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Reactivation of Latent Herpes Simplex Virus by Adenovirus Recombinants Encoding Mutant IE-0 Gene Products

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We have previously shown that adenovirus recombinants expressing functional ICP0 reactivate latent herpes simplex virus type 2 (HSV-2) in an in vitro latency system. This study demonstrated that ICP0, independent of other HSV gene products, is sufficient to reactivate latent HSV-2 in this in vitro system. To assess the effects of defined mutations in the sequence encoding ICP0 (IE-0) on reactivation, seven in-frame insertion and three in-frame deletion mutants were moved into an adenovirus expression vector. Each recombinant directed the synthesis of stable ICP0 of the correct size. The transactivation activity of the mutated sequences in these recombinants was similar to that when they were tested in plasmids. When these recombinants were examined for their ability to reactivate in the in vitro latency system, mutants with dramatic defects in transactivation (Ad-0/125, Ad-0/89, Ad-0/2/7, and Ad-0/88/93) were unable to reactivate latent HSV-2 independent of the multiplicity of infection. An exception to this correlation was the finding that Ad-0/89, which transactivated poorly, was able to reactivate latent virus after prolonged incubation whereas other transactivation-deficient mutants could not. Moreover, the presence of ICP4 did not compensate for the inability of any of the recombinants tested to reactivate HSV-2. These results show that (i) the transactivation domains of ICP0 are also used in reactivation, (ii) the presence of another essential HSV regulatory protein ICP4 does not alter the pattern of reactivation by ICP0, and (iii) mutations in some regions of IE-0 previously shown to affect viral growth and plaque formation did not alter its ability to reactivate in this in vitro system.

The linear double-stranded DNA genome of herpes simplex virus type 1 (HSV-1) has the capacity to encode at least 72 unique proteins (29–31, 40). During the course of productive infection in tissue culture, the synthesis of these proteins is coordinately regulated in a cascade fashion. This temporal program is composed of at least three groups of virus proteins: immediate early (α), early (β), and late (γ) (23, 24). The expression of α genes is required for activation of the β and γ classes of virus genes (43, 59) and autoregulation of α genes (9, 17, 28, 36, 47). The usual outcome of productive infection is cell death. However, HSV is also capable of remaining latent in humans for the life of the host. HSV is retained in a latent form in neurons, during which latency virus gene expression is limited to transcription of LAT (14, 19, 45, 55, 57) and no infectious virus can be found (1, 7, 34, 54). HSV can be reactivated from the latent state by a variety of stimuli, but the mechanism(s) of induction remain to be elucidated.

To study the molecular mechanisms involved in the establishment, maintenance, and reactivation of latent HSV-1, animal models have been developed. However, because of the low number of cells in sensory ganglia that harbor latent virus genomes in the infected animal and the inaccessibility of neurons in situ or after organ culture of ganglia, it is extremely difficult to study latency, particularly at the molecular level. Therefore, in vitro tissue culture systems have been developed. In these model systems, some of the difficulties encountered in working with animals can be overcome, and both the establishment of the latent state and reactivation can be reproduced in vitro.

Russell et al. (49, 50) modified the in vitro latency system of O'Neill (38) and Wigdahl et al. (60, 61). In this model system, infection of human fetal lung (HFL) cells with HSV-2 at the supraoptimal temperature of 42°C results in the establishment of a latent state that is stable following shift down of the infected culture to 37°C. Infectious HSV-2 could be recovered, at high efficiency, by superinfection with HSV-1 temperature-sensitive mutants or human cytomegalovirus but not with adenovirus. Moreover, the HSV-1 mutant *dl*1403, which has a large deletion in the immediate-early gene that encodes ICP0, cannot reactivate the latent HSV-2, suggesting a role for ICP0 in reactivation (50).

ICP0, one of the five HSV immediate-early gene products, is a 110-kilodalton nuclear phosphoprotein (39). Using a transient transfection assay, we and others have shown that ICP0 is a potent transactivator (15, 37, 46) and that it can activate the transcription of other herpesvirus genes in a synergistic manner when present together with ICP4 (10, 16, 46). These results suggested a functional interaction between ICP0 and ICP4. Detailed insertion and deletion mutagenesis of the gene encoding ICP0 identified five functional domains of the protein (11, 12). These include a potential metal-binding "finger," signals that affect the intracellular location of the protein, and the carboxy-terminal region that affects the transcriptional activation in the presence of ICP4. Somewhat different results have been obtained in a similar study from this laboratory (J. Chen, X. Zhu, and S. Silverstein, submitted for publication). We have confirmed the presence of the potential zinc finger domain and shown that the integrity of this domain is essential for transactivation. However, mutations in the carboxy-terminal domain affected transcriptional activation only in HeLa cells in a manner independent of the presence of ICP4. Genetic studies with mutant viruses from which large portions of the IE-0 gene have been deleted indicate that they can be propagated without helper virus (51, 56). These deletion mutants are impaired for growth after infection at a low multiplicity (MOI) but show normal patterns of gene expression at a high MOI. When defined insertion and deletion mutations in ICP0

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were constructed back into HSV-1 and tested, more than one region was shown to affect virus growth and plaque formation; in particular, the potential zinc finger domain was essential for normal virus growth in tissue culture (13).

We have developed an adenovirus vector system that expresses functional ICP0 (64). The ICP0-coding sequences were inserted into the E1 region under the control of either the adenovirus major late promoter (MLP) or the IE-0 promoter itself; these recombinants replicated only in 293 cells, which supplied E1 gene products in *trans*. The level of ICP0 that accumulated in HeLa cells infected with these recombinant viruses was comparable to that of HSV-infected HeLa cells. We have previously shown that these recombinants but not wild-type adenovirus reactivate latent HSV-2 in an in vitro latency system, demonstrating that ICP0 alone of the HSV gene products is sufficient to reactivate latent HSV-2 in this in vitro system (21).

These findings encouraged us to insert some of the defined ICP0 mutations into the adenovirus expression system to test for reactivation to address the following questions: (i) which functional domain(s) defined by the transient transfection assay is responsible for reactivation, (ii) does the reactivation pattern correlate with the transactivation pattern for these mutations, and (iii) does the presence of ICP4 change the reactivation pattern?

MATERIALS AND METHODS

Cells and viruses. HeLa and Vero cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum, and 293 cells, a human embryo kidney cell line expressing functional adenovirus type 5 E1a and E1b functions (18), were grown in the same medium supplemented with 5% fetal calf serum. Monolayer cultures of HFL cells, kindly supplied by A. Gershon, were grown in Dulbecco modified Eagle medium supplemented with 5% calf serum. HSV-1 strain 17 and HSV-2 strain HG52 were propagated and titrated in Vero cells. All of the adenoviruses and adenovirus-ICP0 recombinants were propagated and titrated in 293 cells.

Plasmids. The plasmids mpcv2 and pDS-2, which contains the genomic sequence encoding ICP0 from HSV-1 strain KOS under the control of the MLP of adenovirus, have been described previously (64). pDS-25 contains the genomic segment encoding ICP0 from HSV-1 strain 17 under the control of MLP. The construction of ICP0 linker insertion and deletion mutants has been described elsewhere (Chen et al., submitted).

Construction of adenovirus-ICP0 recombinants. To construct adenovirus-ICP0 recombinants, the ICP0 linker insertion and deletion mutants were first moved into either mpcv2 or pDS-2 by exchanging restriction fragments that span the mutated region. In brief, pCM5 and pCM6 were moved into pDS-2 by *Xho*I-*Sal*I fragment exchange. pCM2/7 and pCM10 were moved into pDS-2 by *Bam*HI-*Sal*I fragment exchange. To move pCM84, pCM88, pCM89, and pCM125, these plasmids were first digested with *Nco*I and *Hin*dIII and then endfilled with the Klenow fragment of DNA polymerase I. These blunt-end fragments containing ICP0 were introduced into mpcv2 that was cut with *Xho*I and *Bgl*II and subsequently endfilled with Klenow fragment. pCM93 and pCM88/93 were introduced into pDS-25 by an exchange of *Mlu*I-*Sal*I fragments. Plasmids with mutations in the IE-0-coding sequences under the control of MLP were then linearized by cleavage with *Cla*I and cotransfected with *Cla*I-cut *dl*327 DNA into 293 cells. Screening and analysis of the recombinants were done as described previously (64). Each recombinant was plaque purified twice on 293 cells.

In vitro latency system. Monolayers of HFL cells were infected with HSV-2 strain HG52 at an MOI of 0.001 and were incubated at 42°C for 6 days, with a change of medium every 2 days. The cultures were then transferred to 37°C and incubated at this temperature for 2 to 3 days. These cultures were then superinfected with adenovirus or adenovirus-ICP0 recombinants. To assay reactivation, cultures were incubated for different periods of time and then harvested and frozen and thawed three times. The released HSV-2 was titrated on Vero cells.

Transfection and CAT assay. HeLa cells seeded at 2×10^6 to 2.5×10^6/10-cm dish in Dulbecco modified Eagle medium containing 10% calf serum were transfected the next day with 10 μg of a thymidine kinase-chloramphenicol acetyltransferase (TK-CAT) reporter (62) and infected 1 day later with adenovirus or recombinant viruses at an MOI of 10. Cells were harvested at 24 to 30 h postinfection, and the CAT activity was determined as previously described (16).

Polypeptide analysis. For analysis of polypeptide synthesis in cells infected with wild-type or recombinant viruses, 35-mm-diameter dishes of HeLa or HFL cells (10^6) were infected with the different viruses at an MOI of 100. At 24 h postinfection, cells were harvested and cellular extracts were prepared by lysing the cells in 100 μl of RIPA buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and 10 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma). Polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose paper. The detection of ICP0 by immunoassay was as previously described (64). The filter was first reacted with H1083 (a mouse monoclonal antibody against ICP0, generously provided by L. Pereira) at a dilution of 1:500 followed by reaction with a second antibody (goat anti-mouse immunoglobulin conjugated to alkaline phosphatase) at a 1:1,000 dilution. In some instances, a rabbit polyclonal antibody prepared against cells infected with HSV-1 (kindly provided by L. Pizer) was used in the first incubation at a dilution of 1:200. In these instances, the second antibody was goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase at a dilution of 1:500.

Analysis of DNA recovered from latently infected cultures by superinfection. Latently HSV-2-infected HFL cells were superinfected with different viruses. The cells were harvested after 3 days of superinfection, and total cytoplasmic nucleic acids were isolated as described previously (50). The cytoplasmic nucleic acids were digested with *Hin*dIII and RNase A and then separated by electrophoresis in a 0.8% agarose gel. Southern blot analysis was performed after the DNA was transferred to a Nytran filter (Schleicher & Schuell, Inc.) and the filter was probed with a ^{32}P-labeled 655-base-pair fragment derived from a 3′ portion of the HSV-2 glycoprotein G gene (32).

RESULTS

In vitro latency system. To study the molecular mechanism of HSV reactivation, we explored the use of an in vitro latency system (49, 50). Monolayers of HFL cells were infected with HSV-2 at an MOI of 0.001 and incubated at 42°C for 6 days. After the latently infected cultures were shifted to 37°C for 2 to 3 days, they were superinfected with

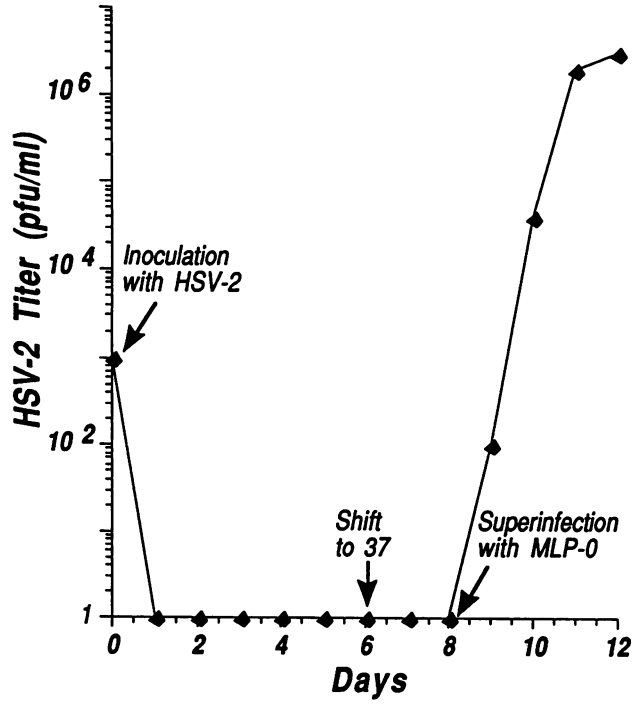

FIG. 1. In vitro latency system. Confluent monolayers of HFL cells infected with HSV-2 strain HG52 at an MOI of 0.001 were incubated at 42°C for 6 days, with a change of medium every 2 days. The cultures were then transferred to 37°C and incubated at this temperature for 2 days, superinfected with MLP-0 at an MOI of 10, and incubated at 37°C. Samples were harvested at 24-h intervals throughout the experiment, and reactivated virus was assayed by titration on Vero cells.

the recombinant adenovirus MLP-0 and harvested at 24-h intervals, and the reactivation of virus was monitored by checking for plaque formation on Vero cells. Low levels of virus were detected as early as 1 day postinfection, and the titer of reactivated HSV-2 increased with time (Fig. 1). Because the yield of reactivated virus was somewhat dependent on the MOI of MLP-0 (see Fig. 6), cultures were routinely infected with the adenovirus-ICP0 recombinants at an MOI of 10 and the reactivated virus was scored at 48 h after superinfection. The latently infected cultures can be sustained for about 1 week after down shift to 37°C, during which time no reactivation can be detected after mock infection or superinfection with either wild-type adenovirus or 434 (21), a parental adenovirus from which the E1 region is deleted (20).

Construction of adenovirus-ICP0 recombinants. To analyze the role of specific domains in ICP0 on reactivation, selected mutated sequences were built into an adenovirus vector by overlap recombination (5). The locations of seven codon insertion and three in-frame deletion mutants used in this study and their transactivation activities (when in plasmid form) on a TK-CAT target are shown in Table 1.

To construct the recombinants, DNA encoding the mutated sequences of ICP0 was first placed behind the adenovirus MLP and a cDNA copy of the complete first two tripartite leaders and two-thirds of the third tripartite leader on vector mpcv2. Following cotransfection into 293 cells of *Cla*I-linearized plasmid DNAs together with *dl*327 DNA cut with *Cla*I, the resulting viable viruses were screened by DNA dot blot hybridization for the presence of an IE-0

TABLE 1. Transactivation on a TK-CAT reporter by plasmids containing mutated IE-0 sequences[a]

Plasmid	Position (aa)	Mutation[b]	Activity[c] (% ± SD)
2/7	106–212	(DE) 106–212	24 ± 14
125	124	(IN) AGIPA	10 ± 6
89	210/211	(IN) PEFR	24 ± 7
84	263	(IN) AGIPA	92 ± 19
5	349/350	(IN) PEFR	94 ± 18
6	400–462	(DE) 400–462	72 ± 22
10	519	(IN) EFPR	65 ± 11
93	628	(IN) AGIPA	109 ± 15
88/93	628–697	(DE) 628–697	49 ± 15
88	697	(IN) AGIPA	64 ± 13

[a] Seven in-frame codon insertion and three in-frame deletion mutants were examined.
[b] Deletion (DE) or insertion (IN) of the indicated amino acids.
[c] Percent of CAT activity induced by a mutant normalized to the level induced by a plasmid encoding wild-type ICP0.

sequence. Positive plaques from each cross were further analyzed by restriction endonuclease mapping and Southern blot hybridization to verify the integrity of the transferred gene and the positions of the mutations. The *Sal*I and *Eco*RI digestion pattern of Hirt DNAs isolated from cultures infected with the putative recombinants and probed with a sequence specific for IE-0 is shown in Fig. 2. After *Sal*I digestion of the codon insertion mutant, the electrophoretic mobilities of these DNAs are indistinguishable from those of wild-type adeno-ICP0 recombinants, whereas the in-frame deletion mutants generated smaller inserts of correct size (Fig. 2A). The *Eco*RI digestion profile was consistent with the predicted pattern based on the position of codon insertion (Fig. 2B). The recombinants were designated Ad-0/2/7, Ad-0/125, Ad-0/89, Ad-0/84, Ad-0/5, Ad-0/6, Ad-0/10, Ad-0/93, Ad-0/88/93, and Ad-0/88, consistent with the nomenclature used to describe the plasmids from which they were derived (Chen et al., submitted). The recombinant MLP-0 expressed functional wild-type ICP0 from HSV-1 strain KOS. A similar recombinant that had the wild-type IE-0 sequence from HSV-1 strain 17 was constructed for this study and was designated Ad-0/25.

ICP0 synthesis in cells infected with the recombinants. Previous studies have shown that the level of ICP0 which accumulated in MLP-0-infected HeLa cells was comparable to that of HSV-infected HeLa cells (64). ICP0 synthesized in cells infected with each recombinant was examined by Western blot (immunoblot) analysis. HeLa cells were infected with each virus at an MOI of 100 and at different times postinfection (HSV-1, 16 h; adenovirus and adenovirus-ICP0 recombinants, 24 h), and total cellular extracts were prepared and examined for the presence of ICP0. When H1083, a monoclonal antibody specific for ICP0, was used, each recombinant except Ad-0/6 was shown to produce ICP0 (Fig. 3A). The level of ICP0 that accumulated in cells infected with each virus was comparable to that in HSV-infected cells. Moreover, mutated forms of ICP0 generated from codon insertions were indistinguishable in electrophoretic mobility from wild-type ICP0 synthesized by either strain KOS or 17. Deletion mutants Ad-0/2/7 and Ad-0/88/93 (deletions of amino acids [aa] 106 to 212 and aa 628 to 697, respectively) directed the synthesis of truncated forms of ICP0 with faster electrophoretic mobilities.

Ad-0/6 specified a protein that lacked amino acids 400 to 462 and was not recognized by H1083. When a polyclonal antibody prepared against cells infected with HSV-1 was

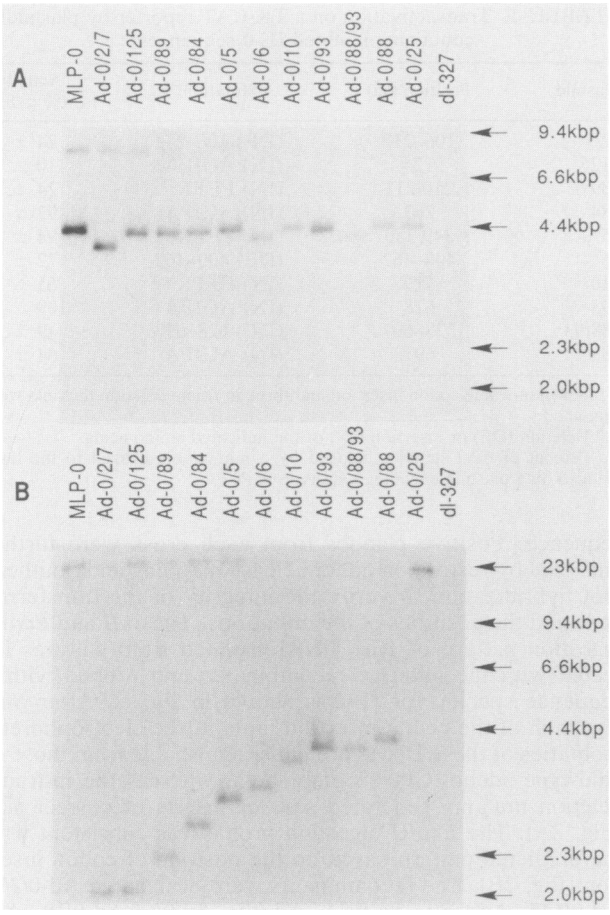

FIG. 2. Southern blot analysis of adenovirus-ICP0 recombinant DNAs. Hirt DNAs were prepared from 293 cells infected with the plaque-purified recombinants. The DNAs were digested with *Sal*I (A) or *Eco*RI (B) and separated by electrophoresis on a 0.8% agarose gel. Blots were prepared and hybridized with an *Nco*I-*Hpa*I probe that spans the coding region for ICP0 (41). kbp, Kilobase pairs.

used, a cross-reacting protein with the expected electrophoretic mobility was detected in infected HeLa cells (Fig. 3B). Therefore, the region deleted in this mutant may contain the reactive epitope recognized by H1083.

Transactivation of TK-CAT by ICP0 produced from the mutant recombinants. ICP0 is a potent transcriptional activator when assayed in a transient transfection assay. Mutagenesis of the IE-0 sequence has identified the transactivation domain(s). To determine whether the recombinant viruses displayed transactivation properties similar to those of the mutated plasmids, each was examined for the ability to transactivate a TK-CAT reporter gene. Twenty-four hours after HeLa cells were transfected with a plasmid containing the reporter, they were infected with the recombinant viruses. At different times postinfection (HSV, 16 h; adenovirus and adenovirus-ICP0 recombinants, 24 h), the cells were harvested and cellular extracts were prepared and assayed for CAT activity. Codon insertion mutants Ad-0/2/7, Ad-0/125, and Ad-0/89 were defective in activating this reporter (Fig. 4). Recombinant viruses carrying mutated IE-0 sequences whose transactivating activity was ≤40% wild-type activity were termed defective in this assay. Although the mutants Ad-0/93 and Ad-0/88 had wild-type transactivating potential, the carboxy-terminal deletion mutant Ad-0/88/93 (deletion of aa 628 to 697), which removed 69 amino acids between these codon insertions, had lower transactivating activity. This suggested that the deleted amino acids are required for proper folding of the protein and that their loss affected the transactivation properties of the protein. In contrast, none of the other codon insertion (Ad-0/84, Ad-0/5, Ad-0/6, Ad-0/10 or Ad-0/25) recombinants were markedly deficient in transactivation of the TK-CAT reporter (Fig. 4). In summary, the transactivation activity of the mutated sequences in these recombinants was similar to that when they were tested in the plasmids (Table 1).

Reactivation of latent HSV. Previously, we showed that superinfection of latently infected cultures with MLP-0 resulted in the reactivation of HSV (21). That study demonstrated that ICP0 alone of the HSV gene products is sufficient to reactivate HSV-2 in this in vitro model system. The adenovirus recombinants permitted us to examine the capacity of the altered ICP0s, in the absence of other HSV

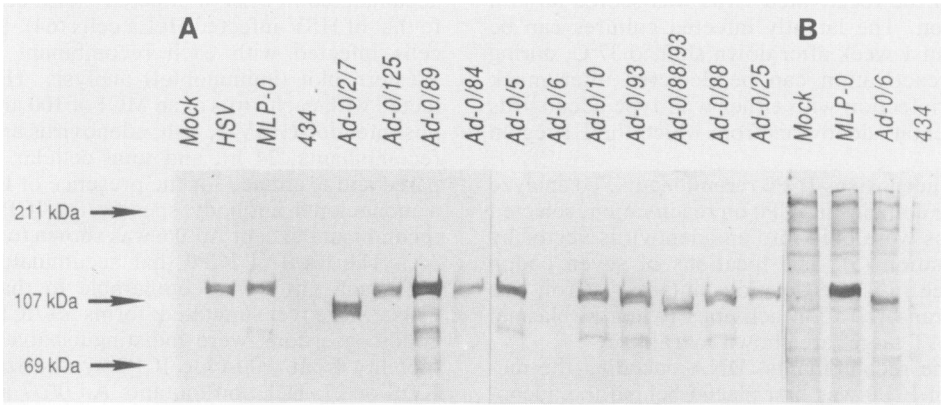

FIG. 3. Western blot analysis of ICP0 synthesis in cells infected with adenovirus-ICP0 recombinants. HeLa cells were infected with adenovirus and adenovirus-ICP0 recombinants or HSV-1 at an MOI of 100. Total cellular extracts were prepared from equal numbers of cells at different times postinfection (24 h for adenovirus or adenovirus-ICP0 recombinants, 16 h for HSV-1) and subjected to electrophoresis in 7.5% sodium dodecyl sulfate-polyacrylamide gels. The separated proteins were electroblotted to nitrocellulose paper and analyzed for the presence and size of ICP0 by Western blot analysis with a monoclonal antiserum (A) or a polyclonal antiserum (B) specific for ICP0, as described in Materials and Methods. kDa, Kilodaltons.

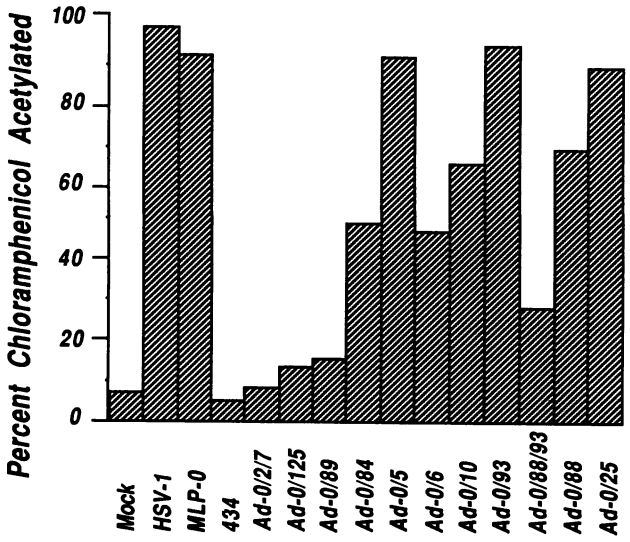

FIG. 4. Transactivation of TK-CAT by ICP0 produced from the mutant recombinants. HeLa cells were transfected with a TK-CAT reporter, and 24 h later, they were infected. When infected with HSV, they were incubated for 16 h, and when infected with adenovirus or an adenovirus-ICP0 recombinant, they were incubated for 24 h. Cell extracts were prepared and analyzed for CAT activity as described previously (16).

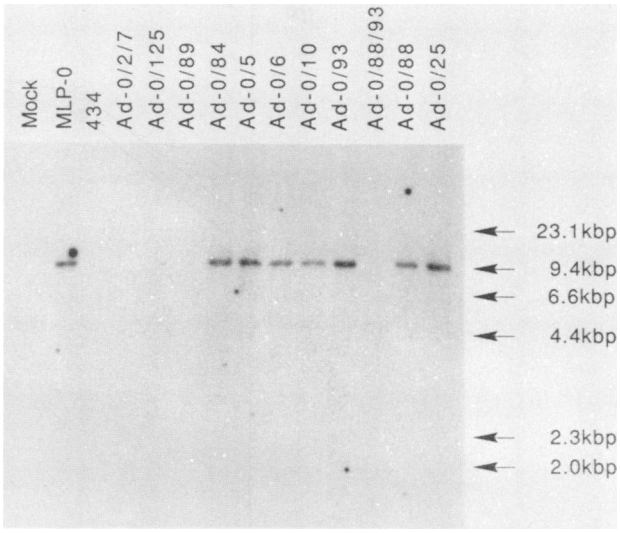

FIG. 5. Analysis of DNA recovered from latently infected cultures after superinfection. Latently HSV-2-infected HFL cells were superinfected with different viruses at an MOI of 10. Cells were harvested 3 days later, and total cytoplasmic nucleic acids were isolated as described previously (50). The cytoplasmic nucleic acids were digested with *Hin*dIII and RNase and separated by electrophoresis in a 0.8% agarose gel. Southern blot analysis was performed by using a probe specific for HSV-2. kbp, Kilobase pairs.

products, to reactivate latent HSV-2. For these analyses, latently infected HFL cultures were established and superinfected with the recombinant viruses at an MOI of 10. At 48 h postinfection, when plaques first appeared, the cells were harvested and viruses were released and titrated on Vero cells. Of the seven linker insertion and three in-frame deletion mutants tested, only Ad-0/2/7, Ad-0/125, Ad-0/89, and Ad-0/88/93 failed to reactivate (Table 2). Recombinants with mutations in other regions of IE-0 did not affect the ability of ICP0 to reactivate (Table 2). The wild-type ICP0s from HSV-1 strains KOS and 17 were equally competent in reactivating virus in this system.

We also tested for reactivation by probing for the appearance of HSV-2 DNA. Cultures latently infected with HSV-2 were infected with the adenovirus-ICP0 recombinants and harvested 3 days later, and the cytoplasmic nucleic acids were isolated. Total cytoplasmic nucleic acids were displayed on an agarose gel after digestion with restriction endonucleases and RNase, and blots were prepared. A cloned 655-base-pair fragment derived from a 3′ portion of the HSV-2 glycoprotein G gene was used to probe for the presence of HSV-2 DNA (Fig. 5). HSV-2 DNA was detected only in those cultures infected with recombinant viruses that generated infectious virus (Table 2).

TABLE 2. Reactivation of latent HSV-2[a]

Superinfecting virus	No.[b] of reactivated cultures	Mean titer (PFU/ml)[c]
Mock	0	ND
MLP-0	3	1.47×10^5
Ad-0/2/7	0	ND
Ad-0/125	0	ND
Ad-0/89	0	ND
Ad-0/84	3	1.37×10^5
Ad-0/5	3	1.07×10^5
Ad-0/6	3	7.95×10^4
Ad-0/10	3	4.67×10^4
Ad-0/93	3	2.24×10^5
Ad-0/88/93	0	ND
Ad-0/88	3	9.22×10^4
Ad-0/25	3	5.33×10^4

[a] Latently infected HFL cells were established as described in the legend of Fig. 1 and superinfected with each of the recombinant viruses at an MOI of 10. At 48 h postinfection, the cells were harvested and viruses were titrated on Vero cells.

[b] Of three total.

[c] ND, Not detected.

Effects of increasing MOI and time on reactivation. The failure of some of these mutants to reactivate latent HSV could have resulted from delayed action of the mutant forms of ICP0. Therefore, we analyzed what effect increasing the MOI and prolonging the time of incubation had on reactivation. Although reactivation by wild-type ICP0 and mutant Ad-0/84 was dependent on an increasing MOI, mutants Ad-0/2/7, Ad-0/125, and Ad-0/88/93 remained defective even when the MOI of the superinfection was increased to 100 (Fig. 6). When the kinetics of reactivation were examined (Fig. 7), cultures infected with wild-type or mutant Ad-0/84 started to produce infectious virus 2 days postinfection and the titer of reactivated HSV-2 reached a plateau by day 3. In contrast, Ad-0/125, Ad-0/2/7, and Ad-0/88/93 failed to reactivate even when the cultures were assayed after 5 days postinfection. Interestingly, Ad-0/89 did not reactivate until day 4 after superinfection, and the titer of reactivated HSV-2 reached a level comparable to that found in cells infected by MLP-0. It appears that the mutation in the IE-0 coding region of Ad-0/89 rendered ICP0 partially defective and that therefore a longer time was required for this mutant to reactivate the latent virus.

Effects of ICP4 on reactivation. In the transient transfection assay, ICP0 could activate the expression of a TK-CAT reporter in a synergistic manner when present with ICP4. The presence of ICP4 can affect the transactivating property of mutations in certain regions of ICP0. To determine

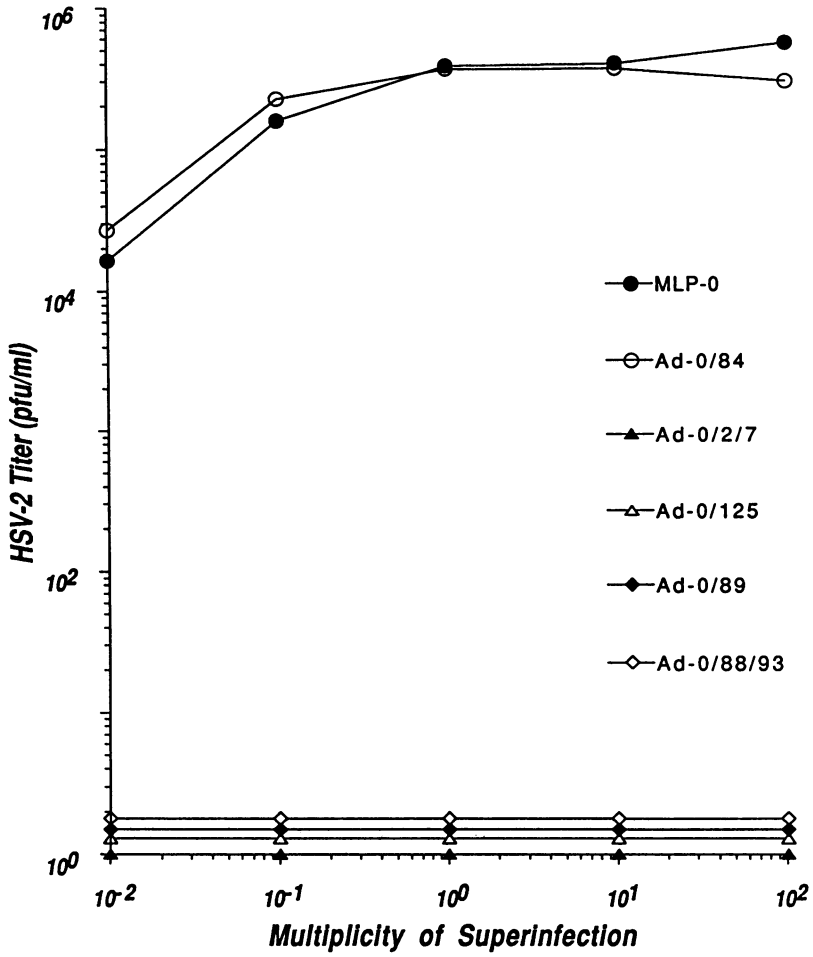

FIG. 6. Effect of increasing MOI on reactivation. Latently infected HFL cells were superinfected with the different recombinant viruses at the indicated MOIs. At 48 h postinfection, the cells were harvested and reactivated virus was titrated on Vero cells.

whether the inability of any of these mutants to reactivate could be compensated for by the presence of ICP4, we took advantage of an adenovirus-ICP4 recombinant (kindly provided by S. Bacchetti) that expresses functional ICP4 upon infection of tissue culture cells (52). Latently infected cultures coinfected with adenovirus-ICP4 and recombinants Ad-0/2/7, Ad-0/125, Ad-0/89, and Ad-0/88/93 for 3 days at 32°C (adenovirus-ICP4 contains a temperature-sensitive IE-4 allele) were analyzed for reactivated virus (Table 3). Adenovirus ICP4 was unable to reactivate the latent HSV-2 in this model system. Moreover, the presence of ICP4 did not compensate for the inability of any of the recombinants tested to reactivate virus.

DISCUSSION

ICP0 was a potent transcriptional activator when assayed in a transient transfection system. However, IE-0 deletion mutants of HSV-1 were competent for replication in tissue culture. Therefore, the biological role of this protein in the HSV life cycle remains to be defined. In this study, we examined the function of ICP0 in the reactivation process by using an in vitro latency system. The results obtained demonstrate that the major transactivation domain of ICP0 is important for the reactivation of virus. In contrast to transient transfection assays in which ICP0 and ICP4 act synergistically to transactivate β promoters, the presence of ICP4 does not alter the pattern of reactivation by ICP0. The close correlation between transactivation and reactivation patterns suggests that ICP0 might reactivate latent HSV by transcriptional activation of other virus or cell genes.

Previous studies from this laboratory of the sequence encoding ICP0 have identified a domain that is essential for transactivation (Chen et al., submitted). This domain, extending from aa 124 through 213, includes a cysteine-rich region, which resembles a zinc finger domain, and also coincides with the major transactivation determinant previously defined by Everett (11, 12). Mutants with deletions in the carboxy terminus also showed an impaired ability to transactivate in HeLa cells. When seven in-frame insertion and three in-frame deletion mutants were moved into the adenovirus expression vector, each recombinant was able to direct the synthesis of stable ICP0 of the correct size. The transactivating properties of the ICP0 mutants in cells infected with the recombinants were similar to those found in cotransfection experiments. When these recombinants were tested for the ability to reactivate in the in vitro latency system, we noted that mutants which did not show dramatic defects in transactivation were able to reactivate latent HSV-2. Only mutants that were defective in transactivation (Ad-0/125, Ad-0/89, Ad-0/2/7, and Ad-0/88/93) failed to reac-

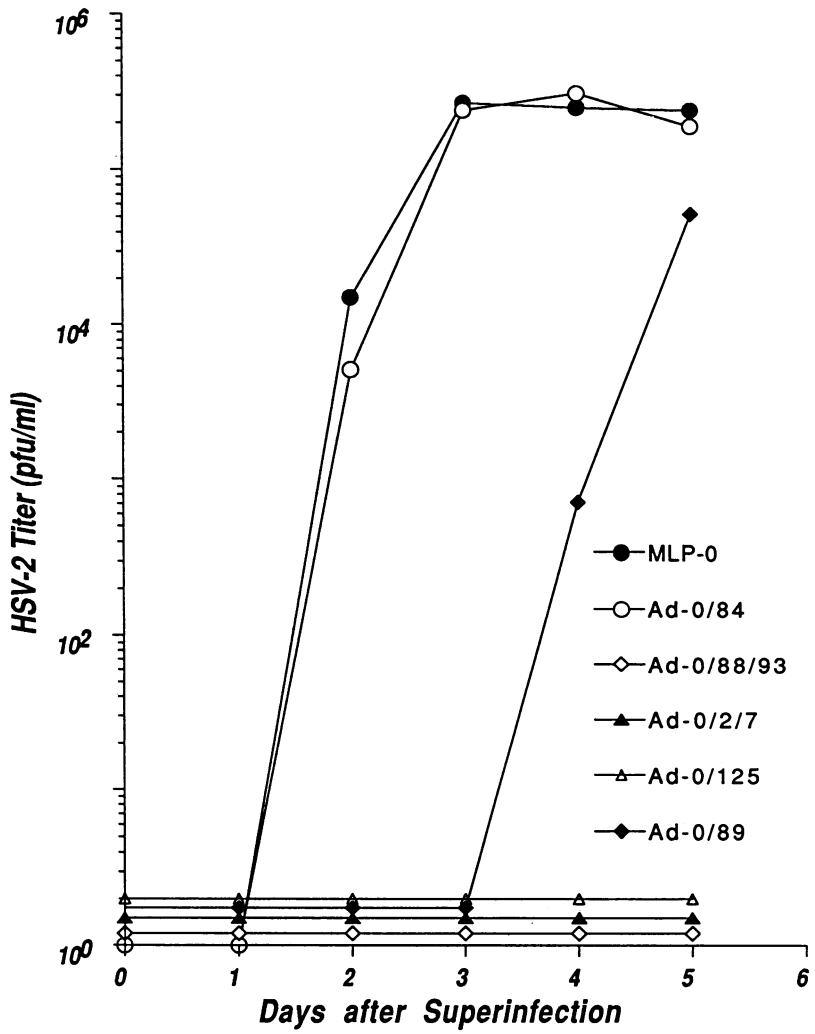

FIG. 7. Effect of prolonged incubation on reactivation. Latently infected HFL cells were established as described in the legend to Fig. 1 and superinfected with each of the recombinant viruses at an MOI of 10. At 24-h intervals, the cells were harvested and reactivated virus was assayed for by titration on Vero cells.

tivate, and the inability to reactivate could not be overcome by increasing the MOI. By prolonging the time till assay postinfection, Ad-0/89 was shown to be able to reactivate whereas the other three mutants could not. Ad-0/125 and Ad-0/2/7 had an insertion and a deletion, respectively, in the potential zinc finger domain; the insertion in Ad-0/89 was at aa 210 of ICP0, which is downstream of the potential zinc finger. In contrast, the defect in the deletion mutant Ad-0/88/93, which is between aa 628 and 697 in the carboxy terminus, was far downstream of the major transactivation domain and did not reactivate. These results establish a strong correlation between the transactivation and reactivation patterns of IE-0 mutants. The only exception was the Ad-0/89 mutant, which reactivated in a delayed fashion in three of four separate experiments. One might argue that evolutionarily one of the reasons HSV has maintained this potent activator protein is to reactivate virus when different stimuli are presented to the latently infected cell.

TABLE 3. Effect of ICP4 on reactivation[a]

Superinfecting virus	No. of reactivated cultures	Mean titer (PFU/ml)[b]
MLP-0	3	1.10×10^4
Adenovirus-ICP4	0	ND
MLP-0 + adenovirus-ICP4	3	1.17×10^4
Ad-0/2/7 + adenovirus-ICP4	0	ND
Ad-0/125 + adenovirus-ICP4	0	ND
Ad-0/89 + adenovirus-ICP4	0	ND
Ad-0/88/93 + adenovirus-ICP4	0	ND

[a] Latently infected cultures were coinfected with adenovirus-ICP4 and the adenovirus-ICP0 recombinants Ad-0/2/7, Ad-0/125, Ad-0/89, and Ad-0/88/93 for 3 days at 32°C. The cultures (three each) were then harvested and assayed for reactivated virus.

[b] ND, Not detected.

In this study, we also examined the effect of the presence of ICP4 on reactivation. When cell cultures latently infected with HSV-2 were coinfected with the recombinants and adenovirus-ICP4, the reactivation-negative mutants remained unable to induce latent virus. It appears that activation of the HSV genome in this system obligatorily requires ICP0. The presence of ICP4 does not compensate for or enhance the action of ICP0. However, from this study we

cannot exclude the possibility that functional interaction between ICP0 and ICP4 exists after the latently infected HSV genome is reactivated by ICP0. Interestingly, the carboxy-terminal mutant Ad-0/88/93 (deletion of aa 628 to 697) fails to reactivate in this system. In a previous study, D14, an HSV-1 mutant in IE-0 (deletion of aa 680 to 720) whose defect overlapped Ad-0/88/93, was able to reactivate. It is plausible that the nonoverlapping region defined by these two mutants (deletion of aa 628 to 680) may account for these different results. Alternatively, deletions in the carboxy terminus can be compensated for in the reactivation assay by other HSV gene products provided during superinfection of latently infected cultures by D14. If this hypothesis is true, the contribution of ICP4 alone can be excluded, because the inability to reactivate by Ad-0/88/93 cannot be overcome by coinfecting with adenovirus-ICP4.

It is noteworthy that a deletion mutant spanning aa 400 to 462 of ICP0 was able to reactivate latent HSV-2. In a previous study, however, a linker insertion at aa 406 rendered the resulting mutant HSV partially defective in a single-step growth experiment and less efficient for plaque formation (13). These results might be interpreted to suggest that different modes of action of ICP0 affect productive infection and reactivation. In productive infection, α-TIF (a 65-kilodalton transcriptional regulatory protein carried within the virus particle) stimulates expression of immediate-early genes (4, 25, 26, 33, 35, 42, 44). These in turn affect the expression of virus early and late genes. Therefore, whatever the target of ICP0 during productive infection, it probably activates genes by interacting with other virus regulatory proteins in conjunction with host transcriptional factors. By contrast, during latency only LAT RNA is expressed, and while it is conceivable that LAT exons encode an as-yet-unidentified regulatory protein, the bulk of the transcripts are nuclear (8, 48, 53, 55, 58). Therefore, no known preexisting virus regulatory proteins are present. Accordingly, ICP0 may mediate the switch from latency to productive infection either by itself or by interacting with cellular transcriptional factors. This further argues that although some regions of ICP0 (e.g., the zinc finger domain and carboxy terminus) may affect the functions of this protein both in productive infection and in reactivation, other regions (e.g., aa 400 to 462) may be involved in only one of these processes.

How ICP0 activates gene expression is still not known. Therefore, how this protein triggers the switch from latency to productive infection remains to be studied. It is interesting to note the findings made by Leib et al. (27) that deletion of ICP0 from strain KOS greatly affects the frequency of reactivation of virus from latency in a mouse model. However, in another study using a different mouse model system, an ICP0 deletion mutant from strain 17 does not appear to affect the establishment or maintenance of latency or reactivation from the latent state upon explantation of ganglia (6). Although these differences between groups may be strain specific, we noted that wild-type ICP0 from both strains 17 and KOS were equally competent to reactivate in the in vitro latency system. On the basis of the observation that ICP0 affects the replication of infectious virus after transfection of virus DNA, Cai and Schaffer suggest that transfection with virus DNA is similar to the situation in reactivation where no preexisting virus regulatory proteins are known to exist (3). Recent findings by C. Preston and J. Russell (unpublished results) have shown that HSV DNA exists as a nonlinear molecule in this in vitro model system. It is worth mentioning that in our study, ICP0 was introduced into latently infected cells in culture by using an adenovirus vector. However, in vivo expression from almost all virus genes other than LAT is repressed in latently infected mouse and human neuronal tissues (14, 19, 45, 55, 57). Therefore, it is reasonable to speculate that in a latently infected animal host, external stimuli trigger expression of IE-0, which then mediates the switch. In a recent study of epinephrine-induced reactivation in the rabbit eye model, Hill et al. (22) demonstrated that a LAT^- $ICP0^+$ virus which spontaneously reactivates normally is deficient in the induced reactivation pathway. Thus, there appears in this model system to be a requirement for LAT in addition to ICP0 in this reactivation pathway.

It seems likely that different stimuli trigger the expression or modification of specific host transcriptional factors which mimic the function of ICP0 or interact with ICP0. In view of the findings that dimethyl sulfoxide can induce the reactivation of an ICP0 deletion mutant from strain KOS, spontaneous reactivation of an ICP0 deletion mutant from strain 17 occurs upon explantation of ganglia (6, 27), and deprivation of nerve growth factor affects the reactivation of HSV-1 from latently infected murine ganglia (63), the latter hypothesis seems more attractive. Further studies should clarify the contributions of host and virus proteins to the reactivation of latent HSV.

Adenovirus has been extensively studied and engineered as a vector system. In this study, we explored the use of an adenovirus vector to study the reactivation of latent HSV in the in vitro latency system. Because of the host range restriction, infecting Ad-0 recombinants did not replicate. Furthermore, by increasing the MOI with different recombinants, either wild-type or mutated forms of ICP0 could be delivered to every cell, thus increasing the sensitivity of the reactivation assay. This is another example of the utility of adenovirus as a delivery system (2).

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LITERATURE CITED

1. **Baringer, J. R., and P. Swoveland.** 1973. Recovery of herpes-simplex virus from human trigeminal ganglions. N. Engl. J. Med. **288**:648–650.
2. **Berkner, K. L.** 1988. Development of adenovirus vectors for the expression of heterologous genes. BioTechniques **6**:616–629.
3. **Cai, W., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J. Virol. **63**:4579–4589.
4. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1–19.
5. **Chinnadurai, G., S. Chinnadurai, and J. Brusca.** 1979. Physical mapping of a large-plaque mutation of adenovirus type 2. J. Virol. **32**:623–628.
6. **Clements, G. B., and N. D. Stow.** 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency-competent in mice. J. Gen. Virol. **70**:2501–2506.
7. **Cook, M. L., V. B. Bastone, and J. G. Stevens.** 1974. Evidence that neurons harbor latent herpes simplex virus. Infect. Immun. **9**:946–951.
8. **Croen, K. D., J. M. Ostrove, L. J. Dragovic, J. E. Smialek, and S. E. Straus.** 1987. Latent herpes simplex virus in human

trigeminal ganglia. N. Engl. J. Med. **317**:1427–1432.
9. **Dixon, R. A. F., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36**:189–203.
10. **Everett, R. D.** 1984. Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
11. **Everett, R. D.** 1987. A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J. **6**:2069–2076.
12. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J. Mol. Biol. **202**:87–96.
13. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J. Gen. Virol. **70**:1185–1202.
14. **Galloway, D. A., C. M. Fenoglio, and J. K. McDougall.** 1982. Limited transcription of the herpes simplex virus genome when latent in human sensory ganglia. J. Virol. **41**:686–691.
15. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:5265–5269.
16. **Gelman, I. H., and S. Silverstein.** 1986. Coordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate early gene products. J. Mol. Biol. **191**:395–409.
17. **Gelman, I. H., and S. Silverstein.** 1987. Herpes simplex virus immediate-early promoters are responsive to virus and cell *trans*-acting factors. J. Virol. **61**:2286–2296.
18. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
19. **Green, M. T., R. J. Courtney, and E. C. Dunkel.** 1981. Detection of an immediate early herpes simplex virus type 1 polypeptide in trigeminal ganglia from latently infected animals. Infect. Immun. **34**:987–992.
20. **Grodzicker, T., and D. F. Klessig.** 1980. Expression of unselected adenovirus genes in human cells co-transformed with the HSV-1 tk gene and adenovirus 2 DNA. Cell **21**:453–463.
21. **Harris, R. A., R. D. Everett, X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein VMW110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J. Virol. **63**:3513–3515.
22. **Hill, J. M., F. Sederati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates *in vivo* reactivation. Virology **174**:117–125.
23. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
24. **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. USA **72**:1276–1280.
25. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **84**:71–75.
26. **Kristie, T. M., and B. Roizman.** 1988. Differentiation and DNA contact points of host proteins binding at the *cis* site for virion-mediated induction of α genes of herpes simplex virus 1. J. Virol. **62**:1145–1157.
27. **Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer.** 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. **63**:759–768.
28. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. **63**:18–27.
29. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
30. **McGeoch, D. J., A. Dolan, S. Donald, and D. M. K. Brauer.** 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res. **14**:1727–1745.
31. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. **181**:1–13.
32. **McGeoch, D. J., H. W. M. Moss, D. McNab, and M. C. Frame.** 1987. DNA sequence and genetic content of Hind III I region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotein G, and evolutionary comparisons. J. Gen. Virol. **68**:19–38.
33. **McKnight, J. L. C., T. M. Kristie, and B. Roizman.** 1987. Binding of the virion protein mediating α gene induction in herpes simplex virus 1-infected cells to its *cis* site requires cellular proteins. Proc. Natl. Acad. Sci. USA **84**:7061–7065.
34. **McLennan, J. L., and G. Darby.** 1980. Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. J. Gen. Virol. **51**:233–243.
35. **O'Hare, P., and C. R. Goding.** 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell **52**:435–445.
36. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
37. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
38. **O'Neill, F. J.** 1977. Prolongation of herpes simplex virus latency in cultured human cells by temperature elevation. J. Virol. **24**:41–46.
39. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733–749.
40. **Perry, L. J., and D. J. McGeoch.** 1988. The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:2831–2846.
41. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. **67**:2365–2380.
42. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of alpha genes of HSV: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell **24**:555–565.
43. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsK*. J. Virol. **29**:275–284.
44. **Preston, C. M., M. C. Frame, and M. E. M. Campbell.** 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell **52**:425–434.
45. **Puga, A., J. D. Rosenthal, H. Openshaw, and A. L. Notkins.** 1978. Herpes simplex virus DNA and mRNA sequences in acutely and chronically infected trigeminal ganglia of mice. Virology **89**:102–111.
46. **Quinlan, M. P., and D. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral factors. Mol. Cell. Biol. **5**:957–963.
47. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific

binding site for the IE175 (ICP4) protein. J. Virol. **62**:4307–4320.
48. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. L. Wechsler.** 1987. Detection of latency related viral RNAs in trigeminal ganaglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61**:3820–3826.
49. **Russell, J., and C. M. Preston.** 1986. An in vitro latency system for herpes simplex virus type 2. J. Gen. Virol. **67**:397–403.
50. **Russell, J., N. D. Stow, E. C. Stow, and C. M. Preston.** 1987. Herpes simplex virus genes involved in latency in vitro. J. Gen. Virol. **68**:3009–3018.
51. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. **61**:829–839.
52. **Spessot, R., K. Inchley, T. M. Hupel, and S. Bacchetti.** 1989. Cloning of the herpes simplex virus ICP4 gene in an adenovirus vector: effects on adenovirus gene expression and replication. Virology **168**:378–387.
53. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61**:3841–3847.
54. **Stevens, J. G., and M. L. Cook.** 1971. Latent herpes simplex virus in spinal ganglia of mice. Science **173**:843–845.
55. **Stevens, J. G., E. K. Wagner, G. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235**:1056–1059.
56. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J. Gen. Virol. **67**:2571–2585.
57. **Stroop, W. G., D. L. Rock, and N. W. Fraser.** 1984. Localization of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by in situ hybridization. Lab. Invest. **51**:27–38.
58. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y.-F. Zhang, W. M. Flanagan, and J. G. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62**:1194–1202.
59. **Watson, R. J., and J. B. Clements.** 1980. A herpes simplex type 1 function continuously required for early and late virus RNA synthesis. Nature (London) **285**:329–330.
60. **Wigdahl, B. L., H. C. Isom, and F. Rapp.** 1981. Repression and activation of the genome of herpes simplex virus in human cells. Proc. Natl. Acad. Sci. USA **78**:6522–6526.
61. **Wigdahl, B. L., A. C. Scheck, E. DeClerq, and F. Rapp.** 1982. High efficiency latency and activation of herpes simplex virus in human cells. Science **217**:1145–1146.
62. **Wigler, M., S. Silverstein, L.-S. Lee, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.
63. **Wilcox, C. L., and E. M. Johnson, Jr.** 1988. Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. J. Virol. **62**:393–399.
64. **Zhu, X., C. S. H. Young, and S. Silverstein.** 1988. Adenovirus vector expressing functional herpes simplex virus ICP0. J. Virol. **62**:4544–4553.