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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

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Derivatives of herpes simplex virus type 1 (HSV-1) have elicited considerable interest as gene transfer vectors because of their ability to infect a wide range of cell types efficiently, including fully differentiated neurons. However, it has been found that infection of many types of cell with vectors derived from replication-defective mutants of HSV-1 is associated with cytopathic effects (CPE). We have previously shown that viral gene expression played an important role in the induction of CPE caused by an HSV-1 mutant deleted for the essential immediate-early gene 3 (IE 3) (P. A. Johnson, A. Miyanohara, F. Levine, T. Cahill, and T. Friedmann, J. Virol. 66:2952–2965, 1992). We have investigated which viral genes might be responsible for CPE by comparing the ability of each of the individual genes expressed by an IE 3 deletion mutant during a nonproductive infection to inhibit biochemical transformation after cotransfection of BHK or CV-1 cells with a selectable marker gene. Transfection of IE genes 1, 2, and 4 individually all caused a marked inhibition of colony formation, while transfection of IE 5 and the large subunit of ribonucleotide reductase had little effect. These results suggested that it would be necessary to mutate or reduce the expression of nearly all HSV-1 IE genes to reduce virus-induced CPE. Therefore, we have used VP16 mutants, which are unable to transinduce IE gene expression (C. I. Ace, T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston, J. Virol. 63:2260–2269, 1989), to derive two replication-defective strains: 14HΔ3, which is deleted for both copies of IE 3, and *in*1850Δ42, which has a deletion in the essential early gene UL42. The IE 3-VP16 double mutant, 14HΔ3, is significantly less toxic than a single IE 3 deletion mutant over a range of multiplicities of infection, as measured in a cell-killing assay, and has an enhanced ability to persist in infected cells in a biologically retrievable form. In contrast, the UL42-VP16 double mutant, *in*1850Δ42, showed reduced toxicity only at low multiplicities of infection. To test the role of the virion host shutoff function as an additional candidate to influence virus-induced CPE, we have introduced a large insertion mutation into the virion host shutoff gene of an IE 3 deletion mutant and the double mutant 14HΔ3. Mutation of this gene did not reduce the cytotoxicity of either strain. These results demonstrate that long-term survival of cells infected with replication-defective HSV-1 mutants can be enhanced through genetic manipulations that reduce viral gene expression.

Approaches to human gene therapy take advantage of the high efficiency of viral vectors in introducing foreign genes into mammalian cells (56). To be useful in most cases, the foreign genes should be expressed stably and efficiently, and the process of gene transfer should not produce cell damage. Some vectors, such as those derived from retroviruses, satisfy most of these requirements, but most retroviral vectors are not capable of gene transfer to postmitotic cells. There are many instances in which gene transfer to nondividing cells would be desirable, whether to permit studies of genetic manipulation in differentiated cell types or for more applied therapeutic purposes. Herpes simplex virus type 1 (HSV-1) has attracted considerable interest for its potential as a gene transfer vector. Much is known about HSV-1 at the molecular biological level, it is able to infect a wide range of replicating and nonreplicating cells, and it can maintain a lifelong latent infection in sensory neurons (for recent reviews on HSV-1 vectors, see references 5 and 35).

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Vectors derived from replication-defective mutants or attenuated strains of HSV-1 have proved successful for the transfer and expression of foreign genes in the mammalian brain in vivo (8, 11, 17) and in primary central nervous system neurons in vitro (30). The target cells for infection with HSV-1 vectors are not limited to neurons, as demonstrated by the high but transient levels of circulating transgene products in mice following injection of the liver with HSV-1 vectors expressing canine factor IX or hepatitis B virus surface antigen (43). However, in most cases, attempts to achieve long-term expression of foreign genes, at least in cells other than those of the peripheral nervous system in vivo, have been hampered by cytotoxicity and/or shutoff of transgene expression.

We have studied the cytopathic effects (CPE) caused by infection with vectors derived from an HSV-1 mutant called D30EBA (49), deleted for nearly the entire coding portion of immediate-early gene 3 (IE 3), whose product, Vmw175 (ICP4), is the major transcriptional activating protein of HSV-1 (51). Since Vmw175 is critically required for expression of viral early and late genes, an IE 3 mutant expresses very few viral genes and is completely replication defective (9, 24, 49, 51). However, we have demonstrated significant CPE, including fragmentation of cellular DNA and cytoplasmic blebbing, 1

to 3 days after infection with an IE 3 mutant (29). We also found that was possible to reduce CPE by two methods which reduced viral gene expression: UV irradiation of the virus and pretreatment of cells with interferon (29). These results suggest that one or more of the few viral genes expressed in the absence of Vmw175 were, at least in part, responsible for the CPE.

The viral genes efficiently expressed by an IE 3 deletion mutant include IE 1, whose product, Vmw110 (ICP0), is a general transactivator of transcription but is not absolutely essential for virus replication (6, 12, 65); IE 2, whose product, Vmw63 (ICP27), is required for expression of true late genes, full levels of viral DNA synthesis, and modulation of early gene expression and which, in transfection experiments, can act as an activator or repressor of transcription in combination with Vmw175 and Vmw110 (38, 55, 60) (Vmw63 has recently been shown to affect mRNA splicing and stimulate viral mRNA 3′-end processing [42, 58]); IE 4, whose product, Vmw68 (ICP22), is required for efficient virus replication in some cell types (59); IE 5, whose product, Vmw12 (ICP47), has recently been shown to inhibit antigen presentation to $CD8^+$ T lymphocytes in infected human fibroblasts (68); and the gene encoding ICP6, which is the large subunit of ribonucleotide reductase, which is not essential for virus replication in tissue culture (20). We have previously tested the cytopathogenicity of HSV-1 mutants bearing lesions in one or more of the five individual IE genes and determined that none of the IE gene products alone are responsible for CPE (29). In addition, not every cell infected with the IE 3 mutant is subject to cell death (29), a finding consistent with the notion that a threshold level of the IE gene products may be required for the induction of CPE and that cell survival may be enhanced by reducing or turning off viral gene expression.

The first part of the present study was aimed at the identification of the HSV-1 IE genes responsible for inducing CPE during infection with an IE 3 mutant. As an assay to measure the potential for CPE, we have cotransfected cells with the bacterial neomycin phosphotransferase gene (*neo*), which confers resistance to the neomycin analog G418, as a selectable marker, together with HSV-1 sequences that may interfere with stable biochemical transformation. We postulated that fewer *neo*-transformed cells would be obtained from cotransfections which include a gene encoding a cytopathogenic product. As a control for promoter competition or for the presence of spurious inhibitory sequences, we have included plasmids bearing mutations within the coding regions of the HSV-1 genes being tested. Our results indicate that IE 1, IE 2, and IE 4 all encode cytopathogenic gene products. We have also found that IE 3 causes inhibition of transformation in CV-1 cells but not in BHK cells. The ICP6 gene and the IE 5 gene did not demonstrate inhibition of transformation in this assay system.

An important regulator of HSV-1 IE gene expression is the virion polypeptide VP16 (Vmw65), which functions to stimulate IE gene transcription at the earliest stages of infection (3, 7) through formation of a complex containing VP16 and cellular transcription factors, including Oct-1, which together specifically bind the IE promoter element TAATGARAT, where R is a purine (45, 52, 66). Ace et al. (2) constructed an HSV-1 mutant, *in*1814, which has a 12-bp insertion in the VP16 gene which abolishes the ability of VP16 to transinduce IE gene expression. However, this virus is still able to replicate, particularly at high multiplicities of infection (MOIs). These results suggest that it might be possible to generate a virus that does not induce CPE during a nonproductive infection if the VP16 mutant *in*1814 were further mutated to render it replication defective. For that reason, we have constructed viruses with deletions in either IE 3 or UL42 as well as the insertion mutation in VP16, designated 14HΔ3 and *in*1850Δ42, respectively. In comparison to their VP16-intact counterparts, we have found that 14HΔ3 has reduced cytotoxicity over a range of MOIs, whereas *in*1850Δ42 is less toxic only at low MOIs.

The virion host shutoff (vhs) function is a component of the virion encoded by gene UL41 (40), which has been thought to contribute to the cytotoxicity of HSV-1 vectors. The vhs function suppresses translation of preexisting mRNAs and increases the turnover of both viral and cellular nascent mRNA (33, 47, 53), although the strength of this function may vary between strains and is weak in HSV-1 strain 17^+ (15). The vhs mutant *vhs-1* is replication competent (53), although in an earlier study, it did not show reduced cytotoxicity compared with its parental wild-type strain (KOS) even when viral DNA replication was inhibited (29). In the present study, we have tested more rigorously the involvement of vhs in the induction of CPE by replication-defective mutants of strain 17^+ by introducing a large insertion mutation into the vhs gene of an IE 3 deletion mutant and also into the vhs gene of the newly constructed IE 3-VP16 double mutant 14HΔ3. Analysis of the resulting mutants indicated that vhs does not contribute to the cytotoxicity induced by this strain of HSV-1 during a nonproductive infection. To determine whether a potential involvement of vhs in virus-induced cytotoxicity was obscured by the effects of viral gene expression, we measured cell viability after infection with UV-irradiated vhs mutants of strains KOS and 17^+ and their parental virus strains, but again, there was no significant difference in cytotoxicity which correlated with the vhs mutation.

MATERIALS AND METHODS

Cells and viruses. All cell lines were propagated in Dulbecco's modification of Eagle's minimal essential medium (DME) containing 10% fetal calf serum. BHK TK^- and CV-1 cells were used to assay the inhibition of colony transformation. T0-119 primary human diploid fibroblasts were used to assay cell killing and the ability of virus to persist in infected cells. Cells used to support growth of IE 3 deletion mutants were RR1 (29) and E5 (obtained from N. A. DeLuca, University of Pittsburgh, Penn.) (10); V9 cells were used to support growth of UL42 deletion mutants (27). RR1, E5, and V9 cells were passaged in medium containing 400 μg of G418 (geneticin; GIBCO BRL, Grand Island, N.Y.) per ml.

All the viruses used in this study were originally derived from HSV-1 strain 17^+ except where stated below. The VP16 mutants *in*1814 and *in*1850 were generously provided by C. M. Preston (Medical Research Council Virology Unit, Glasgow, Scotland). The mutant *in*1850 was derived from *in*1814 (2) by insertion of the *Escherichia coli lacZ* gene into the thymidine kinase (TK) coding sequence (51a). The IE 3 deletion mutant D30EBA was constructed by Paterson and Everett (49). CgalΔ42 contains a deletion in the UL42 coding sequence (27). Viruses containing the *in*1814-specific lesion in the VP16 gene were prepared and titrated by infecting cells in the presence of 5 mM hexamethylene bisacetamide (HMBA; Sigma) for the first 24 h, as described before (39). This step significantly improves both the titer and the particle/PFU ratio of VP16 mutants. An ICP8 deletion mutant derived from strain KOS, *d*301 (19), was used in the superinfection rescue procedure and was kindly provided by D. Knipe (Harvard Medical School, Boston, Mass.). The virion host shutoff mutant *vhs*1 (53) and wild-type strain KOS were kindly provided by G. S. Read (University of Missouri-Kansas City), and the IE 3 deletion

mutant D30EBA was kindly supplied by R. D. Everett (Medical Research Council Virology Unit).

Plasmids. The plasmids used in the colony inhibition assay are shown in Fig. 1. The IE 1 gene contained in plasmid p111, the IE 1 deletion mutant p110del4, the IE 3 gene in p175, and two deletion mutants of IE 3, pD8 and pD9, were obtained from R. D. Everett (48, 50). Plasmid pK1-2 contains IE 3 under the control of its own promoter and was obtained from N. A. DeLuca (10). The IE 2 gene is contained in a 2,448-bp *Bam*HI-*Hpa*I fragment in plasmid pIE63 (29), from which the following derivatives were made: pIE63+ contains an additional 815-bp *Hin*cII-*Bam*HI fragment adjoining the 5′ end of IE 2; pXin63+ contains an *Xho*I linker inserted at the *Sal*I site of pIE63+ (disruption of the IE 2 sequence at this *Sal*I site has been shown to impair the activator and repressor functions of Vmw63 in a transient assay system [22, 55]); and pX63.1 contains an *Xho*I linker inserted at the site of a 28-bp deletion between two *Rsr*II sites in pIE63. The IE 4 gene is contained in plasmid pIE68, and the derivation of pIE68Δ, which contains a 425-bp deletion between the *Sst*I and *Hin*dIII sites of pIE68 and an insertion of 176 bp of Moloney murine leukemia virus DNA, has been described (29). Plasmid pZ4 contains a 4.6-kb human cytomegalovirus (HCMV)-*lacZ* fragment inserted between the *Sst*I and *Hin*dIII sites of pIE68, as shown in Fig. 1. The IE 5 gene is contained in pIE12 as an *Eco*RI-*Bam*HI subfragment of *Bam*HI-*x*. This fragment was inserted between the *Hin*dIII and *Bam*HI sites of p1, replacing the firefly luciferase gene (*luc*) (28), so that IE 5 is terminated by the simian virus 40 (SV40) polyadenylation signal present in p1; pIE12 does not contain complete open reading frames for the overlapping 3′ coterminal genes US10 and US11 (41). Plasmid pZ5 was derived from *Bam*HI-*x* as described before (29). Plasmids pKHF, which contains the large subunit of ribonucleotide reductase (ICP6), and pKHFΔ, which contains a deletion within the ICP6 coding region, were obtained from S. K. Weller (University of Connecticut) (20). All the HSV-1 genes described above were derived from strain 17^+ except those on plasmids pK1-2, pKHF, and pKHFΔ.

The plasmids used to confer resistance to G418 were pSV2neo (62) and pHneo (28), which contain the SV40 enhancer-promoter and the human hypoxanthine phosphoribosyltransferase (HPRT) gene promoter, respectively, driving *neo*.

The plasmid used to delete IE 3 coding sequences from the viral genome was pI11H. This was derived from pdelI11 (49) by insertion of a 587-bp *Hae*III fragment from pUC19 into the *Eco*RI site, marking the IE 3 deletion by blunt-end ligation (see Fig. 3). The plasmid used to disrupt the coding sequence of the vhs gene was pUL41-lacZ. This plasmid contains the 5′ portion of the vhs gene from pBam-i-Sst, into the *Nru*I site of which was inserted, by blunt-end ligation, a 4.6-kb *Bam*HI fragment containing an HCMV-*lacZ* cassette from pON249 (63) (see Fig. 3b).

Other plasmids used in this study were pBaml, which contains the HSV-1 *Bam*HI-*l* fragment in pUC19, and pUL42Δ, which contains a 1,258-bp deletion of the UL42 coding sequence which is marked by an insertion of a 280-bp *Pst*I-*Pvu*II fragment from pBluescript KSII (27). pMC1 contains the coding sequence of VP16 and was kindly provided by C. M. Preston (7).

Transfections and colony inhibition assay. We have used the calcium phosphate method of Graham and van der Eb (21) for all transfections. For the colony inhibition assay, freshly trypsinized cells were seeded on six-well cluster dishes at a density of 10^5 cells per 35-mm well 1 day prior to transfection. The cells were cotransfected in triplicate with 0.25 μg of

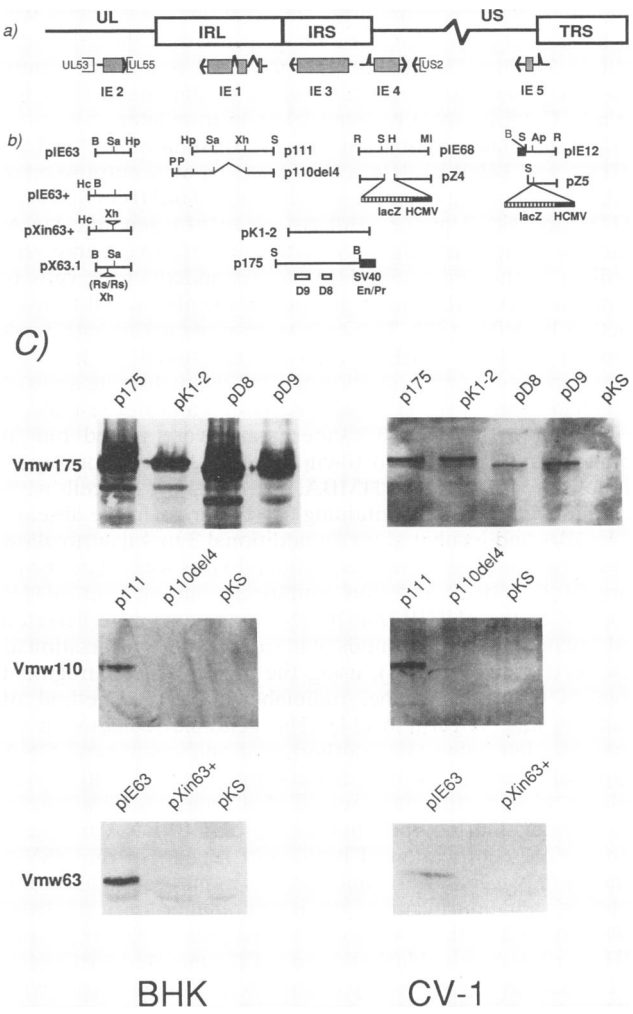


FIG. 1. Location of the HSV-1 IE genes in the viral genome and the structures of IE gene-containing plasmids. (a) The right-hand portion of the viral genome is shown, including part of the long unique region (U_L), the internal long repeat (IR_L), and the short unique region (U_S) bounded by internal and terminal short repeats (IRS and TRS, respectively). The IE transcripts (arrows) and their coding regions (shaded boxes) and additional open reading frames that map close to IE 2 (UL53 and UL55) and IE 4 (US2) are depicted below the genome structure. Note that copies of IE 1 and 3 are present in each repeat (not shown). (b) Structures of the IE gene-containing plasmids and their mutant derivatives. The origins and derivations of these plasmids are described in Materials and Methods. D8 and D9 refer to deletions within p175, contained in plasmids pD8 and pD9, respectively. Ap, *Apa*LI; B, *Bam*HI; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; P, *Pst*I; R, *Eco*RI; Rs, *Rsr*II; S, *Sst*I; Sa, *Sal*I; Xh, *Xho*I; En/Pr, enhancer-promoter. (c) Immunoblot detection of the products of HSV-1 IE genes 1, 2, and 3. BHK and CV-1 cells were transfected with plasmids carrying the individual IE genes as indicated. After 48 h, the cells were collected by scraping, and total-cell lysates were prepared by boiling in sodium dodecyl sulfate (SDS) gel loading buffer (36). Fractions of the cell extracts were run on SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins were electrotransferred onto nitrocellulose membranes. The IE gene products were detected by monoclonal antibodies against HSV-1 Vmw175, Vmw110, and Vmw63, obtained from Advanced Biotechnology, with the enhanced chemiluminescence kit from Amersham.

pSV2neo as a selectable marker to confer resistance to geneticin and 2.5 μg of test plasmid DNA containing HSV-1 sequences. Sixteen hours after transfection, the medium was replaced, and after a further 24 h of incubation, the cells were split by dilution factors of 1:4 and 1:50 and seeded onto 100-mm plates in selection medium containing G418 (400 μg/ml). Colonies were counted 10 to 14 days later.

Generation of recombinant viruses. (i) Isolation of D30HΔ3, 14HΔ3, and 14HRΔ3. RR1 cells (2×10^5) were cotransfected with (i) 2 μg of a 1:1 mixture of intact and *Bam*HI-*Sst*I-cleaved pI11H and (ii) 2 μg of D30EBA or *in*1814 genomic DNA. After 24 h, the cotransfection mixture was removed, and fresh medium containing 5 mM HMBA was added to the cells to stimulate HSV-1 IE gene expression in the absence of transinduction by VP16 (39). The cells were replenished with fresh medium (without HMBA) after an additional 24 h. After several more days of incubation at 37°C, when discrete areas of CPE had formed, the cells were harvested and freeze-thawed, and virus progeny in the supernatant were plated out at appropriate dilutions onto 10-cm dishes of E5 cell monolayers in the presence of 5 mM HMBA. The next day, the cells were overlaid with medium containing 0.8% agarose in the absence of HMBA and incubated for an additional 3 to 4 days to allow the formation of plaques. Recombinant virus which had incorporated the IE 3 deletion mutation contained in pI11H (marked by the pUC19 *Hae*III DNA fragment) were detected and isolated by several rounds of in situ plaque hybridization as described previously (27), using the 587-bp *Hae*III fragment from pUC19 as a probe, radiolabeled by the method of Feinberg and Vogelstein (14). The genomic structure of viruses was determined by Southern blot analysis of total DNA prepared from 16-mm-well cell cultures infected with plaque isolates, picked after each round of plaque hybridization. In this manner, the incorporation of the pUC19 DNA fragment into the correct location of both short repeats of the HSV-1 genome in place of IE 3 sequences was monitored to allow the isolation and purification of recombinants derived from D30EBA and *in*1814, which were designated D30HΔ3 and 14HΔ3, respectively. Marker rescue of the mutated VP16 gene in 14HΔ3 was performed by cotransfection of RR1 cells with 14HΔ3 genomic DNA and pMC1, which contains the wild-type VP16 gene. Larger plaques which grew in the absence of HMBA were picked and purified, and their genomic structure was analyzed as described above for loss of the *Bam*HI linker in the VP16 gene. Three such isolates all exhibited the correct *Bam*HI restriction profile, and a large preparation made from the first of these was designated 14HRΔ3.

(ii) Isolation of *in*1850Δ42. BHK TK^- cells (10^6) were cotransfected with 10 μg of *in*1850 DNA and 10 μg (total) of an equal mixture of intact and *Bam*HI-cleaved pUL42Δ DNA (27). Progeny viruses were plated onto V9 cell monolayers, and the resulting plaques were screened for the presence of the 280-bp fragment of plasmid vector DNA marking the deletion of the UL42 gene, as described above. Stocks were prepared from a plaque-purified isolate which could only form plaques on V9 cells and which exhibited the correct restriction digest profiles corresponding to the UL42 gene deletion and the VP16 gene insertion mutation (26). This virus was named *in*1850Δ42.

(iii) Isolation of Δ3vhsZ and 14HΔ3vhsZ. Disruption of the vhs coding region of D30EBA and 14HΔ3 was achieved by cotransfection of RR1 cells with pUL41-lacZ DNA, linearized by digestion with *Xho*I and the appropriate viral genomic DNA. Recombinant virus in the resulting progeny were detected by the appearance of blue plaques after plating on monolayers of E5 cells and staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described (29). The genomic structures of virus isolates carrying the intended insertion mutation in the vhs genes of D30EBA and 14HΔ3 were confirmed by Southern blot analysis, and these isolates were designated Δ3vhsZ and 14HΔ3vhsZ, respectively.

Yield of progeny virus. E5 or V9 cells (as appropriate) were seeded at a density of 3×10^5 cells per 35-mm dish. After an assumed doubling by 24 h, the cells were infected with virus at an MOI of 1 or 0.01 PFU per cell. Unabsorbed virus was removed by washing 1 h later, and the monolayers were incubated for an additional 19 h at 37°C. Infected cultures were maintained continuously for the 24-h period in either the presence or absence of 5 mM HMBA. The cells were then scraped into the medium and disrupted by freeze-thawing, and virus in the resulting supernatants was titrated by plaque assay on permissive cells in the presence of 5 mM HBMA and 10% pooled human serum.

Cell viability assays. The effect of virus infection on cell survival and growth, as measured by the number of adherent cells at 3 days postinfection, was determined as follows. Human fibroblasts were seeded at a density of 5×10^4 cells per 16-mm well. The cells were infected in triplicate 16 h later with virus at MOIs of 0.1, 0.25, 0.5, 1.0, 5.0, 7.5, 10, and 25 PFU per cell. After a 1-h absorption period, the cells were washed and replenished with fresh medium. At 3 days postinfection, loose cells and debris were removed by washing with phosphate-buffered saline (PBS), and the adherent cells were harvested by trypsinization and counted with a Coulter counter. The titers of the virus stocks used in this cell viability experiment were also determined in terms of infectious genome units (IGU) per ml, as follows. Human fibroblasts (10^6) were infected with either a standard volume of virus stock (25 μl) or a constant MOI (e.g., 5 PFU per cell). The cells were washed three times after a 1-h absorption period and then incubated for 1 h more at 37°C. The cells were then harvested in PBS, and cell nuclei were isolated from outer cell membranes and cytoplasmic components following Nonidet P-40 lysis. Nuclear DNA was prepared, and 2.5-μg aliquots were digested with *Bam*HI alongside uninfected human fibroblast DNA which had been spiked with 3.3 to 825 pg of pBam 1, corresponding to 1 to 250 HSV-1 genome copies per cell in 2.5 μg of human fibroblast DNA. The digested DNAs were separated by agarose gel electrophoresis and analyzed by Southern blotting with *Bam*HI-*l* as a probe. An example of this is shown in Fig. 5c. In some cases, the blot was reprobed with a human DNA sequence (human β-galactosidase cDNA) to normalize the total amount of DNA loaded and transferred in each lane, but it was usually found to be quite constant. Titers in PFU had been determined in the presence of HMBA, which has been shown previously by McFarlane et al. to help overcome the replication defect in *in*1814, so that *in*1814 can be titrated almost as efficiently as wild-type virus (39). The ratios of IGU to PFU in one experiment were as follows: D30HΔ3, 5.4; 14HΔ3, 20.3; and 14HRΔ3, 3.2. Although the difference in the IGU/PFU ratio between D30HΔ3 and 14HΔ3 was fourfold in this experiment, the difference was only twofold in an independent experiment. Some degree of variability in virus titer is inevitable, in part because of differences in the growth state and passage number of permissive cell lines. Since MOI is a very critical parameter for studying cytotoxicity, we have tried to minimize the effects of variation in virus titer by comparing the effects of different viruses using titer values that had been determined at the same time. In some of the experiments described in this paper, we have compared the toxicity of different viruses using PFU rather than IGU as a measure of

virus titer, but we bear in mind that this places more stringent conditions on the VP16 mutants because of at least a two- to fourfold impairment in plaquing efficiency.

Cell viability measurements were also determined by trypan blue exclusion, as follows. Human primary fibroblasts were seeded onto 24-well plates at a density of 5×10^4 cells per well and infected in triplicate the next day with mutant virus strains at an MOI of 10 PFU per cell. Viruses that were inactivated by UV irradiation were exposed to a constant UV source (1,200 $\mu W/cm^2$) for 5 min. We had previously determined that this UV dose would reduce β-galactosidase expression in CgalΔ3 by more than 30-fold and reduce virus titer by more than 100,000-fold (29). One hour after infection, the virus inoculum was removed and replaced with fresh medium containing full serum, and the cells were incubated for 3 days at 37°C. The cells were then harvested by trypinization and stained with 0.5% trypan blue in PBS. The number of viable cells which excluded trypan blue were counted with a hemacytometer.

Superinfection rescue procedure. The ability of different mutant virus strains to persist in infected primary fibroblasts and be "rescued" by a complementing mutant strain 11 days later was measured by a semiquantitative superinfection rescue procedure similar to that described previously (29). Briefly, human fibroblasts in 10% fetal calf serum were seeded onto a 24-well plate at a density of 6×10^4 cells per 16-mm well. After having reached confluency (about 2.5×10^5 cells per well) in 3 days, cultures on duplicate plates were infected in duplicate with each mutant virus strain, at an MOI ranging from 0.005 to 2.5 PFU per cell. After 1 h, the cells were washed three times and either maintained in 0.2% serum for 11 days (with fresh medium added every 2 to 4 days) or processed directly for the superinfection rescue. The rescue was performed by superinfecting cells with the ICP8 mutant *d*301 at an MOI of 5 PFU per cell in the presence of 5 mM HMBA for 4 h. The superinfected cells were then harvested by trypsinization, and appropriate dilutions were plated onto 10-cm plates preseeded with E5 cells (to titer IE 3 deletion mutants) or V9 cells (to titer UL42 deletion mutants), in 5 ml of medium containing 5 mM HMBA and 10% pooled human serum. After 16 h, 5 ml of additional medium was added, and the cells were incubated at 37°C for 3 to 4 days until the resulting plaques could be counted. In control experiments, the highest number of plaques for a replication-defective mutant obtained on day 0 without superinfection was 3.4% of the number of plaques obtained with superinfection, indicating that the vast majority of plaques which do arise do so through complementation with the superinfecting virus and not because of persistence of viable intact viral particles (26). By day 11, no plaques were observed without superinfection. Also, no more than one or two plaques have been detected following superinfection of mock-infected cells; these few plaques probably represent occasional rescue of the ICP8 deletion in *d*301 during preparation of virus stocks. Previous analysis has shown that the plaques which arise from the superinfection rescue procedure represent a mixture of the initial virus persisting in the cells and the superinfecting virus (29).

RESULTS

Inhibition of colony formation with transfected HSV-1 sequences. To determine whether we could identify potential virus genes responsible for cytopathogenicity, we cotransfected BHK TK^- cells and CV-1 cells with plasmids containing each of the candidate viral genes expressed by an IE 3 deletion mutant (Fig. 1) together with pSV2neo in a 10:1 ratio (wt/wt). The cells were then split at various dilutions and placed under selection for neomycin resistance, and the number of colonies obtained was counted 10 to 14 days later. For the sake of completeness, we also examined the effect of IE gene 3 in these experiments. The plasmids containing the IE genes were chosen or constructed so that the presence of additional open reading frames or the presence of extraneous sequences would be very limited. However, the frequency of colony formation could be influenced by promoter competition, the presence of "poison" sequences, or additional open reading frames in the cotransfected test plasmids. To control for these possibilities, we also examined the frequency of colony formation with control plasmids containing mutations in the coding regions of each of the respective IE genes. To ensure that the plasmids encoding IE genes 1, 2, and 3 expressed the expected IE proteins in transfected cells, we also performed Western (immunoblot) analysis with specific monoclonal antibodies raised against these IE proteins (Fig. 1C). In additional control experiments with luciferase as a reporter gene, we have determined that the promoters of IE genes 1, 2, 3, and 4/5 were highly active in transfected BHK TK^- and CV-1 cells (26).

As shown in Fig. 2, plasmids encoding IE 1 (p111), IE 2 (pIE63 and pIE63+), and IE 4 (pIE68) all strongly inhibited the frequency of colony formation in both BHK TK^- and CV-1 cells, in comparison to the number of colonies obtained by the control cotransfection with pBluescript KS. Cotransfection with control plasmids bearing lesions in the coding portions of IE genes 1 (p110del4), IE 2 (pXin63+ and pX63.1), and IE 4 (pZ4) reduced the inhibitory effects observed with the intact IE genes. The inhibitory effect of IE 2 was more completely abolished by pX63.1, which has a 28-bp deletion in the IE 2 coding region, than by pXin63+, which has a single *Xho*I linker insertion mutation. Unexpectedly, both plasmids encoding IE 3 (p175 and pK1-2) strongly inhibited colony formation in CV-1 cells but not in BHK TK^- cells. The inhibition of colony formation in CV-1 cells cotransfected with IE 3 sequences was more effectively abolished by pD8, which encodes an IE 3 deletion product retaining transactivation activity but defective for repression (48), than by pD9, which encodes an IE 3 product with full repression activity but only 4% of the transactivation activity of the wild-type IE 3 protein (48). The plasmid encoding IE 5 (pIE12) did not cause inhibition of colony formation in BHK cells, although a moderate inhibition of transformation frequency (50%) was observed in CV-1 cells. Inhibition of colony formation following cotransfection with plasmids encoding IE genes 1, 2, and 4 has also been observed with a different selectable marker construct, in which the *neo* gene was driven by the human HPRT promoter instead of the SV40 promoter (data not shown). We have also determined that neither plasmid pKHF, which encodes the large subunit of ribonucleotide reductase (ICP6), which is also expressed as an IE gene, nor pKHFΔ, which harbors a deletion within the ICP6 coding region, causes inhibition of colony formation (26). Finally, since the products of IE genes 1, 2, and 3 are known to modulate transcriptional activity, it was possible that they might directly affect the level of neomycin phosphotransferase produced by the selectable marker plasmid, thereby providing a trivial explanation for their effect on the frequency of colony formation. We therefore measured the effect of cotransfecting IE genes 1, 2, and 3 with the *neo* gene on the resulting level of neomycin phosphotransferase activity and determined that while plasmids bearing IE 2 or IE 3 had little effect, IE 1 caused a moderate (twofold) stimulation in activity (data not shown).

Isolation of mutant viruses. (i) IE 3-VP16 double mutant. To reduce the expression of HSV-1 IE genes during a nonproductive infection, we have constructed double mutant viruses

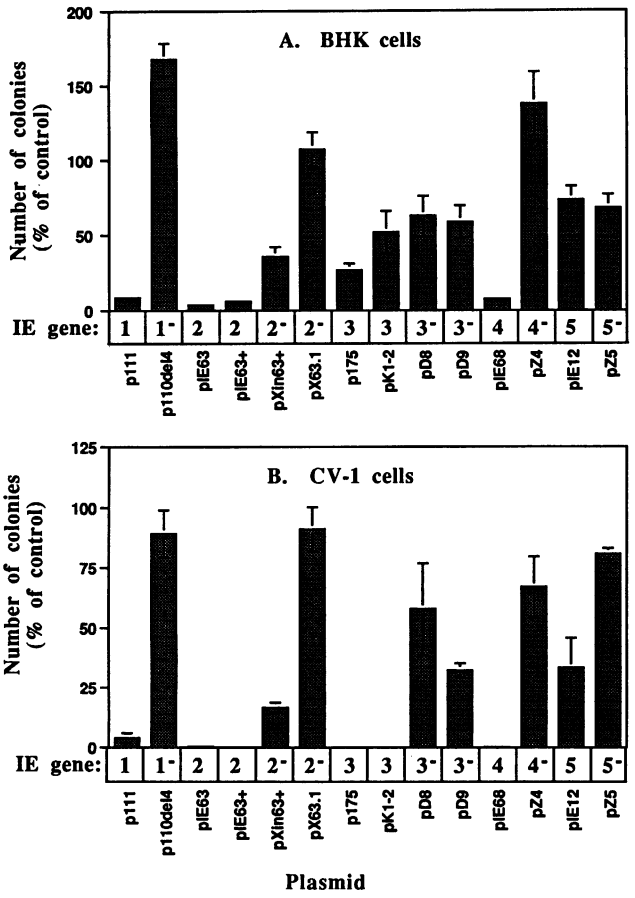


FIG. 2. Effect of HSV-1 IE gene-carrying plasmids on the frequency of colony formation in cotransfected BHK TK$^-$ and CV-1 cells. Approximately 10^5 cells per 35-mm well were cotransfected in triplicate with 0.25 μg of pSV2neo and 2.5 μg of the indicated test plasmid. Cells were washed after 24 h and split the following day at 1:4 and 1:50 dilutions into selection medium containing G418 (400 μg/ml). Colonies were counted 10 to 14 days after transfection. An average of 933 and 2,916 colonies per 0.25 μg of pSV2neo were obtained in the control transfections (with 2.5 μg of pBluescript KS) of BHK TK$^-$ and CV-1 cells, respectively. No colonies developed in the absence of pSV2neo. Error bars indicate standard deviations.

with a mutation in an essential gene to prevent lytic replication and with the insertion mutation in the VP16 gene described by Preston and colleagues (2) to abolish the ability of VP16 to transinduce IE gene expression. The double mutant 14HΔ3 was generated by introducing a deletion into both copies of the IE 3 gene of the VP16 mutant *in*1814. As shown in Fig. 3c, plasmid pI11H, used to generate the IE 3 deletion, contains a DNA fragment from pUC19 (a 587-bp *Hae*III fragment containing the 5′ portion of the ampicillin resistance gene) at the site of the deletion to serve as a tag to aid the identification of viral recombinants by plaque hybridization. This plasmid is otherwise identical to pdelI11, which was used to generate the IE 3 deletion mutant D30EBA (49). Since initially we were unsure how well an IE 3-VP16 mutant would grow, we also isolated a derivative of D30EBA from plasmid pI11H, designated D30HΔ3, to provide a positive control for identification of recombinants by plaque hybridization with the pUC19 DNA fragment and to ensure that recombinants could be identified under conditions that did not require selection against the IE 3 gene. Southern blot analysis of the genomic structure of 14HΔ3 isolates is shown in Fig. 3d (leftmost panel). The presence of the 0.52-kb band in *Bam*HI-*Eco*RI-digested 14HΔ3 DNA, which has been probed with a DNA fragment from the 5′ end of IE 3, indicates that the anticipated deletion of the IE 3 gene has taken place, although overexposure of this blot did reveal the minor presence of a 1.8-kb band, indicative of the wild-type IE 3 *Bam*HI fragment. The viral stock from which this DNA was prepared was also able to form a few plaques on Vero cells, which are noncomplementary for IE 3 mutants. Therefore, this 14HΔ3 virus isolate was further plaque purified until no such IE 3-independent plaques appeared in an inoculum containing at least 10^5 PFU.

To ensure that phenotypic differences between 14HΔ3 and an IE 3 deletion mutant were due to the mutation in the VP16 gene and not to a second mutation, the VP16 lesion was repaired by recombining 14HΔ3 DNA with plasmid pMC1 (7), which contains an intact VP16 gene. Rescued virus could be obtained readily, since it was able to grow much more efficiently than the 14HΔ3 double mutant in the absence of HMBA. The genotypes of three such isolates were examined by Southern blot analysis for the presence of an intact 8-kb *Bam*HI-*f* fragment in place of the 3- and 5-kb bands associated with the *Bam*HI linker insertion present in *in*1814 and 14HΔ3 (Fig. 3d, second panel), and a stock prepared from one of these purified isolates was designated 14HRΔ3.

(ii) UL42-VP16 double mutant. To examine the effect of the VP16 mutation on virus-induced cytotoxicity in the background of a mutant blocked for replication at a step in the lytic cycle different from that of the IE 3 deletion mutant, we also introduced a deletion into an essential early gene of a VP16 mutant (see Materials and Methods). The early gene we chose to mutate was UL42, which encodes a 65-kDa accessory function for the HSV-1 DNA polymerase (18), essential for virus replication (27, 37). The purified UL42-VP16 double mutant isolate was designated *in*1850Δ42.

(iii) IE 3-vhs double mutant and IE 3-vhs-VP16 triple mutant. To assess the role of the vhs function in the cytotoxicity of replication-defective mutants, we introduced an HCMV-*lacZ* cassette into the UL41 coding region of D30EBA and 14HΔ3 (see Materials and Methods). The genomic structures of the recombinants, designated Δ3vhsZ and 14HΔ3 vhsZ, respectively, were examined by Southern blot analysis (Fig. 3d). Digestion of Δ3vhsZ and 14HΔ3vhsZ DNA with *Sst*I gave rise to a 3.2-kb band detectable with a probe from a *Bam*HI-*Sst*I subfragment of *Bam*HI-*i*, which is the size expected from correct insertion of the *lacZ* gene, instead of the 2.2-kb fragment from an intact UL41 5′ region (Fig. 3b, and d). When this blot was reprobed with *lacZ*, 3.2- and 3.5-kb fragments were detected, indicating correct insertion of the *lacZ* gene into the UL41 coding region. We also checked for the presence of the *Bam*HI linker in the VP16 gene of 14HΔ3vhsZ (but not Δ3vhsZ), indicated by cleavage of the 8-kb *Bam*HI-*f* fragment into 3-kb and 5-kb subfragments (Fig. 3d). Smibert and Smiley have shown that insertion of an ICP6-*lacZ* cassette into the *Bam*HI site of the UL41 gene of an HSV-1 KOS strain resulted in inactivation of the vhs function (61). Since their mutation disrupted the UL41 open reading frame after residue 342, and in ours it is disrupted after residue 237 (see Fig. 3b), it is probable that insertion of the *lacZ* gene at the *Nru*I site will also result in inactivation of the vhs function.

Growth characteristics of mutant viruses. The insertion mutation in *in*1814 prevents VP16 from forming the multiprotein complex that normally transactivates transcription of HSV-1 IE genes upon infection, thereby impairing the ability

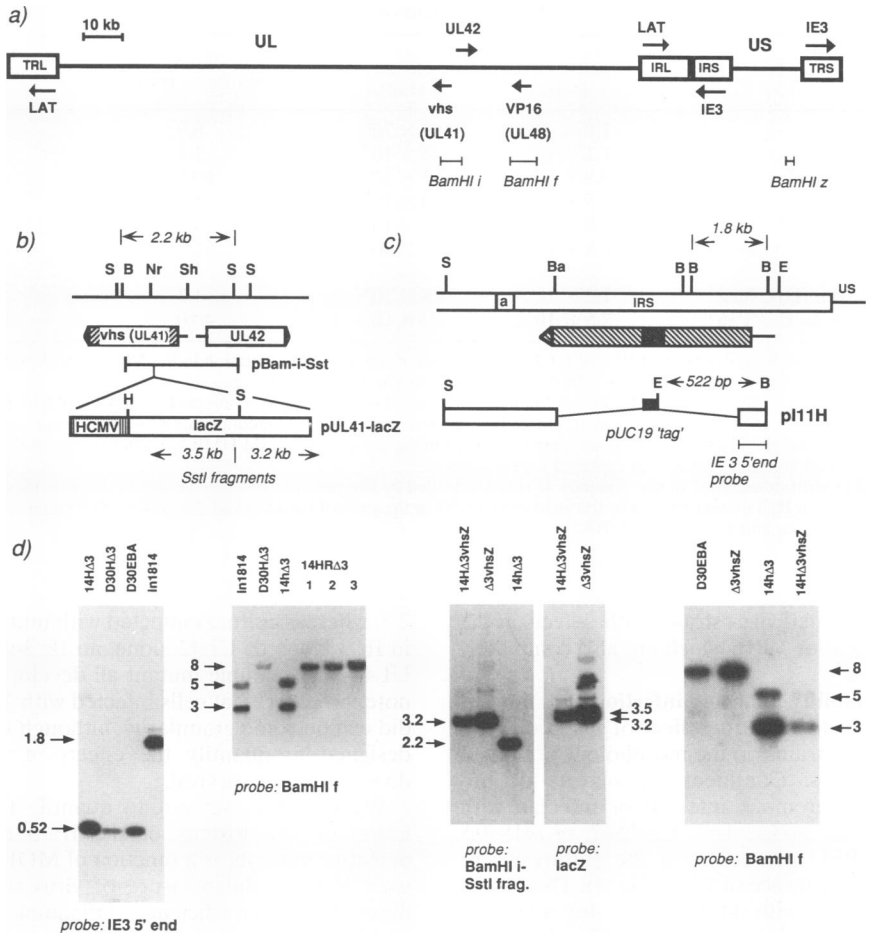


FIG. 3. Construction and analysis of genetic modifications to the HSV-1 genome. (a) Map of the HSV-1 genome, consisting of the unique long (U_L) and unique short (U_S) regions, bound by terminal and internal long (TRL and IRL) and short (TRS and IRS) repeats. Shown are the locations of genes UL41 (vhs), UL42, UL48 (VP16), IE 1, IE 3, and the LAT transcripts, and the locations of *Bam*HI fragments *i*, *f*, and *z* used in the construction and/or analysis of mutants. (b) Construction of vhs mutants. An HCMV-*lacZ* cassette was inserted into the *Nru*I site of UL41, contained on plasmid pBam-i-Sst, creating pUL41-lacZ. The sizes of novel and wild-type *Sst*I fragments detected by *Bam*HI-*i* or *lacZ* probes are given. (c) Deletion of the IE 3 gene. The position of the IRS copy of the IE 3 transcript (line) and coding region (hatched box) is shown, as is the extent of the IE 3 deletion within plasmid pI11H. pI11H was derived from pdelI11 (49) by insertion of a 522-bp *Hae*II fragment from pUC19, to serve as a tag for the detection of recombinants by in situ plaque hybridization. On an *Eco*RI-*Bam*HI digest of unpurified recombinant viral DNAs, the indicated IE 3 5′ probe will detect a 1.8-kb wild-type fragment or a 522-bp novel virus fragment. (d) Southern blot analysis of recombinant viruses. First panel: 14HΔ3, D30HΔ3, D30EBA, and *in*1814 viral genomic DNAs were digested with *Eco*RI and *Bam*HI and probed with the IE 3 5′-end fragment shown in panel c. The presence of the 0.52-kb band in 14HΔ3, D30HΔ3, and D30EBA indicates that deletion of the IE 3 gene has occurred. Second panel: Viral DNAs from *in*1814, D30HΔ3, 14HΔ3, and three isolates of 14HΔ3 which had been rescued with an intact VP16 gene (14HRΔ3) were digested with *Bam*HI and probed with the *Bam*HI-*f* fragment. The presence of the *Bam*HI linker in the VP16 gene results in cleavage of the 8-kb *Bam*HI-*f* fragment into 5-kb and 3-kb subfragments. Third panel: 14hΔ3vhsZ, Δ3vhsZ, and 14HΔ3 DNAs were digested with *Sst*I. The presence of the HCMV-*lacZ* cassette is indicated by a novel 3.2-kb band in place of the wild-type 2.2-kb band when probed with the *Bam*HI-*i*-*Sst*I fragment shown in panel b. Reprobing with *lacZ* gave rise to an additional 3.5-kb band in the vhs mutants. Fourth panel: The indicated viral DNAs were digested with *Bam*HI and probed with *Bam*HI-*f* to confirm the presence of the VP16 mutation in 14HΔ3vhsZ, indicated by the 5- and 3-kb subfragments of *Bam*HI-*f*. Restriction sites: S, *Sst*I; B, *Bam*HI; Sh, *Sph*I; Ba, *Bal*I; E, *Eco*RI; Nr, *Nru*I.

of *in*1814 to initiate infection at low MOI (2). In the presence of HMBA, however, *in*1814 is able to initiate infection almost as efficiently as wild-type HSV-1 (39). To determine whether the new VP16 mutant viruses exhibit a similar phenotype, we measured the yield of progeny virus after 24 h of infection of permissive cells at a low MOI (0.01 PFU per cell), in the presence and absence of HMBA. As shown in Table 1, viruses with the VP16 mutation (14HΔ3, 14HΔ3vhsZ, and *in*1850Δ42) grew very poorly in the absence of HMBA, but their yields were improved by 30- to 60-fold in its presence. HMBA had little effect on the yield of viruses without the VP16 lesion (D30HΔ3, 14HRΔ3, Δ3vhsZ, and CgalΔ42). Viruses with the UL41 mutation were further impaired for growth, since the yield of Δ3vhsZ was 10-fold below that of D30HΔ3, and although in this experiment the yield of 14HΔ3vhsZ with HMBA was only 2-fold below that of 14HΔ3, the titers of stocks of 14HΔ3vhsZ were usually 5- to 10-fold below that of 14HΔ3 (26). These observations are consistent with those of other studies reporting that large insertion or deletion mutations in the vhs gene cause a 5- to 10-fold drop in viral titer (15, 61). Together with the confirmation of genomic structure by Southern analysis, we conclude from these observations that

TABLE 1. Yield of progeny

Virus	Genotype[a]	Yield[b]		Ratio, +HMBA/− HMBA[c]	Yield to D30HΔ3 or CgalΔ42[d]	
		− HMBA	+ HMBA		− HMBA	+ HMBA
D30HΔ3	IE 3$^-$	1.0×10^6	0.9×10^6	0.9	1.0	1.0
D30EBA	IE 3$^-$	1.1×10^6	1.2×10^6	1.1	1.1	1.3
14HΔ3	IE 3$^-$, VP16^{in}	3.8×10^3	2.3×10^5	60.5	0.004	0.25
14HRΔ3	IE 3$^-$	1.5×10^6	2.0×10^6	1.2	1.5	2.0
Δ3vhsZ	IE 3$^-$, vhs-*lacZ*	1.0×10^5	1.2×10^5	1.2	0.1	0.13
14HΔ3vhsZ	IE 3$^-$, VP16^{in}, vhs-*lacZ*	2.8×10^3	1.0×10^5	35.7	0.003	0.11
CgalΔ42	UL42$^-$, *Bam*HI-*z*-*lacZ*	1.2×10^6	1.5×10^6	1.3	1.0	1.0
*in*1850Δ42	UL42$^-$, *tk*-*lacZ*, VP16^{in}	2.5×10^3	1.0×10^5	40.0	0.002	0.067

[a] Genotype indicates whether viruses have deletions in IE 3 or UL42, the presence of the insertion mutation in the VP16 gene described by Ace et al. (2), and the presence of a *lacZ* gene inserted into the *Bam*HI-*z* region or a *lacZ* insertional mutation in the *vhs* or *tk* gene.

[b] Permissive cells that complement growth of mutants (E5 for IE 3 mutants, V9 for UL42 mutants) were infected at an MOI of 0.01 PFU per cell for 20 h in either the absence or continuous presence of 5 mM HMBA, as indicated. The cells were then scraped into the medium and disrupted by freeze-thawing, and the titers of virus in the resulting supernatants were determined on the appropriate permissive cells in the presence of 5 mM HMBA. Values represent the yield of progeny determined from duplicate infected cultures and are representative of repeated experiments.

[c] Values represent the yield of virus determined in the presence of HMBA divided by the yield of virus determined in the absence of HMBA.

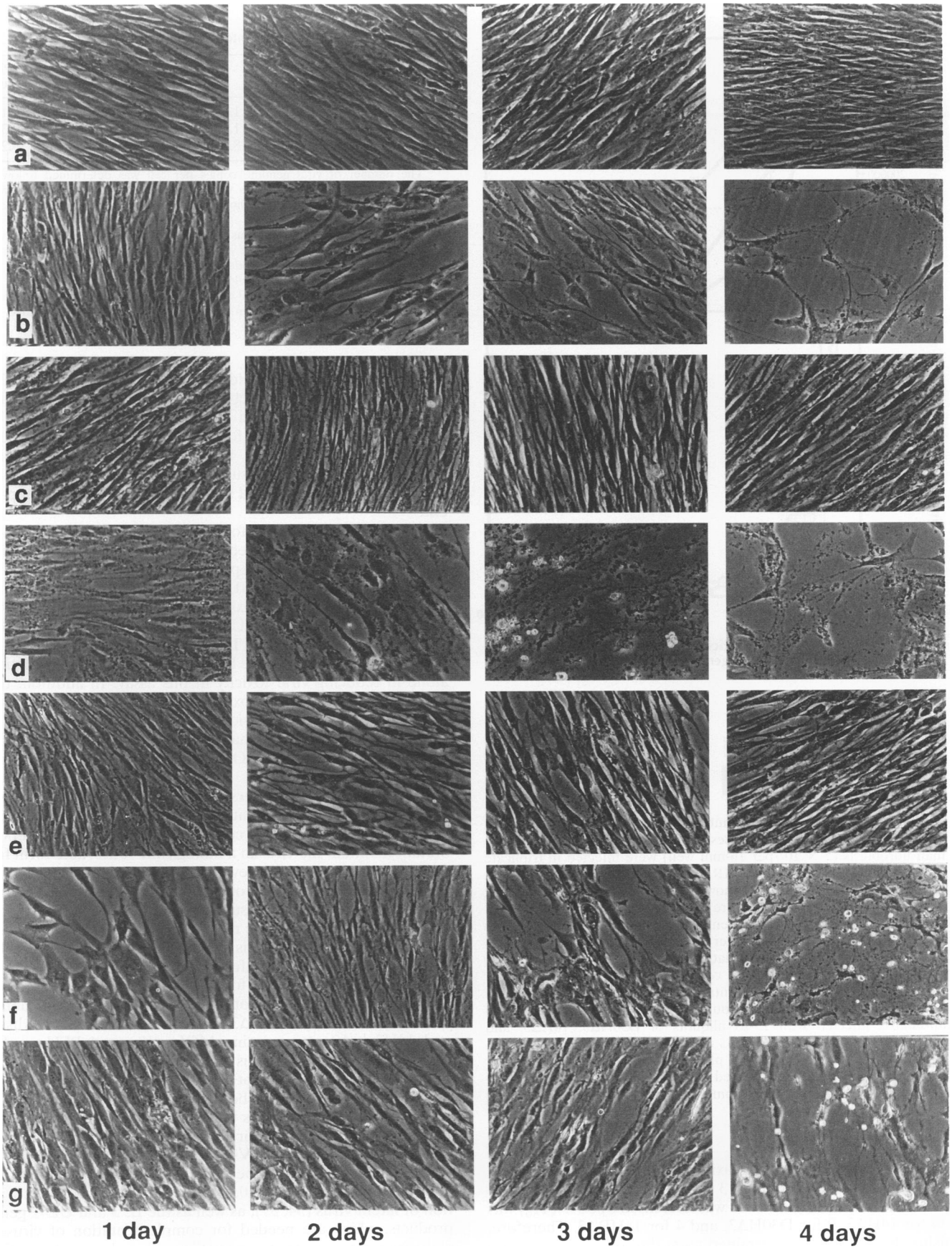
[d] Values represent the yield of each IE 3 mutant divided by the yield of D30HΔ3 or the yield of CgalΔ42 and the UL42-VP16 mutant *in*1850Δ42 divided by the yield of CgalΔ42, determined in the presence and absence of HMBA.

we have successfully generated virus strains with defects in the virion components vhs and/or VP16 which are also completely defective for replication.

Comparison of cell viability following infection with mutant virus strains. We have examined the effect of infection with the different mutant virus strains on the morphology of human primary fibroblast cultures. Confluent monolayers of human fibroblasts were either mock infected or infected with D30HΔ3, 14HΔ3, Δ3vhsZ, 14HΔ3vhsZ, CgalΔ42, or *in*1850Δ42 at an MOI of 2.5 PFU per cell, and the cultures were examined daily for the appearance of CPE (Fig. 4). The results show that cultures infected with 14HΔ3 (Fig. 4c) remained almost as healthy as the mock-infected control (Fig. 4a), while those infected with D30HΔ3 (Fig. 4b) exhibited increasingly severe CPE with increased time postinfection. Therefore, as a result of the VP16 lesion in 14HΔ3, the cytopathogenicity of an IE 3 deletion mutant has been significantly reduced. In contrast, Δ3vhsZ (Fig. 4d), which has a defective vhs function in addition to the IE 3 deletion, did not appear to be any less cytopathogenic than D30HΔ3. Cultures infected with the triple mutant 14HΔ3vhsZ (Fig. 4e), which has a VP16 lesion as well as the IE 3 and UL41 mutations, also remained healthy for the duration of the experiment. However, *in*1850Δ42 (Fig. 4g), which has a VP16 lesion and is deleted for the essential early gene UL42, did cause generalized CPE in infected cultures at this MOI, as did CgalΔ42 (Fig. 4f), which is deleted for UL42 but has an intact VP16 gene. In these experiments, the cultures were infected with the same number of PFU, even though the plaque-forming ability of the VP16 mutant strains (determined in the presence of HMBA) was likely to represent a two- to fourfold underestimate of the relative number of infectious viral genomes (see Materials and Methods) and thereby place a more stringent test on the ability of the VP16 mutants not to cause CPE. Nevertheless, these results show that as a result of the VP16 mutation, the cytopathogenicity of an IE 3 deletion mutant has been sufficiently reduced so that an infected culture does not develop extensive CPE when infected at an MOI of 2.5, whereas cultures infected with mutants carrying mutations in IE 3 alone or UL42 alone, an IE 3-vhs double mutant, or a UL42-VP16 double mutant all develop CPE at this MOI. We note, however, that cells infected with 14HΔ3 and 14HΔ3vhsZ did exhibit some granularity, although this experiment was not designed to quantify the effects of viral infection on cell division and/or survival.

We therefore set out to quantify the effect of the VP16 lesion on the toxicity of the IE 3 and UL42 replication-defective mutants as a function of MOI. In this experiment, we used IGU as the measure of virus titer to compensate for differences in the efficiency of plaquing between VP16 mutants and VP16-intact virus. The determination of IGU titer for the stocks of IE 3 mutants used in this experiment is shown in Fig. 5c and Table 2. To assay toxicity, we counted cell number at 3 days postinfection, to allow sufficient time for virus-induced cytotoxicity to occur. We used a mitotic fibroblast cell line because CPE occurs faster and there is less ambiguity in distinguishing living cells than if we had used nondividing cells. Cell number therefore reflects both the survival and replication of cells over the 3-day period after the infection. Subconfluent monolayers of human fibroblasts were infected with D30HΔ3, 14HΔ3, 14RHΔ3, CgalΔ42, and *in*1850Δ42 over a range of MOIs from 0.1 to 25 PFU per cell. The titers of the virus stocks used in this experiment were determined at the same time in terms of IGU per ml to compensate for the reduced plaquing efficiency of the VP16 mutants (as shown for the IE 3 mutants in Fig. 5c and Table 2). As shown in Fig. 5a, the curve produced from the number of adherent cells at 3 days postinfection with increasing amounts of 14HΔ3 is shifted significantly to the right of the curves corresponding to D30HΔ3 and 14HRΔ3, indicating that 14HΔ3 is less toxic over a wide range of MOIs. In the case of 14HΔ3, the curve was steepest after an MOI of 10 IGU per cell, which indicates the point at which virus toxicity is measurably affecting cell growth and survival, whereas for infections with 14HRΔ3 and D30HΔ3, significant loss in cell number occurred after an MOI of approximately 3

FIG. 4. Infection of human primary fibroblasts with HSV-1 mutants. Confluent monolayers of human fibroblasts were either mock infected (a) or infected with D30HΔ3 (b), 14HΔ3 (c), Δ3vhsZ (d), 14HΔ3vhsZ (e), CgalΔ42 (f), or *in*1850Δ42 (g) at an MOI of 2.5 PFU per cell. Monolayers were observed daily for the appearance of CPE and photographed.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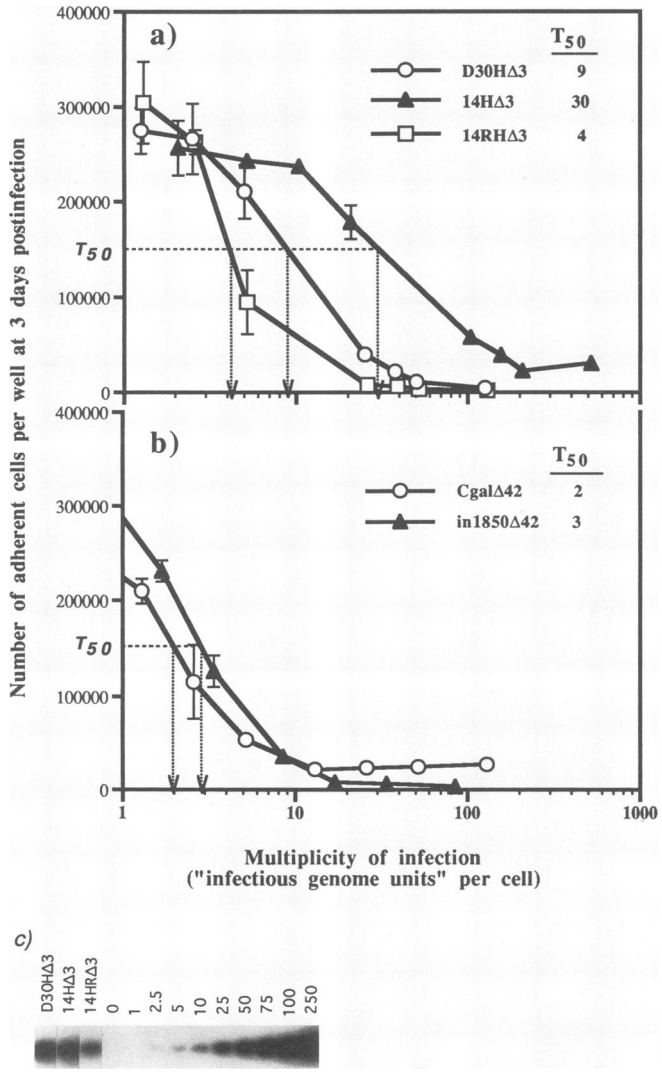

FIG. 5. Effect of the VP16 mutation on cell survival and growth after infection with mutant viruses. Subconfluent monolayers of human fibroblasts (5×10^4 per 16-mm well) were infected in triplicate with (a) D30HΔ3, 14HΔ3, or 14HRΔ3 or (b) CgalΔ42 or *in*1850Δ42 at increasing MOIs. The MOI is shown here in terms of IGU per cell. The IGU titers of virus stocks were determined as described for panel c. The average number of adherent cells remaining at 3 days postinfection, determined with a Coulter counter, are plotted against MOI (error bars indicate standard deviations). T_{50} is defined as the MOI at which the number of adherent cells was reduced by 50% of the maximum number of cells on a control uninfected plate (IGU per cell). (c) Southern blot showing the amount of viral DNA detected in human fibroblasts infected with 25-μl aliquots of D30HΔ3, 14HΔ3, and 14HRΔ3. The infected-cell DNAs and cellular DNA spiked with the equivalent of 0 to 250 copies of pBaml plasmid DNA per cell were digested with *Bam*HI and probed with radiolabeled *Bam*HI-*l* fragment. The calculation of IGU from densitometric analysis of this blot is shown in Table 2.

IGU per cell. Taking the maximum number of cells from a control uninfected well at day 3 to be 300,000, the titer (in IGU per cell) at which cell number was decreased by 50% (T_{50}) was 30 for 14HΔ3, 9 for D30HΔ3, and 4 for 14HRΔ3. Therefore, using the T_{50} values determined from this assay as a measure of virus toxicity, the IE 3-VP16 double mutant 14HΔ3 is between three- and sevenfold less toxic than either of the IE 3 mutants which have an intact VP16 gene. Since in two separate determinations 14HΔ3 had a two- to fourfold-higher IGU/PFU ratio than D30HΔ3, it follows that if PFU was used as the measure of virus titer in this cell-killing experiment, the reduction in toxicity as a result of the VP16 mutation in the IE 3 mutant background would be less pronounced or not apparent.

TABLE 2. Infectious genome titer

Virus	No. of copies/cell[a]	IGU[b]	PFU[c]	IGU/PFU ratio
D30HΔ3	45.3	1.8×10^9	3.5×10^8	5.1
14HΔ3	32.3	1.3×10^9	6.4×10^7	20.3
14HRΔ3	20.0	8.0×10^8	2.5×10^8	3.2

[a] The number of viral genome copies per cell was determined following infection of 10^6 T0-119 fibroblasts with 25 μl of each virus stock and from densitometry of the Southern blot shown in Fig. 5c, as described in Materials and Methods.

[b] The titer of virus in infectious genome units (IGU) per milliliter was calculated from the copy number of viral genomes per cell (N) multiplied by the total number of cells (10^6) and divided by the volume of the virus inoculum (V, in milliliters) as follows: $(N \times 10^6)/V$.

[c] The titer of virus in PFU per ml was determined from the same virus stocks at the same time following infection of E5 cells in the presence of HMBA.

In contrast, cell survival following infection with *in*1850Δ42 was not markedly improved over that after infection with CgalΔ42 (Fig. 5b). A steep reduction in cell number was apparent at an MOI of 2 IGU per cell for both UL42 mutants. The titers at which the maximum cell number (300,000) was reduced by 50% were 2 IGU per cell for CgalΔ42 and 3 IGU per cell for *in*1850Δ42. Therefore, the VP16 lesion in *in*1850Δ42 appeared to have little benefit over a UL42 deletion alone in reducing viral toxicity in this assay. In addition, the UL42 mutants were more toxic than the IE 3 deletion mutants, irrespective of the VP16 lesion. However, these results do not rule out the possibility that *in*1850Δ42 is less toxic than CgalΔ42 at much lower MOIs, as examined in the following experiment.

Rescue of virus from persistently infected cells. We have previously used an in vitro latency system to show that at a low MOI, a small percentage of cells infected with an IE 3 deletion mutant maintain the virus in a biologically retrievable form for at least 2 weeks (29). However, it was not possible to significantly increase the number of cells which could maintain virus by increasing the MOI, presumably because of increased viral toxicity at higher MOIs. We reasoned that if any of the mutant virus strains described in this study have reduced toxicity, cells should be able to sustain infection at a higher MOI and more virus should be available for rescue a week or later after infection. To test this hypothesis, we infected human fibroblasts with D30HΔ3, 14HΔ3, CgalΔ42, *in*1850Δ42, Δ3vhsZ, and 14H3vhsΔ3 at increasing MOIs. We used PFU as the measure of virus titer in this experiment since the method of titer determination will not ultimately affect the maximum number of virus that can be rescued over a wide range of MOIs. The amount of virus that could be recovered immediately (1 h postadsorption) or 11 days after infection by rescue with a complementing HSV-1 strain deleted for ICP8, *d*301 (19), is shown in Fig. 6. Superinfection with *d*301 provides the IE 1 gene product, Vmw110, which is important for reactivation of latent virus (34, 57), as well as the IE 3 and UL42 gene products, which are needed for complementation of viruses with mutations in these genes. Since the plaques from the rescue procedure have arisen from infected human fibroblasts

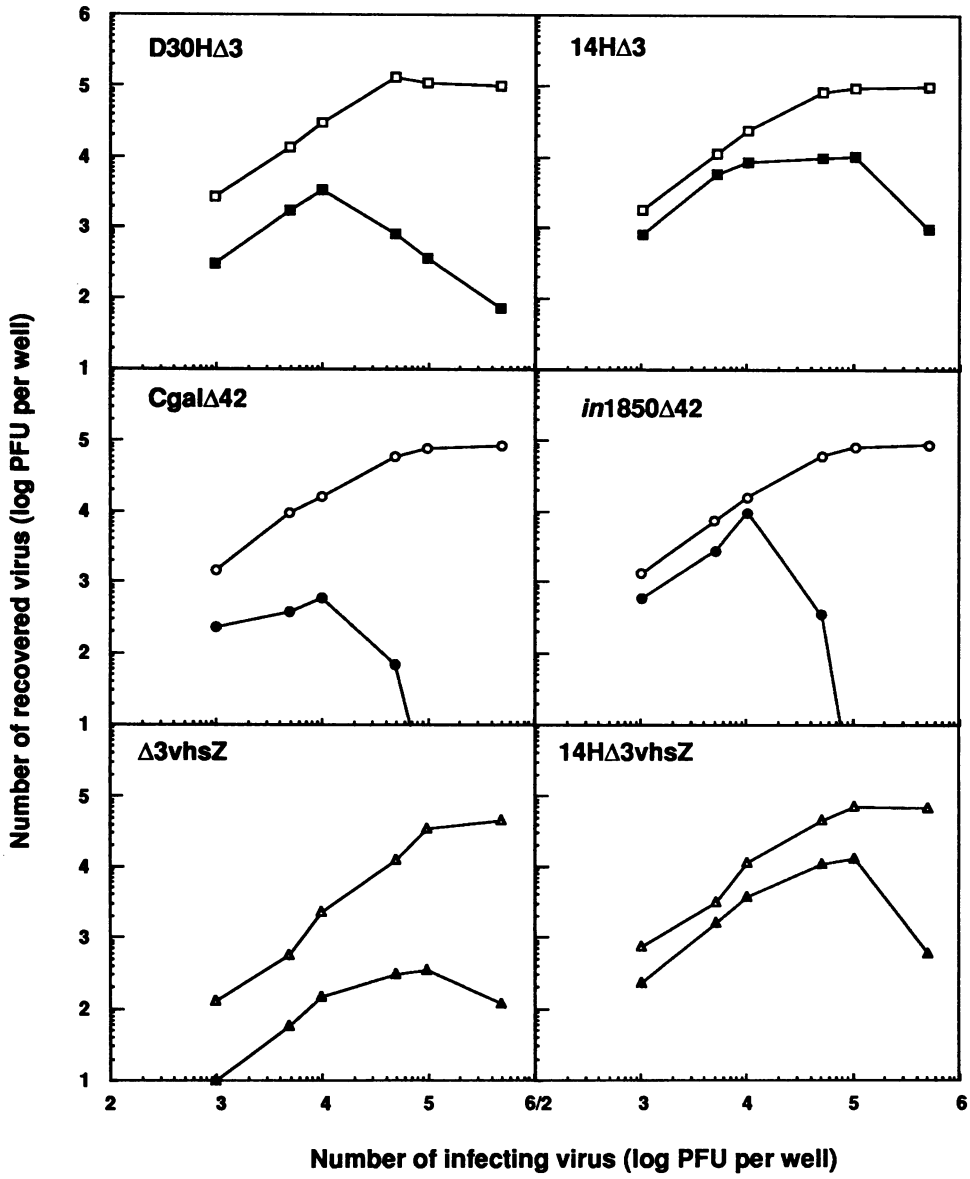

FIG. 6. Recovery of virus mutants from infected fibroblasts. Confluent monolayers of human fibroblasts (approximately 2.5×10^5 cells per 16-mm well) were infected at MOIs of from 1×10^3 to 5×10^5 PFU per well with each of the mutants D30HΔ3, 14HΔ3, CgalΔ42, *in*1850Δ42, Δ3vhsZ, and 14HΔ3vhsZ, as indicated. Graphs show the average number of plaques from duplicate cultures that could be recovered 1 h (open symbols) or 11 days (solid symbols) postinfection. Recovery was performed by superinfection with the ICP8 mutant *d*301 (19). Superinfected cells were plated out at various dilutions onto monolayers of E5 cells (for IE 3 mutants) or V9 cells (for UL42 mutants) as appropriate and incubated at 37°C in the presence of 5% human serum until plaques could be counted.

plated on permissive cell monolayers and not from free virus, they should not exceed the total number of cells available for infection, which was approximately 1×10^5 to 2×10^5, as determined by counting with a Coulter counter. Therefore, since cells which were infected by more than one virus can still give rise to only one plaque, the actual amount of persistent virus in this experiment cannot be determined above a maximum of 1 PFU per cell. In some cases, the number of rescued plaques at day 0 exceeded the number of PFU in the infecting inoculum (for instance, 1×10^3 PFU of D30HΔ3 resulted in 2.5×10^3 rescued plaques), which probably reflects the fact that the actual titer of infectious genomes able to complement *d*301 is severalfold higher than the PFU titer.

As shown in Fig. 6, infection with either D30HΔ3 or 14HΔ3 at increasing MOIs led to a maximum number of plaques obtainable on day 0 of approximately 10^5, which correlates reasonably well with the total number of cells available for infection. Striking differences were observed between the numbers of D30HΔ3 and 14HΔ3 which could be rescued at day 11. First, at low MOIs (10^3 to 10^4 PFU per 10^5 cells), less than 10% of the input D30HΔ3 could be recovered, compared with 45 to 50% for 14HΔ3. Second, the maximum number of rescuable D30HΔ3 reached a sharp peak of 3.5×10^3 PFU at an MOI of 10^4 PFU per well and then rapidly declined at higher MOIs. In contrast, the number of rescuable 14HΔ3 began to plateau at 9×10^3 PFU at an MOI of 10^4 PFU per

well and reached a peak of 1.2×10^4 PFU at an MOI of 10^5 PFU per well. The number of rescuable 14HΔ3 declined above an infecting inoculum of 10^5 PFU per well. Therefore, by the following two criteria, (i) the proportion of infecting virus that can be recovered at a later time point and (ii) the maximum amount of virus that can be recovered after sustained infection of cells, 14HΔ3 has a considerably less toxic profile than D30HΔ3. Comparison of the UL42 mutants CgalΔ42 and *in*1850Δ42 revealed that a greater proportion of *in*1850Δ42 than of CgalΔ42 could be recovered at low MOIs. Also, the maximum amount of *in*1850Δ42 that could be recovered (1×10^4 PFU) was substantially higher than the maximum amount of CgalΔ42 (6×10^2 PFU). However, above an infecting inoculum of 10^4 PFU per well, the recovery of both viruses dropped precipitously, indicating that both UL42 mutants were more toxic than their corresponding IE 3 mutant counterparts, in agreement with the results described earlier. Comparison of the IE 3-vhs double mutants Δ3vhsZ and 14HΔ3vhsZ showed again that inclusion of the VP16 mutation (in 14HΔ3vhsZ) allowed an increased recovery of virus at 11 days postinfection, in terms of both the proportion of infecting virus recovered (less than 10% for Δ3vhsZ versus 30 to 50% for 14HΔ3vhsZ) and the maximum sustainable virus load (3×10^2 PFU for Δ3vhsZ versus 1×10^4 PFU for 14HΔ3vhsZ). Interestingly, Δ3vhsZ appeared to be more toxic than D30HΔ3 by this assay. The recovery of Δ3vhsZ was less efficient than that of D30HΔ3 on day 0, and the peak amount of Δ3vhsZ that could be recovered was 10-fold below that for D30HΔ3. In terms of virus recovery and maximum sustainable virus load, the triple mutant 14HΔ3vhsZ was similar to the double mutant 14HΔ3, indicating that inclusion of the vhs mutation in this mutant background had little or no benefit in reducing virus toxicity.

Finally, we tested the possibility that the vhs function does contribute to CPE but its effects are usually masked by a greater level of cytopathogenicity caused by viral gene expression. We therefore infected human primary fibroblasts at an MOI of 10 PFU per cell with D30EBA, Δ3vhsZ, and 14HΔ3vhsZ which had either been untreated or irradiated with UV light to abolish viral gene expression. It has been shown previously that the vhs function is not inactivated by such UV treatment (16, 44). Cells were harvested by trypsinization at 3 days postinfection, and cell viability was determined by counting cells which excluded trypan blue dye (Fig. 7). In the absence of UV treatment, the number of viable cells in comparison to that in a mock-infected culture was reduced to less than 3% for Δ3vhsZ and D30EBA and to 15% for 14HΔ3vhsZ. After UV irradiation of these mutants, cell viability following infection was much improved but very similar for each mutant (about 42%), indicating that factors other than vhs are causing cytotoxic effects in the absence of gene expression. In a previous study, we found that cells infected with strain KOS and the vhs mutant of strain KOS called *vhs*1 (53) suffered cytopathic effects at an MOI of 2, even in the presence of phosphonoacetic acid to prevent viral DNA replication, whereas infection with UV-irradiated viruses at the same MOI abolished CPE (29). We therefore infected cells with UV-irradiated KOS and *vhs*1 at an MOI of 10 to determine whether differences in the induction of CPE can be detected at a higher MOI. Cell viability following infection with these viruses in two separate experiments was reduced to between 52 and 62% (Fig. 7), and there was not a significant difference between *vhs*1 and KOS, indicating that the vhs function of strain KOS, like that of strain 17^+, does not contribute in a major way to the induction of CPE.

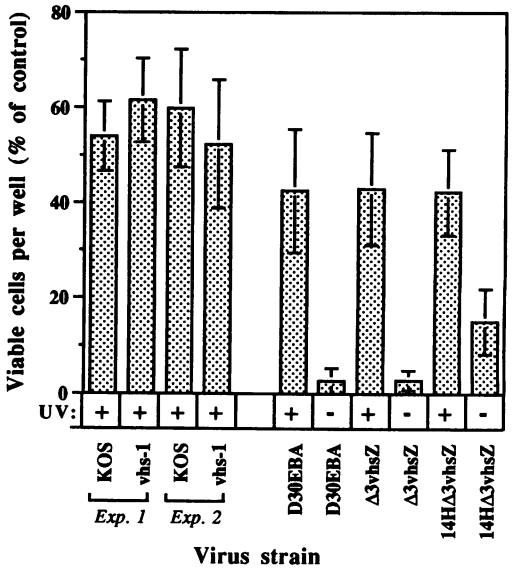

FIG. 7. Effect of vhs mutation on cell viability after infection with UV-irradiated viruses. Human primary fibroblasts were seeded at a density of 5×10^4 cells per well and infected in triplicate the following day with KOS, *vhs*1, D30EBA, Δ3vhsZ, or 14HΔ3vhsZ at an MOI of 10 PFU per cell. At 3 days postinfection, the cells were harvested by trypsinization, and the number of viable cells in each culture was estimated after staining with 0.5% trypan blue and counting the unstained cells. The average number of viable cells is shown as the percentage of viable cells in a mock-infected culture, determined from counting in eight fields of a hemacytometer for each of three infected cultures. Error bars indicate standard deviations.

DISCUSSION

Replication-defective mutants of HSV-1 retain a strong potential to induce CPE and cause cell death during infection of nonpermissive cells. The results of a previous study had indicated that viral gene expression was a major factor causing CPE (29). In an attempt to construct rather more benign vectors for gene transfer, we have in this study examined individual HSV-1 genes and virion components which might be responsible for CPE. We have shown that at least three HSV-1 genes, which are efficiently expressed by an IE 3 deletion mutant during infection of nonpermissive cells, cause a marked inhibition of colony formation in cotransfection experiments. As a means to reduce IE gene expression globally in a replication-defective mutant, and as an alternative to trying to produce a mutant defective for three or four IE genes, we constructed viruses which were deleted for either of the essential genes IE 3 or UL42 and included the mutation in the VP16 gene of *in*1814 described by Ace et al. (2). This mutation abolishes the ability of VP16 to transinduce IE gene expression. The IE3-VP16 double mutant 14HΔ3 is significantly less cytotoxic than a single mutant deleted for IE 3, whereas the reduction in toxicity of the UL42-VP16 mutant is less marked. The vhs function has also been considered a potentially cytotoxic component of the HSV-1 virion. However, inclusion of a mutation in the vhs gene does not reduce the cytopathogenicity of either an IE 3 deletion mutant or the double IE 3-VP16 mutant.

Virally encoded cytopathogenic gene products. Plasmids bearing HSV-1 IE genes 1, 2, and 4 had a marked inhibitory effect on the ability to obtain colonies from a cotransfected selectable marker gene (Fig. 2). Plasmids bearing IE 3 also

caused a marked inhibition of transformation in CV-1 cells but not in BHK TK$^-$ cells, for reasons that are unclear. Inhibition of colony formation was not caused by promoter competition and was unlikely to be due to sequences outside of the specified IE gene open reading frames, since inhibitory activity was reduced or abolished in plasmids bearing mutations in the IE coding regions. Neither was it due to direct effects on the level of neomycin phosphotransferase produced from the cotransfected *neo* gene (26). In additional studies in which we examined inhibition of colony formation at different molar ratios of test plasmid to selectable marker, we found that a plasmid encoding IE 2 caused a greater than 90% inhibition in colony formation at a 1:1 molar ratio, whereas IE 4 and IE 1 required cotransfection ratios of at least 5:1 and 10:1, respectively, to cause this level of inhibition (26). Unfortunately, since it is hard to compare the actual amounts of IE protein made from the transfected plasmids or to determine whether the level of protein expressed by plasmid-borne IE genes reflects the level produced during a viral infection and to what extent the activity of the IE proteins is modified by the presence of other viral proteins, it is not possible to say which IE products are the most cytopathogenic either in the transfection experiment or, by extrapolation, during a viral infection. However, our results are consistent with the hypothesis that, above a certain threshold level, the products of IE genes 1, 2, and 4 are cytopathogenic and reduce the ability of cells to survive if they are overexpressing these gene products.

Farkas et al. have examined the HSV-1 genome for sequences that inhibit stable biochemical transformation and identified two regions that possess inhibitory activity (13). One of these regions was a 6-kb subclone of the EK *Eco*RI fragment of the HSV-1 genome, which included the IE 2 gene, yet they showed that sequences outside of the IE 2 gene were required for this effect and that IE 2 itself was not solely responsible for inhibition of transformation. In a separate study, it was found that this 6-kb "antitransformation" sequence could inhibit transient gene expression from a cotransfected HSV-1 TK promoter or the Rous sarcoma virus promoter (4). Why does transfection with the IE 2 gene cause inhibition of colony formation in our study but not the previously published one? It is possible that strain differences are responsible, since Farkas et al. used an IE 2 gene from strain KOS, whereas our IE 2 gene was subcloned from strain 17$^+$. Another more likely reason is that the phenomenon they studied occurred at very low concentrations of test plasmid DNA (less than a 1:1 molar ratio with the selectable marker plasmid DNA) and the test sequences caused a repression of transcriptional activity. In our study, we used cloned HSV-1 DNA fragments which contained IE transcription units but as little extraneous sequence as possible, and thus we have probably observed a phenomenon different from that reported by Farkas et al.

How is a cytopathogenic gene product identified? We have been looking for HSV-1 genes responsible for virus-induced CPE, and therefore the products of the genes that we have identified might not be considered "toxic" in the traditional sense, like a bacterially encoded toxin. For instance, the product of IE 1 has the potential to transactivate cellular promoters (for a review, see reference 12) and has been shown to activate the cellular transcription factor AP-1 during viral infection (25), which could have deleterious effects on the host cell if aberrantly expressed. The product of IE 2 can disrupt host cell splicing mechanisms and alter polyadenylation site usage (42, 58) and therefore has the potential to drastically alter normal patterns of cellular gene expression. Rice et al. recently reported that HSV-1 infection results in rapid and aberrant phosphorylation of the host cell RNA polymerase II, dependent on the expression of an as yet unidentified HSV-1 IE and/or early protein(s) (54). While it has not yet been shown what effect this has on host cell transcription, it could clearly be part of a general mechanism through which the virus subverts the host cell transcriptional machinery for expression of its own genes at the expense of most cellular genes. The expression of viral gene products which alter the normal pattern of cellular gene expression may thus eventually impair the ability of a cell to survive. In our study, we have restricted our search to cytopathogenic genes expressed by an IE 3 deletion mutant, but it is probable that many other HSV-1 gene products, including those of early and late genes, have cytopathic potential under some conditions. The induction of CPE by HSV-1 vectors could have many routes.

Reduced cytotoxicity of VP16 mutants. VP16 is a major component of the virion, located in the tegument layer between the capsid and the envelope. It is required for virus assembly as well as for induction of IE gene expression (67). Preston and colleagues made a series of in-frame *Bam*HI linker insertion mutations within the gene encoding VP16 and identified one such mutant that was defective in its transinducing activity in transfection assays but not in its ability to complement an HSV-2 mutant with a temperature-sensitive lesion in VP16 (1). They incorporated this VP16 insertion mutation into HSV-1 strain 17$^+$, and the phenotype of the resulting virus, *in*1814, included the following: it is unable to transinduce IE gene expression; it expresses IE genes at a reduced efficiency; it has reduced plaquing ability and a high particle/PFU ratio; and it is essentially avirulent in mice (2). Even though *in*1814 does not replicate well in vivo, it is capable of establishing a latent infection and can reactivate from explanted ganglia (64). In in vitro experiments, it was found that *in*1814 is much less toxic than wild-type virus and is predisposed to latency rather than lytic growth (23). The reduced cytotoxicity of *in*1814 is an important feature, desirable of a gene transfer vector. But because *in*1814 can still initiate infection, particularly at a high MOI, it was necessary to construct viruses which had additional mutations in essential genes to prevent lytic growth.

One of the mutants described in this study, 14HΔ3, defective for IE 3 and VP16, was significantly less toxic than a single mutant deleted for IE 3. This result is consistent with the hypothesis that overexpression of IE genes in an IE 3 deletion mutant is a major contributing factor to cytotoxicity. However, the data in Fig. 6 show that the critical dose for the recovery of D30HΔ3 at 11 days postinfection is 0.1 PFU per cell and for 14HΔ3 it is 1 PFU per cell, above which the amount of recoverable virus decreases. Such low MOIs suggest that structural components may also make an important contribution to cytotoxicity, possibly by "priming" cells for death if the cell is also infected with a transcriptionally active viral genome. The CPE caused by IE 3-VP16 mutants at higher MOIs could be due to a number of factors, including the vhs function, residual expression of HSV-1 IE genes in the absence of transinduction by VP16, or toxicity of other virion components such as glycoproteins. However, we have found no contribution of vhs to the induction of CPE, and mutation of the vhs genes of D30EBA and 14HΔ3 (creating mutants Δ3vhsZ and 14HΔ3vhsZ, respectively) did not detectably reduce cytotoxicity, as discussed further below. What is the possibility that residual IE gene expression in 14HΔ3 is responsible for inducing CPE at higher MOIs? Ace et al. found that during infection with *in*1814, there was a four- to fivefold reduction in accumulation of RNA of IE genes 1 and 2 in comparison to a VP16-intact virus, but only a twofold drop in IE 4 RNA (and

the accumulation of IE 3 RNA was unaffected) (2). Since IE 4 has been shown to have cytopathogenic potential (Fig. 2, plasmid pIE68), and since *in*1814 can still initiate infection at a high MOI, there is probably also sufficient IE gene expression in the background of IE 3-VP16 mutants to cause CPE at high MOIs. Interestingly, the UL42-VP16 mutant was more cytotoxic than the IE 3-VP16 mutant at a given MOI. This could be due to the ability of a UL42 mutant to express more viral gene products than an IE 3 mutant and therefore increase the chance of expressing gene products which are incompatible with cell survival.

No role for vhs in cytotoxicity? The vhs function encoded by gene UL41 causes a general destabilization of both host and viral mRNAs, an effect thought to contribute to preferential synthesis of viral proteins during a lytic infection because viral messages are being made more rapidly (31, 33, 46, 53). It has been suggested that the vhs function could contribute to the cytotoxicity of HSV-1 vectors (5, 35), although our results indicate otherwise. To assess the role of the vhs function in the cytotoxicity of replication-defective viruses, we inserted an HCMV-*lacZ* cassette into the UL41 coding region of D30EBA and 14HΔ3. Analysis of the genomic structure of the resulting viruses, Δ3vhsZ and 14HΔ3vhsZ, respectively, confirmed the anticipated disruption of gene UL41, and growth of these viruses following infection of permissive cells at low MOI was impaired by 2- to 10-fold (Table 1), similar to the reported growth impairment of other vhs mutants (53, 61). These data, plus the fact that Smibert and Smiley (61) showed that insertion of a *lacZ* expression cassette into the UL41 gene at a site 105 codons downstream of our mutation resulted in functional disruption of vhs, would strongly indicate that the vhs function should also be disrupted in our mutants. However, in cell-killing and virus rescue experiments, we detected no reduction in the toxicity of Δ3vhsZ and 14HΔ3vhsZ in comparison to their vhs-intact counterparts (Fig. 4 and 6). A number of factors could contribute to this somewhat surprising observation. First, HSV-1 strain 17^+ causes only a slight inhibition of host cell polypeptide synthesis. This has been elegantly shown by Fenwick and Everett (15), who inserted the gene encoding the strong shutoff function of HSV-2 (G) into the genome of HSV-1 17^+, converting the phenotype of strain 17^+ to one including a strong shutoff and reduced stability of its IE RNA. Second, the degree of virion-associated host shutoff is dependent on a threshold level of input virions. Kwong and Frenkel (32) showed that host shutoff by infection with HSV-1 strain KOS, which has a stronger shutoff function than 17^+, required an MOI of 10 or more PFU per cell, and no effect was observed at an MOI of 2 PFU per cell or less. Therefore, even if vhs does cause cytotoxicity at high MOIs, it is probably less important than the more potent effects of viral gene expression in both VP16-intact and VP16-defective mutants. However, when we tested this possibility by using a high MOI to infect cells with UV-irradiated virus to abolish CPE due to viral gene expression, we were still unable to discern differences in cytotoxicity between vhs mutants of HSV-1 strain 17^+ and strain KOS and their parental strains (Fig. 7). Finally, there are several reasons why it may be advantageous to avoid including a vhs mutation in HSV-1 vectors. Since vhs mutants have increased stability of IE messages (31, 46), a corresponding increase in IE protein synthesis could actually contribute to cytotoxicity rather than reduce it. Furthermore, the vhs mutation impairs virus growth and may also result in a higher particle/PFU ratio (15, 53), which makes vhs mutants more difficult to prepare and to manipulate genetically and increases the load of foreign particles that need to be applied to cells to achieve a given level of gene transfer.

In conclusion, we have provided evidence that expression of at least three different HSV-1 IE genes (IE genes 1, 2, and 4) could contribute to the cytopathogenicity of a replication-defective mutant of HSV-1 deleted for IE 3 during a nonproductive infection. By combining an HSV-1 IE 3 deletion with the VP16 insertion mutation described by Ace et al. (2), which abolishes VP16-mediated transinduction of HSV-1 IE genes, it was possible to substantially reduce virus-induced CPE and enhance cell survival. This mutant, 14HΔ3, should prove useful for further studies involving long-term gene transfer mediated by HSV-1 and for studies of the regulation of gene expression during a latent infection.

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