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Cytotoxicity of a Replication-Defective Mutant of Herpes Simplex Virus Type 1

PAUL A. JOHNSON,[1]* ATSUSHI MIYANOHARA,[1] FRED LEVINE,[1]
TIM CAHILL,[2] AND THEODORE FRIEDMANN[1]

Departments of Pediatrics[1] and Medicine,[2] University of California San Diego, La Jolla, California 92093-0634

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Replication-defective mutants of herpes simplex virus type 1 (HSV-1) may prove useful as vectors for gene transfer, particularly to nondividing cells. CgalΔ3 is an immediate-early gene 3 (IE 3) deletion mutant of HSV-1 that expresses the *lacZ* gene of *Escherichia coli* from the human cytomegalovirus immediate-early control region but does not express viral early or late genes. This vector was able to efficiently infect and express *lacZ* in cells refractory to traditional methods of gene transfer. However, 1 to 3 days postinfection, CgalΔ3 induced cytopathic effects (CPE) in many cell types, including neurons. In human primary fibroblasts CgalΔ3 induced chromosomal aberrations and host cell DNA fragmentation. Other HSV-1 strains that caused CPE, tested under conditions of viral replication-inhibition, included mutants of the early gene UL42, the virion host shutoff function, single mutants of IE 1, IE 2, and IE 3, and double mutants of IE 3 and 4 and IE 3 and 5. Inhibition of viral gene expression by UV irradiation of virus stocks or by preexposure of cells to interferon markedly reduced the CPE. We conclude from these studies that HSV-1 IE gene expression is sufficient for the induction of CPE, although none of the five IE gene products appear to be solely responsible. After infection of human fibroblasts with CgalΔ3 at a low multiplicity of infection, we were able to recover up to 6% of the input virus 2 weeks later by a superinfection-rescue procedure, even though the virally transduced human cytomegalovirus-*lacZ* transgene was not expressed at this time. It is therefore likely that inhibition or inactivation of viral IE gene expression, either for establishing latency or for the long-term transduction of foreign genes by HSV-1 vectors, is essential to avoid the death of infected cells.

Postmitotic cells such as neurons are refractory to common methods of gene transfer. Consequently, there is a growing interest in the potential use of vectors based on herpes simplex virus type 1 (HSV-1) to overcome this limitation in gene transfer technology (for a review, see reference 2). Characteristics of HSV-1 which make it desirable as a vector include its wide host cell range, its capacity for up to 30 kb of foreign DNA, the ability to prepare high-titer virus stocks, the fact that it does not usually integrate into the host cell genome, and the fact that it can persist in a state of latency in at least some cells, i.e., sensory neurons (2). In addition, the extensive characterization of HSV-1 at the molecular level greatly facilitates the design of HSV-1 vectors and further manipulation of the HSV-1 genome.

To investigate the potential usefulness of replication-defective mutants of HSV-1 as vectors for gene transfer, we have studied the properties of CgalΔ3, a vector derived from an HSV-1 mutant deleted for nearly the entire coding portion of immediate-early gene 3 (IE 3) (D30EBA [57]). The product of the IE 3 gene, Vmw175 (ICP4), is the major transcriptional regulatory protein of HSV-1 and is required for early and late viral gene expression. Lytic growth of an IE 3 mutant is therefore dependent upon complementation with functional Vmw175. Infection of nonpermissive cells with an IE 3 deletion mutant is associated with expression of the remaining four HSV-1 IE genes and the large subunit of ribonucleotide reductase but not of other early genes or late genes (9, 57). The vector CgalΔ3 contains the *Escherichia coli lacZ* gene under control of the human cytomegalovirus (HCMV) IE gene regulatory region introduced into an intergenic site of the short unique region of D30EBA. We chose to insert the *lacZ* gene into our vector since its product, β-galactosidase (β-gal), is easily detectable in many cell types, including neurons, by histochemical staining and has proven to be a useful nontoxic marker, for example, in transgenic animal studies of the expression of the neuron-specific enolase promoter (19) or in lineage analysis of the vertebrate nervous system following retrovirus-mediated gene transfer (82). In primary cultures of central nervous system neurons infected with CgalΔ3 the virally transduced *lacZ* gene was efficiently expressed 1 day postinfection (37). However, by 2 to 3 days postinfection the β-gal-positive cells demonstrated extensive cytopathic effects (CPE), suggesting that the HSV-1 vector itself was toxic to the cells and potentially limiting the usefulness of this strain of HSV-1 as a vector for stable gene transfer (37).

An important feature of HSV-1 is its ability to establish a latent infection, a phenomenon in which the viral genome in the infected cell remains episomal (48) and is transcriptionally silent except for the production of the latency-associated transcripts whose function is uncertain (72, 76, 83). The latent infection is nondestructive to the host cell, at least until the virus is reactivated, leading to its reentry into the lytic cycle (47, 75). Although the natural site of latency is within neurons of the sensory ganglia, it has been possible to establish latency in vitro in neuronal (84) and nonneuronal (55, 64) cells, mostly through the use of drugs or elevated temperature to inhibit the initial lytic infection and below a critical multiplicity of infection (MOI). With an improved understanding of the factors that lead to either destruction of the infected cell or the establishment of latency, it may become possible to modify and routinely use HSV-1 as an

* Corresponding author.

efficient vector for stable gene transfer to a variety of cell types in addition to sensory neurons.

A permissive infection with HSV-1 results in profound alterations in the host cell at a number of different levels. Most wild-type strains of HSV-1 induce cell rounding early in infection; this event is associated with a breakdown of components of the cytoskeleton, including actin-containing microfilaments (31), and a concomitant loss of cell surface matrix proteins such as fibronectin, leading to cell detachment (13). Rapid shutoff of host cell polypeptide synthesis is mediated by the virion host shutoff function (vhs) encoded by gene UL41, which induces disaggregation of cellular polyribosomes and suppresses translation of preexisting mRNAs (18, 39, 51, 60, 81). The vhs function does not play an essential role in virus replication or a vital structural role in the virion (15, 60, 69). Variability in the strength of the shutoff function has been demonstrated in different HSV strains (17), and although the effects of vhs are best observed with infection at a high MOI, an optimum HSV-1 strain for use as a vector would probably have a weak or deficient shutoff function.

Infection with HSV-1 also leads to rapid inhibition of host cell DNA synthesis (63) and to the induction of chromosomal damage (29). The subsequent occurrence of unscheduled repair synthesis of cellular DNA, even during inhibition of viral DNA replication, may be a response to the HSV-induced chromosomal damage (44, 52). Chromosomal aberrations induced by HSV-1 include chromatid breaks, fragmentation, enhanced secondary constrictions, and pulverization, which become more severe with increasing time after infection. Interesting features of this cytogenetic damage are the nonrandom distribution and the requirement for at least limited viral gene expression for its induction (5, 56, 58, 77).

We report here on the mechanism of cytotoxicity induced by a nonproductive infection with HSV-1. The induction of CPE by CgalΔ3 was characterized by the appearance of chromosomal damage, fragmentation of cellular DNA, and cytoplasmic blebbing; it was inhibited by UV irradiation of the virus or by pretreatment of cells with interferon (IFN), both of which significantly reduce viral gene expression. Under conditions of DNA replication inhibition, both wild-type HSV-1 and single mutants of IE 1, IE 2, and IE 3 or double mutants of IE 3 and 4, and IE 3 and 5 were cytotoxic. Therefore, although viral IE gene expression is more likely to be responsible for the CPE caused by CgalΔ3 than the action of a virion component such as vhs, no single IE gene product appears to be totally responsible. We note also that not all cells in an infected culture were killed by CgalΔ3, and it was possible to recover some of the infecting virus from surviving fibroblasts 2 weeks after infection. Furthermore, the virally transduced HCMV-*lacZ* hybrid gene was not stably expressed, indicating that the HCMV IE promoter is down-regulated along with HSV IE genes in surviving cells. We conclude that limiting cytotoxic viral gene expression is required for maintaining a long-term infection with HSV-1.

MATERIALS AND METHODS

Cells and viruses. The HSV-1 strain 17^+ IE 3 deletion mutant D30EBA (57) was kindly provided by T. Paterson and R. D. Everett, Medical Research Council Virology Unit, Glasgow, Scotland. The virion host shutoff mutant *vhs*1 (60) was provided by G. S. Read, Loyola University, Chicago, Ill.; and wild-type KOS and the IE 2 deletion mutant, 5*dl*1.2 (45), were provided by P. A. Schaffer, Dana-Farber Cancer Institute, Boston, Mass. An ICP8 deletion mutant, *d*301, and a permissive cell line for its growth, S2 (20), were obtained from D. Knipe, Harvard Medical School, Boston, Mass. The IE 1 deletion mutant, *dl*1403 (79), was provided by N. D. Stow, Medical Research Council, Glasgow. We have previously described the UL42 deletion mutant, CgalΔ42, and the V9 cell line permissive for its growth (34).

Unless otherwise stated, all cells were maintained in Dulbecco's modification of Eagle's minimal essential medium (DME) containing 10% fetal calf serum. Stocks of viruses were grown and titers were determined in Vero cells or in the appropriate permissive cell type. Mutants of IE 3 were grown on E5 cells, provided by N. DeLuca (10), or on RR1 cells, derived from a BHK TK^- cell line by cotransformation with pK1-2 (10) and pHneo (35) and selection for neomycin resistance. RR1 cells contain approximately three copies of IE 3. E5 and RR1 cells, which support D30EBA with similar one-step growth and plaque-forming efficiencies, produced IE 3-intact recombinants in virus stocks at a frequency of less than 1 in 10^6 (36). E5, RR1, S2, and V9 cells were passaged in medium containing 400 μg of G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml. M64A cells (8), kindly supplied by N. Stow, were used for the initial derivation of CgalΔ3 and were passaged in medium containing hypoxanthine (10^{-4} M), aminopterin (5×10^{-7} M), and thymidine (5×10^{-5} M) (HAT medium). However, since M64A cells gave rise to IE 3-intact recombinants at a comparatively high rate (1 in 10^4), we replaced these cells with E5 and RR1 cells. TO-119 cells are primary human diploid fibroblasts. PC12 cells were obtained from D. Schubert, Salk Institute, La Jolla, Calif., and cultured as described previously (66) in DME containing 10% fetal calf serum plus 5% horse serum. PC12 cells were differentiated after seeding on collagen (type 1, rat; Sigma)-coated dishes by treatment with 50 ng of mouse 2.5S nerve growth factor (NGF; Boehringer) per ml in DME containing 10% horse serum and 5% fetal calf serum. The cells were infected with virus 3 days after treatment with NGF. Dorsal root ganglia were isolated from newborn rats and dissociated by treatment with 1 mg of collagenase-dispase (Boehringer) per ml at 37°C for 30 min followed by extensive trituration. The cells were plated onto a collagen-coated dishes and fed every 2 days with DME containing 10% fetal calf serum and 50 ng of mouse 2.5S NGF per ml. Four days after plating, the cultures were treated for 48 h with 20 μM each fluorodeoxyuridine and uridine to reduce the growth of nonneuronal cells. The cells were infected with CgalΔ3 at an MOI of 0.25 PFU per cell 10 days after plating.

Plasmids. Plasmid pON249 containing the *E. coli lacZ* gene of pON1 driven by the HCMV IE control region was supplied by E. S. Mocarski, Stanford University, Stanford, Calif. Plasmid pGXA contains the HSV-1 *Bam*HI *z* fragment from pGX40Xba (61; kindly supplied by F. J. Rixon, Medical Research Council Virology Unit), which has an *Xba*I linker inserted at the *Rsa*I site lying between the 3′ cotermini of genes US 8 and 9 and US 10, 11, and 12 (46), now in the background of pBluescript KS (Stratagene, La Jolla, Calif.). pGXCgal was created by blunt-end ligation of a *Bam*HI fragment containing the HCMV-*lacZ* hybrid gene from pON249 into the XbaI site of pGXA. Plasmid pZ5 contains an HCMV-*lacZ* hybrid gene within the middle of the Vmw12 coding region of IE 5 and was constructed as follows. A 1,769-bp *Eco*RI-*Sst*I fragment containing IE 5 sequences was subcloned from the HSV-1 *Bam*HI *x* fragment into the *Eco*RI-*Sst*I sites of pBluescript SK (Stratagene). The *Apa*LI site lying over codons 44 and 45 of Vmw12 (46) was filled in

with Klenow fragment and converted to a *Bam*HI site, into which the 4.6-kb HCMV-*lacZ* *Bam*HI fragment from pON249 was inserted (in the same orientation as Vmw12). Other plasmids used in this study were as follows. pK1-2, containing IE 3, was supplied by N. DeLuca (10); pIE63 contains IE 2 sequences in a 2,448-bp *Bam*HI-*Hpa*I fragment cloned into pUC19; pIE68 contains IE 4 sequences in a 2,920-bp *Eco*RI-*Mlu*I fragment cloned into pUC19; pIE68Δ was derived from pIE68 by deletion of the 425-bp *Hin*dIII-*Sst*I fragment from the coding region of IE 4 and its substitution by a 176-bp *Hin*dIII (originally *Nhe*I)-*Sst*I fragment from the Moloney murine leukemia virus long terminal repeat; and pUCA1 contains chicken β-actin DNA in a 2.0-kb *Pst*I fragment derived from pA1 (7), cloned into pUC19.

Generation of recombinant virus. M64A cells (2×10^5) were transfected by the method of Graham and van der Eb (25) with 10 μg of pGXCgal which had been cut with *Bam*HI to excise the HCMV-*lacZ* hybrid gene flanked by *Bam*HI-*z* DNA. At 24 h after transfection, the cells were washed with DME and infected with D30EBA at an MOI of 1.0. Following the development of extensive CPE, progeny virus was harvested and the virus titer was determined on monolayers of E5 cells under 0.5% agarose on 10-cm plates. Recombinant viruses were detected by the method of Chakrabarti et al. (4). Once plaques had formed, they were stained with a second 0.5% agarose overlay containing 1.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution (2% in dimethylformamide). Blue plaques were usually detectable by 12 h following incubation at 37°C and were plaque purified three times before final stocks were made. We chose one such isolate, designated CgalΔ3, for further study. We confirmed by Southern blot analysis that CgalΔ3 contained the *lacZ* gene at the expected position and that the IE 3 deletion of D30EBA was maintained (data not shown). Stocks of CgalΔ3 produced on E5 or RR1 cells contained fewer than 1 in 10^6 replication-competent recombinants that had regained the IE 3 gene, and in practice, infections with CgalΔ3 did not result in a productive infection on noncomplementary cells. Cgal$^+$ was derived from CgalΔ3 by marker transfer of the IE 3 gene and was indistinguishable from the parental strain 17$^+$ in terms of growth properties, production of viral DNA, and pattern of expression of viral genes, as previously reported (34).

Two double IE gene mutants based on the IE 3 deletion mutant D30EBA, carrying additional mutations in either IE 4 or IE 5, were generated as follows. (i) Δ3Z5 contains an HCMV-*lacZ* hybrid gene within the coding region of IE 5 and was derived from D30EBA and plasmid pZ5 by using the same method as for the isolation of CgalΔ3 described above. No differences have been observed in growth properties between Δ3Z5 and CgalΔ3. (ii) CgalΔ3/4 is deleted for 425 bp of the coding region of IE 4 (but carries, for marker purposes, an insertion of 176 bp of foreign DNA at the site of the deletion) and was derived from CgalΔ3 and plasmid pIE68Δ. RR1 cells (1×10^5) were transfected with 2.5 μg each of intact and cleaved pIE68Δ DNA (*Eco*RI and *Bam*HI). At 16 h posttransfection, the cells were washed with DME and infected with CgalΔ3 (5 PFU per cell); they were harvested 48 h later. The total virus yield was then determined on E5 cells in 10-cm dishes which were overlaid with medium containing 1% agarose. Recombinant virus which had incorporated the IE 4 mutation contained in pIE68Δ were detected and isolated by multiple rounds of in situ plaque hybridization as described previously (34), with the 176-bp *Nhe*I-*Sst*I murine leukemia virus DNA fragment present in pIE68Δ as a probe. Growth and titer determination of CgalΔ3/4 were routinely performed with E5 cells rather than RR1 cells since the replication of this mutant was significantly impaired in the BHK-derived cell line, in agreement with the findings of Sears et al. (67), and in contrast to the results with CgalΔ3 (data not shown). The structures of Δ3Z5 and CgalΔ3/4 shown in Fig. 1 were confirmed by Southern blot analysis (data not shown).

DNA and RNA analysis. To examine DNA isolated from intact cells, the cells were removed from dishes in Versene and embedded in agarose blocks after resuspension in 30 μl of Versene–30 μl of 1.5% low-melting-point agarose (Bethesda Research Laboratories). The blocks were incubated in 0.5 M EDTA–1% Sarkosyl–2 mg of proteinase K per ml at 50°C for 16 h, washed five times in 10 mM Tris · HCl (pH 8.0)–50 mM EDTA, and loaded onto a 1% agarose gel. The gel was placed in a pulsed-field apparatus (CHEF DRII; Bio-Rad, Richmond, Calif.), and electrophoresis was carried out in Tris-borate-EDTA buffer by using 200-V 30-s pulses at 15°C for 12 h. Viral and cellular DNAs were also prepared from whole cells or from pelleted nuclei following lysis by Nonidet P-40 and purified by phenol-chloroform extraction and ethanol precipitation. For Southern analysis (70), DNA was digested with restriction endonucleases and separated by agarose gel electrophoresis in a Tris-borate-EDTA buffer. The separated DNA was transferred to Nytran filters (Schleicher & Schuell) by vacuum blotting. Cytoplasmic RNA was prepared by Nonidet P-40 lysis of cells followed by extensive phenol-chloroform extraction and was transferred to Nytran by slot blotting for analysis. RNA and DNA blots were hybridized with probes radiolabeled by the method of Feinberg and Vogelstein (16) and washed as specified by the manufacturer (Schleicher & Schuell). The following probes were used: IE 1, a 752-bp *Bam*HI-*Kpn*I fragment from p111 (59); IE 2, a 1,195-bp *Bam*HI-*Sal*I fragment from pIE63; IE 4, a 425-bp *Hin*dIII-*Sst*I fragment from pIE68; *Bam*HI-*z*, the 1.85-kb *Bam*HI fragment from pGX40Xba; UL42, a 738-bp *Pst*I fragment from pUL42; *lacZ*, a 3.1-kb *Hin*dIII-*Eco*RI fragment from pON249; and β-actin, a 2.0-kb *Pst*I fragment from pUCA1.

UV irradiation of virus and IFN treatment. Stocks of CgalΔ3 were irradiated with a constant UV source (1,200 μW/cm^2) for up to 10 min, and viral titers were determined on E5 cells. A 5-min dose of UV irradiation reduced the viral titer by 5 log units (36). The effect of UV irradiation on viral gene expression was determined at the same time by enzymatic assay of β-gal activity (53). Protein extracts were made from Vero cells following a 24-h infection with aliquots of irradiated virus equivalent to an initial MOI of 8×10^6 PFU per 5×10^5 cells per 35-mm dish. A 5-min dose of UV irradiation reduced the β-gal activity more than 30-fold, essentially to background levels. The ability of viral DNA to reach the cell nucleus following a 5-min dose of UV irradiation was found to be unimpaired as determined by Southern blot analysis of viral DNA from infected-cell nuclei (36). For experiments with IFN, human leukocyte IFN-α (product no. I 1008; Sigma) was added to cells at the time of plating (1,000 U/ml). The IFN-α was removed 16 h later by washing, at which time the cells were infected.

Morphological and cytogenetic analysis. The cells were photographed in situ by phase-contrast microscopy with a Sedival (JENA) light microscope. The cells were stained for *lacZ* expression as described previously (37). Cytogenetic analysis was performed on cells which had been infected for 6 h at an MOI of 5 PFU per cell and arrested in metaphase by exposure to colcemid (0.1 μg/ml) for 30 min at 37°C. The

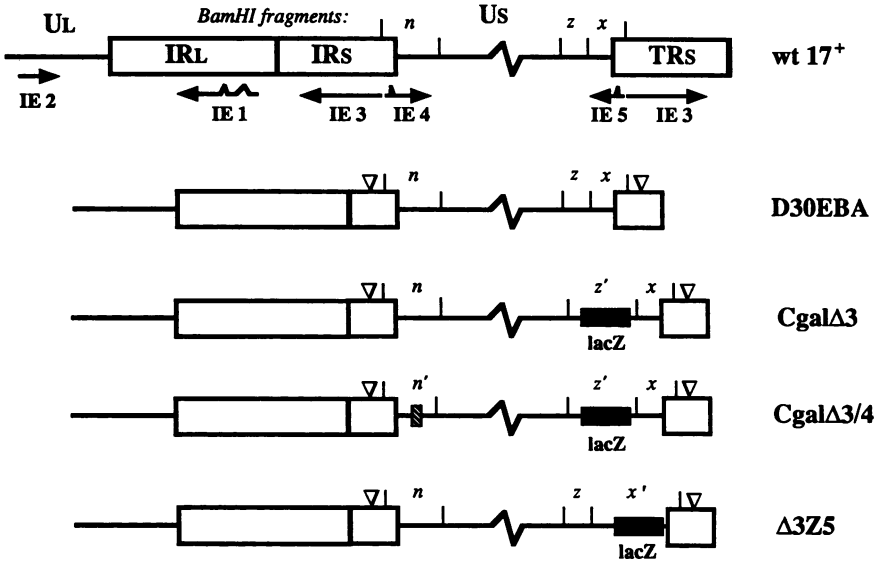

FIG. 1. Schematic detail of the structures surrounding the short-unique (U_S) portion of D30EBA and related viral genomes. Boxed regions represent the short repeat sequences (IR_S and TR_S), which flank U_S (not to scale), and the internal long repeat (IR_L), which adjoins the unique long (U_L) portion of the genome. The locations of the five IE gene transcripts in wild-type HSV-1 (wt 17^+) are shown, as are *Bam*HI fragments *n*, *z* and *x* (modifications in these *Bam*HI fragments are indicated below by *n'*, *z'*, and *x'*). D30EBA is deleted for 3.6 kb of the coding region of IE 3 (indicated by an inverted triangle), as described by Paterson and Everett (57). The construction details of CgalΔ3, CgalΔ3/4 and Δ3Z5 are outlined in Materials and Methods. CgalΔ3 was derived from D30EBA and contains a 4.6-kb insert comprising the HCMV-*lacZ* hybrid gene (indicated by the black/stippled box) within an intergenic site in *Bam*HI-*z*. CgalΔ3/4 was derived from CgalΔ3 and contains an additional deletion-substitution mutation in IE 4 (located in *Bam*HI-*n*), shown by the stripped box. Δ3Z5 was derived from D30EBA and contains the 4.6-kb HCMV-*lacZ* hybrid gene inserted between codons 44 and 45 of IE 5 (located in *Bam*HI-*x*).

cells were harvested in 0.05% trypsin–0.02% EDTA, resuspended in hypotonic solution (0.075 M KCl) for 30 min at room temperature, and then fixed in methanol-acetic acid (3:1, vol/vol). Chromosome spreads were prepared by the drop method and analyzed by light microscopy following staining with 5% Giemsa.

Superinfection-rescue procedure. TO-119 cells were seeded at a density of 6×10^4 cells in 0.2% serum per 16-mm well in 24-well plates. After 3 days the cells had grown to confluency and had become growth arrested, having reached a density of approximately 2×10^5 cells per well. Triplicate wells of cells in the presence of 400-μg/ml phosphonoacetic acid (PAA) were then mock infected or infected with CgalΔ3 or $Cgal^+$ at an MOI of 0.025. At various times cells were fixed with 1.2% glutaraldehyde and stained with X-Gal to determine the number of *lacZ*-expressing cells. At the same time, the cells on another plate were washed with medium to remove residual PAA and mock infected or infected with an ICP8 deletion mutant, *d*301, for 8 h (note that similar results had also been obtained with 5*dl*1.2 [45] as the superinfecting virus). The superinfected cells were then dissociated with trypsin and plated onto 10-cm plates preseeded with E5 monolayers to allow CgalΔ3 or $Cgal^+$ but not *d*301 alone to form plaques, since the latter requires complementation with functional ICP8. The monolayers were incubated either in the presence of 10% human serum for enumeration of plaques or under an agarose overlay to allow plaque isolation for subsequent viral DNA analysis.

RESULTS

CgalΔ3 causes cell death. The HSV-1 vector CgalΔ3 contains the *E. coli lacZ* gene under control of the HCMV IE regulatory region inserted into an intergenic site of the U_S portion of a mutant viral genome deleted for IE 3 (Fig. 1). We studied the effects of CgalΔ3 infection in cultured primary sensory neurons derived from rat dorsal root ganglia and in PC12 cells, a rat pheochromocytoma cell line which can be induced to differentiate and resemble sympathetic neurons following treatment with NGF (26). Sensory neurons and differentiated PC12 cells were infected with CgalΔ3 at an MOI of 0.25 and stained for *lacZ* expression 1 and 3 days postinfection. At 1 day postinfection, blue (β-gal-positive) cells exhibiting neuronlike morphology were observed in both types of cell culture (Fig. 2A and B). However, by 3 days postinfection most of the β-gal-positive cells were pyknotic or showed evidence of extensive degeneration, although many of the β-gal-negative cells retained normal morphology (Fig. 2C and D). Infection with CgalΔ3 therefore resulted in extensive cytotoxicity in those sensory neurons and differentiated PC12 cells which expressed the HSV-1-transduced *lacZ* gene. Because the β-gal-positive cells showed damage while the nonstaining cells retained an unaltered normal morphology when infections were performed at a low MOI, cell death was a direct result of virus infection rather than of non-virion-associated factors in the vector stock which would have affected infected and noninfected cells equally. Similar cytotoxic effects have been observed following infection of primary cultures of central nervous system neurons with CgalΔ3 (37).

Time course of induction of CPE in human fibroblasts. To facilitate characterization of the virus-induced cytotoxicity, we conducted the remaining studies with a human primary fibroblast line, TO-119, rather than with cultured neurons. The time course of induction of CPE by CgalΔ3 was compared with that induced by the replication-competent strain $Cgal^+$ at an MOI of 10 PFU per cell (Fig. 3). Perinuclear granularity was first detected in CgalΔ3-infected cultures at

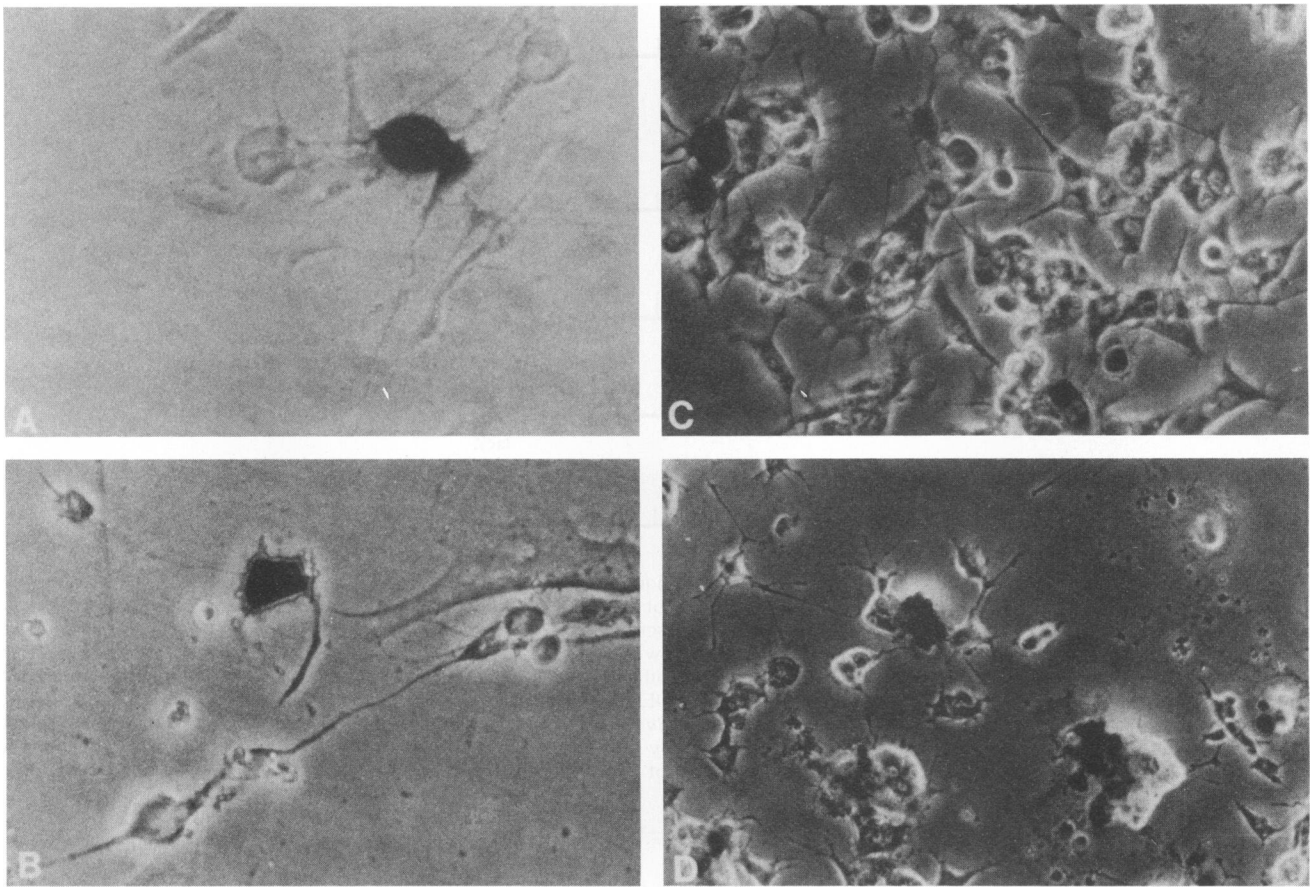

FIG. 2. Infection of dorsal root ganglia neurons and PC12 cells with CgalΔ3. Sensory neurons from neonatal rat dorsal root ganglia maintained in the presence of NGF (A and B) and PC12 cells differentiated with NGF (C and D) were infected with CgalΔ3 at an MOI of 0.25 and stained with X-Gal to detect *lacZ* expression 1 day (panels A and C) and 3 days (panels B and D) postinfection.

12 h postinfection, after which time an increasing proportion of cells exhibited granularity or suffered more obvious morphological changes, becoming rounded and pyknotic. Perinuclear granularity was detected only infrequently in cells infected with $Cgal^+$ or in mock-infected cells. Between 24 and 48 h postinfection, the fraction of rounded cells in both CgalΔ3- and $Cgal^+$-infected cultures increased until few if any cells remained with normal fibroblast morphology.

Role of viral gene expression in CgalΔ3-induced cytotoxicity. In principle, the cytotoxicity caused by infection with CgalΔ3 could be mediated by components of the virion or could be a result of viral gene expression. To distinguish between these possibilities, we examined the effect of UV irradiation of vector stocks on the survival of infected cells. The dose of UV irradiation necessary to inhibit viral gene expression without reducing the infectivity of the virus was determined as outlined in Materials and Methods. TO-119 human fibroblasts infected with UV-irradiated CgalΔ3 did not show signs of CPE (Fig. 3). These results suggest that viral gene expression is involved in initiating cell death, although we have not excluded the possibility that the UV treatment damaged a structural component of the virion.

Because IFN is able to inhibit HSV-1 replication in susceptible cells by inhibiting transcription of viral IE genes (12, 49, 54), we have examined the ability of human IFN-α to protect cells against virus-induced cytotoxicity. Cultures of TO-119 fibroblasts were pretreated for 16 h with 1,000 U of IFN-α per ml and then infected with CgalΔ3 at MOIs ranging from 1 to 100 PFU per cell and maintained in the absence of IFN. Untreated control cultures were similarly infected. At 3 days after infection, we determined the number of cells in the infected cultures compared with the mock-infected control (Fig. 4). Although this assay does not measure absolute cell survival, since the surviving cells continue to replicate during the 3 days it takes for the dying cells to detach from the plate, it does provide a reproducible estimate of virus toxicity. Pretreatment with IFN-α did not affect cell growth in the mock-infected cultures in this experiment. As shown in Fig. 4, IFN markedly enhanced the resistance of the fibroblast cultures to the cytotoxic effects of CgalΔ3. Up to an MOI of 10, IFN gave virtually complete protection, although at higher MOIs the protection was less complete. In contrast, in the absence of IFN, the cell number was reduced to less than 30% of that of the mock-infected control at an MOI of 10 and less than 2.5% at an MOI of 50 to 100.

To determine the effect of IFN-α pretreatment on viral gene expression in CgalΔ3-infected cells, we infected pretreated or untreated TO-119 fibroblasts with CgalΔ3 at an MOI of 10 PFU per cell and determined the accumulation of viral transcripts by slot blot analysis (Fig. 5A). In the IFN-negative cultures, accumulation of RNA transcripts corresponding to viral IE 1, IE 2, and IE 4 was substantial over the first 24 h and then declined to background levels by 72 h postinfection (Fig. 5B). As expected for an IE 3 deletion

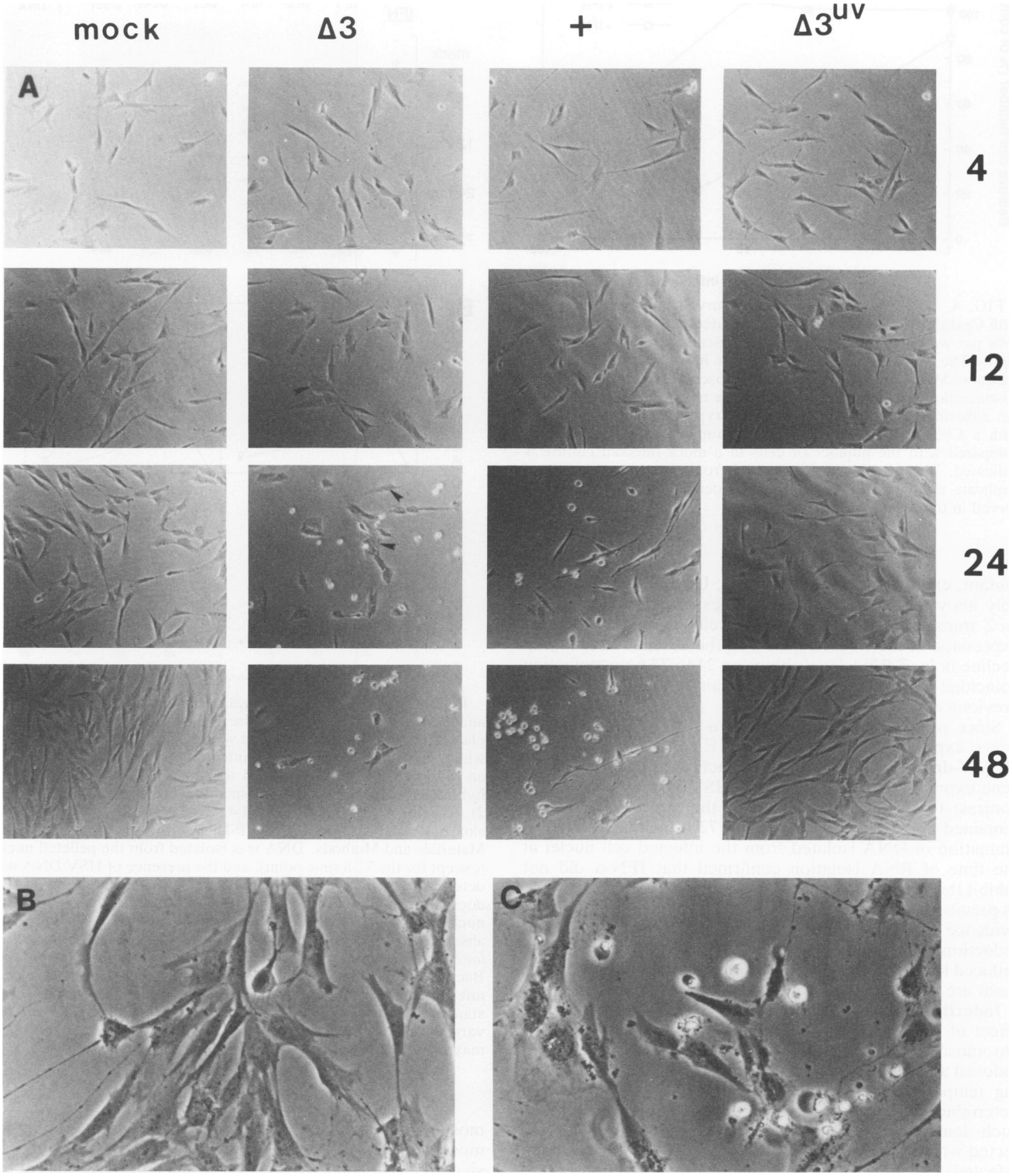

FIG. 3. Induction of CPE in human primary fibroblasts. (A) TO-119 cells were mock infected or infected with CgalΔ3, Cgal$^+$, or CgalΔ3 which had been exposed to UV irradiation (1,200 μW/cm^2) for 5 min. The cells were photographed at 4, 12, 24, and 48 h postinfection as indicated. Arrowheads indicate cells with perinuclear granularity. Higher magnification of mock-infected cells (B) and CgalΔ3-infected cells which at 24 h postinfection show blebbing, pyknosis, and granularity (C) are also shown.

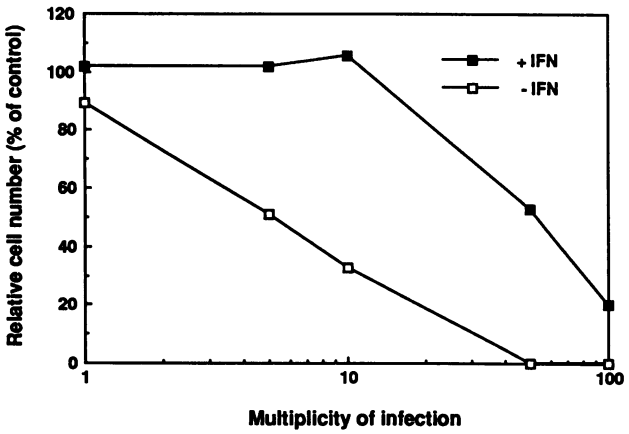

FIG. 4. Influence of IFN-α on cell survival following infection with CgalΔ3. TO-119 fibroblasts were plated at a density of 1×10^4 cells per well in the presence (■) or absence (□) of IFN-α (1,000 U/ml). The cells were washed, infected 16 h later with CgalΔ3 at the indicated MOIs, and maintained in the absence of IFN-α. At 3 days postinfection, loose cells and debris were removed by washing and the adhering cells were harvested by trypsinization and counted with a Coulter Counter. The relative number of cells remaining compared with the number of cells in a mock-infected culture is indicated. These results were derived from the averaged data of duplicate cultures and are essentially identical to the trends observed in three separate experiments.

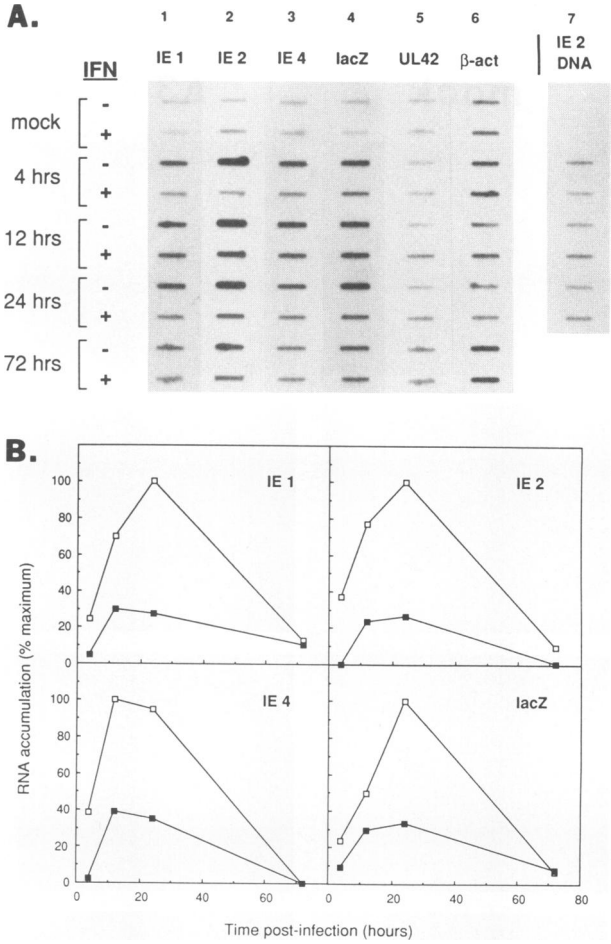

FIG. 5. Effect of IFN-α on CgalΔ3 gene expression. (A) Slot blot analysis. TO-119 fibroblasts were grown to confluency in 10-cm plates. The cells were pretreated with IFN-α (1,000 U/ml) for 16 h where indicated and then mock infected or infected with CgalΔ3 at an MOI of 5 PFU per cell for 4 to 72 h. RNA was prepared and hybridized with sequences corresponding to IE 1 (lane 1), IE 2 (lane 2), IE 4 (lane 3), *lacZ* (lane 4), UL42 (lane 5), and β-actin (lane 6) by slot blot analysis, with the ^{32}P-labeled fragments described in Materials and Methods. DNA was isolated from the pelleted nuclei (except for the 72-h time point), and the presence of HSV DNA was determined by hybridization with the IE 2 probe to confirm that IFN does not inhibit the entry or retention of viral DNA in the cell nucleus (lane 7). (B) RNA accumulation in the presence (■) or absence (□) of IFN-α. The intensities of bands corresponding to the *lacZ* and the three IE genes were determined by densitometry. Background levels of hybridization determined from the mock-infected samples were subtracted. The RNA values were then standardized to the signal corresponding to β-actin (which did not vary with IFN treatment or time of infection) and normalized to the maximum-intensity band (taken as 100%) on each blot.

mutant, expression of an early gene, UL42, was not detectably above background. The kinetics of expression of the *lacZ* transgene in CgalΔ3-infected cells were similar to the expression of the viral IE genes. In the absence of IFN, the decline in *lacZ* RNA levels between 24 to 72 h postinfection coincided with the death of β-gal-staining cells observed in previous experiments.

Since pretreatment of cells with α-IFN substantially reduced expression not only of viral IE genes but also of the HCMV-driven *lacZ* transgene, the action of IFN on HSV-1 gene expression is not specific to HSV-1 IE promoters. In contrast to IFN-negative cultures, the pretreated cultures remained apparently healthy up to 72 h postinfection. Examination of DNA isolated from the infected cell nuclei at the time of RNA isolation confirmed that IFN-α did not inhibit the uptake of stability or viral DNA (Fig. 5A, lane 7), as previously reported (49, 54). These results provide further evidence that viral IE gene expression is involved in the induction of cell death by CgalΔ3 infection and that the reduced levels of IE gene expression following IFN pretreatment are apparently too low to produce CPE.

Induction of host cell chromosomal damage. A well-known effect of HSV-1 infection on host cells is the induction of chromosomal damage (29), an effect which can also be induced at the nonpermissive temperature by mutants bearing temperature-sensitive lesions in Vmw175 (5, 58). To determine whether a deletion mutant of Vmw175 also causes such damage, TO-119 human primary fibroblasts were infected with D30EBA at an MOI of 5 PFU per cell or mock infected and then arrested at metaphase for chromosomal analysis (Table 1). Chromosomal damage was apparent by 6 h postinfection with D30EBA. The damage was frequently characterized by an uncoiling in chromosome 1 region 1q12-21, as previously reported for *ts*K (58), and by separation of sister chromatids as shown in Fig. 6A. Some cells showed more extensive cytogenetic damage, including chromosome breakage and pulverization. The induction of chromosomal damage was not specific for human cells. Extensive breakage and pulverization induced in BHK cells at 6 h postinfection with CgalΔ3 is shown in Fig. 6B. The cytogenetic damage became more severe with increasing MOI and length of exposure to the virus (data not shown). In some experiments, no metaphases at all were obtained in infected cultures, possibly because chromosomal or cellular damage was so severe that no cells were able to enter metaphase.

TABLE 1. Percentage of cells demonstrating chromosomal damage 6 h after infection with D30EBA

Chromosomal aberration	% of cells damaged in[a]:			
	Expt 1		Expt 2	
	Δ3	Mock	Δ3	Mock
Uncoiling[b]	10.6	0.0	10.0	1.8
Uncoiling and/or chromatid breaks[c]	4.2	0.0	5.0	0.0

[a] A total of 2×10^5 primary human fibroblasts (TO-119) were infected with D30EBA (Δ3) or mock infected at an MOI of 5 PFU per cell for 6 h, after which colcemid was added for the preparation of metaphase chromosomes (see Materials and Methods). The percentage of cells demonstrating cytogenetic aberrations in approximately 50 spreads in two independent experiments is shown.

[b] Minor damage characterized by uncoiling in chromosome 1 and occasional evidence of centromere separation. In the mock-infected control cells, 1 cell in 53 showed a single break in chromosome 9.

[c] More extensive damage characterized by centromere separation, uncoiling, and breaks in more than one chromosome.

Host cell DNA fragmentation induced by CgalΔ3. We have examined TO-119 fibroblasts for evidence of DNA damage following viral infection. Since the damage seen at the chromosomal level may correspond to infrequent cleavage of chromatin, we examined DNA for the presence of very high molecular weight degradation products by pulsed-field gel electrophoresis. Duplicate cultures of TO-119 fibroblasts were infected at an MOI of 10 with Cgal$^+$, CgalΔ3, or UV-irradiated CgalΔ3 or were mock infected, and the cells were harvested and embedded in agarose blocks for pulsed-field agarose gel electrophoresis. The results illustrated in Fig. 7 are derived from studies with stationary-phase fibroblasts, although similar results have been obtained following infection of logarithmically growing cells. By 12 to 24 h postinfection, differences between CgalΔ3- and Cgal$^+$-infected samples on one hand and mock- and UV-treated-virus-infected samples on the other were detectable; they were especially apparent at 48 h postinfection, as shown in Fig. 7. A prominent band of high-molecular-weight DNA running at the limiting mobility in this gel system (630 kb) and a smear of DNA in the size range from 40 to 400 kb were present in samples derived from cells infected with CgalΔ3 and Cgal$^+$ but not in samples derived from mock-infected or UV-irradiated CgalΔ3-infected cells. However, much of the DNA was also apparently largely intact since it failed completely to enter the gel. In samples infected with Cgal$^+$ there was also a band of approximately 150 kb corresponding to progeny HSV-1 genomic DNA, whose identity was confirmed by Southern blot analysis (data not shown).

Fragmentation of cellular DNA into oligonucleosome-sized fragments is characteristic of cells undergoing apoptosis (85, 86). We have examined DNA isolated from infected cells for the presence of such fragments on 2% agarose gels. Although we were able to detect oligonucleosomal ladders in some DNA samples from CgalΔ3-infected Vero cells, we also detected similar fragmentation in some mock-infected samples, indicating that this type of DNA fragmentation was not specifically caused by virus infection but could also occur as a result of cell turnover in tissue culture (data not shown).

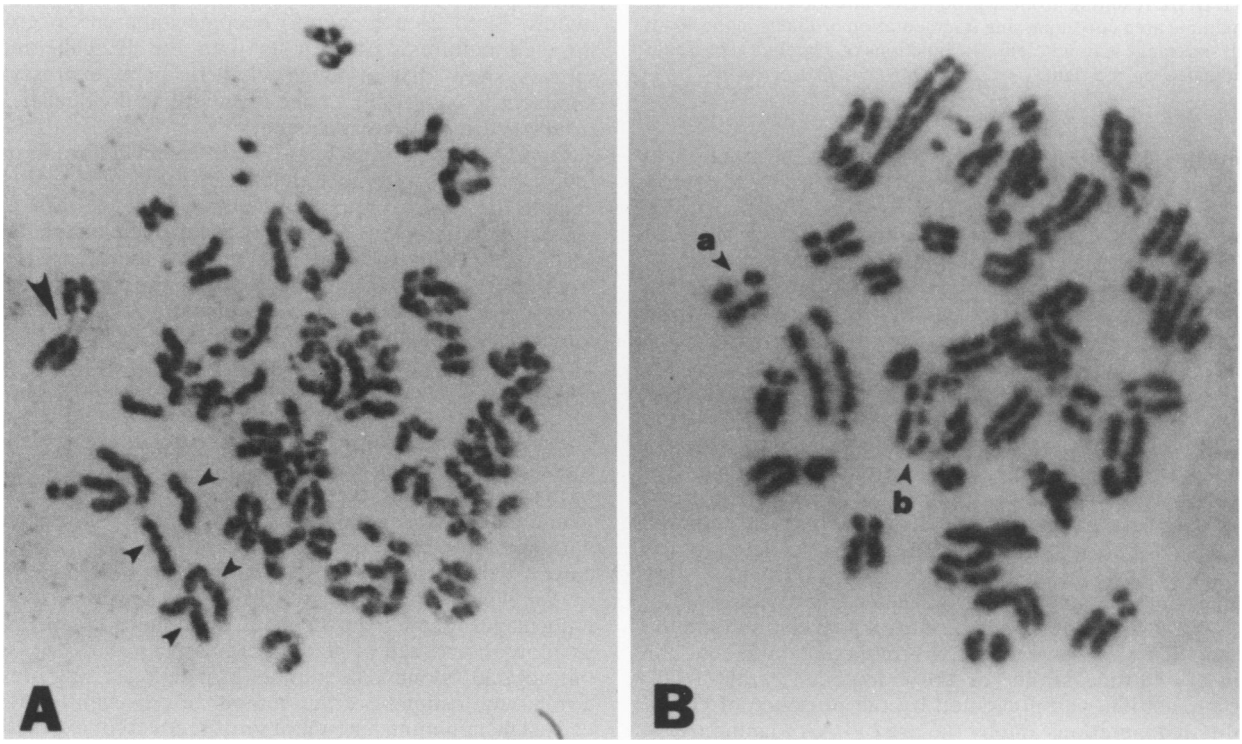

FIG. 6. Induction of chromosomal aberrations. (A) Metaphase spread from the TO-119 human primary fibroblast line, infected with D30EBA at an MOI of 5 PFU per cell for 6 h. The large arrowhead indicates the uncoiling in chromosome 1. Small arrowheads indicate examples of sister chromatid separation. (B) Metaphase spread from a BHK cell infected with CgalΔ3 at an MOI of 5 PFU per cell for 6 h. All the chromosomes are pulverized, and examples of uncoiling (a) and multiple breaks (b) are indicated.

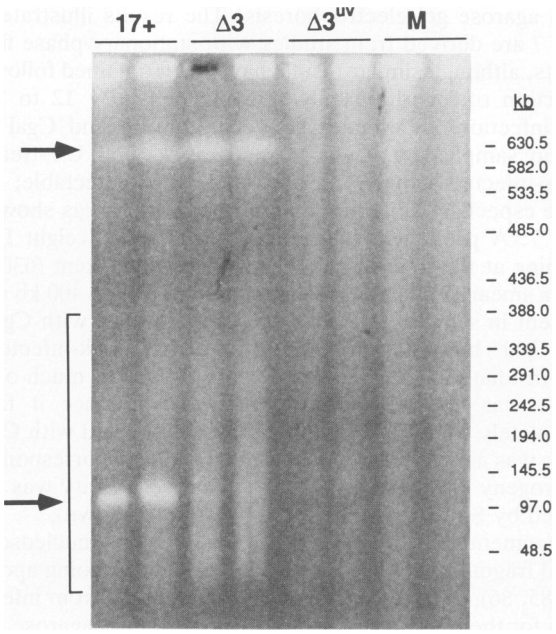

FIG. 7. Pulse-field gel showing the distribution of high-molecular-weight DNA fragments produced by infection. Duplicate lanes show DNA from growth-arrested TO-119 cells that were mock infected (M) or infected at an MOI of 10 PFU per cell for 48 h with wild-type 17^+ (17^+), CgalΔ3 (Δ3), or UV-irradiated CgalΔ3 ($\Delta 3^{uv}$). The cells were embedded intact in agarose blocks (note that one block was washed out of a Δ3 well after electrophoresis), and electrophoresis was performed with 30-s pulses at 200 V for 12 h at 15°C. Arrows indicate the positions of large fragmentation products running at the limit of separation (630 kb) and of the wild-type HSV-1 DNA resulting from a productive infection (150 kb). The approximate area encompassing fragmentation products in the 40- to 400-kb size range is bracketed. The positions of a lambda size ladder are indicated on the right.

Induction of CPE by other HSV-1 strains. In addition to CgalΔ3, we have examined other HSV-1 strains for their ability to induce generalized CPE, with and without prior exposure to UV irradiation. These additional viruses were *dl*1403, an IE 1 deletion mutant (79); CgalΔ3/4, which is derived from CgalΔ3 and carries an additional deletion mutation within IE 4 (Fig. 1); Δ3Z5, which is derived from D30EBA (ΔIE 3) and contains a 4.6-kb HCMV-*lacZ* insertion between codons 44 and 45 of IE 5 (Fig. 1); *ts*K, which has a temperature-sensitive lesion in Vmw175 (it has been reported that the *ts*K mutant does not cause CPE at the nonpermissive temperature [13]); CgalΔ42, which has a deletion in the UL42 gene, whose product is essential for viral DNA replication and consequently for late gene expression (34); and the parental wild-type strain for these mutants, strain 17^+. We also examined 5*dl*1.2, which is deleted for the essential gene IE 2 and overexpresses viral early gene products but does not express all late gene products (45); *vhs*1, which has a mutation in the virion host shutoff function of strain KOS but is replication competent (60); and the parental wild-type strain for these mutants, strain KOS. Lytic replication was prevented by the presence of conditional-lethal mutations and by the use of PAA to inhibit viral DNA replication. As shown in Table 2, all virus strains tested caused generalized CPE at an MOI of 2 PFU per cell, in the presence or absence of PAA. We found no difference in the induction of CPE between *vhs*1 and its parental strain, KOS, further indicating that the vhs function is not an important factor in the development of CPE in infected cells at the MOIs used in this study. Prior UV irradiation of these virus strains abolished their ability to induce CPE, consistent with the interpretation that viral gene expression causes cell death. Similar results were obtained with both proliferating and serum-starved cultures of Vero cells and with human primary fibroblasts, although the CPE took approximately 12 to 24 h longer to become apparent in growth-arrested cultures. The results with the IE gene mutant viruses show that although viral IE gene expression is sufficient to cause CPE, none of the five individual IE gene products are solely responsible.

TABLE 2. Cytotoxicity of HSV-1 strains

Strain	Gene(s)	CPE following treatment[a]		
		None	PAA[b]	UV[c]
Mock		−	−	ND[d]
17^+	*wt*	+	+	−
CgalΔ3	IE 3	+	+	−
*dl*1403	IE 1	+	+	−
CgalΔ3/4	IE 3, IE 4	+	+	−
Δ3Z5	IE 3, IE 5	+	+	−
KOS	*wt*	+	+	−
5*dl*1.2	IE 2	+	+	−
vhs-1	*vhs*	+	+	−
*ts*K[e]	IE 3	+	ND	ND
CgalΔ42	UL42	+	ND	ND

[a] Vero cells were infected with the indicated virus strains at an MOI of 2 PFU per cell and examined for up to 3 days postinfection for the appearance (+) or absence (−) of generalized CPE.
[b] Cells were treated with 400 μg of PAA per ml for 1 h prior to and throughout infection.
[c] Stocks of virus were exposed to UV irradiation as outlined in Materials and Methods.
[d] ND, not done.
[e] Infection with *ts*K and a mock-infected control was performed at the nonpermissive temperature of 39.5°C.

CgalΔ3 can establish a long-term infection in fibroblasts. To investigate the possibility that CgalΔ3 can persist in some cells in the absence of transgene or IE gene expression, we used a superinfection-rescue procedure similar to that described by Leib et al. (42) to recover virus quantitatively from infected cells (see Materials and Methods). Triplicate cultures of fibroblasts that had reached confluency were infected with CgalΔ3 or $Cgal^+$ at an MOI of 0.025 PFU per cell. Cells were infected and maintained in the presence of 400 μg of PAA per ml for the first 3 days to inhibit replication of $Cgal^+$. The number of β-gal-positive cells at 1 day postinfection was determined since this correlates very well with Cgal virus titer and therefore provides an accurate indication of the number of infecting virus units. Virus which had been maintained in the cells was reactivated by superinfection with the HSV-1 deletion mutant of ICP8, *d*301 (20), which can provide Vmw110 to rescue latent virus (30) and Vmw175 to complement the growth of CgalΔ3. Cells were harvested 8 h postsuperinfection and replated on monolayers of E5 cells to allow the growth of plaques containing CgalΔ3 or $Cgal^+$, but not *d*301 alone. The number of plaques arising provides a minimum estimate of the number of persistently infected cells. The minimum amount of virus recovered, expressed as a percentage of the infecting virus, was calculated from the number of β-gal-positive cells at 1 day postinfection and the number of plaques obtained at later times. Table 3 shows the results of a typical experiment performed with TO-119 cells

TABLE 3. Recovery of CgalΔ3 and Cgal$^+$ from TO-119 fibroblasts

Virus	No. of β-gal-positive cells on[a]:		No. of plaques from virus rescue on[b]:		Minimum % recovery on[c]:	
	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14
CgalΔ3	1,029	0	797	60	77	6
Cgal$^+$	1,559	0	372	186	24	12
None (mock)	0	0	0	0	0	0

[a] TO-119 cells were infected 3 days after seeding (6×10^4 cells per 16-mm well) with the indicated virus at an MOI of 0.025 PFU per cell in the presence of 400 μg of PAA per ml. After 3 days PAA was removed, and the medium was replaced every 2 to 3 days thereafter. At the indicated time points, the cells in three wells were fixed in 1.25% glutaraldehyde and stained with X-Gal indicator dye (see Materials and Methods), and the average number of β-gal-positive cells per 16-mm well was calculated from the number of blue-staining cells in nine 1.5-mm^2 fields per well. At 14 days postinfection it was still possible to discern blue-staining debris in the infected cultures, but no intact β-gal-positive cells were present. Standard errors of the mean number of β-gal-positive cells per well were 12% or less.

[b] Indicates the average number of plaques per 16-mm well arising after superinfection of triplicate wells with *d*301 and subsequent plating of superinfected cells on monolayers of E5 cells. No plaques were obtained at 14 days postinfection in the absence of superinfection with *d*301. Standard deviations of the mean number of plaques were less than 10%.

[c] Calculated from the number of rescued plaques at 14 days postinfection compared with the number of β-gal-positive cells at 1 day postinfection.

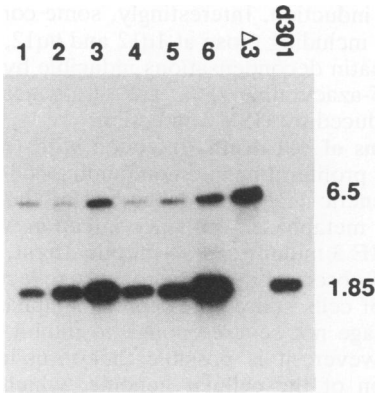

FIG. 8. Southern blot analysis of viral DNA from six plaques isolated from the superinfection-rescue procedure. Virus persisting in cells infected with CgalΔ3 at an MOI of 0.025 PFU per cell was rescued by superinfection with *d*301 at an MOI of 20 PFU per cell. After 8 h, the superinfected cells were suspended in medium and replated on monolayers of E5 cells under a 0.8% agarose overlay to allow plaques to form. DNA from individual isolated and expanded plaques was cleaved with *Bam*HI, subjected to electrophoresis, and transferred to a Nytran filter. The blot was hybridized with the *Bam*HI-*z* fragment (shown in Fig. 1) to distinguish between CgalΔ3 DNA containing the *lacZ* insert (6.5 kb) and *d*301 DNA without an insert in *Bam*HI-*z* (1.85 kb). *Bam*HI digests of *d*301 and CgalΔ3 (Δ3) DNA are indicated, with hybridizing fragment sizes shown in kilobases.

maintained for 14 days postinfection. Between 1 and 14 days postinfection with CgalΔ3, the number of β-gal-positive cells dropped from 1,029 to 0, indicating either loss or shutdown of the *lacZ* transgene. At 1 day postinfection almost 80% of the input CgalΔ3 was recovered by the superinfection procedure. At 14 days postinfection, we were able to recover 6% of input CgalΔ3, demonstrating that CgalΔ3 does not kill every cell it infects. Similar results were obtained with Cgal$^+$, except that the efficiency of virus rescue at 1 day postinfection was somewhat lower than with CgalΔ3 although at 14 days postinfection it was possible to recover more of the input Cgal$^+$ virus than CgalΔ3. We have also found that recovery of CgalΔ3 from E5 cells at 1 week postinfection was approximately double that obtained with Vero cells (36), further indicating that Vmw175 or early genes such as ICP8 which can be expressed as a result of Vmw175 complementation may help to inhibit threshold levels of IE gene products which cause cytotoxicity.

To confirm the presence of both CgalΔ3 and *d*301 viral genomes in plaques on E5 cells following the superinfection-rescue procedure, we performed Southern blot analysis of *Bam*HI-restricted DNA on randomly picked individual plaque isolates from the rescue procedure. The blot was hybridized with radiolabeled *Bam*HI-*z* DNA to distinguish between the 1.85-kb *Bam*HI-*z* fragment of *d*301 and the 6.5-kb novel *Bam*HI fragment of CgalΔ3 containing the *lacZ* gene insert (Fig. 8). The higher intensity of the *d*301 band than of the CgalΔ3 band probably reflects the high MOI used in the rescue procedure (each plaque would arise from approximately one copy of CgalΔ3 and 20 PFU of *d*301).

DISCUSSION

Host cell chromosomal aberrations. Early damage to HSV-1-infected mitotic cells often involves uncoiling in specific regions of human chromosomes 1, 9, and 16 (5, 56, 58, 77). One of the first detectable effects of infection with an IE 3 deletion mutant in our study was the induction by 6 h postinfection of chromosomal damage, characterized by chromosome uncoiling and, less frequently, more extensive damage including chromosome pulverization (Fig. 6, Table 1). With the use of a temperature-sensitive IE 3 mutant, *ts*K, and chemical agents or UV irradiation to block viral replication at various stages, Peat and Stanley concluded that HSV-1 IE gene expression was necessary and sufficient to induce chromosomal uncoiling, but that more severe damage required viral early protein synthesis (58). However, in agreement with our results, Chenet-Monte et al. determined that *ts*B21, also a temperature-sensitive IE 3 mutant, could induce a range of effects on host cell chromosomes from uncoiling to pulverization, with pulverization occurring less frequently with the mutant than with wild-type virus (5). These authors also demonstrated that the extent of chromosomal damage was directly correlated with the multiplicity of infection and the duration of the infection. Studies of adenovirus type 12 mutants have indicated that expression of the E1B 55-kDa protein is responsible for the occurrence of both random cytogenetic damage and uncoiling at specific sites in chromosomes 1 and 17 (65). It is interesting that the induction of chromosomal aberrations by HSV-1 and adenovirus type 12 requires only immediate-early viral gene expression, but does not specifically involve either of the major transcriptional-activating proteins, Vmw175 or E1A, respectively.

Chromosome regions uncoiled by CgalΔ3 infection resemble fragile sites—regions of the chromosome that fail to condense during mitosis and have a tendency to break. The fragile X site is perhaps the best-characterized fragile site in the human genome and is associated with the most common inherited form of mental retardation (see reference 1 and references therein). Fragile sites usually require biochemical induction, and many sites occur in only a small fraction of the metaphases of a chromosome preparation (80). Therefore, infection with an HSV-1 IE 3 mutant may induce fragility at some sites more frequently than is possible by

biochemical induction. Interestingly, some common human fragile sites, including those at 1q12 and 9q12, representing heterochromatin decondensations inducible by treatment of cells with 5-azacytidine (80), are at or near regions of uncoiling induced by HSV-1 infection.

Mechanisms of cell death. Infection with CgalΔ3 caused CPE in both proliferating and nondividing cells. For mitotic cells, cytogenetic analysis is dependent on the ability of the cell to enter metaphase, and since infection with wild-type virus or an IE 3 mutant causes mitotic arrest, the type and frequency of observed damage probably reflect only a small proportion of cells still able to enter metaphase with cytogenetic damage not severe enough to inhibit mitosis completely. However, it is possible that virus-induced DNA fragmentation of the cellular genome, which occurred in proliferating and growth-arrested cultures (Fig. 7), is sufficiently severe to disrupt a number of homeostatic cell functions as well as mitosis.

Cell death is thought to generally occur through one of two routes, apoptosis or necrosis (85, 86), although it has been pointed out that there may be some overlap between these two processes (43). Apoptosis is an active form of cell death, characterized by DNA condensation around the cell nucleus, cell shrinkage, membrane blebbing, and DNA fragmentation through activation of a cellular endonuclease generating oligonucleosome-sized DNA fragments. Necrotic cell death is characterized by disruption of the mitochondria and swelling of the cytoplasm, followed by cell lysis. There has been a recent report that a nonproductive infection of activated bovine T lymphocytes by bovine herpesvirus type 1 causes cell death by a process morphologically resembling apoptosis (28). In our study, cells infected with CgalΔ3 produced apoptic-like membrane-bound bodies observable by light microscopy (Fig. 2D and 3C) and demonstrated perinuclear granularity (Fig. 3C), which may represent chromatin condensation around the nucleus. However, studies looking for additional indicators of apoptosis, including the appearance of oligonucleosome-sized-DNA fragmentation and the induction of TRPM-2 RNA (3), have suggested that neither of the classical forms of apoptosis or necrosis can fully account for the death of cultured fibroblasts infected with CgalΔ3 (36).

HSV-1 IE gene expression causes cytotoxicity. Our studies indicate that CPE is caused by viral gene expression rather than by structural components of the virion and that only IE gene expression is required. Conditions which inhibited viral gene expression, either treatment of cells with IFN-α prior to infection or UV irradiation of virus stocks, reduced the induction of CPE. It has been shown that IFN-α inhibits HSV-1 IE gene transcription in infected cells and suppresses the activity of the HSV-1 IE 1 promoter in transfected cells. It does not decrease viral penetration of the cytoplasm or viral DNA stability in the nucleus, nor does it prevent HSV-1 virion-mediated host shutoff of protein synthesis (12, 49, 54). Since IFN-α also inhibited expression of the HCMV-*lacZ* transgene resident in CgalΔ3 in these studies (Fig. 5), its action may not be specific to the transactivation event mediated by the virion component Vmw65, as previously proposed (12, 40). Further support of the role of viral gene expression in causing cell death after infection with HSV-1 is provided by a report from Harris and Preston (30) showing that the HSV-1 mutant *in*1814, with a lesion in Vmw65 rendering it defective in transinduction of IE transcription, is less cytotoxic and is predisposed to latency rather than lytic growth in human fetal lung cells. The identities of the genes which could cause cytotoxicity in cells infected with an IE 3 deletion mutant are limited to those still expressed in the absence of Vmw175, i.e., the four remaining IE genes and the large subunit of ribonucleotide reductase (9, 57). It seems unlikely that the large subunit of ribonucleotide reductase would be toxic in the absence of the small subunit, which is necessary for enzymatic activity, and yet mutations in none of the five IE genes appear to render the virus less toxic (Table 2), which suggests that more than one gene product may cause toxicity. It is also possible that early gene expression in cells infected by HSV-1 strains which have an intact IE 3 gene contributes to the induction of CPE, particularly since IE 3-expressing strains often induce cell rounding very early after infection, similarly to wild-type virus (Fig. 3, $Cgal^+$), rather than showing the characteristic cellular granularity (Fig. 3, CgalΔ3) which preceded pyknosis in cells infected with the IE 3 deletion mutant (36). Although the virion host shutoff function would be an obvious candidate function that could affect cell survival, the mutant *vhs*1 under conditions of DNA replication inhibition appeared as toxic as the parental wild-type strain (Table 2). It may be that at very high MOIs (e.g., greater than 25 PFU per cell) or during infection with strains that have a strong shutoff function, vhs might play a more determinant role in cell death.

Implications for HSV-1 vectors. There are two different types of HSV-1 vectors in current use: helper-virus-dependent plasmid vector systems (71, 78) and vectors based on recombinant viral genomes (50, 62, 68). Plasmid vectors contain HSV-specific sequences for replication and packaging in the presence of helper virus. This type of vector has been used to transfer the *lacZ* reporter gene to cultured neurons of the peripheral and central nervous systems where transgene expression was reported for up to 2 weeks (21, 22). Theoretically, if a cell is infected with a plasmid vector in the absence of coinfection with helper virus, it should not suffer CPE as a result of HSV-specific viral gene expression. It is therefore important that the ratio of plasmid vector to helper virus be high for this system to offer advantages over the recombinant virus approach. Replication-defective recombinant viral vectors have been used to transfer the *lacZ* reporter gene to the mammalian brain in vivo (6, 14) and to primary central nervous system neurons in culture (37). Chiocca et al. injected an IE 3 deletion mutant vector into rat brain but observed only transient expression of the *lacZ* gene under the control of the HSV-1 promoter for the large subunit of ribonucleotide reductase (6). The inability to detect long-term stable expression of *lacZ* in this system could be due to toxicity of the vector, shutdown of *lacZ* expression, or both. Similarly, infection of CNS neuronal cultures with the vector CgalΔ3 resulted in transient expression of the *lacZ* gene from the HCMV IE promoter and, after several days, caused cytopathology to *lacZ*-expressing neurons and glia (37). Dobson et al. placed *lacZ* under the control of the Moloney murine leukemia virus long terminal repeat in an HSV-1 mutant containing deletions in IE 3 and the LAT region (14). In this case, stable expression of *lacZ* was detected in a small number of neurons in sensory ganglia in vivo. This demonstrates that an IE 3 deletion mutant can stably transduce and express a foreign gene, although it is difficult to determine the proportion of infected cells which survived and expressed the *lacZ* gene stably.

In the present study, up to 6% of infected primary fibroblasts can retain the CgalΔ3 mutant for at least 2 weeks (Table 3), even though most of the infected cells probably die. It is also possible that neurons in vivo are less susceptible to the cytotoxic consequences of infection with an IE 3

deletion mutant than they or other cell types are in vitro. However, one would wish to construct a more ideal HSV-1 vector that is noncytopathic in a variety of cell types both in vitro and in vivo and in which long-term stable transgene expression can occur. If our hypothesis that IE gene expression causes CPE is correct, the design of less cytotoxic recombinant viral vectors might be based on mutants that fail to activate or that shut down the expression of IE genes in infected target cells.

Implications for the establishment of latency. The absence of HSV-1 IE gene expression during a latent infection may be no coincidence, given that the unregulated expression of the remaining four IE genes in an IE 3 deletion mutant appears to lead to cell death. The role of the latency-associated transcripts in latency is unclear, although they are not absolutely essential for any part of the latency process (32, 33, 41, 74). Conversely, Epstein-Barr virus protects latently infected human B cells from undergoing cell death by expressing virus-encoded latent proteins (27). It may be advantageous for cells to have mechanisms that lead to self-destruction upon viral infection, so the phenomenon of DNA fragmentation in infected cells would help to protect adjacent phagocytosing cells against subsequent infection or gene transfer. It might therefore be advantageous for HSV-1 to avoid triggering host cell death during a latent infection and for it to escape or suppress this protective process during a lytic infection.

Mechanisms that negatively regulate HSV-1 gene expression to prevent entry into the lytic cycle or to avoid causing cell death during the establishment of latency are probably mediated through a combination of viral and cellular factors. Variations in the availability or activity of Vmw65 (30, 73), the amount and activity of cellular transcription factors and cellular repressor factors (38), the infected-cell type, the uptake of viral DNA into a chromatin structure (11), whether cells are infected in vivo or in vitro, the multiplicity of infection, and the ability to express HSV-1 early gene products such as ICP8 (23, 24) may all affect IE gene expression and thereby affect the proportion of cells able to survive infection with HSV-1 and its derivatives.

In summary, our studies have shown that infection with an HSV-1 mutant deleted for IE 3 causes cytotoxicity and cell death in a variety of cultured cells including sensory neurons. HSV-1 and vectors derived from it are thus able to cause CPE in the absence of a lytic cycle and without production of early and late viral gene products. Experiments with UV-irradiated virus or pretreatment of infected cells with IFN-α indicate the involvement of viral IE gene expression in the induction of cytotoxicity. The shutoff of HSV IE gene expression is therefore probably required during latency to avoid killing the host cell as much as to avoid entry to the lytic cycle, and is probably necessary for developing efficient and safe HSV-1 vectors for gene transfer.

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REFERENCES

1. **Bell, M. V., M. C. Hirst, Y. Nakahori, R. N. MacKinnon, A. Roche, T. J. Flint, P. A. Jacobs, N. Tommerup, L. Tranebjaerg, U. Froster-Iskenius, B. Kerr, G. Turner, R. H. Lindenbaum, R. Winter, M. Pembrey, S. Thibodeau, and K. E. Davies.** 1991. Physical mapping across the fragile X: hypermethylation and clinical expression of the fragile X syndrome. Cell **64**:861–866.
2. **Breakefield, X. O., and N. A. DeLuca.** 1991. Herpes simplex virus for gene delivery to neurons. New Biol. **3**:203–218.
3. **Buttyan, R., C. A. Olsson, J. Pintar, C. Chang, M. Bandyk, P.-Y. Ng, and I. S. Sawczuk.** 1989. Induction of the *TRPM-2* gene in cells undergoing programmed death. Mol. Cell. Biol. **9**:3473–3481.
4. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
5. **Chenet-Monte, C., F. Mohammad, C. M. Celluzi, P. A. Schaffer, and F. E. Farber.** 1986. Herpes simplex virus gene products involved in the induction of chromosomal aberrations. Virus Res. **6**:245–260.
6. **Chiocca, E. A., B. B. Choi, W. Cai, N. A. DeLuca, P. A. Schaffer, M. DiFiglia, X. O. Breakefield, and R. L. Martuza.** 1990. Transfer and expression of the *lacZ* gene in rat brain neurons mediated by herpes simplex virus mutants. New Biol. **2**:739–746.
7. **Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner.** 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell **20**:95–105.
8. **Davidson, I., and N. D. Stow.** 1985. Expression of an immediate early polypeptide and activation of a viral origin of replication in cells containing a fragment of herpes simplex virus DNA. Virology **141**:77–88.
9. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. **56**:558–570.
10. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. Nucleic Acids Res. **15**:4491–4511.
11. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. **63**:943–947.
12. **De Stasio, P. R., and M. W. Taylor.** 1990. Specific effect of interferon on the herpes simplex virus type 1 transactivation event. J. Virol. **64**:2588–2593.
13. **Dienes, H. P., A. Knoblich, and D. Falke.** 1985. Loss of surface fibronectin after infection of cultured cells by HSV-1 and 2. Arch. Virol. **86**:223–237.
14. **Dobson, A. T., T. P. Margolis, F. Sedarati, J. G. Stevens, and L. T. Feldman.** 1990. A latent, nonpathogenic HSV-1-derived vector stably expresses β-galactosidase in mouse neurons. Neuron **5**:353–360.
15. **Everett, R. D., and M. L. Fenwick.** 1990. Comparative DNA sequence analysis of the host shutoff genes of different strains of herpes simplex virus: type 2 strain HG52 encodes a truncated UL41 product. J. Gen. Virol. **71**:1387–1390.
16. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
17. **Fenwick, M. L., and R. D. Everett.** 1990. Transfer of UL41, the gene controlling virion-associated host cell shutoff between different strains of herpes simplex virus. J. Gen. Virol. **71**:411–418.
18. **Fenwick, M. L., and M. J. Walker.** 1978. Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41**:37–51.
19. **Forss-Petter, S., P. E. Danielson, S. Catsicas, E. Battenberg, J. Price, M. Nerenberg, and J. G. Sutcliffe.** 1990. Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. Neuron **5**:187–197.

20. **Gao, M., and D. M. Knipe.** 1989. Genetic evidence for multiple nuclear functions of the herpes simplex virus ICP8 DNA-binding protein. J. Virol. **63**:5258–5267.
21. **Geller, A. I., and X. O. Breakefield.** 1988. A defective HSV-1 vector expresses *Escherichia coli* β-galactosidase in cultured peripheral neurons. Science **241**:1667–1669.
22. **Geller, A. I., and A. Freese.** 1990. Infection of cultured central nervous system neurons with a defective herpes simplex virus 1 vector results in stable expression of *Escherichia coli* β-galactosidase. Proc. Natl. Acad. Sci. USA **87**:1149–1153.
23. **Godowski, P. J., and D. M. Knipe.** 1985. Identification of a herpes simplex virus function that represses late gene expression from parental viral genomes. J. Virol. **55**:357–365.
24. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA **83**:256–260.
25. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
26. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73**:2424–2428.
27. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordan, and A. B. Rickinson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature (London) **349**:612–614.
28. **Griebel, P. J., H. Bielefeldt Ohmann, M. J. P. Lawman, and L. A. Babiuk.** 1990. The interaction between bovine herpesvirus type 1 and activated bovine T lymphocytes. J. Gen. Virol. **71**:369–377.
29. **Hampar, B., and S. A. Ellison.** 1961. Chromosomal aberrations induced by an animal virus. Nature (London) **192**:145–147.
30. **Harris, R. A., and C. M. Preston.** 1991. Establishment of latency *in vitro* by the herpes simplex virus type 1 mutant *in* 1814. J. Gen. Virol. **72**:907–913.
31. **Heeg, U., H. P. Dienes, S. Muller, and D. Falke.** 1986. Involvement of actin-containing microfilaments in HSV-induced cytopathology and the influence of inhibitors of glycosylation. Arch. Virol. **91**:257–270.
32. **Hill, J. M., F. Sedarati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates *in vivo* reactivation. Virology **174**:117–125.
33. **Ho, D. Y., and E. S. Mocarski.** 1989. Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA **86**:7596–7600.
34. **Johnson, P. A., M. G. Best, T. Friedmann, and D. S. Parris.** 1991. Isolation of a herpes simplex virus type 1 mutant deleted for the essential UL42 gene and characterization of its null phenotype. J. Virol. **65**:700–710.
35. **Johnson, P. A., and T. Friedmann.** 1990. Limited bidirectional activity of two housekeeping gene promoters: human HPRT and PGK. Gene **88**:207–213.
36. **Johnson, P. A., A. Miyanohara, and T. Friedmann.** Unpublished results.
37. **Johnson, P. A., K. Yoshida, F. H. Gage, and T. Friedmann.** 1992. Effects of gene transfer into cultured CNS neurons with a replication-defective herpes simplex virus type 1 vector. Mol. Brain Res. **12**:95–102.
38. **Kemp, L. M., C. L. Dent, and D. S. Latchman.** 1990. Octamer motif mediates transcriptional repression of HSV immediate-early genes and octamer-containing cellular promoters in neuronal cells. Neuron **4**:215–222.
39. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. HSV virion host shutoff function. J. Virol. **62**:912–921.
40. **LaMarco, K. L., and S. L. McKnight.** 1989. Purification of a set of cellular polypeptides that bind to the purine-rich *cis*-regulatory elements of herpes simplex virus immediate early genes. Genes Dev. **3**:1372–1383.
41. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. **63**:2893–2900.
42. **Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer.** 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. **63**:759–768.
43. **Lockshin, R. A., and Z. F. Zakeri.** 1990. Programmed cell death: new thoughts and relevance to aging. J. Gerontol. **45**:135–140.
44. **Lorentz, A. K., K. Munk, and G. Darai.** 1977. DNA replication in human embryonic lung cells infected with herpes simplex virus. Virology **82**:401–408.
45. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. **63**:18–27.
46. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. **181**:1–13.
47. **McLennan, J. L., and G. Darby.** 1980. Herpes simplex virus latency: the cellular location of the virus in dorsal root ganglia and the fate of the infected cell following virus activation. J. Gen. Virol. **51**:233–243.
48. **Mellerick, D. M., and N. W. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology **158**:265–275.
49. **Mittnacht, S., P. Straub, H. Kirchner, and H. Jacobsen.** 1988. Interferon treatment inhibits onset of herpes simplex virus immediate-early transcription. Virology **164**:201–210.
50. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22**:243–255.
51. **Nishioka, Y., and S. Silverstein.** 1978. Requirement of protein synthesis for the degradation of host mRNA in Friend erythroleukemia cells infected with herpes simplex virus 1. J. Virol. **27**:619–627.
52. **Nishiyama, Y., and F. Rapp.** 1981. Repair replication of viral and cellular DNA in herpes simplex virus type 2-infected human embryonic and xeroderma pigmentosum cells. Virology **110**: 466–475.
53. **Norton, P. A., and J. M. Coffin.** 1985. Bacterial β-galactosidase as a marker of Rous sarcoma virus gene expression and replication. Mol. Cell. Biol. **5**:281–290.
54. **Oberman, F., and A. Panet.** 1988. Inhibition of transcription of herpes simplex virus immediate early genes in interferon-treated human cells. J. Gen. Virol. **69**:1167–1177.
55. **O'Neill, F. J., R. J. Goldberg, and F. Rapp.** 1972. Herpes simplex virus latency in cultured human cells following treatment with cytosine arabinoside. J. Gen. Virol. **14**:189–197.
56. **O'Neill, F. J., and F. Rapp.** 1971. Early events required for induction of chromosomal abnormalities in human cells by herpes simplex virus. Virology **44**:544–553.
57. **Paterson, T., and R. D. Everett.** 1990. A prominent serine-rich region in Vmw175, the major regulatory protein of herpes simplex virus type 1 is not essential for virus growth in tissue culture. J. Gen. Virol. **71**:1775–1783.
58. **Peat, D. S., and M. A. Stanley.** 1986. Chromosome damage induced by herpes simplex virus type 1 in early infection. J. Gen. Virol. **67**:2273–2277.
59. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. **67**:2365–2380.
60. **Read, G. S., and N. Frenkel.** 1983. Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate early) viral polypeptides. J. Virol. **46**:498–512.
61. **Rixon, F. J., and J. McLauchlan.** 1991. Insertion of DNA sequences at a unique restriction enzyme site engineered for vector purposes into the genome of herpes simplex virus type 1. J. Gen. Virol. **71**:2931–2939.
62. **Roizman, B., and F. J. Jenkins.** 1985. Genetic engineering of

novel genomes of large DNA viruses. Science **229:**1208–1214.
63. **Roizman, B., and P. R. Roane, Jr.** 1964. The multiplication of herpes simplex virus. II. The relationship between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology **22:**262–269.
64. **Russell, J., N. D. Stow, E. C. Stow, and C. M. Preston.** 1987. Herpes simplex virus genes involved in latency in vitro. J. Gen. Virol. **68:**3009–3018.
65. **Schramayr, S., D. Caporossi, I. Mak, T. Jelinek, and S. Bacchetti.** 1990. Chromosomal damage induced by human adenovirus type 12 requires expression of the E1B 55-kilodalton viral protein. J. Virol. **64:**2090–2095.
66. **Schubert, D., N. Ling, and A. Baird.** 1987. Multiple influences of a heparin-binding growth factor on neuronal development. J. Cell Biol. **104:**635–643.
67. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the α 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. **55:**338–346.
68. **Shih, M.-F., M. Arsenakis, P. Tiollais, and B. Roizman.** 1984. Expression of hepatitis B virus S gene by herpes simplex virus type 1 vectors carrying α- and β-regulated gene chimeras. Proc. Natl. Acad. Sci. USA **81:**5867–5870.
69. **Smibert, C. A., and J. R. Smiley.** 1990. Differential regulation of endogenous and transduced beta-globin genes during infection of erythroid cells with a herpes simplex type 1 recombinant. J. Virol. **64:**3882–3894.
70. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
71. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon: a new eukaryotic defective-virus cloning-amplifying vector. Cell **30:**295–304.
72. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61:**3841–3847.
73. **Steiner, I., J. G. Spivack, S. L. Deshmane, C. I. Ace, and C. M. Preston.** 1990. A herpes simplex virus type 1 mutant containing a nontransducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J. Virol. **64:**1630–1638.
74. **Steiner, I., J. G. Spivack, R. P. Lirette, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8:**505–511.
75. **Stevens, J. G.** 1989. Human herpesviruses: a consideration of the latent state. Microbiol. Rev. **53:**318–332.
76. **Stevens, J. G., E. K. Wagner, G. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to herpesvirus α-gene mRNA is predominant in latently infected neurons. Science **235:**1056–1059.
77. **Stich, H. F., T. C. Hsu, and F. Rapp.** 1964. Viruses and mammalian chromosomes. 1. Localization of chromosome aberrations after infection with herpes simplex virus. Virology **22:**439–445.
78. **Stow, N. D., M. D. Murray, and E. C. Stow.** 1986. Cis-acting signals involved in the replication and packaging of herpes simplex virus type 1 DNA, p. 497–507. *In* M. Botchan, T. Grodzicker, and P. A. Sharp (ed.), Cancer cells, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
79. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J. Gen. Virol. **67:**2571–2585.
80. **Sutherland, G. R.** 1988. The role of nucleotides in human fragile site expression. Mutat. Res. **200:**207–213.
81. **Sydiskis, R. J., and B. Roizman.** 1967. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virology **32:**678–686.
82. **Turner, D. L., and C. L. Cepko.** 1987. A common progenitor for neurons and glia persists in rat retina late in development. Nature (London) **328:**131–136.
83. **Wagner, E. K., W. M. Flanagan, G. Devi-Rao, Y.-F. Zhang, J. M. Hill, K. P. Anderson, and J. G. Stevens.** 1988. The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. **62:**4577–4585.
84. **Wilcox, C. L., R. L. Smith, C. R. Freed, and E. M. Johnson, Jr.** 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. J. Neurosci. **10:**1268–1275.
85. **Wyllie, A. H.** 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature (London) **284:**555–556.
86. **Wyllie, A. H., J. F. R. Kerr, and A. R. Currie.** 1980. Cell death: the significance of apoptosis. Int. Rev. Cytol. **68:**251–306.