

## Persistence and Expression of the Herpes Simplex Virus Genome in the Absence of Immediate-Early Proteins

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**The immediate-early (IE) proteins of herpes simplex virus (HSV) function on input genomes and affect many aspects of host cell metabolism to ensure the efficient expression and regulation of the remainder of the genome and, subsequently, the production of progeny virions. Due to the many and varied effects of IE proteins on host cell metabolism, their expression is not conducive to normal cell function and viability. This presents a major impediment to the use of HSV as a vector system. In this study, we describe a series of ICP4 mutants that are defective in different subsets of the remaining IE genes. One mutant, *d109*, does not express any of the IE proteins and carries a green fluorescent protein (GFP) transgene under the control of the human cytomegalovirus IE promoter (HCMVIEp). *d109* was nontoxic to Vero and human embryonic lung (HEL) cells at all multiplicities of infection tested and was capable of establishing persistent infections in both of these cell types. Paradoxically, the genetic manipulations that were required to eliminate toxicity and allow the genome to persist in cells for long periods of time also dramatically lowered the level of transgene expression. Efficient expression of the HCMVIEp-GFP transgene in the absence of ICP4 was dependent on the ICP0 protein. In *d109*-infected cells, the level of transgene expression was very low in most cells but abundant in a small subpopulation of cells. However, expression of the transgene could be induced in cells containing quiescent *d109* genomes weeks after the initial infection, demonstrating the functionality of the persisting genomes.**

The temporal regulation of herpes simplex virus type 1 (HSV-1) gene expression during permissive infection commences with the induction of the immediate-early (IE) genes by the virion protein VP16 (3, 8). VP16 activates transcription by binding, along with the cellular factors Oct1 and host cell factor (HCF), to the TAATGARAT elements present in all IE promoters (56, 57, 80, 113, 116a). The virus encodes five IE proteins, designated infected cell polypeptides (ICP) 0, 4, 22, 27, and 47 (13, 43, 84). With the exception of ICP47, these IE proteins are known to have regulatory functions demonstrated to affect the coordinated expression of the HSV genome. ICP4 and ICP27 are absolutely essential for virus replication (18, 24, 71, 87, 96, 115), while the growth of ICP0 mutants is significantly impaired (97, 110). These three IE proteins have the most profound effects on subsequent viral gene expression.

ICP4 is the major regulatory protein of the virus, and it is necessary for the transition of viral gene transcription from the IE to the E phase (24, 87, 115). It functions as a repressor (19, 38, 76, 82) or activator (19, 27, 33, 81, 90) of transcription by forming multiple contacts with basal transcription factors (9, 37, 38, 105).

ICP0 is a potent transactivator of viral and cellular promoters in transient assays (27, 33, 81, 90), and it provides for efficient viral gene expression and growth, in vitro and in vivo (5, 7, 62). ICP0 enhances gene expression, in part by increasing the transcription rates of viral genes (50, 99), although the exact mechanism by which ICP0 accomplishes this is not yet clear. These functions most likely underlie the requirement for ICP0 in lytic viral growth and the efficient reactivation of the virus from latent infections as shown in the mouse and rabbit

models (5, 14, 28, 35, 62, 95). ICP0 has also been shown to interact with ND10 nuclear structures (29, 67) and components of the transcription (61), translation (51), cell cycle (52), and proteolytic (30) machinery of the cell. Therefore, it has the potential to affect many aspects of host cell metabolism.

ICP27 regulates the processing of viral and cellular mRNAs (39, 73, 74, 102, 106). It may also act by modulating the activities of ICP4 and ICP0 (75, 104), as well as the modification state of ICP4 (75, 92, 112). The combined activities of ICP27 contribute to efficient late-gene expression (71, 96). Recent studies have shown that ICP27 also significantly contributes to elevated levels of early-gene expression, explaining the requirement for ICP27 in viral DNA synthesis (98, 114).

ICP22 and ICP47 can be deleted from the genome without greatly affecting viral growth and viability in most cell types (70, 86, 103). ICP22 promotes efficient late-gene expression in a cell type-dependent manner (103). It has been reported to give rise to a novel phosphorylated form of RNA polymerase II (pol II) (93) as well as to regulate the stability and splicing pattern of the ICP0 mRNA (10). Its regulatory effects may thus be mediated through these activities. The function of ICP47 may be more relevant in vivo, where it may help the virus escape immune surveillance on the basis of its ability to block the presentation of antigenic peptides to CD8<sup>+</sup> cells (118).

In ICP4 mutant backgrounds, expression of viral early and late genes is dramatically reduced. This has led to the consideration of such backgrounds as starting points for the construction of gene transfer vehicles. However, despite the limited expression of the HSV genome in ICP4 mutant backgrounds, such mutants are very toxic to cells. This is most likely because of the overexpression of the remaining IE proteins in the absence of ICP4. Several of the IE genes reduce the transformation of cells when cotransfected with a selectable marker, implying that the protein products of the transfected IE genes are not conducive to cell survival (48). Given this observation, and the findings of numerous groups on the effects of IE

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proteins on different aspects of host cell metabolism, it seems reasonable that the elimination of IE protein activity is essential for the efficient and safe use of HSV as a gene transfer vehicle. Lending support to this hypothesis are the observations that elimination of the VP16 activation function in an ICP4 mutant background (48, 88) and the deletion of subsets of IE genes (99, 117) reduce toxicity. Both ICP0 and ICP22 contribute to toxicity, as demonstrated by the improved survival of infected cells when these activities were individually abrogated in the background of viruses already deleted for ICP4 and ICP27 (99, 117). However, both of these triple mutants remained toxic at high multiplicities of infection (MOI). For one of these studies, we hypothesized that the elimination of ICP22 activity from the ICP4<sup>-</sup> ICP27<sup>-</sup> ICP0<sup>-</sup> background would further reduce toxicity.

Another point of consideration with viral vectors is the degree of transgene expression. The IE proteins of HSV have multiple and varied effects on the expression of viral genes, mostly, but not entirely, acting to increase gene expression. Similar effects are to be expected for transgenes inserted into the viral genome. Therefore, the elimination of viral IE genes may be expected to decrease toxicity but also might significantly reduce transgene expression. In support of this hypothesis, elimination of VP16, ICP4, and ICP0 activity reduced cytotoxicity but also reduced gene expression (88, 89). The same observation was made in studies of an ICP4<sup>-</sup> ICP27<sup>-</sup> ICP0<sup>-</sup> mutant (99). In the latter study it was concluded that ICP0 could significantly increase the transcription rates of genes in the absence of ICP4 and ICP27. The genes of HSV are standard pol II transcription units (15). Therefore, heterologous pol II promoters used to express transgenes may be affected by HSV IE genes in the same way as HSV promoters are affected.

In the current study, we set out to further assess the toxicity of different IE mutant backgrounds and examine the level of gene expression as a consequence of reduced toxicity and elimination of viral *trans*-acting factors. In all the mutants that are deleted for ICP4 and ICP27, an expression cassette containing the green fluorescent protein (GFP) reporter gene under the control of the human cytomegalovirus (HCMV) IE promoter-enhancer was inserted into the ICP27 deletion locus as a model transgene. The following points were addressed, with the stated general outcomes.

(i) **Vector toxicity.** Both ICP0 and ICP22 contribute to the toxicity of a virus deleted for ICP4 and ICP27. Elimination of these functions in addition to ICP47 resulted in a virus that was by several criteria nontoxic to Vero and human embryonic lung (HEL) cells at high MOI.

(ii) **Genome persistence.** The genome of the virus (*d109*) that did not express any IE proteins was capable of long-term persistence in both HEL and Vero cells.

(iii) **Gene expression.** The abundant expression of the HCMVIEp-GFP transgene was dependent on the ICP0 protein. In the absence of ICP0, the level of transgene expression was very low in most cells but abundant in a subpopulation of cells. Expression of the transgene could also be induced weeks after infection in cells containing persistent *d109* genomes.

## MATERIALS AND METHODS

**Cells and viruses.** Vero cells and the complementing cell lines E5 (ICP4<sup>+</sup>) (20), E26 (ICP4<sup>+</sup> ICP27<sup>+</sup>) (98), L7 (ICP0<sup>+</sup>) (99), and FO6 (ICP4<sup>+</sup> ICP27<sup>+</sup> ICP0<sup>+</sup>) (99) were maintained by standard cell culture procedures as previously described (17, 24). Tissue culture medium for maintaining L7 cells was supplemented with 400 µg of G418 (GIBCO-BRL)/ml. For maintaining FO6 cells, the medium was supplemented with 400 µg of G418 and 300 µg of hygromycin B (Boehringer Mannheim)/ml. Viruses carrying single or multiple IE mutations were propagated and subjected to titer determination on the appropriate cell

lines. The viruses *d92* (ICP4<sup>-</sup> ICP27<sup>-</sup>), *d96* (ICP4<sup>-</sup> ICP22<sup>-</sup>), and *d95* (ICP4<sup>-</sup> ICP27<sup>-</sup> ICP22<sup>-</sup>) were described previously (98, 117). The viruses with mutations in capsid genes, including K23Z (22), K5ΔZ (22), KUL26ΔZ (23), and KΔ19C (85), were generously provided by Stanley Person (Johns Hopkins University, Baltimore, Md.). In experiments that compare *d107*, *d106*, *d104*, and *d109*, the infectivities of the virus stocks were normalized on the basis of the number of genomes in infected nuclei at 6 h postinfection (p.i.) as determined by PCR amplification.

**Plasmid constructions.** The plasmid pEBΔAE contains the *EcoRI*-B genomic fragment with deleted copies of ICP4 (*d120*) (18) and ICP0 (0Δ) and a wild-type (wt) copy of ICP27. The boundaries of the deletion in the ICP0 gene are defined by the *BsmI* site at nucleotide (nt) 120142 and the *AseI* site at nt 124775. pEBΔAE was constructed by inserting the 2.7-kb *XbaI* fragment isolated from pAE into the unique *XbaI* site of pEB. Both pEB and pAE derived their cloned HSV sequences from *pd/EB*, which contains the *EcoRI*-B genomic fragment isolated from the virus mutant *d120* ligated into the unique *EcoRI* site of pBR325 (21). To construct pEB, *pd/EB* was cleaved with *BsmI* and the ends of the resulting fragments were modified by ligation of adapter oligonucleotides (5'-CGTCTAGACGCG-3') to convert the *BsmI* sites to *XbaI* sites. This pool of fragments was digested simultaneously with *EcoRI* and *XbaI* and then fractionated in a 0.8% agarose gel to isolate the 10-kb *EcoRI-XbaI* fragment. After being isolated by electroelution, this 10-kb fragment was cloned into the corresponding sites of *puc19* to generate pEB. To construct pAE, *pd/EB* was cleaved simultaneously with *AseI* and *EcoRI*. After the ends of the resulting fragments were filled in with DNA polymerase I Klenow fragment, they were modified with *XbaI* linkers. From this pool of fragments, a 2.7-kb *XbaI* fragment was isolated by electroelution and ligated into the *XbaI* site of *puc19* to produce pAE.

In plasmid pGFP27, the GFP gene, under the control of the HCMV IE promoter (HCMVIEp), is substituted between the *BamHI* and *SalI* sites in the ICP27 locus. pGFP27 was constructed by insertion of the 1.6-kb HCMVIEp-GFP cassette into the unique *PacI* site of pAT2. The insert corresponded to an *AseI-MluI* fragment derived from pGFPX whose ends were modified with *PacI* linkers. pGFPX was derived from pEGFP-C1 (Clontech) by deletion of the multiple cloning sites between the *BamHI* and *BglII* restriction enzyme sites. pAT2 resulted from the conversion of the unique *XbaI* site in pAT1 into a *PacI* site. Both pAT2 and pAT1 contain the *EcoRI*-to-*SalI* (nt 110095 to 120902) HSV sequence with the 1.2-kb deletion between the *BamHI* and *SalI* sites in the ICP27 locus. At the site of the ICP27 deletion is a unique *XbaI* site in pAT1. Construction of pAT1 was done by cloning the 3.2-kb *EcoRI-BamHI* fragment isolated from pKEB-S1 (4) into the corresponding site in *pucSF*. The plasmid *pucSF* contains the 6.4-kb *SalI* fragment derived from pKSF (4) inserted into the corresponding site in *puc19*.

The plasmid pTGTΔ contains the joint fragment of the HSV genome (P isotype) extending from the *AseI* site (nt 124775) 5' of ICP0 to the proximal *BamHI* site (nt 136289) 3' of ICP22, with the *d120* mutation and the 270-bp TGTΔ deletion in the ICP22 promoter located in the *c'* repeat sequence. The TGTΔ mutation removes the TAATGARAT elements in the ICP22 promoter and is defined by the *EcoRI* and *BssHIII* restriction enzyme sites. pTGTΔ was generated by insertion of the 2.7-kb *XbaI* fragment isolated from pAE into the unique *XbaI* site of *pucBNΔXB*. To construct *pucBNΔXB*, *pucBN* (99) was digested with *XbaI* and *BssHIII*. The large vector fragment isolated from this digest was then ligated in the presence of the oligonucleotide adapters 5'-CGC GCTTAATTAAT-3' and 5'-CTAGATTAATTAAG-3' to allow joining of the *XbaI*- and *BssHIII*-restricted ends. Both of these restriction enzyme recognition sites remained intact in the resulting plasmid, *pucBNΔXB*, and the oligonucleotide adapters also introduced a *PacI* site.

The *pucGFP* plasmid used as the source for the GFP fragment probe carries the 1.6-kb *PacI* fragment derived from pGFP27 and inserted into the unique *PacI* site of pNEB193 (New England Biolabs). The orientation of the insert is such that a *HindIII-NheI* double digestion results in a 1-kb fragment containing the GFP coding sequence.

**Viruses constructions.** The DNA-calcium phosphate precipitates used for transfections were prepared essentially as described by Graham and van der Eb (36). A typical mixture used to transfect approximately 10<sup>6</sup> cells plated in two 60-mm-diameter dishes contained 3 µg of viral DNA, 1 to 3 µg of the linearized plasmid carrying the mutant IE alleles, 1 µg of the plasmid pW3ΔHS8 (20), and 1 µg of pGreen Lantern-1 (GIBCO-BRL). pW3ΔHS8 provides ICP0, which improves the transfection efficiency of recombinant virus mutants deficient in this gene product. The plasmid pGreen Lantern-1 was added to allow convenient monitoring of transfection efficiency. It was not added for the construction of *d104*. Generation of mutants by crossing two viruses was done by coinfecting cells with approximately 5 PFU of each parent virus per cell.

**Southern blot analysis.** DNA, isolated from the IE mutant viruses as previously described (98), was cleaved with *HpaI* to detect the GFP substitution in ICP27, with *PstI* and *SacI* to detect the 0Δ deletion in ICP0, with *BamHI* and *SacI* to detect the *d120* mutation in ICP4 (18), and with *NcoI* to detect the TGTΔ mutation in the ICP22 and ICP47 promoters. Digested viral DNA was fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to nick-translated probes as previously described (100, 107). Gel-purified DNA fragments that were nick translated and used as probes included the 2.4-kb *BamHI-SacI* fragment derived from pKH-X-BH (4) for detecting ICP27, the 1.3-kb *PstI* fragment from pEB for detecting ICP0, the 1.8-kb *BamHI-Y* frag-

ment from pKBY (98) for detecting ICP4, and the 1.6-kb *PvuII* fragment from pucBN $\Delta$ XB for detecting the TGTA mutation.

**Isolation of infected-cell RNA and Northern blot analysis.** Total infected-cell RNA was prepared as previously described (98). For infections performed in the presence of cycloheximide (CHX), the medium was supplemented with the inhibitor (100  $\mu$ g/ml) 1 h prior to and during infection. Fractionation of the RNA samples in 1.3% agarose-formaldehyde gels and the conditions for blotting, hybridization, and washing have also been previously described (45). Gel-purified DNA fragments that were nick translated and used as probes included the 2-kb *HindIII* fragment from pucBN (99) for detecting the ICP22 mRNA, the 2.4-kb *BamHI-SacI* fragment from pKHX-BH (4) for detecting ICP27, and the 1-kb *NheI-HindIII* fragment from pucGFP for detecting GFP.

**Analysis of viral proteins.** Viral polypeptides were radiolabeled by incubating approximately  $6 \times 10^5$  cells infected at the indicated MOI with 100  $\mu$ Ci of [ $^{35}$ S]methionine per ml of medium at 6 to 9 h p.i. The CHX-treated cells were incubated in the presence of the inhibitor (100  $\mu$ g/ml) for 1 h prior to infection until 6 h p.i. The treated monolayers were then washed twice with Tris-buffered saline and further incubated for 3 h in the presence of actinomycin D (10  $\mu$ g/ml) and [ $^{35}$ S]methionine (100  $\mu$ Ci per plate). The labeled viral proteins were solubilized in sodium dodecyl sulfate (SDS) sample solution and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (60, 66). For Western blot analysis of GFP, proteins separated by SDS-PAGE were electroblotted to nitrocellulose and analyzed using an anti-GFP ( $\alpha$ -GFP) monoclonal antibody (Clontech) diluted 1:500 and a chemiluminescence Western blotting kit (Boehringer Mannheim).

**Colony formation inhibition assays.** Inhibition of colony formation by the different viral mutants was assayed as previously described (117). Briefly, Vero cells infected at different MOI and control uninfected monolayers were trypsinized at 6 h p.i. to prepare single-cell suspensions that were then serially diluted and plated in medium supplemented with 20% fetal bovine serum. Ten to 14 days after plating, the colonies were stained with crystal violet and counted.

**PCR analysis.** For normalizing virus stocks,  $2 \times 10^6$  cells plated in 60-mm-diameter dishes were infected at an MOI of 10 based on titers determined by plaque assay. At 6 h p.i., the cultured cells were washed with cold Tris-buffered saline and scraped from the plates. The cells were pelleted by low-speed centrifugation and resuspended in 0.5 ml of RSB buffer (10 mM NaCl, 10 mM Tris-HCl [pH 7.4], 3 mM MgCl<sub>2</sub>). Nonidet P-40 (1%, 0.5 ml in RSB) was then added, and the mixture was vigorously vortexed. The infected nuclei were pelleted, resuspended in lysis buffer (1.2% Nonidet P-40 and 0.4 mg of proteinase K per ml, prepared in Tris-EDTA buffer) and incubated overnight at 45°C. The mixtures were then heated at 95°C to inactivate the proteinase. Aliquots (2.5  $\mu$ l) of these nuclear lysates were used in PCRs. For determining the number of genomes in persistently infected cells, total cell DNA was prepared from the infected cells as previously described (98) and equivalent aliquots from all the samples were used in PCRs.

The PCR conditions, with minor modifications, and the primers used to amplify glycoprotein C (gC) sequences were described previously (55). A typical PCR mixture made up to 100  $\mu$ l contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10% glycerol, 3 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleoside triphosphate, 50 pmol of each upstream and downstream primer (gC-1, 5'-GGTCCGTCCCC CCAAT-3'; gC-2, 5'-CGTTAGGTTGGGGCGCT-3') (55), 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim), and the sample or standard DNA. Amplifications were done with a Perkin-Elmer GeneAmp PCR System 2400 with an initial denaturation step (94°C, 5 min) followed by 25 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 30 s) and a final extension step (72°C, 7 min). For detection of the 109-bp gC PCR product, equal aliquots of each reaction mixture were run on a 1% agarose-1% NuSieve GTG (FMC BioProducts) gel. The gel was treated with 0.4 N NaOH for 30 min and blotted on a GeneScreen Plus membrane (NEN Life Science Products) in 0.4 N NaOH. After being subjected to UV cross-linking for 30 s (UV Stratilinker 2400; Stratagene), the membrane was prehybridized (6 $\times$  SSC [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% SDS, 5 $\times$  Denhardt's solution, 8  $\mu$ g of salmon sperm DNA per ml) at 50°C for 1 h and hybridized (6 $\times$  SSC, 1% SDS, 10<sup>5</sup> to 10<sup>6</sup> cpm/ml probe) at 50°C for 1 h. The end-labeled oligonucleotide probe (gC-3, 5'-TAGAGGAGGTCCTGACGAACA-3') (55) was used at a concentration of approximately 10 ng/ml of hybridization solution. After hybridization, the blots were washed (6 $\times$  SSC, 0.001% SDS) with several changes of buffer, rinsed with 2 $\times$  SSC, allowed to dry, and exposed to film for autoradiography. The radioactive signals were quantitated with an AMBIS 4000 radioanalytic imaging system (AMBIS, Inc., San Diego, Calif.).

**Immunofluorescence.** Infected and uninfected cells were prepared on circular coverslips. For detection of promyelocytic leukemia antigen (PML), cells were fixed with methanol as previously described (117). For simultaneous detection of ICP0 or ICP4 with GFP, the infected cells were fixed with paraformaldehyde as previously described (40). Briefly, the cells were washed with phosphate-buffered saline (PBS), incubated in 4% paraformaldehyde solution for 10 min, and then rinsed with PBS. Following paraformaldehyde fixation, the cells were permeabilized with 0.2% Triton X-100 for 2 min and then rinsed twice with PBS. Staining of the cells for PML, ICP0, or ICP4 was done as previously described (117). The monoclonal antibodies against PML (Santa Cruz Biotechnology, Santa Cruz, Calif.), ICP0, and ICP4 (no. 1112 and no. 1011, respectively; Goodwin Institute for Cancer Research, Inc., Plantation, Fla.) were used at dilutions of 1:30,

1:1,000, and 1:1,000, respectively. The stained antigens were visualized at a magnification of  $\times 100$  (PML) or  $\times 60$  (ICP0 and ICP4) with the appropriate cubes for fluorescent imaging in conjunction with a Nikon FXA photomicroscope.

## RESULTS

We have previously described mutants inactivated for ICP22 or ICP0 in an ICP4<sup>-</sup> ICP27<sup>-</sup> background (99, 117). Cells infected with *d95* (ICP4<sup>-</sup> ICP27<sup>-</sup> ICP22<sup>-</sup>) exhibited prolonged cell survival and high-level expression of ICP0 as well as some early genes (117). Although *d95*-infected cell monolayers maintained their normal morphology for an extended period of time, the cells stopped dividing and eventually died. Gene expression from the genome was severely restricted upon inactivation of ICP0 in the same ICP4<sup>-</sup> ICP27<sup>-</sup> background. In this virus, *d97* (ICP4<sup>-</sup> ICP27<sup>-</sup> ICP0<sup>-</sup>), the level of expression of an inserted *lacZ* transgene under the control of the ICP0 promoter was initially high but declined soon thereafter to low levels that could still be detected up to 2 weeks p.i. The survival of *d97*-infected cells was improved relative to that of *d92*- or *d95*-infected cells, although the virus was still toxic to cells at high MOI. The characteristics of both *d95* and *d97* demonstrate the contributions of ICP0 and ICP22 to virus toxicity. The studies detailed herein examine genome persistence, vector toxicity, and transgene expression in the absence of IE protein functions.

**Generation of mutants inactivated for multiple IE genes.** Construction of the virus *d109* (ICP4<sup>-</sup> ICP27<sup>-</sup> ICP0<sup>-</sup> ICP22<sup>-</sup> ICP47<sup>-</sup>) proceeded in a series of steps that generated and utilized a set of mutants defective in sets of IE genes. All mutants deficient in ICP4 carried the *d120* allele of the gene. ICP0 was inactivated by a substantial deletion (0 $\Delta$ ) that removed the entire coding region along with the promoter (Fig. 1C). The sequences deleted in ICP27 are the same as those deleted in the *5dl1.2* allele (71) of the gene. In place of the deletion in ICP27, an HCMVIEP-GFP transgene cassette containing the GFP gene under the control of the HCMV IE promoter was inserted (Fig. 1B).

To inactivate ICP22, a 270-bp sequence containing the TAA TGARAT elements was deleted from its promoter (Fig. 1C). Since this promoter is the same as that upstream of the ICP47 gene, the mutation (TGTA) affects expression of both ICP22 and ICP47. The mutated promoter, which retains the TATA box and several Sp1 sites, closely resembles some of the early and late promoters activated by ICP4. It was assumed that removal of the TAATGARAT elements would eliminate the responsiveness of the promoter to induction by VP16 but perhaps not to activation by ICP4. The TGTA mutation was intended to abrogate ICP22 and ICP47 expression under the noncomplementing conditions in which these experiments are conducted but also to allow expression of functional ICP22 under the complementing conditions used to isolate and grow the mutants. The latter takes into account the apparent growth advantage that ICP22 provides to multiple IE mutants.

The virus *d100* (Fig. 1D) was generated by cotransfecting E26 cells with *d92* viral DNA and pEB $\Delta$ AE linearized by *HindIII*. The *d92* virus carries the *d120* (ICP4) and *5dl1.2* (ICP27) mutant alleles. The plasmid pEB $\Delta$ AE contains a genomic fragment that carries the *d120* (ICP4) and 0 $\Delta$  (ICP0) mutations as well as a wt copy of ICP27. Progeny from the cotransfection were plated on FO6 cells, and individual plaques were initially screened by Southern blot analysis for incorporation of the  $\Delta$ 0 allele. Plaque isolates whose ICP0 loci were both converted to the 0 $\Delta$  allele were further analyzed to determine the state of the ICP27 gene. None of the isolates retained the deletion in ICP27. One isolate carrying the in-

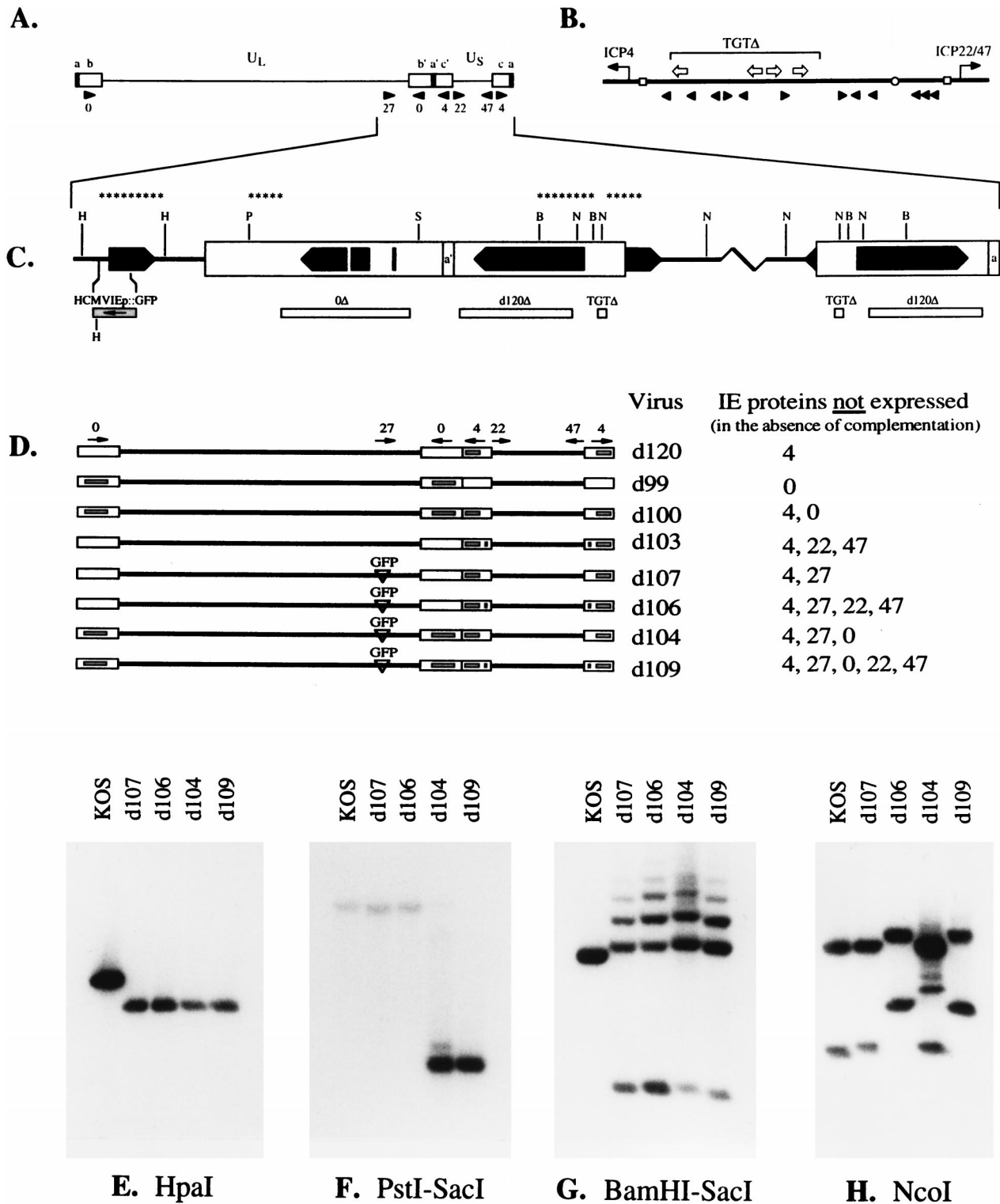


FIG. 1. Structures of isogenic mutants defective in combinations of IE genes. (A) The viral genome is represented with the unique long ( $U_L$ ) and short ( $U_S$ ) regions bounded by terminal and internal repeats (white boxes). The genomic locations and directions of transcription of the IE genes are indicated (arrowheads). (B) The TAATGARAT deletion, TGTA (bracketed), introduced into the promoter regions of ICP22/47. The positions of the TAATGARAT elements (open arrows), binding sites for the transcription factor SP1 (arrowheads), TATA boxes (open squares), and oriS (open circle) are shown relative to the transcription start sites of ICP4 and ICP22/47. (C) Expanded map of the right end of the *d109* genome, showing the coding sequences of the IE genes (solid arrows) and the deletion mutations (white bars) in ICP4 (*d120*), ICP0 ( $0\Delta$ ), and the ICP22/47 promoter (TGTA). The transgene cassette containing the GFP reporter gene under the control of the HCMV IE promoter substituted into the deletion in ICP27 is represented by a shaded bar, with the arrow inside indicating the direction of transcription. The relevant restriction sites are indicated and are abbreviated as follows: H, *HpaI*; P, *PstI*; S, *SacI*; B, *BamHI*; and N, *NcoI*. The line of asterisks represent the nick-translated DNA fragments used as probes for Southern blot analysis. (D) Structures of the isogenic mutants. The deletion mutations in ICP4, ICP0, and the ICP22/47 promoter present in the different virus mutant strains are indicated by the shaded bars inside the white boxes representing the repeat sequences. The GFP substitution in ICP27 is also shown (inverted triangles). The mutant designations are indicated on the right along with a list of the IE proteins not synthesized by the viruses in the absence of complementation. Viral DNA isolated from cells infected with the indicated viruses were digested with *HpaI* (E), *PstI-SacI* (F), *BamHI-SacI* (G), or *NcoI* (H) and analyzed by Southern hybridization. The probes used are shown in panel C and described in Materials and Methods.

tended deletions in both loci of ICP4 and ICP0 was further purified and designated *d100*. The virus *d99* (Fig. 1D) was derived by rescuing the *d120* mutation of *d100* to produce a virus that carried only the  $0\Delta$  mutation.

To construct *d104* (Fig. 1D), FO6 cells were cotransfected with pGFP27 cleaved with *HindIII* and *d100* viral DNA. The plasmid pGFP27 contains the HCMVIEp-GFP transgene inserted in the ICP27 locus. Viruses derived from the cotransfection were plated on FO6 cells, and well-isolated green fluorescent plaques were further purified and analyzed by Southern blotting to confirm incorporation of the GFP insert into the ICP27 locus. The virus resulting from this construction, *d104*, has the *d120* (ICP4) and  $0\Delta$  (ICP0) mutations as well as the GFP substitution in ICP27.

To generate virus *d103* (Fig. 1D), E5 cells were cotransfected with *d96* viral DNA and *EcoRI*-cleaved pTGTA. The *d96* virus carries the *d120* (ICP4) and *n199* (ICP22) mutant alleles. The pTGTA plasmid contains a wt ICP22 gene, the *d120* mutation, and the TGTA deletion that removes the TAA TGARAT elements from the ICP22 promoter (Fig. 1C). Progeny from the cotransfection were plated on E5 cells, and individual plaques were screened by Southern hybridization for both the incorporation of the TGTA deletion and repair of the *n199* nonsense mutation in ICP22. One isolate carrying the *d120* mutation in ICP4, the TGTA mutation in the ICP22 and ICP47 promoters, and a wt copy of the ICP22 gene was propagated and designated *d103*.

Viruses *d106* and *d107* (Fig. 1D) were derived from a cross between *d103* and *d104*. The virus *d109* (Fig. 1D) was generated by crossing *d106* and *d104*. Isolated green fluorescent plaques resulting from these coinfections were analyzed by Southern blotting to determine their genotypes. The mutant *d107* carried the *d120* deletion in ICP4 and the GFP substitution in ICP27. *d106* carried both of these mutations in addition to the TGTA deletion in the ICP22 and ICP47 promoters. The isolate designated *d109* carried the *d120* (ICP4),  $0\Delta$  (ICP0), and TGTA (ICP22 and ICP47 promoters) deletions as well as the GFP substitution in ICP27.

Figure 1E to H shows Southern blot analyses in which restriction digests of KOS, *d107*, *d106*, *d104*, and *d109* were probed to demonstrate the structures of the IE genes in *d109*. Figure 1B shows an expanded map of the right end of the genome, highlighting the sequences used to probe the Southern blots (asterisks), the locations of the coding sequences for the IE genes, relevant restriction sites, and the mutant alleles. To visualize the ICP27 sequences in the viruses, electrophoretically separated *HpaI* fragments of the viral DNA were probed with the indicated fragment. The substituted HCMVIEp-GFP transgene provides an additional *HpaI* site (Fig. 1B), and as a consequence of this substitution in the ICP27 locus, all the mutant viruses contained the shortened *HpaI* fragment (Fig. 1E). To visualize the ICP0 sequences in the viruses, separated *PstI-SacI* fragments of viral DNA were probed with the indicated fragments. The 4.6-kb deletion ( $0\Delta$ ) present in *d104* and *d109* resulted in the shorter *PstI-SacI* fragment relative to the wt allele of the gene (Fig. 1F). To visualize the ICP4 sequences in the viruses, fractionated *BamHI-SacI* fragments of viral DNA were probed with the indicated fragments. All the mutant viruses carried the *d120* allele, as indicated by the fusion of *BamHI*-Y sequences (corresponding to the probe fragment) to the *BamHI-SacI* fragment spanning the joint, by virtue of the deletion in ICP4 (Fig. 1B). This 2.2-kb *BamHI-SacI* fragment spanning the joint is detected running slightly behind the 1.8-kb *BamHI* fragment characteristic of the wt ICP4 allele (Fig. 1G). Variation in the number of copies of the *a'* repeat sequence in a given popu-

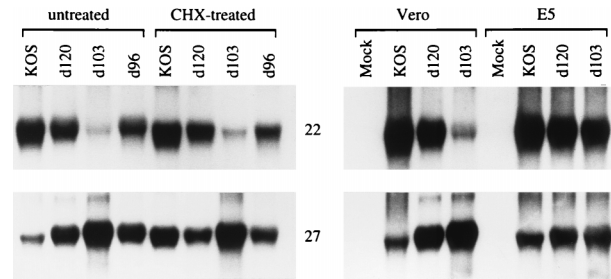


FIG. 2. Effect of the TGTA mutation on expression of ICP22. Total cell RNA, isolated at 6 h p.i. from Vero or E5 cells (ICP4<sup>+</sup>) infected (MOI = 10) with the indicated viruses, was processed for Northern blot analysis. For infections of Vero cells done in the presence of CHX, the medium was supplemented with the inhibitor 1 h prior to and during infection. The blots were hybridized to probes specific for ICP22 and ICP27 mRNAs as indicated (22 and 27, respectively). KOS is the wt virus control, and *d96* (ICP4<sup>-</sup> ICP22<sup>-</sup>) (117) carries the *d120*Δ deletion in ICP4 and the *n199* nonsense mutation in ICP22 (93).

lation produces the multiple bands observed above the 2.2-kb fragment. The smaller, 1.3-kb fragment detected by the probe corresponds to the S-terminal *BamHI* of *d120*. To demonstrate the presence of the TGTA mutation in the ICP22 and ICP47 promoters of the different viruses, electrophoretically separated *NcoI* fragments of viral DNA were probed with the indicated fragment. Incorporation of the TGTA mutation in *d106* and *d109* is indicated by the loss of an *NcoI* site located within the 270-bp deleted sequence (Fig. 1B). As a consequence of this deletion in *d106* and *d109*, two adjacent *NcoI* fragments are fused, resulting in larger *NcoI* fragments of 4.6 and 2.8 kb encompassing ICP22 and ICP47, respectively (Fig. 1H). The wt *NcoI* fragments containing ICP22 and ICP47 are 4.1 and 2.3 kb in size, respectively, and these were not detected in *d106* and *d109*, demonstrating the presence of the TGTA mutation in both copies of the *c'* repeat. Therefore, *d109* contains the *d120* (ICP4),  $0\Delta$  (ICP0), and TGTA (ICP22 and ICP47 promoters) deletions as well as the GFP substitution in ICP27.

The TGTA mutation introduced into the ICP22 and ICP47 promoters was intended to restrict expression of these genes under noncomplementing conditions but also to allow for expression of functional ICP22 under the complementing conditions used to isolate and grow the mutants. Figure 2 shows the effect of the TGTA deletion present in *d103* on the expression of ICP22. As a consequence of the TGTA mutation, accumulation of the ICP22 mRNA in *d103*-infected Vero cells was significantly reduced compared to that in *d120*- or *d96*-infected cells (top left). The levels of the ICP27 mRNA in cells infected for the *d103* infections was in fact larger (bottom left), thereby suggesting that the actual level of ICP22 mRNA made in *d103*-infected cells is lower than that observed. Moreover, the *d103* mutant still expresses the other IE activator, ICP0. In the absence of ICP0, such as in the mutant *d109*, expression of ICP22 is expected to be even further reduced. This prediction was borne out at the level of protein synthesis, as shown below. In the presence of ICP4 provided by the complementing cell line E5, ICP22 mRNA expressed by *d103* accumulated to levels comparable to those in *d120*-infected cells (Fig. 2, top right). These results indicate that the TGTA mutation can effectively reduce expression of ICP22. In the absence of the TAATGARAT elements, induction by VP16 does not occur and ICP22 is no longer expressed as an IE gene. However, the mutated ICP22 promoter remains responsive to activation by ICP4, so that under complementing conditions in which ICP4 is present,

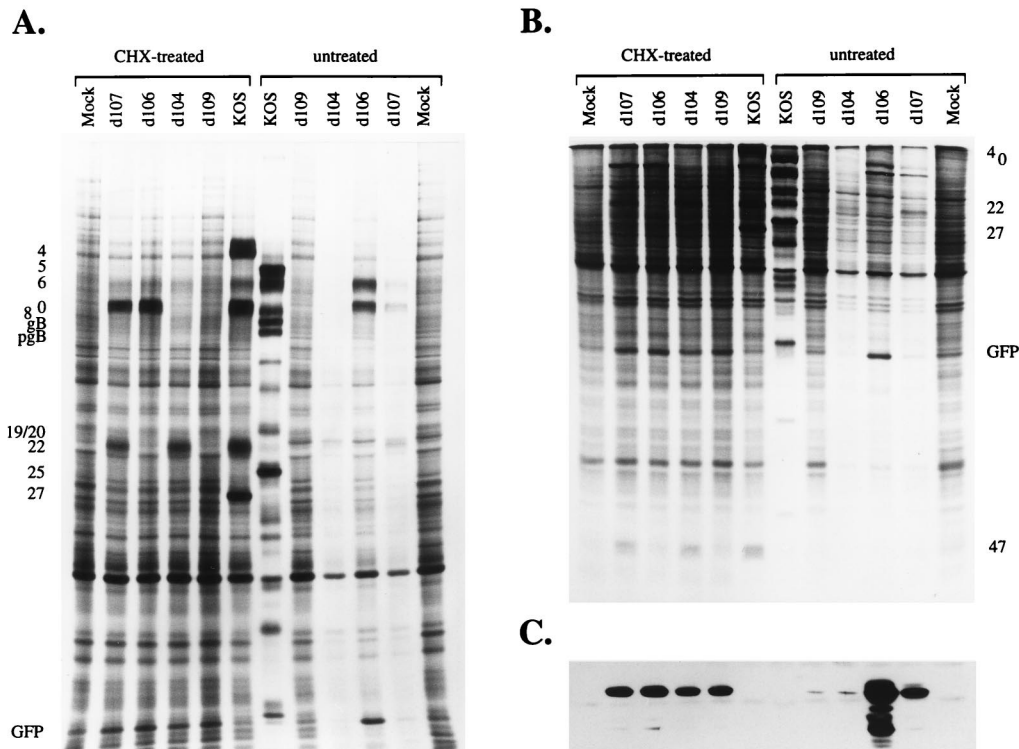


FIG. 3. Synthesis of viral proteins in cells infected with *d109*. Vero cells were infected (MOI = 20) with the indicated viruses in the presence or absence of CHX, pulsed with [<sup>35</sup>S]methionine at 6 to 9 h p.i., and processed for SDS-PAGE. The CHX-treated cultures were incubated in the presence of actinomycin D during labeling. The samples were run on 9% (A) and 18% (B) SDS-polyacrylamide gels (the latter to better visualize the smaller viral proteins). The positions of the IE proteins and GFP, as well as some early and late proteins, are indicated. (C) The same samples were also transferred to a membrane for Western blot analysis with an  $\alpha$ -GFP monoclonal antibody. Mock, uninfected cells.

the gene is expressed, perhaps with the same kinetics as an early gene. Consistent with this interpretation, the relative abundance of the ICP22 mRNA in *d103*-infected Vero and E5 cells is reminiscent of that of thymidine kinase in the two cell types (45). As predicted, expression of functional ICP22 under these conditions gives the virus a growth advantage since *d103* can be propagated to higher titers than can *d96* (data not shown).

The viruses *d106*, *d107*, *d104*, and *d109* carry mutations that should abrogate the expression of specific sets of IE proteins. To demonstrate that this is the case, a CHX reversal experiment (42) was performed on cells infected with the multiple IE mutants. This type of experiment predominantly limits viral gene expression to that of the IE genes. The results of this experiment are shown in Fig. 3. In addition to the SDS-9% polyacrylamide gel routinely used for these experiments (Fig. 3A), the same samples were also run on an 18% gel (Fig. 3B) to allow the detection of the smaller 27-kDa GFP and 11-kDa ICP47 proteins. The mobility of GFP coincides with that of a cellular protein. To verify the identity of the band corresponding to GFP, a Western blot analysis was also performed (Fig. 3C). As expected from the genotypes of these viruses, none of the mutants expressed ICP4 or ICP27. ICP0 was not synthesized in *d104*- or *d109*-infected cells. ICP22 and ICP47 were not synthesized in *d106*- or *d109*-infected cells. Also, under conditions of CHX reversal, all four mutants synthesized GFP to similar levels. These results show that in infected Vero cells, *d109* does not express ICP4, ICP27, ICP0, ICP22, or ICP47, and it synthesizes GFP in the absence of prior viral protein synthesis.

**Expression of the viral genome in the absence of IE proteins.** In the absence of regulatory functions encoded by the IE proteins, viral gene expression from *d109* was expected to be severely restricted and solely a function of cellular factors. The contribution of the virion component, VP16, in this case is irrelevant since none of the endogenous IE promoters remain intact. Figure 3 shows the results of a parallel experiment conducted in the absence of inhibitors (untreated). Under these conditions, the effects of expressed IE proteins on subsequent gene expression can be observed. A typical permissive pattern of viral protein synthesis from 6 to 9 h p.i. is seen in cells infected with the wt virus KOS. Except for *d106*, which synthesized abundant amounts of ICP0 and ICP6, none of the mutants expressed the viral gene products to a significant degree. The polypeptide profile of *d109* was indistinguishable from that of uninfected cells, thus showing that little or no HSV protein is made in this background.

The GFP transgene was synthesized to abundant levels in *d106*- or *d107*-infected cells, in which ICP0 was present (Fig. 3C). In cells infected with a mutant that does not express ICP0 (*d104* or *d109*), the abundance of GFP was greatly reduced. These results clearly indicate that in the absence of ICP4, ICP0 can be a major determinant of gene expression driven from the HCMV IE promoter. Another notable observation is that the amount of GFP made in untreated cells infected with *d104* or *d109* was very low compared to that expressed in similarly infected CHX-treated cells. Preston and Nicholl previously observed that the heterologous HCMV promoter is active in CHX-treated cells infected with the mutant *in1332*, which is defective for the viral activators VP16, ICP0, and ICP4 (89). In

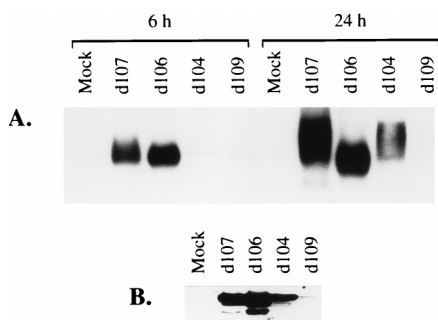


FIG. 4. Accumulation of GFP mRNA and protein. (A) Total cell RNA, isolated at 6 and 24 h p.i. from Vero cells infected (MOI = 10) with the indicated viruses, was processed for Northern blot analysis with a GFP-specific probe. (B) Proteins extracted at 24 h p.i. from infected cells (MOI = 10) were separated on an SDS-18% polyacrylamide gel and transferred to a membrane for Western blot analysis with an  $\alpha$ -GFP monoclonal antibody. Mock, uninfected cells.

the absence of the inhibitor, the activity of the HCMV promoter in this background was reduced. These observations suggest that an active mechanism represses the HCMV promoter in the absence of viral activators. This inhibition may involve a cellular factor that is transiently expressed and therefore not made in the presence of CHX.

The greater reduction in the levels of cell and viral proteins seen in *d104*- or *d107*-infected cells suggests an increase in shutoff of protein synthesis and correlates with the presence of ICP22 in these viruses. The *vhs* gene product (UL41) of HSV functions in attenuating host protein synthesis by destabilizing preexisting host mRNAs (31, 91, 111) and ensures the rapid turnover of viral mRNAs after the onset of viral transcription (58, 83). Similar activities have not been described for ICP22, although it is possible that the effect of ICP22 in this case is exerted indirectly. Previous studies with *d92* and *d97*, viruses with IE mutations analogous to those of *d107* and *d104*, respectively, did not show this same effect on cellular protein synthesis (99). However, these previous experiments were conducted at a lower MOI that may be below the threshold level at which the effects of ICP22 are observed. Additional studies are required to determine the contribution of ICP22 to this effect and the status of *vhs* or its ability to function in these mutant backgrounds.

The accumulation of stable GFP mRNA at 6 h p.i. (Fig. 4A) was consistent with the levels of GFP synthesized by the different mutants early in infection (Fig. 3C). GFP mRNA was abundantly expressed in *d106*- or *d107*-infected cells, accumulating to a lesser degree in the latter. This minor difference may be due to the higher level of ICP0 synthesized in *d106*-infected cells (Fig. 3A). In *d104*- or *d109*-infected cells, GFP mRNA was barely detectable at 6 h p.i. However, at 24 h p.i., the relative level of GFP mRNA seen in *d104*-infected cells increased, while in *d109*-infected cells it remained very low (Fig. 4A). This difference suggests a possible contribution of ICP22 to the transcription of GFP late in infection. The abundance of the GFP mRNA in *d106*-infected cells relative to that in *d107*-infected cells at 24 h p.i. is difficult to assess because of the heterogeneity in the size of the mRNA in the latter. This effect was also observed with *d104*, and it correlates with the presence of ICP22 in these two mutants. The GFP mRNA is not spliced, so this heterogeneity may reflect differential processing of the mRNA at the 3' end. Rice et al. have previously noted that ICP22 phosphorylates the carboxy-terminal domain (CTD) of pol II, resulting in a novel modified form of the enzyme (93). A possible explanation for the above-described

results is suggested by recent findings of McCracken et al. showing a functional linkage between the CTD and 3' RNA processing (72). They further demonstrated a specific interaction of the CTD with the cleavage-polyadenylation factors CPSF and CstF. An alteration in the polyadenylation process may thus be a consequence of the CTD modification mediated by ICP22. Further studies are necessary to determine the connection between the activity of ICP22 and the coupled processes of transcription and mRNA processing.

The relative levels of GFP synthesized by the different mutants at 24 h p.i. (Fig. 4B) correlated with the amounts of GFP mRNA they express late in infection (Fig. 4A). GFP was abundant in cells infected with *d107* or *d106* (Fig. 4B). The increased amount of GFP mRNA expressed by *d104* late in infection was also reflected proportionally at the protein level. With *d109*, the amount of GFP expressed was very small. The amount of GFP expressed by the different mutants late in infection was consistent with the level of green fluorescence exhibited by the infected monolayers when viewed under a UV microscope (data not shown). At 24 h p.i., all cells in *d106*- or *d107*-infected monolayers fluoresce, with those in the latter exhibiting significant cytopathic effects, consistent with previous observations on analogous mutants *d95* and *d92*, respectively (117). A smaller proportion of fluorescing cells was observed in *d104*-infected monolayers, and with *d109* there were even fewer of these GFP-expressing cells.

Results of the above-described studies show that in the absence of the IE proteins, the level of gene expression from the *d109* genome is very low, even from the HCMV promoter, which is generally associated with high levels of transcriptional activity. Possible explanations for this apparent repression of the genome in the absence of viral activators are discussed below.

**Toxicity and persistence of *d109*.** Previous observations suggested that both ICP0 and ICP22 contribute to virus toxicity and that simultaneous elimination of these gene products in an ICP4<sup>-</sup> ICP27<sup>-</sup> background would improve the survival of infected cells. To evaluate the toxicity of the different multiple IE mutants, Vero cell monolayers were infected at different MOI. At 6 h p.i., the monolayers were trypsinized to generate single-cell suspensions, and dilutions of these suspensions were plated to enumerate CFU. The abilities of *d107* and *d106* to inhibit colony formation by infected cells were quite similar (Fig. 5). In comparison, *d104*-infected cells showed enhanced survival, although virus toxicity was still observed at the higher MOI. These results are consistent with previous observations on the analogous mutants *d92*, *d95*, and *d97* (99). The survival of *d109*-infected cells was indistinguishable from that of uninfected cells, even at the highest MOI of 30 PFU/cell. Therefore, from the standpoint of colony-forming ability of the infected cells, *d109* is nontoxic.

As another potential measure of perturbation of normal host cell function, the different IE mutants were assessed for their ability to disrupt the punctate nuclear bodies known as ND10. Early in infection, ICP0 localizes to these discrete nuclear structures (ND10), which are thought to be involved in the proliferative or differentiation state of the cell (26, 53, 116). As infection progresses, ICP0 perturbs the structure and the composition of these nuclear bodies, which are known to contain a number of cellular proteins, including PML (29, 67, 68). Similar activities have also been described for the IE proteins E4-ORF3 of adenovirus and IE1 of HCMV (25, 54). The association of viral genomes and replication proteins with ND10 early in infection led to the suggestion that these nuclear structures serve as sites where initial stages of HSV replication occur (46, 69). Consistent with previous observations, the dis-

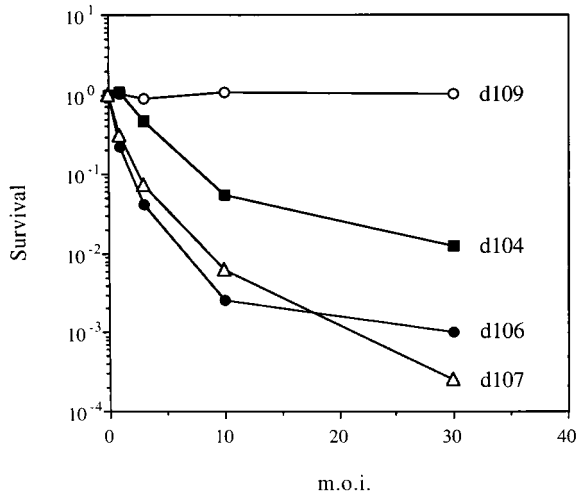


FIG. 5. Survival of cells infected with IE mutants. Vero cell monolayers were infected with the IE mutant viruses at the indicated MOI. The monolayers were harvested at 6 h p.i. and plated for determination of CFU. Colonies were counted 10 days after cell plating. Points plotted represent the surviving fractions of the infected cells relative to uninfected cells.

crete PML-containing ND10 structures were not observed in cells infected with mutants that express ICP0 (*d107* and *d106*) (Fig. 6). In contrast, the characteristic punctate staining, similar to that seen in mock-infected cells, was readily observed in *d104*- or *d109*-infected cells. There was a small perturbation in the quality and quantity of staining in *d104*-infected cells late after infection, suggesting that ICP22 may directly or indirectly affect ND10 structure and composition.

Considering that *d109* was nontoxic to cells, we thought it likely that the virus could establish a long-term or persistent infection. To address this possibility, uninfected and *d109*-infected Vero cells were maintained and monitored for a period of 28 days. Even when infected at an MOI of 20, only a subpopulation of *d109*-infected monolayers expressed GFP (Fig. 7), and the number of these fluorescing cells decreased with time. Given the abundant expression of GFP in the presence of ICP0, it was reasonable to expect that providing ICP0

to the *d109*-infected monolayers would induce GFP and thus cause cells harboring *d109* to fluoresce. Therefore, a parallel set of *d109*-infected cultures was superinfected with *d95* 1 day prior to each time point. The only IE regulatory protein that *d95* expresses is ICP0, and this virus does not encode GFP. With the induction of GFP upon superinfection with *d95*, *d109*-infected cells were more readily identified, and these could be observed for the duration of the experiment (Fig. 7). Considering that the cells doubled in number about three times during this period, as deduced from the amount of total DNA isolated (Fig. 8), a significant number of *d109*-infected cells were maintained for up to 28 days p.i. This observation was also seen in HEL cells infected with *d109* for up to 6 weeks (data not shown). PCR quantitation of the number of *d109* genomes in the nuclei of infected Vero cells showed a gradual decline over time, although the number of viral genomes present at 28 days p.i. still constituted about one-third of the genomes present at 1 day p.i. (Fig. 8).

The number of *d109* genomes present in the nuclei at 1 day p.i. exceeded the expected value by about 10-fold. Jamieson et al. found that the number of viral genomes in the nuclei 1 day after infection with *in1833* exceeded the number present at 1 day p.i. because capsid uncoating proceeded relatively slowly (47a). It may be that since our input inoculum was normalized to the number of genomes present in the nuclei at 6 h p.i., the larger number detected at 1 day p.i. reflected additional genomes that had not been uncoated by 6 h p.i. It is also noteworthy that at 28 days p.i. the number of genomes present in the cells exceeded the number of cells in the culture. There were approximately  $7 \times 10^7$  genomes of *d109* (Fig. 8) and  $1.5 \times 10^7$  cells, assuming that the cell number doubled three times over the 28-day period. However, only about one-third of the cells appeared to express detectable GFP upon infection with *d95* at 28 days (Fig. 7). The reason for this is unclear. The cells were infected at an MOI of 20; however, at present we do not know how these genomes partition from the initially infected cell into daughter cells following cell division. It may be that some daughter cells do not contain genomes while others retain multiple genomes. Alternatively, the infection of overly confluent monolayers with *d95* may not result in efficient infection, or some of the quiescent genomes may not respond to ICP0. With respect to these last two points, when monolayers

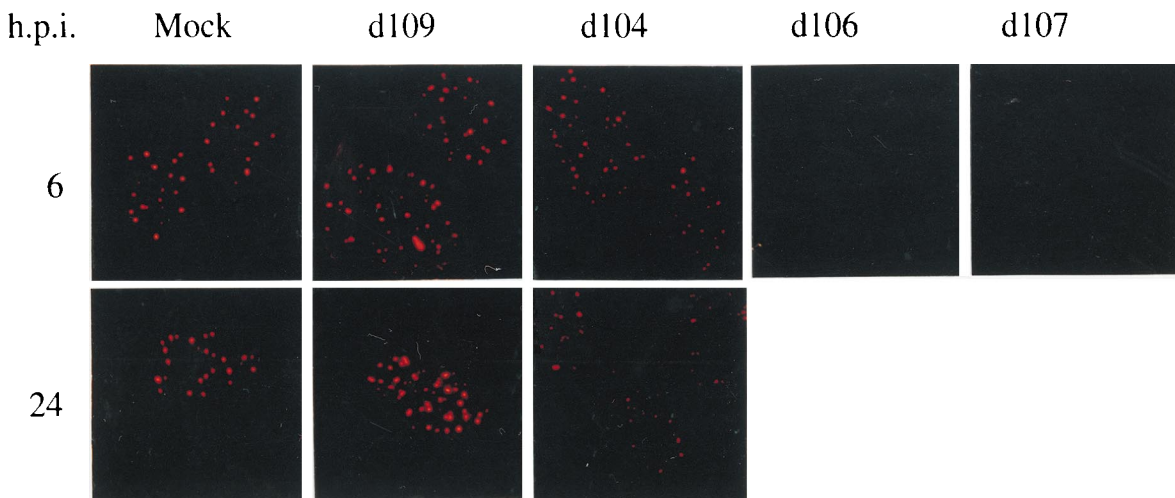


FIG. 6. Nuclear distribution of PML in cells infected with IE mutants. Human fetal lung cells infected (MOI = 10) with the indicated viruses were processed at 6 and 24 h p.i. for immunofluorescence with an  $\alpha$ -PML antibody. Shown are stained infected nuclei (magnification,  $\times 100$ ).



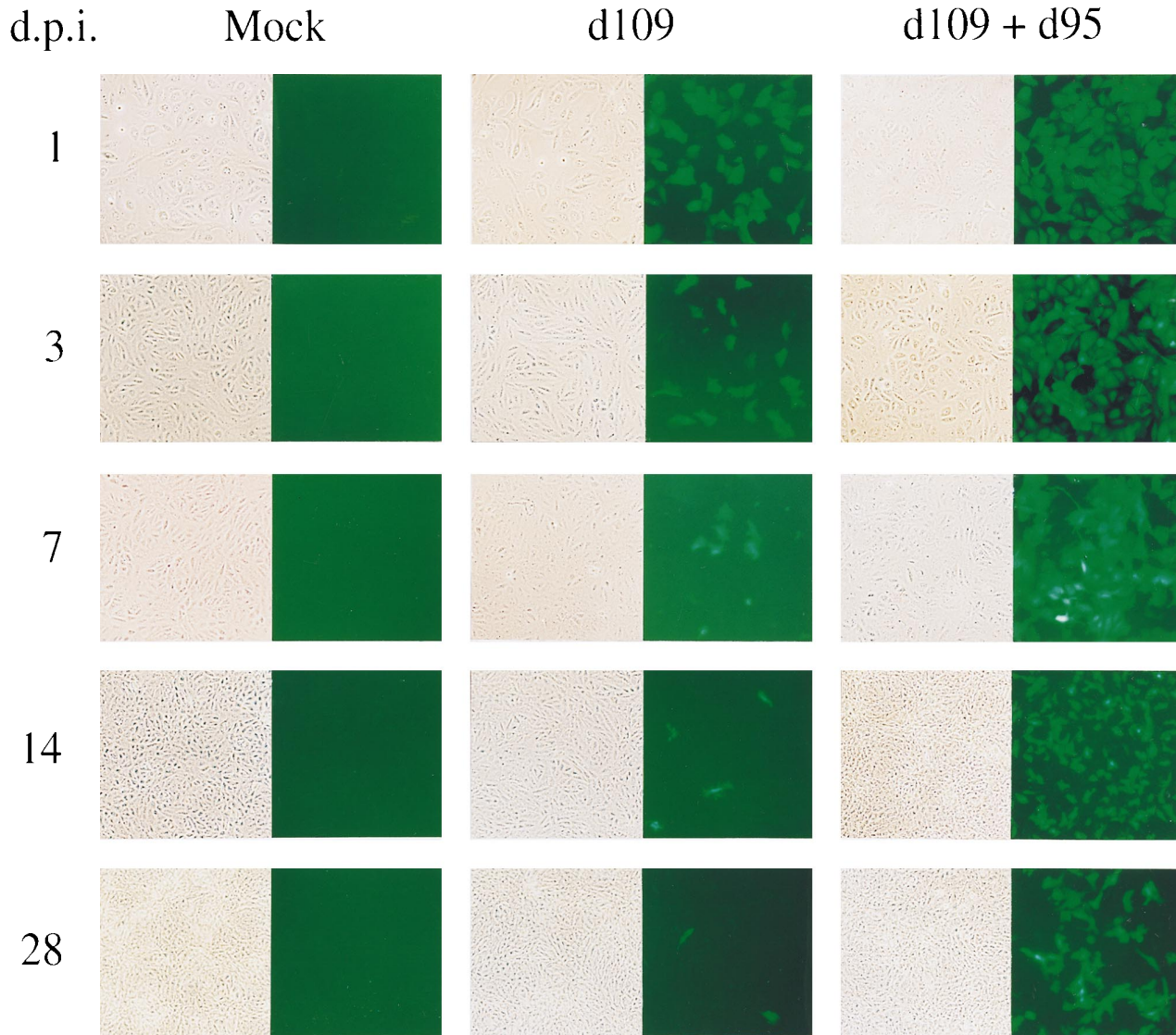


FIG. 7. Persistence and induction of *d109*. Mock-infected (Mock) and *d109*-infected (MOI = 20) Vero cells were maintained for a period of 28 days in 2% serum at 34°C to limit cell division. At different time points (in days) after infection (indicated on the left), the monolayers were photographed under phase-contrast and fluorescence microscopes. A separate set of *d109*-infected cultures was superinfected with *d95* (MOI = 20) 1 day prior to the time points indicated on the left.

of *d109*-infected (28 days p.i.) HEL cells were infected with *d95*, nearly all of the cells fluoresced (data not shown). HEL cells are more prone to contact inhibition than are Vero cells and therefore did not divide to the same extent as Vero cells over the 28-day period.

Taken together, the results presented thus far demonstrate that *d109* is a noncytotoxic virus that can efficiently establish a persistent infection. Furthermore, the results show that gene expression from the persisting quiescent viral genomes remains inducible after long-term maintenance of the infected cultures. In the absence of induction, the number of green-fluorescing cells in *d109*-infected monolayers varies, but it is generally low. The reason for the high level of activity of the HCMV promoter regulating GFP expression in this subpopulation of cells is not known at the present time. To rule out the possibility that this small fraction of *d109*-infected cells expressing GFP was due to a low-level contamination with viruses that synthesize ICP0 or ICP4, Vero cells were infected with *d109* and at

24 h p.i. were processed for immunofluorescence staining with  $\alpha$ -ICP0 and  $\alpha$ -ICP4 monoclonal antibodies. The results show that the *d109*-infected cells, which expressed GFP, did not express ICP4 or ICP0 (Fig. 9). Therefore, the expression of GFP from the HCMV IE promoter was not due to contamination of the infected cultures by viruses expressing ICP4 or ICP0.

The properties of *d109* suggest that it is capable of transducing functional genes into cells with minimal effects on cell survival. As an immediate test of this capability, we explored the possibility that *d109*-infected monolayers could serve as complementing cells for plaque formation by HSV mutants defective in essential genes. This prediction would hold true assuming that the region of the *d109* genome required for complementation is maintained in the infected cells. To address this possibility, Vero monolayers were infected with *d109* and, at 24 h p.i., the infected monolayers were used to determine the titers of virus mutants with lesions in capsid genes.

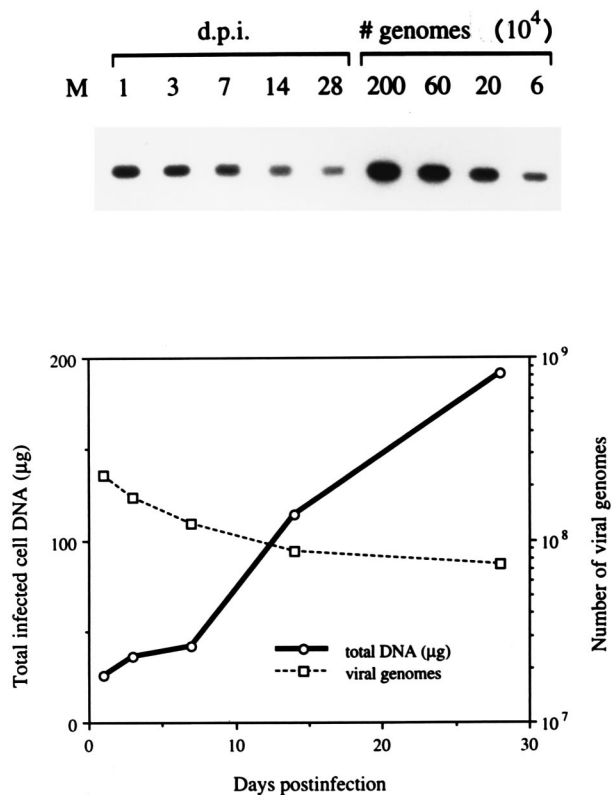


FIG. 8. Persistence of *d109* genomes in infected cells. A separate set of *d109*-infected monolayers was maintained in parallel to those described in the legend to Fig. 7. At the same time points indicated, total infected cell DNA was isolated. Equivalent aliquots of the DNA samples, along with standards, were used in PCRs to amplify sequences from the *gC* gene (top). The amounts of total infected cell DNA (in micrograms) isolated at different time points (in days) after infection with *d109* are plotted along with the number of viral genomes derived from quantitation of the *gC* PCR products.

The titers of the capsid mutants obtained on *d109*-infected Vero cells were not significantly different from their known titers determined on dedicated cell lines that contain and express complementing levels of the genes mutated in the virus (Fig. 10). Induction of GFP from the resident *d109* genomes was evident in all of the cells of the plaques (data not shown). It is important to note that the formation of the plaques on the monolayer was possible because *d109* is sufficiently nontoxic that the nonsuperinfected areas of the monolayer maintained their integrity. These results further underscore the persistence and subsequent functionality of the *d109* genomes resident in noncomplementing cells.

## DISCUSSION

This study was undertaken to determine the effects of the HSV IE proteins expressed from virus mutants defective in ICP4 on cell survival and gene expression. The information gained from these studies is relevant to the activities of the IE genes and the utility of replication-defective viruses as gene transfer vehicles. The results of this study clearly demonstrate that both ICP0 and ICP22 are toxic to cells. We found it necessary to abrogate all IE gene expression to completely eliminate the toxicity associated with HSV infection. Furthermore, both ICP0 and ICP22 affected transgene expression, with ICP0 having the greater effect. One virus, *d109*, which did not express any IE proteins in noncomplementing cells, was

capable of establishing a long-term relationship with the cell, in which the genome persisted in the nucleus in a relatively inactive but potentially functional state.

**Toxicity of viral mutants.** The toxicity associated with ICP4 mutants has long been documented. Chromosomal damage occurs (49), and the infected cells die, probably due to both necrotic and apoptotic modes of cell death (49, 63). In the absence of ICP4, the remaining IE proteins are overexpressed. Considering the large number of cellular proteins and metabolic systems that the IE proteins have been found to interact with or modify, it is probable that the observed toxicity is a complex superposition of many deleterious effects.

A UV-irradiated virus is not toxic to cells (78), suggesting, but not proving, that the virus particle itself is not toxic. Elimination of toxicity with the *d109* mutant clearly demonstrates that the virion is not toxic to cells. Considering that the virus particle contains enzymatic functions, including a protein involved in the attenuation of host protein synthesis, UL41 or vhs (59, 79, 91), it is surprising that cells infected with *d109* at a high multiplicity (30 PFU/cell) are indistinguishable from uninfected cells with respect to morphology, protein synthesis, and the ability to divide. Perhaps *d109* virions contain smaller numbers of particular proteins, such as vhs, relative to wt virions. This could occur as a consequence of suboptimal complementation during propagation on FO6 cells. Alternatively, as suggested from the studies with UV-irradiated virus, the effects of the virion proteins in the absence of IE proteins or metabolic inhibitors are not sufficient to adversely affect cell function.

This study complements and extends previous studies from our lab demonstrating that elimination of either ICP22 (117) or ICP0 (99) from an ICP4<sup>-</sup> ICP27<sup>-</sup> virus (98) reduced toxicity. While these reports do not elucidate the mechanism by which ICP0 and ICP22 cause cell death, all three studies clearly demonstrate that mutants expressing ICP0 inhibit colony formation, probably by affecting cell cycle progression. The phenotypes of *d95* (ICP4<sup>-</sup> ICP27<sup>-</sup> ICP22<sup>-</sup>)- and *d106* (ICP4<sup>-</sup> ICP27<sup>-</sup> ICP22<sup>-</sup> ICP47<sup>-</sup>)-infected cells are similar in that the cells do not divide. They grow in size and continue to be otherwise metabolically active until they die. Further experiments are necessary to determine if and how the ability of ICP0 to affect transcription (50, 99), translation (51), and the activity of DNA-dependent protein kinase (61) and to interact with ubiquitin proteases (30), translation factors (51), cyclins (52), and PML oncogenic domains (PODs) (29, 67, 68) contributes to this phenotype. Considering this, and the observed phenotype of viruses expressing ICP0, such vectors may be of limited utility. Finally, it should be noted that these studies do not directly address the potential contribution of ICP47 to toxicity, since the abrogation of its expression was obligatory to the abrogation of ICP22 expression by construction. However, the results of previous transfection experiments imply that ICP47 does not significantly contribute to toxicity (48).

**Gene expression.** ICP0 is a promiscuous transactivator of gene expression (27, 33, 81, 90). Viral gene expression is also generally reduced in ICP0 mutants (5, 6). In accord with these observations, we found that GFP expression from the HCMV IE promoter was highly dependent on ICP0. In the absence of all of the HSV IE proteins, the level of expression was extremely low. Like HSV IE promoters, the HCMV IE promoter can be activated by an IE protein (12, 108) and a virion component (65, 109). Both the HSV and HCMV genomes localize to ND10 structures early in infection, in the absence of viral protein synthesis (47, 69). Both establish latent infections in which the viral genomes, including the IE promoters, are relatively silent (77, 94). HSV and HCMV also express IE pro-

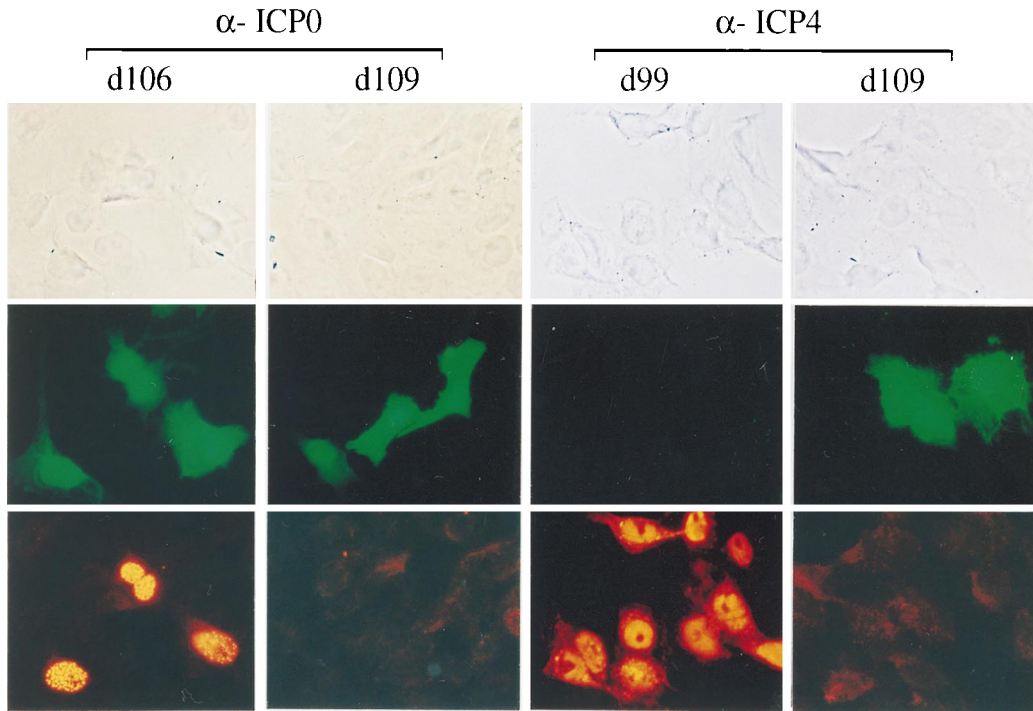
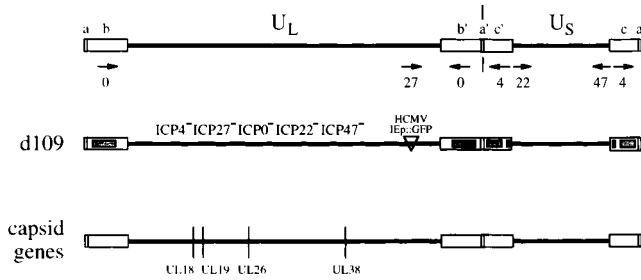


FIG. 9. Absence of ICP0 and ICP4 in GFP-expressing *d109*-infected cells. Vero cells were infected with *d109* (MOI = 10) and, at 24 h p.i., processed for immunofluorescence with  $\alpha$ -ICP0 and  $\alpha$ -ICP4 monoclonal antibodies. Also included were *d106*- and *d99*-infected cells as positive controls for ICP0 and ICP4 staining, respectively. The secondary antibody used was conjugated to rhodamine. Photomicrographs in each column represent the same field viewed by phase-contrast microscopy (top) or fluorescence microscopy with the appropriate filter blocks for observing green (middle) or red (bottom) fluorescence.

teins, ICP0 by HSV and IE1 by HCMV, which are transactivators that localize to ND10 and dissociate PML from the POD structures. In the case of HSV, ICP0 has been shown to be involved in reactivation events in vivo and in tissue culture



mutant	gene inactivated	titer ( $10^8$ ) PFU/ml	
		complementing cells	<i>d109</i> -infected Vero cells
K23Z	UL18	6.0	4.9
K5ΔZ	UL19	2.0	2.0
KUL26ΔZ	UL26	1.0	1.3
KΔ19C	UL26 & UL38	1.0	1.1

FIG. 10. Efficiency of plating of capsid gene mutants on *d109*-infected cells. Vero cell monolayers were infected with *d109* (MOI = 10), and at 24 h p.i. the infected monolayers were used to determine the titers of virus mutants with lesions in capsid genes. The structure of the *d109* genome is shown, along with the genomic locations of the capsid genes mutated in the viruses whose titers were measured. Virus stock titers of the mutants, determined with the appropriate complementing cell lines, were provided by S. Person. See the legend to Fig. 1 for definitions of symbols and abbreviations.

model systems (5, 14, 35, 41, 95, 119). Our studies showed that a virus expressing ICP0 will activate a previously silent HCMV promoter on persisting *d109* genomes weeks after infection. HCMV infection will also activate GFP expression in *d109*-infected HEL cells (data not shown). Perhaps the observed low level of HCMV promoter activity in *d109*-infected cells represents the true "ground state" of genomes localized at ND10 in the absence of viral activator proteins, and it may reflect some of the events that occur in latency. The observations of Preston and Nicholl are consistent with ours, and they have proposed that HSV and HCMV IE promoters are repressed in the absence of VP16, ICP4, and ICP0 (89).

GFP expression in *d109*-infected cells was not uniform, being abundant in a subpopulation of cells and undetectable in most cells. It may be that the HCMV promoter is active in the absence of ICP0 only in cells which are in a particular stage of the cell cycle or state of activation by signaling pathways. Cai and Schaffer have shown that at a particular point in the cell cycle, the intracellular environment reduces the requirement for ICP0 (6). It is possible that the occurrence of GFP-expressing *d109*-infected cells is a reflection of cells in this state. Alternatively, the fluorescent cells may represent those in which the appropriate signaling pathways for specific targets in the HCMV promoter are activated. The HCMV promoter contains sites for NF- $\kappa$ B, CREB, retinoic acid receptor, and AP-1 (1, 34, 44, 101), all of which are components of regulated pathways. It remains to be tested whether the HCMV promoter on the *d109* genome can be activated by one of these pathways.

***d109* as a gene transfer vector.** From the standpoints of toxicity, genome persistence, and efficiency of gene delivery, *d109* is clearly an optimal replication-defective herpesvirus

vector. However, the very manipulations that conferred these attributes resulted in low-level and perhaps repressed gene expression. It may be that this level of gene expression is sufficient for some applications. Most cellular genes are not expressed at the level of an ICP0-induced viral gene. However, it is likely that systems for cell- or tissue-specific expression of transgenes will have to be incorporated into *d109*.

It is interesting that the development of adenovirus vectors has proceeded along parallel lines, with analogous results. E1-deleted adenovirus exhibits high-level expression of transgenes from the HCMV promoter; however, other viral proteins are also expressed, and the virus is somewhat toxic to cells (16). The additional deletion of the E4 region resulted in a virus that was less toxic and persisted in cells for long periods of time (2, 32). However, expression from the HCMV promoter was greatly reduced. E4 contains seven open reading frames (64). One of them, Orf3, like ICP0, perturbs ND10 structures (11, 25). The adenovirus genome also localizes to ND10 structures in the absence of viral protein synthesis (96). Considering these parallels as well as the similar phenotypes of E1-E4-deleted adenovirus and *d109*, it is likely that approaches for promoting or regulating gene expression from the vectors will have similar outcomes in both viruses.

The development of HSV strains has systematically progressed (18, 98, 99, 117) to the point where we now have a virus that has no detectable harmful effects on cells, does not express its own genes, and efficiently delivers the genome to the nucleus, where it persists for a prolonged period of time in a functional form. The lack of toxicity does not appear to be constrained by the input MOI. The results of this study allow future efforts to be aimed at understanding the association between persisting HSV genomes and the cell nucleus and determining how this association affects transcription. These considerations have obvious relevance to expression of transgenes from HSV vectors and may reflect events occurring during latency, when the genome is relatively silent but persists in a potentially functional state.

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