes simplex virus; HSV-1, HSV-2, HSV types 1 and 2; Ara-C, 1- β -D-arabinofuranosylcytosine; ts, temperature sensitive; BVdUrd, (E)-5-(2-bromovinyl)-2'-deoxyuridine; HEL, human embryo lung; PRK, primary rabbit kidney; HCMV, human cytomegalovirus; PFU, plaque-forming unit(s).

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unit (PFU) of HSV-1 per cell, and treated every 24 hr with maintenance medium containing 25 μg of Ara-C per ml for 7 days to establish repressed HSV-1 infection. Cultures were then washed twice with Tris-buffered saline (pH 7.4), maintenance medium without Ara-C was added, and the incubation temperature was increased from 37°C to 40.5°C to maintain repressed HSV-1 infection. In addition, cultures were prepared by pretreatment of HEL cells with maintenance medium containing 25 μg of Ara-C per ml, mock infection with fluid prepared from HEL cells, and treatment with Ara-C (25 $\mu\text{g}/\text{ml}$) for 7 days before the shift from 37°C to 39.5°C or 40.5°C. Such cultures were referred to as repressed mock-infected cultures.

HCMV-Mediated Activation of HSV Synthesis. HSV-1- or HSV-2-infected cultures repressed by Ara-C treatment and temperature increase were superinfected at several times after Ara-C removal with 0.2 PFU of HCMV (AD169 or Towne) per cell or mock-infected with fluid prepared from uninfected Flow 5000 cells. After adsorption for 1 hr, superinfected cultures were overlaid with maintenance medium and incubated at 40.5°C until harvest and determination of infectious virus.

Purification of HSV Virion DNA. Parental HSV-1 (Patton) and HSV-2 (186) DNAs were obtained by infection of HEL cultures at 37°C and repressed mock-infected cultures at 40.5°C and 39.5°C, respectively, with 0.2 PFU of either virus per cell. Activated virion DNA was obtained by superinfecting repressed HSV-1- and HSV-2-infected cultures maintained at 40.5°C and 39.5°C, respectively, with 0.2 PFU of HCMV (AD169) per cell after maintenance at elevated temperature for 14 days. After adsorption for 1 hr, infected cells were overlaid with maintenance medium containing 10 μCi ($1 \text{ Ci} = 3.7 \times 10^{10}$ becquerels) of [^3H]thymidine (80.1 Ci/mmol, New England Nuclear) per ml. Infected cell cultures were harvested at 24–60 hr after infection or superinfection. Intracellular virions were obtained by sonic cell disruption and treatment with 0.5% Nonidet P-40 and 0.5% sodium deoxycholate (7). Extracellular virions were obtained by precipitation with 1.0 M zinc acetate and treatment with 0.5% sodium deoxycholate and 0.5% Nonidet P-40 as described for purification of rabies virus (14) and recently modified for use with HSV (ref. 7; M. K. Howett, personal communication). The detergent-treated intracellular and extracellular preparations were combined, and virus was purified by centrifugation to the interface of a step CsCl gradient (1.2–1.4 g/ml) at $82,000 \times g$ for 2.5 hr at 10°C in a Beckman SW27 rotor. The virus band was removed, dialyzed against 0.01 M Tris·HCl/0.001 M EDTA, pH 7.8, and digested with 2 mg of proteinase K per ml (EM Biochemicals, Elmsford, NY) in 0.5% NaDodSO₄ for 2 hr at 37°C. Yeast tRNA (Schwarz/Mann) was added to 50 $\mu\text{g}/\text{ml}$ and the nucleic acid was precipitated and resuspended in Tris/EDTA, placed in CsCl (initial average density of 1.70 g/ml), and centrifuged to equilibrium at $87,000 \times g$ for 72 hr at 20°C. Fractions containing HSV [^3H]DNA, as determined by density in CsCl, were pooled, dialyzed against Tris/EDTA buffer, extracted twice with an equal volume of Tris/EDTA-saturated redistilled phenol/chloroform/isoamyl alcohol, 25:25:1 (vol/vol), precipitated, and resuspended in Tris/EDTA buffer.

Digestion with Restriction Endonucleases. For digestion with restriction endonuclease *EcoRI*, 0.2–0.1 μg (20,000–40,000 cpm) of purified virus DNA was incubated twice for 3 hr at 37°C in 20 μl of 50 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM MgSO₄/0.1 mM EDTA/2 mM 2-mercaptoethanol/10 units (total) of *EcoRI* (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The digestion was terminated by the addition of 5 μl of 0.25 M Tris·HCl, pH 8.0/30% (wt/vol) Ficoll/0.5% NaDodSO₄/60 mM EDTA/0.2% bromphenol blue, heated for 3 min at 70°C, and immediately placed on ice. Digestion with 10 units of *HindIII* (Boehringer Mannheim) and 18 units of *Xba*

I (Bethesda Research Laboratories, Rockville, MD) was accomplished in a similar manner under the conditions suggested by the manufacturer.

Electrophoretic Resolution. Digested DNA was subjected to electrophoresis through a submerged 0.5% agarose (Bio-Rad), horizontal slab gel at 3.1 V/cm for 13–22 hr in a 40 mM Tris·HCl, pH 7.8/5 mM sodium acetate/1 mM EDTA running buffer. The DNA fragments were visualized by fluorography with sodium salicylate (Mallinckrodt) as described for enhancement for polyacrylamide gels (15) and modified for agarose gels (16). For molecular weight markers, ^3H -labeled phage λ cI857 DNA digested with *EcoRI* or *HindIII* was used.

RESULTS

Establishment and Maintenance of HSV-1 in a Repressed Form in HEL Cells and Activation by HCMV Superinfection. Restricted HSV-1 gene expression has been studied in cell culture systems (1, 2, 17); therefore, we wanted to determine whether a repressed HSV-1 infection of HEL cells analogous to that previously reported with HSV-2 (3, 5–7) could be established. HEL cell cultures were pretreated with Ara-C, infected with HSV-1 (Patton) at multiplicities of infection ranging from 0.01 to 0.5 PFU per cell, and treated for 7 days with Ara-C. When cultures were infected with more than 0.1 PFU per

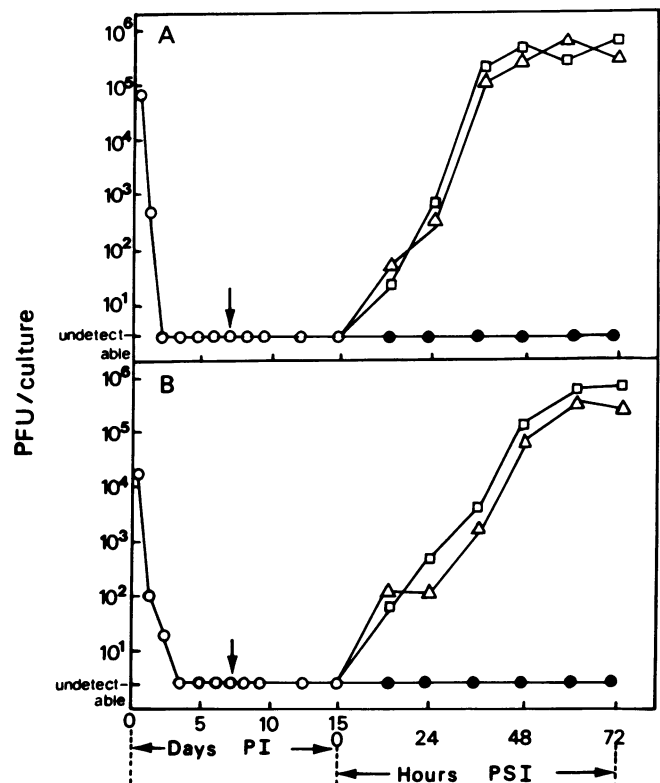


FIG. 1. Establishment and maintenance of repression interval and HCMV-mediated activation. HEL cell cultures were pretreated with Ara-C, infected with HSV-1 (A) or HSV-2 (B), and shifted to the higher temperature after a 7-day Ara-C treatment interval. Arrow, day-7 Ara-C removal; PI, time after infection; PSI, time after superinfection. At 15 days PI, HSV-1- (A) or HSV-2-infected (B) cultures maintained in the repressed interval were superinfected with HCMV strain AD169 (Δ) or strain Towne (\square) or mock-superinfected (\bullet), maintained at 40.5°C, and subsequently assayed for infectious virus at the indicated time PSI. Mock infecting fluid was prepared from uninfected Flow 5000 cells in a manner similar to preparation of HCMV stocks. \circ , Cultures assayed for infectious virus during the Ara-C treatment interval and after temperature shift to either 40.5°C (A) or 39.5°C (B).

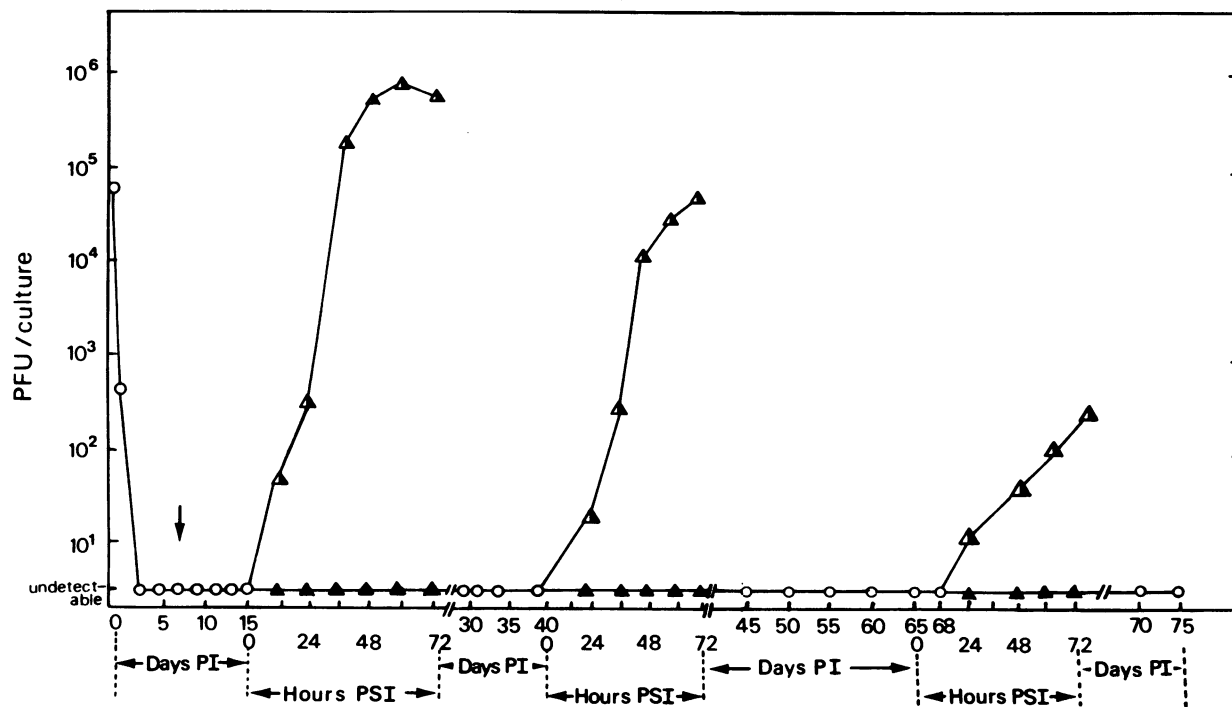


FIG. 2. HCMV-mediated activation of the HSV-1 system after increasing the repression time interval. HEL cell cultures were pretreated with Ara-C, infected with HSV-1, and shifted to 40.5°C after a 7-day Ara-C treatment interval. Arrow, day-7 Ara-C removal; PI, time after infection; PSI, time after superinfection. At the indicated times PI, parallel HSV-1-infected HEL cultures maintained in the repression interval were superinfected with HCMV (AD169) at 15 days (Δ), 38 days (Δ), or 68 days (Δ) or were mock infected (Δ), maintained at 40.5°C and subsequently assayed for infectious virus at the indicated times PSI. Mock infecting fluid was prepared from uninfected Flow 5000 cells in a manner similar to the preparation of HCMV stocks. Cultures were also assayed for infectious virus during the Ara-C treatment interval and after the temperature shift to 40.5°C (○).

cell and treated with Ara-C (25 μg/ml), surviving cell fractions after 7 days of drug treatment were reduced to unacceptable levels (50% to >90% cell death). To maintain drug-induced cytotoxicity at a minimum level, attain greater than 80% cell survival after HSV-1 infection, and maximize the fraction of cells retaining virus genetic information, Ara-C-pretreated HEL cultures were infected with 0.1 PFU of HSV-1 per cell and treated with 25 μg of Ara-C per ml. Under these conditions, infectious virus generally became undetectable by plaque assay 3–5 days after infection. These cultures were then shifted from 37°C to 39.5°C after Ara-C removal. Infectious virus remained undetectable for 10–20 days, after which infectious virus reappeared and cell destruction ensued. Because HSV-1 has been shown to be more thermally stable than HSV-2 (18, 19), it was theorized that increasing the incubation temperature to more than 39.5°C might extend the repression interval. When Ara-C-treated HSV-1-infected cultures were shifted from 37°C to 40.5°C after drug removal (Figs. 1 and 2), infectious virus remained undetectable for several months. At any time during the repression interval, infectious virus could be reactivated by decreasing the incubation temperature from 40.5°C to 37°C. In addition, decreasing incubation temperature to 39.5°C also resulted in infectious virus production; however, virus reappearance and culture destruction were slower than after the decrease to 37°C. Studies with another HSV-1 isolate (KOS) yielded similar results (data not shown).

Reports (6, 7) have demonstrated that superinfection of HEL cultures harboring HSV-2 in repressed form with 0.2 PFU of HCMV per cell activates synthesis of infectious HSV-2 (Fig. 1B). Because HSV-1 and HSV-2 are partially homologous viruses (20, 21), it was of particular interest to establish whether HCMV superinfection could activate virus replication in HEL

cells maintaining repressed HSV-1 genetic information (Fig. 1A). Kinetics of virus replication after HCMV superinfection of either repressed HSV-1 (Fig. 1A) or HSV-2 (Fig. 1B) cultures

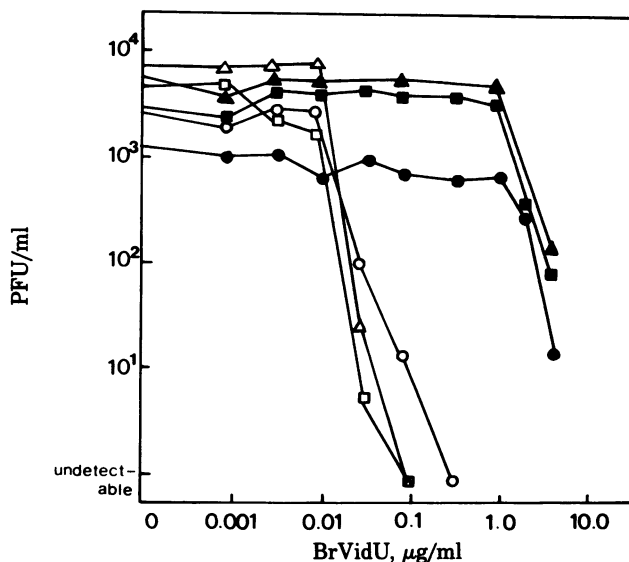


FIG. 3. Effect of BVdUrd on HSV plaque formation in PRK cell cultures. Confluent PRK cell cultures were infected with each virus type, overlaid with a methylcellulose nutrient medium containing BVdUrd, and incubated at 37°C. □, Parental HSV-1; ■, parental HSV-2; ○, parental HSV-1 replicated in repressed mock-infected HEL cultures; ●, parental HSV-2 replicated in repressed mock-infected HEL cultures; Δ and ▲, virus replicated after HCMV-mediated activation of repressed HSV-1 or HSV-2 cultures, respectively.

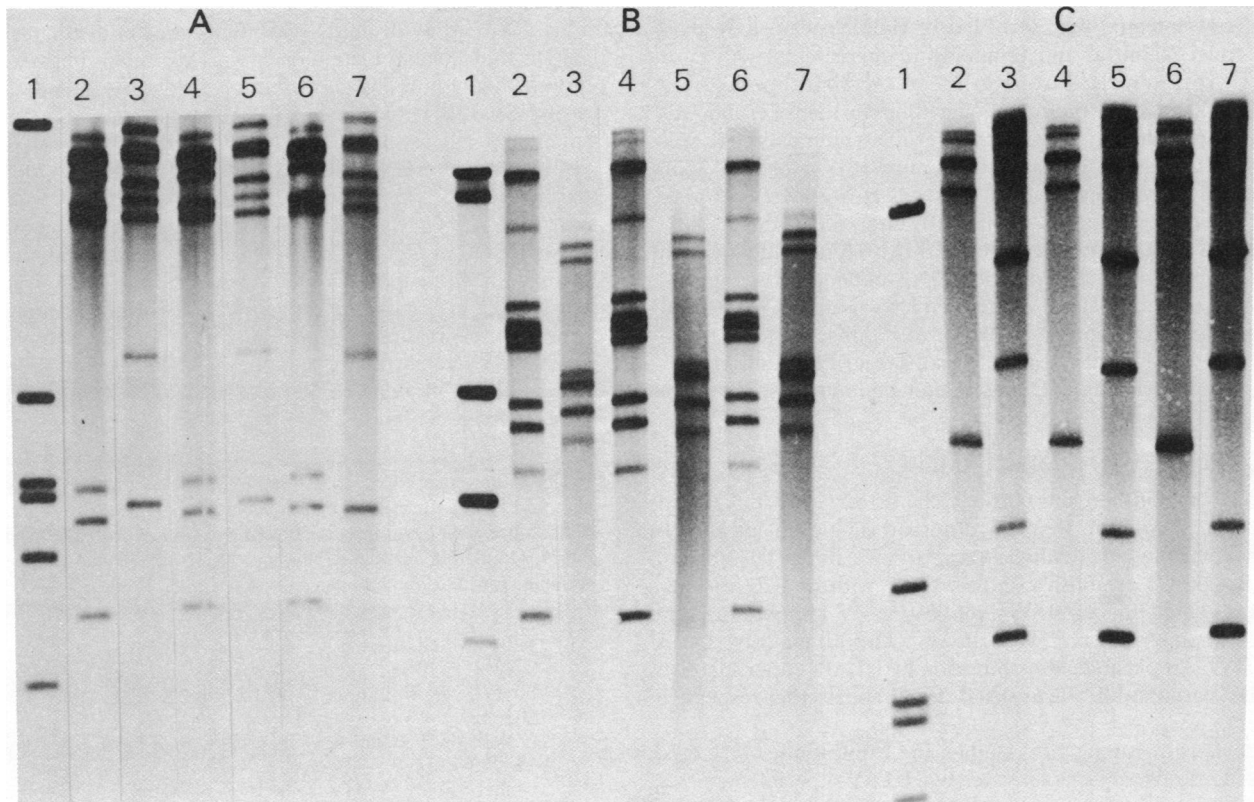


FIG. 4. Restriction endonuclease analysis of virus DNA purified from virus replicated after HCMV-mediated activation. HSV-infected cultures maintained in repression interval were superinfected with HCMV and radiolabeled, and virus was harvested after 50 hr. Virion DNA was purified by CsCl equilibrium centrifugation, digested with restriction endonuclease, and subjected to electrophoresis through 0.5% agarose. Fragments were visualized by sodium salicylate-enhanced fluorography: Lanes: 1, phage λ DNA; 2, parental HSV-1 DNA; 3, parental HSV-2 DNA; 4, DNA obtained from parental HSV-1 replicated in repressed mock-infected HEL cultures; 5, DNA obtained from parental HSV-2 replicated in repressed mock-infected HEL cultures; 6 and 7, virus DNA purified from virus replicated after HCMV-mediated activation of either the repressed HSV-1 or HSV-2 cultures, respectively. (A) *EcoRI* digestion. (B) *HindIII* digestion. (C) *Xba I* digestion, except phage λ DNA was digested with *EcoRI*.

were very similar. There was no significant difference in the production of infectious virus in either system after superinfection with either HCMV (AD169) or (Towne). Mock superinfection with fluid prepared from Flow 5000 cells did not result in activation of infectious virus.

Effect of Repression Time Interval on HCMV-Mediated Activation. The effect of length of repression interval on the capability of HCMV to activate virus synthesis was studied by superinfecting with HCMV at increasing times of incubation at 40.5°C (Fig. 2). The time at which infectious HSV-1 was first detected after HCMV superinfection was unaffected by the length of incubation of repressed cultures at 40.5°C (which included time intervals up to 61 days after Ara-C removal). However, in general, maximum infectious virus production after HCMV superinfection declined with increasing time of incubation at 40.5°C. The decrease in maximum virus production is most likely because of a deterioration in the ability of repressed cultures to support virus replication after maintenance of cultures for extended periods of time at 40.5°C rather than an actual loss in HSV genetic information. Results from two studies support this hypothesis. (i) Although infectious center titers, as determined on PRK cells, fluctuated to some degree and demonstrated that only a small fraction of the cells contains HSV genetic information (0.0001–0.01%), there was no significant downward trend with increasing time of repression interval. (ii) Yields of infectious HSV-1 replicated in repressed mock-infected HEL cultures were progressively lower the longer the cultures were incubated at the elevated temperature prior to superinfection (data not shown).

Characterization of Virus Replicated Subsequent to HCMV-Mediated Activation. Two different techniques, sensitivity to inhibition by the HSV-1 strain-specific agent BVdUrd and restriction endonuclease cleavage analysis, were used (i) to extend previous immunological studies (6) which suggested that virus replicated after HCMV superinfection of the repressed HSV-2 system was parental-like input HSV-2, (ii) to show that virus replicated after HCMV superinfection of repressed HSV-1 system was parental-like input HSV-1, and (iii) to compare virus reactivated by HCMV in either repressed HSV-1 or HSV-2 cultures with the respective parental virus used to establish the system. BVdUrd has been shown to inhibit the replication of HSV-1 at concentrations $1/100$ th to $1/1000$ th those required to inhibit either HSV-2, HSV-1 deficient in thymidine kinase, or normal cell metabolism (10, 11). To confirm these results with HSV-1 (Patton) and HSV-2 (186), plaque reduction assays in the presence of BVdUrd were carried out (Fig. 3). At 0.1 μg of BVdUrd per ml, a concentration at which HSV-2 plaque production was only minimally inhibited, HSV-1 plaque production was reduced by more than 3 logarithms. In addition, the same strains of HSV-1 and HSV-2 that were previously replicated in repressed mock-infection HEL cultures demonstrated BVdUrd sensitivity similar to that of the parental viruses. Virus replicated after HCMV activation of HSV-1 or HSV-2 responded in a parental-like fashion to BVdUrd exposure. These results suggest that viruses activated by HCMV superinfection maintained parental-like, virus-coded thymidine kinase activity.

To further confirm the identity of the activated viruses, restriction endonuclease cleavage analysis was utilized (Fig. 4).

HEL cultures harboring HSV-1 or HSV-2 in repressed form were superinfected with 0.2 PFU of HCMV per cell 14 days after Ara-C removal and temperature increase to 40.5°C or 39.5°C, respectively. Virus was harvested from superinfected cultures 50 hr after superinfection without further passage. This enabled examination of activated virus DNA after only 3–5 virus replication cycles. Replication of parental HSV-1 and HSV-2 in repressed mock-infected HEL cultures at the increased temperatures did not result in significantly altered *EcoRI* (Fig. 4A), *HindIII* (Fig. 4B), or *Xba I* (Fig. 4C) restriction endonuclease cleavage profiles. Purified virion DNA obtained from viruses isolated from either HCMV-activated HSV-1 or HSV-2 retained the parental-like genome structure of input virus used to establish the system with respect to *EcoRI* (Fig. 4A), *HindIII* (Fig. 4B), and *Xba I* (Fig. 4C) restriction endonuclease cleavage patterns.

DISCUSSION

We have established and partially characterized (3, 6, 7) an *in vitro* system wherein HSV-2 synthesis was blocked and the virus genome was maintained in a repressed form. The studies in this report (i) establish a similar system with HSV-1, (ii) demonstrate activation of HSV-1 replication by temperature decrease or superinfection with HCMV, and (iii) identify HSV-1 and HSV-2 replicated after activation by HCMV superinfection as the parental-like virus used to establish the respective system.

Previous immunological studies and replication characteristics suggested that virus activated by HCMV superinfection was parental-like virus (6); two additional criteria have now been used to extend these results. Plaque reduction assays in the presence of BVdUrd, (Fig. 3) showed that virus replicated after HCMV activation of either HSV-1 or HSV-2 responds in a manner analogous to the parental virus. Because BVdUrd antiviral activity and selectivity are most likely dependent on phosphorylation by HSV-induced thymidine kinase (12, 13, 22), these results suggest that the coding region for HSV thymidine kinase has been retained by the virus after the repression interval and activation process. The restriction endonuclease cleavage analysis also confirmed that the virus replicated after HCMV-mediated activation was similar to parental virus. In addition, the similarity between reactivated virus and parental virus DNA restriction endonuclease cleavage sites suggests that the DNA is not extensively altered during repression. However, if alterations do occur, they are either undetectable by the resolution of the technique or the restriction endonucleases employed in this study or are reversible and are, therefore, not detectable in purified reactivated virus DNA.

Previous studies have attempted to utilize HCMV ts mutants to delineate the HCMV-specific gene function(s) involved in the activation of HSV-2 (7). Although these studies have shown that HCMV DNA synthesis is not required for activation of synthesis of infectious virus (suggesting direct or indirect involvement of an early or immediate-early HCMV function in the reactivation), the limited availability of numerous well-characterized HCMV ts mutants necessitates the development of alternative approaches. Unpublished preliminary results with HSV-2 (18) and HSV-1 (19) ts mutants have suggested that HSV-2 or HSV-1, respectively, may activate virus replication in repressed HSV-1 or HSV-2 cultures. Because BVdUrd is a highly strain-specific nucleoside analog (10, 11) and was shown to inhibit ac-

tivated virus like parental input virus (Fig. 3), it may be possible to use BVdUrd as an initial screening agent to distinguish activation from recombination or complementation between superinfecting ts mutants and the repressed virus genome. The use of HSV-1 (19) and HSV-2 (18) ts mutants deficient in specific gene functions may be useful in characterizing the block in the lytic interaction of HSV with the host cell. It could be postulated that because the repressed cultures were established by treatment with Ara-C, they are blocked at some point in the expression of HSV α or β genes (23, 24). An early gene product(s) synthesized by the superinfecting virus, either HCMV or HSV, may be able to circumvent the block and activate the repressed virus gene expression. A function(s) necessary for synthesis of infectious virus may be directly provided by the superinfecting virus or the interaction could be at the level of transcription of the repressed genome.

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