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Truncation of the C-Terminal Acidic Transcriptional Activation Domain of Herpes Simplex Virus VP16 Renders Expression of the Immediate-Early Genes Almost Entirely Dependent on ICP0

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The herpes simplex virus (HSV) proteins VP16 and ICP0 play key roles in stimulating the onset of the viral lytic cycle. We sought to explore the regulatory links between these proteins by studying the phenotypes of viral mutants in which the activation functions of both were simultaneously inactivated. This analysis unexpectedly revealed that truncation of the C-terminal transcriptional activation domain of VP16 (allele V422) in an ICP0-deficient background almost completely eliminated immediate-early gene expression and virus replication in Vero and HEL cells. The doubly mutant viral genome persisted in a quiescent state for at least 10 days in HEL cells infected at high multiplicity and could be reactivated by superinfection with wild-type HSV. In contrast, the *in*1814 VP16 mutation produced a markedly less severe phenotype in the same ICP0-deficient background. These data demonstrate that expression of the immediate-early genes requires ICP0 when the C-terminal activation domain of VP16 is deleted and raise the possibility that the *in*1814 form of VP16 retains a residual ability to stimulate gene expression during virus infection.

Herpes simplex virus type 1 (HSV-1) is a large nuclear DNA virus that induces both lytic and latent infections in its natural human host (60). HSV-1 gene expression occurs during lytic infection in three phases, termed immediate-early (IE), early, and late. The IE genes are the first to be transcribed, and the resulting IE polypeptides are essential for early- and late-gene expression (31). Transcription of the IE genes is coordinately activated by the virion protein VP16, which is targeted to IE promoters through the TAATGARAT (R = purine) element (10, 44, 55, 70, 71). VP16 is a 65-kDa phosphoprotein that is synthesized late in infection and packaged into the tegument of HSV-1 virions (41, 45, 49). It contains an extremely potent C-terminal transcriptional activation domain (62, 71, 72) and forms a complex with the cellular factors Oct-1 and HCF that binds TAATGARAT with high affinity (reviewed in references 51 and 60). VP16-induced activation of IE gene expression plays an important role in triggering the onset of the HSV-1 lytic cycle, as illustrated by the phenotypes of viral mutants encoding transactivation-deficient forms of VP16. For example, *in*1814, a mutant in which the ability of VP16 to form a complex with Oct1, HCF, and DNA is disrupted by an in-frame linker insertion, displays a greatly increased particle-to-PFU ratio and substantially reduced IE gene expression during infection (2). Truncation of the C-terminal acidic transcriptional activation domain produces a similar phenotype (66). These data demonstrate that VP16 greatly increases the probability that cells infected with a single HSV-1 virion enter the lytic cycle.

Four of the IE genes induced by VP16 encode nuclear phosphoproteins (ICP0, ICP4, ICP22, and ICP27) that act at a variety of levels to regulate IE-, early-, and late-gene expression (reviewed in reference 60). The IE protein ICP0 occupies an unusual position in this regulatory cascade, because it is required for efficient expression of the IE genes: ICP0 mutants display a greatly increased particle-to-PFU ratio and reduced levels of IE gene expression during infection (4, 5, 42, 43, 67, 68, 76), and ICP0 activates the expression of IE, early, and late genes in transient-transfection assays (7, 14, 24, 42, 52, 53, 59). Taken in combination, these observations indicate that VP16 is unable to fully activate IE gene expression in the absence ICP0. In this sense, the regulatory function of ICP0 appears to lie "upstream" of those of the other IE gene products.

The mechanism of action of ICP0 has yet to be precisely defined. ICP0 behaves as a promiscuous activator in transient-cotransfection assays, stimulating expression from a variety of HSV and heterologous promoters (reviewed in reference 22). Nuclear runoff transcription assays indicate that it acts at the transcriptional or pretranscriptional level (35, 64), and Lium et al. have shown that it contains a promoter-specific amino-terminal acidic transcriptional activation domain (42). ICP0 localizes to nuclear ND10 domains and disperses their constituent proteins (17, 47), an activity that correlates with activation function in mutational studies (20, 46). ICP0 also directly interacts with a variety of cellular proteins including the G_1-phase cell cycle regulator cyclin D3 (37), translation elongation factor 1δ (36), and a ubiquitin-specific protease, HAUSP (19, 50). The association between ICP0 and HAUSP correlates with ICP0 function (18), suggesting a link to ubiquitin-mediated protein turnover pathways. Consistent with this view, ICP0 induces proteasome-dependent degradation of the catalytic subunit of the DNA-dependent protein kinase (39, 54), some isoforms of the ND10-associated PML protein (16), and the kinetochore binding protein CENP-C (15). Moreover, ICP0 activation function is blocked by proteasome inhibitors (21). These data have led to the emerging hypothesis that ICP0 acts by altering the stability of specific cellular proteins, thus enhancing the environment for HSV-1 replication (3, 16, 18). This hypothesis may explain how ICP0 plays a key role in the

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establishment and reactivation phases of latency (4, 8, 28, 40, 73, 77), as well as the lytic replication cycle.

Although VP16 and ICP0 appear to stimulate gene expression through very different mechanisms, viral mutants lacking VP16 or ICP0 activation function display a number of striking similarities, including (i) severely impaired replication at low multiplicities of infection (2, 61, 69), (ii) increased particle-to-PFU ratio (2, 61, 69), (iii) efficient growth on U2OS osteosarcoma cells (66, 76), and (iv) cell cycle-dependent variation in the severity of the mutant phenotype (6, 9). In addition, Ace et al. reported that the expression of ICP0 in *trans* at least partially complements the defect of a VP16 mutation (2). These findings suggest that the functions of VP16 and ICP0 are interlinked and/or overlap. One interpretation is that the primary physiological role of the transactivation function of VP16 is to stimulate expression of ICP0, which then suffices to activate the other IE genes. We sought to explore the regulatory links between VP16 and ICP0 by studying the phenotypes of viral mutants in which the activation functions of both proteins were simultaneously inactivated. This analysis unexpectedly revealed a major phenotypic difference between the *in*1814 and V422 VP16 alleles when these mutations were placed in an ICP0-deficient background and demonstrated that accumulation of IE RNAs is rendered almost completely dependent on ICP0 when the C-terminal activation domain of VP16 is deleted.

MATERIALS AND METHODS

Cells and viruses. Vero and U2OS cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5 and 10% fetal bovine serum, respectively. Human embryonic lung fibroblasts (HEL cells) were generously provided by C. Spencer and maintained in DMEM–10% fetal bovine serum. HSV-1 KOS and 17syn+ were propagated on Vero cells. HSV-1 *in*1814 (2), V422 (38), *n*212 (7), KM100, and KM110 (see below) were propagated on U2OS cells in the presence of 3 mM hexamethylene bisacetamide (HMBA; Sigma).

Construction of recombinant viruses. Two ICP0/VP16 double mutants that combine the *n*212 ICP0 mutation with either the *in*1814 or V422 VP16 mutation (KM100 and KM110, respectively) were constructed. U2OS cells were coinfected with each parental virus at a multiplicity of infection (MOI) of 5, and plaque-purified progeny were screened by Southern blot hybridization for the *Spe*I, *Bam*HI, and *Nhe*I linkers that define the *n*212, *in*1814, and V422 mutations respectively. Doubly mutant recombinants were then plaque purified three times to obtain a final working stock.

Southern blot analysis. Monolayers growing in wells of a six-well plate were infected with the indicated virus at an MOI of 10, and harvested 24 h postinfection directly into 1× lysis buffer (0.6% sodium dodecyl sulfate [SDS], 10 mM Tris [pH 7.5], 10 mM EDTA, 100 μg of proteinase K per ml). Following incubation at 37°C for 4 h, DNA was precipitated with 95% ethanol and resuspended in 10 mM Tris (pH 7.6)–1 mM EDTA. DNA was cleaved with the indicated restriction endonuclease, separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled probe generated by random priming in ExpressHyb (Clontech) buffer as specified by the manufacturer. The VP16 probe was a 2.9-kb *Bam*HI fragment spanning the entire VP16 open reading frame, and the ICP0 probe was a 0.5-kb *Xho*I-*Bam*HI fragment internal to the ICP0 gene.

Northern blot analysis. Cells growing in 100-mm dishes were infected at the indicated MOI. Where indicated, cycloheximide (100 μg/ml) was added 1 h prior to infection and maintained continuously. Total cellular RNA was extracted from infected monolayers by using Trizol (Gibco-BRL). Aliquots (5 μg) of RNA were prepared in MOPS buffer (20 mM morpholinepropanesulfonic acid [MOPS], 5 mM sodium acetate, 1 mM EDTA) containing 50% formamide and 20% formaldehyde, incubated at 55°C for 15 min, cooled on ice, and then loaded onto a 1% agarose gel containing 1× MOPS buffer, 2% formaldehyde, and 0.5 μg of ethidium bromide per ml. Following electrophoresis, RNA was transferred to a nylon membrane and hybridized as described above. Probes for ICP22 and ICP8 were 1.2- and 1.9-kb fragments, respectively, derived from the 5′ portions of these genes.

Western blot analysis. Cellular lysates harvested directly into 1× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer were separated on 9% polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with 5% skim milk in TBS-T (1× Tris-buffered saline, 0.2% Tween 20). The membranes were washed extensively in TBS-T and incubated with both primary and secondary antibodies for 30 min each in TBS-T. Protein was visualized by enhanced chemiluminescence (Gibco-BRL). VP16 was detected with a 1:5,000 dilution of LP1 (kindly provided by A. Minson), and VP5 was detected with a 1:20,000 dilution of NC-1 (kindly provided by G. H. Cohen and R. J. Eisenberg).

Purification of virions. Roller bottles of U2OS cells were infected at an MOI of 1 in the presence of 3 mM HMBA. Cells were harvested 2 days postinfection, pelleted at 1,700 × *g* for 10 min, and resuspended in 1 mM sodium phosphate buffer (pH 7.4). The cells were Dounce homogenized on ice, and nuclei were pelleted at 3,000 × *g* for 5 min at 4°C. Supernatants were loaded onto dextran gradients and centrifuged at 50,000 × *g* for 1 h at 4°C. Linear dextran gradients were made by mixing solutions of dextran (Sigma) prepared in 1 mM phosphate buffer (pH 7.5) in a gradient maker (18.4 ml of the lighter solution [ρ = 1.04] was mixed with 17.6 ml of the denser solution [ρ = 1.09]). Banded virus was removed from the gradient, resuspended in serum-free DMEM, and pelleted by centrifugation at 78,000 × *g* for 2 h at 4°C.

RESULTS

Construction of HSV-1 ICP0/VP16 double mutants. HSV-1 mutants bearing lesions in ICP0 and VP16 display similar multiplicity- and cell cycle-dependent defects in viral gene expression and are "complemented" to approximately the same extent by growth on U2OS osteosarcoma cells. Our initial objective was to explore the degree to which the functions of these two activators overlap. To this end, we constructed two ICP0/VP16 double mutants (Fig. 1). These isolates harbor the *n*212 ICP0 mutation (7) and the *in*1814 (2) or V422 (38) VP16 mutations (isolates KM100 and KM110, respectively). KM100 and KM110 were produced by in vivo recombination between *n*212 and *in*1814 or V422 in coinfected U2OS cells in the presence of 3 mM HMBA (see Materials and Methods). Following plaque purification, recombinants were identified by Southern blot analysis of the ICP0 and VP16 loci. As diagrammed in Fig. 1A, all three parental mutations are marked by a diagnostic restriction endonuclease cleavage site: *n*212 was derived by inserting a synthetic *Spe*I linker bearing an in-frame termination codon into the second exon of the ICP0 gene (truncating the protein after amino acid residue 212), *in*1814 bears an in-frame *Bam*HI linker that inserts four extra amino acids into VP16 following residue 397, and V422 is marked by a chain-terminating *Nhe*I linker that truncates VP16 after residue 422 (removing the majority of the C-terminal acidic transcriptional activation domain). Southern blot analysis confirmed the status of the ICP0 and VP16 alleles in recombinants KM100 and KM110 (Fig. 1B). We also generated derivatives of KM100 and KM110 in which the VP16 and ICP0 mutations were individually rescued to the wild type (data not shown).

Plaquing efficiency of mutant viruses. Mutations that inactivate the *trans*-inducing activity of VP16 or ICP0 greatly reduce the probability that cells infected with a single HSV virion will enter the lytic cycle (2, 69). As a result, these mutations lead to an increased particle-to-PFU ratio (reduced titer) in plaque assays conducted under noncomplementing conditions. The defect of VP16 mutants can be largely overcome by adding HMBA to the culture medium (48), and ICP0 and VP16 mutants are efficiently "complemented" on U2OS cells (66, 76). We subjected stocks of our VP16/ICP0 double mutants to titer determination on Vero and U2OS cells in the presence or absence of 3 mM HMBA, to determine if simultaneous inactivation of ICP0 and VP16 produces a more severe defect than loss of only one of these activators (Fig. 2). As previously described (48, 66, 76), wild-type strains KOS and 17syn+ plaqued with similar efficiency under all four conditions, while *n*212, *in*1814, and V422 displayed a titer that was ca. 2 log units (*n*212 and *in*1814) to 3 log units (V422) lower on Vero cells than on U2OS cells (Fig. 2). HMBA markedly stimulated the VP16 mutants (and had a marginal effect on *n*212) in Vero cells but had a smaller effect in U2OS cells. The KM100 and KM110 double mutants displayed a much more severe defect

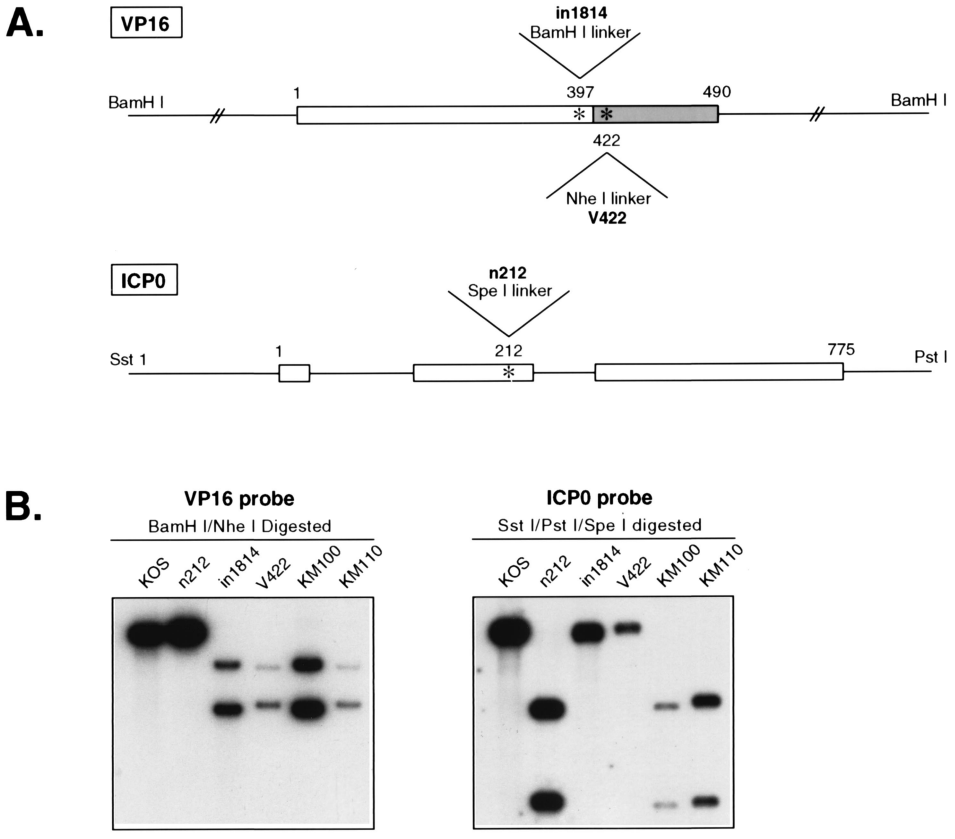

FIG. 1. Construction of ICP0/VP16 double mutants KM100 and KM110. (A) Schematic diagram of the VP16 and ICP0 loci, indicating the locations of the linkers corresponding to the *in*1814, V422, and *n*212 mutations. The C-terminal acidic activation domain of VP16 is displayed as a shaded box. Numbers refer to amino acid residues. Diagrams are not to scale. (B) Southern blot analysis of viral DNA from wild-type and mutant viruses. Viral DNA was digested with the indicated restriction endonucleases and subjected to Southern blot hybridization as described in Materials and Methods.

under "noncomplementing" conditions than did either of their singly mutated parents (the titer was 4 and >5 log units lower on Vero cells minus HMBA than on U2OS cells plus HMBA, respectively). Indeed, KM110 was essentially incapable of forming plaques on Vero cells in the absence of HMBA. Derivatives of KM100 and KM110 in which the VP16 and ICP0 mutations were individually rescued to wild type could not be distinguished from their respective single-mutant parental counterparts in this assay (data not shown), indicating that the severe defect displayed by the double mutants stems from simultaneous inactivation of ICP0 and VP16.

These data provide genetic evidence that ICP0 and VP16 make largely independent contributions to plaquing efficiency on Vero cells and that U2OS cells are able to bypass the requirement for both proteins. Moreover, they demonstrate that the V422 VP16 allele produces a more severe phenotype than does the *in*1814 allele, especially in an ICP0-deficient background.

KM110 is severely defective in IE gene expression. The remarkably severe defect exhibited by KM110 in plaque assays on Vero cells prompted us to examine viral IE gene expression during infection at higher input MOIs (Fig. 3). Vero and U2OS cells were infected at 0.5 and 5 PFU/cell in the presence or absence of cycloheximide, and total RNA harvested at 6 h postinfection was scored for ICP22 mRNA by Northern blot hybridization. Input MOIs were based on the titers obtained in U2OS cells in the presence of HMBA. Consistent with previous work, Vero cells infected with *n*212, *in*1814 and V422 showed reduced levels of ICP22 RNA relative to the wild-type strains KOS and 17syn+, particularly at the lower input MOI (Fig. 3). In contrast, these mutants displayed little if any defect in U2OS cells. Perhaps surprisingly, KM100 was not obviously impaired in this assay relative to its single-mutant parents (*in*1814 and *n*212), a result which may reflect the higher MOIs used in this experiment. In striking contrast, KM110 produced virtually no ICP22 mRNA during infection of Vero cells at either MOI. However, the same aliquot of KM110 induced high levels of ICP22 RNA in U2OS cells, confirming that biologically active virus was present. Moreover, cycloheximide increased the level of ICP22 RNA in Vero cells infected with KM110 to roughly that observed with *in*1814, V422, and KM100. Preston et al. (58) have shown that cycloheximide actively stimulates HSV IE gene transcription under conditions where the major viral transactivators are absent. Our data support this conclusion and argue that the KM110 genome is delivered to infected Vero cells in a potentially expressible state.

Entirely analogous results were obtained when probes for ICP4 and ICP27 RNA were used (data not shown). Taken in combination, these data indicate that KM110 displays a severe defect in IE gene expression in Vero cells, even at high MOIs.

KM110 does not inhibit IE gene expression from KM100. The data presented in Fig. 2 and 3 demonstrate that the V422 and *in*1814 VP16 mutations produced readily distinguishable

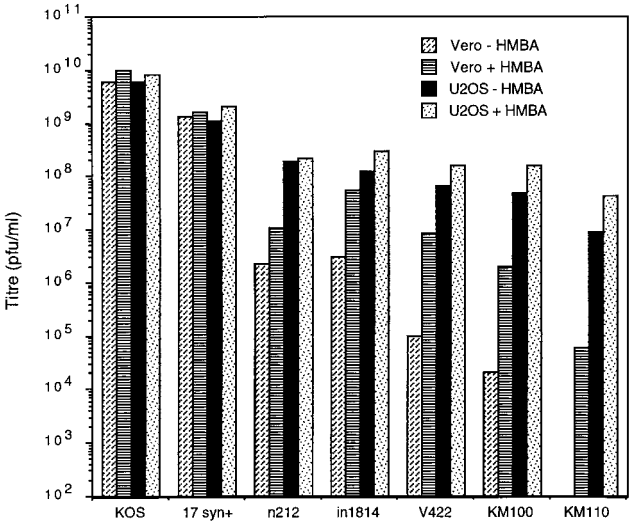

FIG. 2. Plaquing efficiency of mutant viruses. Virus stocks were subjected to titer determination on Vero and U2OS cells in the presence or absence of 3 mM HMBA, as indicated.

phenotypes when combined with the *n*212 ICP0 mutation, with V422 displaying a more severe defect. This observation was somewhat surprising, because the *in*1814 mutation is thought to completely eliminate the transactivation function of VP16 (by preventing the assembly of VP16 into the complex with Oct1, HCF, and DNA [1, 2, 27, 30]). We therefore considered the possibility that the V422 protein actively represses the VP16-independent basal activity of IE promoters and thus produces a more severe phenotype than that encoded by a simple loss-of-function mutation. Consistent with this hypothesis, the V422 mutation truncates the C-terminal acidic activation domain of VP16 but leaves the region of the protein required for promoter recognition intact (25, 26). Indeed, Greaves and O'Hare have shown that VP16 truncated at residue 422 (as in V422) retains the ability to assemble into a complex with Oct1, HCF, and DNA but is incapable of activating IE promoters (26). Moreover, McKnight and colleagues have shown that a similarly truncated VP16 derivative blocks transactivation mediated by wild-type VP16 (71) and serves as a *trans*-dominant inhibitor of HSV-1 replication (23). Alternatively, it was conceivable that the *in*1814 form of VP16 retains one or more residual functions that marginally stimulate IE gene expression in the context of a viral infection. As one approach to distinguishing between these two scenarios, we asked if KM110 inhibits viral gene expression in cells coinfected with KM100 (Fig. 4). The results demonstrated that cells coinfected with KM100 and KM110 accumulated approximately the same amount of ICP22 and ICP8 RNA as did cells singly infected with KM100. Similar results were obtained for ICP4 and ICP27 transcripts (data not shown). Inasmuch as purified KM110 virions appear to contain roughly the same amount of VP16 as wild-type HSV-1 KOS does (Fig. 5), these results suggest that the V422 VP16 protein present in KM110 virions does not act as a strong *trans*-acting repressor of VP16-independent IE gene expression. This interpretation is further supported by our finding that KM110 expresses easily detectable levels of IE RNAs during infection of U2OS cells and in Vero cells treated with cycloheximide (Fig. 3).

KM110 is markedly less cytotoxic than KM100 and persists in a quiescent state in restrictive cells. Early studies by Johnson et al. established that expression of HSV-1 IE proteins is cytotoxic to cultured cells (33, 34). More recently, the groups of Preston and DeLuca have shown that the cytotoxicity of HSV-1 can be reduced or eliminated by introducing multiple mutations into the viral genome that prevent synthesis of the

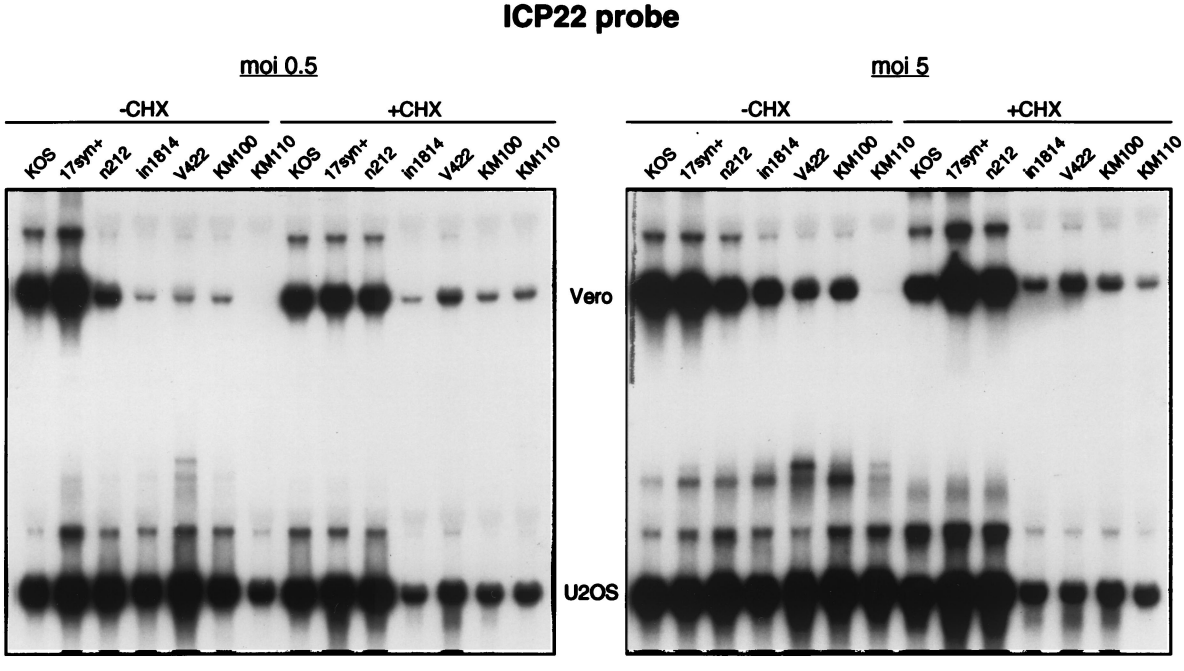

FIG. 3. Northern blot analysis of ICP22 RNA levels. Vero and U2OS cells were infected with either 0.5 or 5 PFU of the indicated virus per cell in the presence or absence of 100 μg of cycloheximide (CHX) per ml. The same inoculum was used to infect both cell types. At 6 h postinfection, RNA was extracted and analyzed for ICP22 RNA levels by Northern blot hybridization.

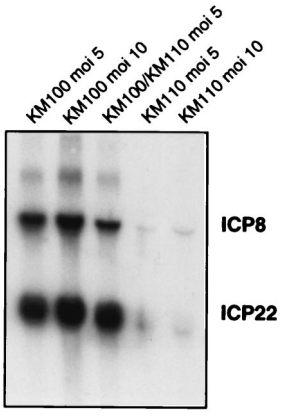

FIG. 4. ICP22 and ICP8 RNA levels in cells coinfected with KM100 and KM110. Vero cells were singly infected or coinfected with KM100 or KM110 at the indicated MOI. RNA extracted 6 h postinfection was analyzed for ICP22 and ICP8 RNA by Northern blot hybridization.

IE proteins of HSV (29, 32, 56, 57, 63). The substantial defect in IE gene expression exhibited by KM110 suggested that this isolate might display a similar reduction in cytotoxicity. To assess this possibility, we infected Vero cells and HEL cells with varying input MOIs of KM110 and KM100 and examined the cultures 3 days postinfection (Fig. 6). The results demonstrated that KM110 is much less toxic than KM100, particularly on HEL cells. Vero cell monolayers tolerated infection with 1 and 5 PFU of KM110 and produced only small isolated foci of cytopathic effect, while HEL cells could be infected with 10 and 20 PFU/cell and showed no detectable cytotoxicity. In contrast, KM100 induced virtually complete destruction of monolayers of both cell types at 1 PFU/cell. These data provide additional evidence that KM110 is substantially more impaired than KM100 and indicate that KM110 is essentially incapable of entering the lytic cycle in HEL cells.

Previous studies have demonstrated that similarly compromised viral mutants bearing multiple lesions in several viral transactivators persist for extended periods in nonproductively infected cells, in a quiescent form that can be induced into the lytic cycle by superinfection with wild-type HSV (29, 32, 56, 57, 63). To clarify the nature of the defect exhibited by KM110, we investigated whether potentially expressible copies of the

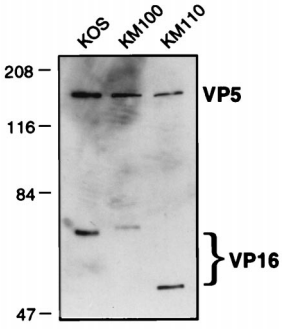

FIG. 5. VP16 levels in purified virions. KOS, KM100, and KM110 virions were purified by centrifugation through dextran gradients (see Materials and Methods), pelleted, resuspended in SDS-PAGE sample buffer, and subjected to electrophoresis through an SDS–9% polyacrylamide gel. The relative amounts of the capsid protein (VP5) and VP16 were then visualized by Western blot analysis with antibodies NC-1 and LP1, respectively. Note the differences in the electrophoretic mobilities of the wild-type, *in*1814, and V422 forms of VP16.

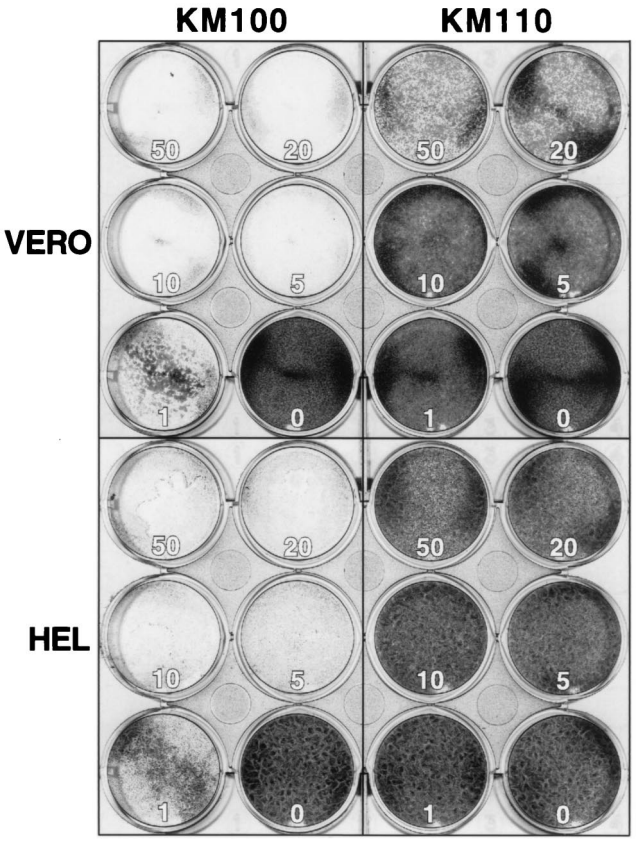

FIG. 6. KM110 is less cytotoxic than KM100. Monolayers of Vero and HEL cells were infected at the indicated MOIs, fixed, and stained 72 h postinfection.

KM110 genome persist in infected HEL cells. These experiments used the altered electrophoretic mobility of VP16 encoded by KM110 (Fig. 5) as a means of specifically monitoring expression from the KM110 genome in the presence of superinfecting wild-type HSV-1.

Confluent monolayers of HEL cells were infected with 10 PFU of KM110 per cell or left untreated. Three days later the cells were either superinfected with wild-type HSV-1 KOS or mock infected. At the same time, control monolayers were infected with KOS, KM110, or both viruses. The cells were then harvested 24 h later (i.e., 4 days after the initial infection with KM110), and expression of VP16 was examined by Western blot analysis (Fig. 7A). HEL cells singly infected with KM110 expressed little if any VP16. However, high levels of VP16 arising from the KM110 genome were detected in cells that were either coinfected or superinfected with KOS. Further analysis revealed that the resident KM110 genome remained susceptible to activation by superinfecting KOS for at least 10 days (Fig. 7B). Taken in combination, these data indicate that the KM110 genome persists in a quiescent but inducible form in infected HEL cells. These results provide additional evidence that KM110 displays a severe defect in launching the viral lytic cycle and argue that the defect operates at the level of gene expression (as opposed to a defect in adsorption or penetration). Thus, this recombinant will probably provide a useful tool for analyzing the quiescent state adopted by HSV genomes in the absence of IE gene expression.

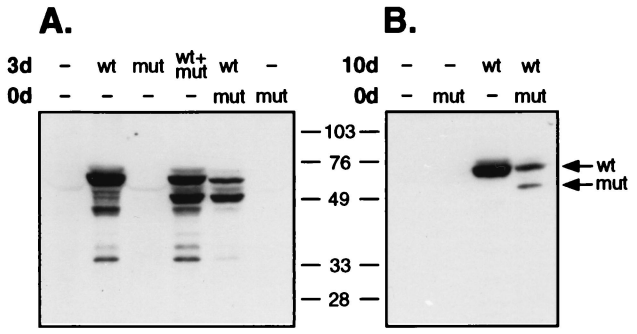

FIG. 7. Persistence of quiescent KM110 genomes in infected HEL cells. Confluent monolayers of HEL cells were either left untreated (0d, −) or infected with KM110 at an MOI of 10 (0d, mut). At 3 days (A) or 10 days (B) later, the cells were superinfected with 10 PFU of KOS (wt) or KM110 (mut) per cell or mock infected (−). Monolayers were harvested 24 h later, and VP16 was detected by Western blot analysis. Arrows indicate the mobility of VP16 arising from either the KOS (wt) or KM110 (mut) genome.

DISCUSSION

Several lines of evidence indicate that VP16 and ICP0 play interrelated roles in stimulating the onset of the HSV lytic cycle. First, mutations that eliminate the activation functions of ICP0 and VP16 produce similar multiplicity- and cell cycle-dependent defects in viral gene expression (2, 6, 9, 13, 61, 69). Second, expression of ICP0 in *trans* at least partially alleviates the effects of a VP16 mutation (2). Similarly, ICP0 stimulates the production of virus from transfected protein-free viral DNA (7). Third, ICP0 and VP16 mutants are both "complemented" on U2OS cells (66, 76), which have been proposed to express a cellular ICP0-like function (76). Fourth, ICP0 contains an amino-terminal transcriptional activation domain that selectively activates IE promoters (42). Taken in combination, these data suggest that, once expressed, ICP0 can at least partially substitute for VP16 activation function. This in turn raises the possibility that the primary role of the transactivation function of VP16 is to stimulate expression of ICP0, which then suffices to activate the other IE genes. If activation of ICP0 was the only physiologically relevant function of VP16, VP16/ICP0 double mutants should display a phenotype similar to that of an ICP0 mutant. However, we found that such double mutants exhibit a 4- to >5-log-unit reduction in titer under noncomplementing conditions while their singly mutant parents display only a 2- to 3-log-unit reduction relative to wild-type virus. These data indicate that VP16 and ICP0 make largely independent contributions to plaquing efficiency on Vero cells. Preston et al. (56) have reported similar results with another *in*1814-based VP16/ICP0 double mutant (*in*1820), in which ICP0 expression was reduced by placing the gene under the control of the murine leukemia virus long terminal repeat. The simplest interpretation of these findings is that although ICP0 can partially substitute for VP16 function, VP16-induced transactivation of other IE genes plays a major role in triggering the onset of the lytic cycle when ICP0 is inactivated.

We found that the VP16/ICP0 double mutant bearing the V422 VP16 allele (KM110) displayed a markedly more severe phenotype than did the double mutant harboring the *in*1814 allele (KM100). Thus, KM110 was essentially incapable of forming plaques on Vero cells and displayed a much more severe defect in IE gene expression during high-MOI infection. In addition, HEL cell monolayers survived infection with 20 PFU of KM110 per cell without obvious cytopathic effect, while KM100 induced extensive cell death at 1 PFU/cell. The large difference between the two double mutants was surprising, because the *in*1814 mutation is thought to eliminate the transactivation function of VP16. One possible explanation was that the V422 protein actively represses IE expression, thereby reducing expression below the level obtained with a simple loss-of-function mutant (*in*1814). However, although similarly truncated derivatives of VP16 can block transactivation mediated by wild-type VP16 (23, 71), KM110 did not interfere with IE gene expression in cells coinfected with KM100. This result argues that the V422 form of VP16 does not serve as a strong *trans*-acting inhibitor of the VP16-independent activity of IE promoters. Consistent with this view, KM110 expressed readily detectable levels of IE transcripts during infection of U2OS cells and in Vero cells treated with cycloheximide. In our view, the simplest interpretation of our data is that the V422 mutation eliminates the ability of VP16 to stimulate IE gene expression in the context of an HSV infection whereas the *in*1814 protein retains residual function. How can one reconcile this hypothesis with previous data that clearly demonstrate that the *in*1814 mutation abolishes the ability of VP16 to stimulate IE transcription in the absence of other HSV proteins, by preventing the assembly of VP16 into the complex with Oct1, HCF, and DNA (1, 2, 27, 30)? One possibility is that VP16 influences the packaging or activity of other tegument proteins that facilitate IE gene expression, in addition to directly stimulating IE transcription. According to this hypothesis, the V422 lesion inactivates both of these stimulatory functions whereas the *in*1814 mutation affects only direct transactivation. In this context, it is interesting that VP16 directly binds to at least two tegument proteins, the virion host shutoff (vhs) protein (65) and VP22 (12). Intriguingly, the interaction with VP22 occurs through the C-terminal transcriptional activation domain (12).

KM110 exhibited a much more severe defect in IE gene expression than did either KM100 or V422 at high MOI (Fig. 3). This finding indicates that IE gene expression becomes almost completely dependent on ICP0 when the activation domain of VP16 is deleted. Inasmuch as ICP0 is itself an IE gene product, an interesting question arises: what is the source of the ICP0 protein that allows V422 to express its IE genes more efficiently than KM110 does? One possibility is that sufficient quantities of ICP0 are produced in the newly infected cell to launch the infection. However, an alternative explanation is suggested by the finding that small amounts of ICP0 are packaged into the tegument of HSV virions produced in Vero and HEp-2 cells (74, 75). In this context, Dargan et al. have provided evidence that ICP0 delivered into cells by noninfectious L particles is biologically active in that it can enhance the infectivity of transfected viral DNA (11). Thus, it is possible that ICP0 delivered by the infecting virion plays a key role in initiating the V422 infection. Further experiments are required to test this hypothesis.

Although KM110 is effectively unable to replicate in Vero and HEL cells, it can be readily propagated on U2OS osteosarcoma cells (Fig. 2), which have been previously shown to "complement" ICP0 and VP16 mutants (66, 76). This result demonstrates that U2OS cells can compensate for the simultaneous loss of both ICP0 and VP16 activation functions. The molecular basis for this "complementation" is unknown. Yao and Schaffer have suggested that U2OS cells express a functional homologue of ICP0, and our data are consistent with this hypothesis. However, an equally plausible explanation is that these cells lack one or more inhibitors of IE gene expression that are present in most other cell types. In this context, it is interesting that current evidence suggests that ICP0 stimulates viral gene expression by inducing proteosome-dependent

degradation of one or more cellular proteins (15, 16, 18, 21, 54). Defining the mechanism of "complementation" by U2OS cells will probably enhance our understanding of the regulation of HSV IE gene expression. It will also be important to determine if other human tumor-derived cell lines exhibit similar complementing properties. If so, KM110 may have considerable potential for viral antitumor therapy.

Vero and HEL cells tolerated infection with KM110 at quite high MOIs without showing major cytopathic effects, and the KM110 genome persisted in a quiescent form in infected HEL cells for at least 10 days. These findings demonstrate that it is possible to effectively prevent the onset of the HSV lytic cycle by mutating two viral regulatory proteins: VP16 and ICP0. KM110 differs from previously described HSV constructs displaying similar properties (56, 63), in that it retains functional copies of four of the viral IE genes. We therefore believe that KM110 and derivatives will serve as useful tools for investigating the quiescent state adopted by HSV genomes in the absence of IE gene expression and the process of reactivation from this state. KM110 or related constructs may also prove to be useful platforms for the development of HSV vectors for gene therapy.

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REFERENCES

1. **Ace, C. I., M. A. Dalrymple, F. H. Ramsay, V. G. Preston, and C. M. Preston.** 1988. Mutational analysis of the herpes simplex virus type 1 transinducing factor Vmw65. J. Gen. Virol. **69:**2595–2605.
2. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J. Virol. **63:**2260–2269.
3. **Burkham, J., D. Coen, and S. K. Weller.** 1998. ND10 protein PML is recruited to herpes simplex virus type 1 prereplicative sites and replication compartments in the presence of viral DNA polymerase. J. Virol. **72:**10100–10107.
4. **Cai, W., T. L. Astor, L. M. Lipak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J. Virol. **67:**7501–7512.
5. **Cai, W., and P. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J. Virol. **66:**2904–2915.
6. **Cai, W., and P. A. Schaffer.** 1991. A cellular function can enhance gene expression and plating efficiency of a mutant defective in the gene for ICP0, a transactivating protein of herpes simplex virus type 1. J. Virol. **63:**4578–4590.
7. **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:**4570–4589.
8. **Clements, G. B., and N. D. Stow.** 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latent-competent in mice. J. Gen. Virol. **70:**2501–2506.
9. **Daksis, J. I., and C. M. Preston.** 1992. Herpes simplex virus immediate early gene expression in the absence of transinduction by Vmw65 varies during the cell cycle. J. Gen. Virol. **189:**196–202.
10. **Dalrymple, M. A., D. J. McGeoch, A. J. Davison, and C. M. Preston.** 1985. DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate-early promoters. Nucleic Acids Res. **13:**7865–7879.
11. **Dargan, D. J., and J. H. Subak-Sharpe.** 1997. The effects of herpes simplex virus type 1 L-particles on virus entry, replication, and the infectivity of naked herpesvirus DNA. Virology **239:**378–388.
12. **Elliott, G., G. Mouzakitis, and P. O'Hare.** 1995. VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells. J. Virol. **69:**7932–7941.
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. **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.
15. **Everett, R. D., W. C. Earnshaw, J. Findlay, and P. Lomonte.** 1999. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein vmw110. EMBO J. **18:**1526–1538.
16. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection with the Vmw110- and proteasome-dependent loss of several PML isoforms. J. Virol. **72:**6581–6591.
17. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. EMBO J. **13:**5062–5069.
18. **Everett, R. D., M. Meredith, and A. Orr.** 1999. The ability of herpes simplex virus type 1 immediate-early protein Vmw110 to bind to a ubiquitin-specific protease contributes to its roles in the activation of gene expression and stimulation of virus replication. J. Virol. **73:**417–426.
19. **Everett, R. D., M. Meredith, A. Orr, A. Cross, M. Kathoria, and J. Parkinson.** 1997. A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. EMBO J. **16:**1519–1530.
20. **Everett, R. D., P. O'Hare, D. O'Rourke, P. Barlow, and A. Orr.** 1995. Point mutations in the herpes simplex virus type 1 Vmw110 ring finger helix affect activation of gene expression, viral growth, and interaction with PML-containing structures. J. Virol. **69:**7339–7344.
21. **Everett, R. D., A. Orr, and C. M. Preston.** 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. EMBO J. **17:**7161–7169.
22. **Everett, R. D., C. M. Preston, and N. D. Stow.** 1991. Functional and genetic analysis of the role of Vmw110 in herpes simplex virus replication, p. 49–76. *In* E. K. Wagner (ed.), The control of herpes virus gene expression. CRC Press Inc., Boca Raton, Fla.
23. **Friedman, A. D., S. J. Triezenberg, and S. L. McKnight.** 1988. Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. Nature **333:**452–454.
24. **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.
25. **Goding, C. R., and P. O'Hare.** 1989. Herpes simplex virus Vmw65-octamer binding protein interaction: a paradigm for combinatorial control of transcription. Virology **173:**363–367.
26. **Greaves, R., and P. O'Hare.** 1989. Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. J. Virol. **63:**1641–1650.
27. **Greaves, R. F., and P. O'Hare.** 1990. Structural requirements in the herpes simplex virus type 1 transactivator Vmw65 for interaction with the cellular octamer-binding protein and target TAATGARAT sequences. J. Virol. **64:**2716–2724.
28. **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.
29. **Harris, R. A., and C. M. Preston.** 1991. Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814. J. Gen. Virol. **72:**907–913.
30. **Hayes, S., and P. O'Hare.** 1993. Mapping of a major surface-exposed site in herpes simplex virus protein Vmw65 to a region of direct interaction in a transcription complex assembly. J. Virol. **67:**852–862.
31. **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.
32. **Jamieson, D. R. S., L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston.** 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus Vmw65 mutants. J. Gen. Virol. **76:**1417–1431.
33. **Johnson, P. A., A. Miyanohara, F. Levine, T. Cahill, and T. Friedmann.** 1992. Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. J. Virol. **66:**2952–2965.
34. **Johnson, P. A., M. J. Wang, and T. Friedmann.** 1994. Improved cell survival by the reduction of immediate-early gene expression in replication-defective mutants of herpes simplex virus type 1 but not by mutation of the virion host shutoff function. J. Virol. **68:**6347–6362.
35. **Jordan, R., and P. A. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J. Virol. **71:**6850–6862.
36. **Kawaguchi, Y., R. Bruni, and B. Roizman.** 1997. Interaction of herpes simplex virus 1 α regulatory protein ICP0 with elongation factor 1δ: ICP0 affects translational machinery. J. Virol. **71:**1019–1024.

37. **Kawaguchi, Y., C. V. Sant, and B. Roizman.** 1997. Herpes simplex virus 1 α regulatory protein ICP0 interacts with and stabilizes the cell cycle regulator cyclin D3. J. Virol. **71:**7328–7336.
38. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. EMBO J. **15:** 2575–2581.
39. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type I transactivator ICP0. J. Virol. **70:**7471–7477.
40. **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.
41. **Lemaster, S., and B. Roizman.** 1979. Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. J. Virol. **35:**798–811.
42. **Lium, E. K., C. A. Panagiotidis, X. Wen, and S. J. Silverstein.** 1998. The NH2 terminus of the herpes simplex virus type 1 regulatory protein ICP0 contains a promoter-specific transcription activation domain. J. Virol. **72:**7785–7795.
43. **Lium, E. K., and S. Silverstein.** 1997. Mutational analysis of the herpes simplex virus type 1 ICP0 C_3HC_4 zinc ring finger reveals a requirement for ICP0 in the expression of the essential α27 gene. J. Virol. **71:**8602–8614.
44. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44:**939–949.
45. **Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilkie.** 1978. Physical mapping of herpes simplex virus-induced polypeptide. J. Virol. **28:**624–642.
46. **Maul, G. G., and R. D. Everett.** 1994. The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J. Gen. Virol. **75:**1223–1233.
47. **Maul, G. G., H. H. Guldner, and J. G. Spivack.** 1993. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). J. Gen. Virol. **74:**2679–2690.
48. **McFarlane, M., J. I. Daksis, and C. M. Preston.** 1992. Hexamethylene bisacetamide stimulates herpes simplex virus immediate-early gene expression in the absence of trans-induction by Vmw65. J. Gen. Virol. **73:**285–292.
49. **McLean, G., F. Rixon, N. Langeland, L. Haarr, and H. Marsden.** 1990. Identification and characterization of the virion protein products of herpes simplex virus type 1 gene UL47. J. Gen. Virol. **71:**2953–2960.
50. **Meredith, M., A. Orr, and R. Everett.** 1994. Herpes simplex virus type 1 immediate-early protein Vmw110 binds strongly and specifically to a 135-kDa cellular protein. Virology **200:**457–469.
51. **O'Hare, P.** 1993. The virion transactivator of herpes simplex virus. Semin. Virol. **4:**145–155.
52. **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 promoter. J. Virol. **53:**751–760.
53. **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.
54. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein Vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J. Virol. **73:**650–657.
55. **Pellett, P. E., J. L. C. McKnight, F. J. Jenkins, and B. Roizman.** 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing α genes. Proc. Natl. Acad. Sci. USA **82:**5870–5874.
56. **Preston, C. M., R. Mabbs, and M. J. Nicholl.** 1997. Construction and characterization of herpes simplex virus type 1 mutants with conditional deletions in immediate early gene expression. Virology **229:**228–239.
57. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J. Virol. **71:**7807–7813.
58. **Preston, C. M., A. Rinaldi, and M. J. Nicholl.** 1998. Herpes simplex virus type 1 immediate early gene expression is stimulated by inhibition of protein synthesis. J. Gen. Virol. **79:**117–124.
59. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5:**957–963.
60. **Roizman, B., and A. E. Sears.** 1996. Herpes simplex viruses and their replication, p. 1043–1107. *In* B. N. Fields, D. M. Knipe, P. M. Howley et al. (ed.), Fundamental virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
61. **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.
62. **Sadowski, I., J. Ma, S. J. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature **335:**563–564.
63. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J. Virol. **72:**3307–3320.
64. **Samaniego, L. A., N. Wu, and N. A. DeLuca.** 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. J. Virol. **71:** 4614–4625.
65. **Smibert, C. A., B. Popova, P. Xiao, J. P. Capone, and J. R. Smiley.** 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. J. Virol. **68:**2339–2346.
66. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the *in*1814 linker insertion mutation. J. Virol. **71:**6191–6193.
67. **Stow, E. C., and N. D. Stow.** 1989. Complementation of a herpes simplex virus type 1 Vmw110 deletion mutant by human cytomegalovirus. J. Gen. Virol. **70:**695–704.
68. **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.
69. **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.
70. **Thompson, C. C., and S. L. McKnight.** 1992. Anatomy of an enhancer. Trends Genet. **8:**232–236.
71. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev. **2:**718–729.
72. **Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight.** 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. **2:**730–742.
73. **Wilcox, C. L., R. L. Smith, R. Everett, and D. Mysofski.** 1997. The herpes simplex virus type 1 immediate-early protein ICP0 is necessary for the efficient establishment of latent infection. J. Virol. **71:**6777–6785.
74. **Yang, T. Y., and R. J. Courtney.** 1995. Influence of the host cell on the association of ICP4 and ICP0 with herpes simplex virus type 1. Virology **211:**209–217.
75. **Yao, F., and R. Courtney.** 1992. Association of ICP0 but not ICP27 with purified virions of herpes simplex virus type 1. J. Virol. **66:**2709–2716.
76. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J. Virol. **69:**6249–6258.
77. **Zhu, X., J. Chen, C. Young, and S. Silverstein.** 1990. Reactivation of latent herpes simplex virus by adenovirus recombinants encoding mutant IE-0 gene products. J. Virol. **64:**4489–4498.