ga menziesii, Abies concolor, and *Pinus ponderosa* are reported in this study.
9. A. M. Phillips, thesis, University of Arizona (1977); J. I. Mead and A. M. Phillips, *Southwest. Nat.* **26**, 257 (1981).
10. R. A. Watson and H. E. Wright, *Boreas* **9**, 153 (1980).
11. H. E. Wright *et al.*, *Geol. Soc. Am. Bull.* **84**, 1155 (1971); P. S. Martin and P. Mehringer, Jr., in *The Quaternary of the United States*, H. E. Wright, Jr., and D. G. Frey, Eds. (Princeton Univ. Press, Princeton, N.J., 1965); P. J. Mehringer, *Nev. State Mus. Anthropol. Pap. 13* (1967), pp. 129–200.
12. R. H. Whittaker, *Biol. Rev. Biol. Proc. Cambridge Philos. Soc.* **42**, 207 (1967); R. H. Whittaker and W. A. Niering, *Ecology* **46**, 4 (1965).
13. W. D. Billings, *Am. Midl. Nat.* **42**, 87 (1949); A. Cronquist, *Great Basin Nat. Mem. 2* (1972); M. G. Lepper, thesis, University of California, Davis (1974); J. C. Beatley, *Am. Midl. Nat.* **93**, 53 (1975).
14. The base level for most mountain ranges in northern Utah is above 1500 m.
15. V. L. Mitchell, *J. Appl. Meteorol.* **15**, 920 (1976).
16. W. G. Spaulding, thesis, University of Arizona (1981); R. S. Thompson and J. I. Mead, *Quat. Res. (N.Y.)* **17**, 39 (1982); P. V. Wells, *Bull. Ecol. Soc. Am.* **61**, 106 (1980).
17. The Sonoran and Mojave deserts are mostly below 1300 m, and the Great Basin Desert and Colorado Plateau are mostly above 1500 m. Also the Sierra Nevadan rain shadow is present from 36° to 39° latitude.
18. J. E. Kutzbach, *Science* **214**, 59 (1981).
19. L. V. Benson, *Quat. Res. (N.Y.)* **9**, 300 (1978).
20. The retention of the Wisconsin soil layer and the stability of climax communities may also have retarded species migration upslope following the Wisconsin period.
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High Efficiency Latency and Activation of Herpes Simplex Virus in Human Cells

Abstract. *Herpes simplex virus (HSV) exists in humans in a latent form that can be activated. To characterize the molecular basis of the cell-virus interactions and to analyze the state of the latent HSV genome, an in vitro model system was established. In this system a large fraction of the latently infected cells contain an HSV genome that can be activated. Cell survival was reduced minimally after repression of high multiplicity HSV type 1 (HSV-1) infection of human fibroblast cells with (E)-5-(2-bromovinyl)-2'-deoxyuridine in combination with human leukocyte interferon (IFN-α). A minimum of 1 to 3 percent of the surviving cells contained an HSV genome that could be activated either by human cytomegalovirus superinfection or reduction in incubation temperature.*

Herpes simplex virus (HSV) can be harbored in animals and humans without signs of clinical disease (*1*). The establishment and maintenance of the latent state, the latent form of the HSV genome, and the mechanism of reactivation are foci of current research. In addition to in vivo studies of HSV latency (*1*), in vitro model systems have made it possible to study the maintenance and expression of virus genetic information in latent HSV infections (*2, 3*). We previously described infection of human embryo lung (HEL) fibroblast cells with either HSV type 1 (HSV-1) or type 2 (HSV-2) wherein virus replication was blocked by treatment of infected cultures for 7 days with arabinosyl cytosine (ara-C); (*4–6*). Infectious virus was maintained in a repressed form by elevating the incubation temperature after removal of ara-C from 37° to 39.5°C for HSV-2 (*7*) or to 40.5°C for HSV-1 (*8*). Replication of HSV-1 or HSV-2 was activated by reducing incubation temperature, or by superinfecting with human cytomegalovirus (HCMV) (*5, 6, 8*) or HSV temperature-sensitive mutants (*9*).

Repressed HSV infections were established by infection of HEL cells with low levels [0.01 to 0.2 plaque-forming unit (PFU) per cell] of virus followed by treatment with ara-C and an increase in temperature. Assays of infectious centers were performed on ara-C–treated, HSV-2–infected cells at several times after removal of inhibitor (6 to 15 days); these assays indicated that less than 0.0002 to 0.02 percent of the cells contained a virus genome that could be activated.

To characterize the state of the virus genome and the virus activation process, it was essential to increase the number of surviving cells containing a virus genome that could be activated. Inhibitors of DNA synthesis interfere with expression of late (γ) genes of HSV and at least a subset of HSV early (β) genes, while presumably allowing complete expression of the immediate early (α) genes. Reports that human interferon blocks the deleterious effects of HSV on human chromosomes (*10*) and that interferon of fibroblast origin in combination with acyclovir inhibits HSV-1 or HSV-2 replication in an additive to synergistic manner (*11, 12*), prompted the use of human leukocyte interferon (IFN-α) in combination with the relatively noncytotoxic compound (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) (*13*) to obtain a significant increase in surviving cells after infection with an increased multiplicity of HSV-1.

HEL cells were first treated for 24 hours with BVDU and IFN-α and infected with HSV-1 (Fig. 1). HSV-1 was not detected after the 7-day inhibitor treatment or after incubation at 40.5°C for 8 days. Infectious virus (determined by plaque assay on primary rabbit kidney cells) was not detectable in control uninfected HEL cells treated with BVDU plus IFN-α for analogous time intervals. Surviving cells obtained after BVDU plus IFN-α treatment have been maintained for at least 50 days at 40.5°C without detection of infectious virus. Virus can be activated at this time by

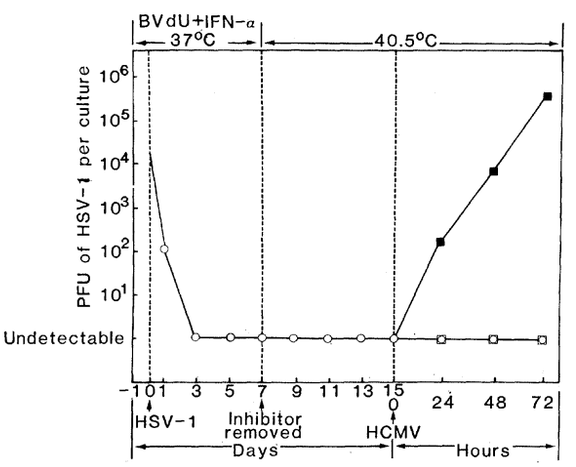

Fig. 1. Establishment and maintenance of repression interval and HCMV-mediated activation. Confluent HEL cell cultures were first treated with BVDU (10 μg/ml) and IFN-α (125 IU/ml) for 24 hours, infected with HSV-1 (2.5 PFU/cell); they were then treated at 24-hour intervals with maintenance medium containing BVDU (10 μg/ml) and IFN-α (125 IU/ml) for 7 days. The cultures were then washed twice with tris-buffered saline (*p*H 7.4); maintenance medium without inhibitors was added, and cultures were shifted from 37° to 40.5°C. Maintenance medium without inhibitors was changed every third day at the elevated temperature. Cultures were assayed for infectious virus during the treatment with BVDU plus IFN-α and after the inhibitor was removed and the temperature was increased (○). BVDU plus IFN-α-repressed, HSV-1–infected cells maintained at 40.5°C for 8 days, were superinfected with 1.0 PFU of HCMV (■) per cell or treated with mock-infecting fluid (□) prepared from uninfected (Flow 5000) cells. After adsorption, superinfected cultures were overlaid with maintenance medium at 40.5°C until the indicated harvest time and infectious virus was determined. All cultures were harvested and assayed for infectious virus by plaque assay in primary rabbit kidney cells (*5*).

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temperature reduction or HCMV superinfection (data not shown).

To quantify survival after infection of cells that had been treated with BVDU and IFN-α at 0.1 to 2.5 PFU per cell, the viable cell number and percent viability were determined after the 7-day treatment with the inhibitor and subsequent incubation for 8 days at 40.5°C. The viable number of BVDU plus IFN-α–treated cells (determined by trypan blue dye exclusion) obtained after infection with 2.5 PFU per cell was 76 percent that of uninfected, BVDU plus IFN-α–treated controls and 72 percent that of uninfected, untreated controls. Use of multiplicities higher than 2.5 PFU per cell diminished survival. Studies with BVDU (10 μg/ml) alone, ara-C (25 μg/ml) alone, IFN-α (125 IU/ml) alone, or ara-C (25 μg/ml) in combination with IFN-α (125 IU/ml) resulted in 10 percent or less cell survival at lower multiplicities (1.0 PFU per cell; data not shown). These data suggest that BVDU in combination with IFN-α may act synergistically to inhibit HSV-1 replication.

To determine whether BVDU plus IFN-α treatment of cells infected with a high multiplicity of HSV-1 resulted in elimination of virus from the surviving cells or maintenance of the virus in a repressed form that can be activated, BVDU plus IFN-α–treated cells were either superinfected with HCMV or shifted from 40.5° to 37°C. The HSV-1 was activated by superinfection with HCMV (Fig. 1) and by lowering the incubation temperature from 40.5° to 37°C (data not shown). Treatment with mock-infecting fluid did not activate HSV-1. Virus activated from BVDU plus IFN-α–treated cells was shown to be parental-like HSV-1 by analysis of purified virus DNA with Eco RI, Hind III, and Xba I restriction endonucleases (data not shown).

Cell death in HCMV-superinfected BVDU plus IFN-α–treated cell cultures infected with 2.5 PFU of HSV-1 per cell was more apparent 24 hours after superinfection than that of superinfected cells infected with a low multiplicity of HSV-1. This suggested that an increased number of surviving cells containing an HSV genome was attained after high multiplicity infection. To investigate this possibility, the infectious center titer of BVDU plus IFN-α–treated HSV-1–infected cells was determined before and shortly after HCMV superinfection (Table 1). The number of infectious centers formed by BVDU plus IFN-α–treated, HSV-1–infected cells increased in direct correlation with the multiplicity of infection used. After infection with 2.5 PFU of HSV-1 per cell, approximately 1 in 100 BVDU plus IFN-α–treated cells contained a virus genome that could be activated (Table 1). This represented at least a 20-fold increase over that obtained after infection with 0.1 PFU per cell.

As demonstrated with BVDU plus IFN-α–treated, HSV-1–infected cells before superinfection, the infectious center titer of HCMV-superinfected cells also increased in direct correlation with the multiplicity of infection (Table 1). Values of 3 and 2 percent were attained by infectious center assays performed at 37° and 40.5°C, respectively, in superinfected cells initially infected with 2.5 PFU of HSV-1 per cell (Table 1). These values are slightly higher than those obtained with BVDU plus IFN-α–treated, HSV-1–infected cells that had not been superinfected with HCMV. It is not known whether HCMV superinfection resulted in activation of a subpopulation of cells not activated by temperature reduction, or in an increase in the efficiency of activation of HSV-1 by temperature reduction. The infectious center titer may represent the minimum number of cells that contain an HSV-1 genome since the BVDU plus IFN-α–treated cell population may include cells with an HSV-1 genome that cannot be activated.

Results with indirect immunofluorescence techniques have indicated that 10 hours after HCMV superinfection (1.0 PFU per cell), approximately 10 percent of the surviving cells are positive for HSV-1 antigens. To characterize this system in greater detail, the BVDU plus IFN-α–treated cells are being analyzed by nucleic acid hybridization with molecularly cloned HSV-1 DNA labeled to high specific activity as a probe. Results have demonstrated the presence of HSV-1 DNA in the latent cell populations. It should now be possible to analyze the state of the virus genome during establishment and maintenance of the quiescent phase and during reactivation of virus synthesis.

Table 1. Infectious center titer of BVDU plus IFN-α–treated HSV-1–infected cells. UD, undetected.

HSV-1* (PFU per cell)	Superinfection	Infectious centers (No. per 10^5 cells)	
		37°C	40.5°C
0	None†	UD	UD
	HCMV‡	UD	UD
	Mock§	UD	UD
0.1	None	54	UD
	HCMV	79	30
	Mock	52	UD
0.5	None	78	UD
	HCMV	310	200
	Mock	70	UD
1.0	None	300	UD
	HCMV	1100	310
	Mock	480	UD
2.0	None	660	UD
	HCMV	880	520
	Mock	600	UD
2.5	None	1220	UD
	HCMV	3000	2000
	Mock	1500	UD

*HEL cells were first treated with BVDU (10 μg/ml) and IFN-α (125 IU/ml; 6.5×10^5 IU per milligram of protein), mock-infected or infected with HSV-1 (strain Patton) at the indicated multiplicities of infection, treated with BVDU and IFN-α at the same concentrations for 7 days at 37°C, and incubated for 8 days at 40.5°C after removal of inhibitors on day 7. †After the 8-day incubation at 40.5°C, the cultures were harvested by treatment with Versene and trypsin, and infectious center assays were performed in Vero cells at either 37° or 40.5°C. ‡After the 8-day incubation at 40.5°C, cultures were superinfected with HCMV (strain AD169; 1.0 PFU per cell), overlaid with maintenance medium containing 1 percent rabbit antiserum to HSV-1, and harvested by treatment with Versene and trypsin after 3 hours and infectious center assays were performed in Vero cells at either 37° or 40.5°C. §After the 8-day incubation at 40.5°C, cultures were treated with mock-infecting fluid and harvested and assayed as described for HCMV superinfected cultures.

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References and Notes

1. J. G. Stevens, in *Oncogenic Herpesviruses*, F. Rapp, Ed. (CRC Press, Boca Raton, Fla., 1980), pp. 1–11.
2. J. C. Gerdes, H. S. Marsden, M. L. Cook, J. G. Stevens, *Virology* **94**, 430 (1979).
3. M. Levine, A. L. Goldin, J. C. Glorioso, *J. Virol.* **35**, 203 (1980).
4. F. J. O'Neill, R. J. Goldberg, F. Rapp, *J. Gen. Virol.* **14**, 189 (1972).
5. A. M. Colberg-Poley, H. C. Isom, F. Rapp, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5948 (1979).
6. ———, *J. Virol.* **37**, 1051 (1981).
7. F. J. O'Neill, *ibid.* **24**, 41 (1977).
8. B. L. Wigdahl, H. C. Isom, F. Rapp, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6522 (1981).
9. B. L. Wigdahl, H. C. Isom, E. DeClercq, F. Rapp, *Virology* **116**, 468 (1982).
10. F. J. O'Neill and F. Rapp, *ibid.* **44**, 544 (1971); *J. Virol.* 7, 692 (1971).
11. T. L. Stanwick, R. F. Schinazi, D. E. Campbell, A. J. Nahmias, *Antimicrob. Agents Chemother.* **19**, 672 (1981).
12. M. J. Levine and P. L. Leary, *Infect. Immun.* **32**, 995 (1981).
13. E. De Clercq, J. Descamps, P. DeSomer, P. J. Barr, A. S. Jones, R. T. Walker, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2947 (1979).

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