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The Herpes Simplex Virus Immediate-Early Protein ICP0 Affects Transcription from the Viral Genome and Infected-Cell Survival in the Absence of ICP4 and ICP27

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ICP4, ICP0, and ICP27 are the immediate-early (IE) regulatory proteins of herpes simplex virus that have the greatest effect on viral gene expression and growth. Comparative analysis of viral mutants defective in various subsets of these IE genes should help elucidate how these proteins affect cellular and viral processes. This study focuses on the mutant d97, which is defective for the genes encoding ICP4, ICP0, and ICP27 and expresses the bacterial β-galactosidase (β-gal) gene from the ICP0 promoter. Together with the d92 virus (ICP4$^-$ ICP27$^-$) and the ICP0-complementing cell line L7, d97 provided a unique opportunity to evaluate ICP0 function in the absence of the regulatory activities specified by ICP4 and ICP27. The pattern of protein synthesis in d97-infected cells was unique relative to other IE gene mutants in that it was similar to that seen in the absence of prior viral protein synthesis, possibly approximating the effect of cellular factors and virion components alone. Inactivation of ICP0 in the absence of ICP4 produced a significant decrease in the levels of the early mRNAs ICP6 and thymidine kinase (*tk*). There was also a marginal reduction in the levels of the IE ICP22 mRNA, and this was most notable at low multiplicity of infection (MOI). In d97-infected L7 cells, the levels of the viral mRNAs were mostly restored to those observed in infections with d92. Nuclear runoff transcription analysis demonstrated that the presence of ICP0 resulted in an increase in the transcription rates of the analyzed genes. The transcription rates of the early genes were dramatically reduced in the absence of ICP0. At low MOI, the transcription rates of ICP6 and *tk* were comparable to the rate of transcription of a cellular gene. Relevant to the potential use of d97 as a transfer vector, it was also determined that the absence of ICP0 reduced the cellular toxicity of the virus compared to that of d92. The β-gal transgene expressed from an IE promoter was detected for up to 14 days postinfection; however, the level of β-gal expression declined dramatically after 1 day postinfection. In the presence of ICP0, the level of expression of β-gal was increased; however the infected monolayer was destroyed by 3 days postinfection. Therefore, deletion of ICP0 in the absence of ICP4 and ICP27 reduces toxicity and lowers the level of expression of genes from the viral genome.

When herpes simplex virus (HSV) infects cells, the first viral genes to be expressed are referred to as the immediate-early (IE) or α genes (42). IE genes are operationally defined as those that are transcribed in the absence of prior viral protein synthesis (42, 43). The transcription of IE genes in the absence of prior viral protein synthesis is a function of *cis*-acting sites located in the promoters of IE genes for cellular transcription factors and VP16 (8, 45, 53, 54). VP16 is a component of the virion (3, 8), which activates transcription by binding to TAA TGARAT elements with cellular oct 1 and C1 (30, 31, 36, 48, 49, 65, 91). There are five IE genes, which encode infected-cell polypeptides 4, 0, 22, 27, and 47 (ICP4, ICP0, ICP22, ICP27, and ICP47, respectively) (15, 43, 68, 93). ICP4, -0, and -27 have been shown to have a profound effect on subsequent viral gene expression and viral growth (17, 22, 58, 61, 73, 74, 85, 88).

ICP4, ICP0, and ICP27 are nuclear phosphoproteins (68, 97). ICP4 is a large multifunctional protein that either represses (18, 38, 64, 67, 72) or activates (18, 24, 28, 66, 70) transcription through different sets of contacts with the general transcription machinery (9, 37, 38, 84). ICP4 is largely responsible for the transition from the IE to the E phase of viral gene transcription (22, 69, 94). ICP0 will activate most test promoters in transient assays (24, 28, 66, 70) and has been shown to elevate the levels of viral gene expression and growth in tissue culture and in the trigeminal ganglia of mice (6, 7, 51). ICP0 also facilitates the reactivation of virus from latency in the mouse model (7, 51). The level at which ICP0 functions and the mechanism by which it works to increase expression levels are not known. However, ICP0 has been shown to associate with a 135-kDa cellular protein (62, 63) and has also been shown to dissociate the nuclear structures known as ND10 (27, 56, 57), which are thought to be involved in the proliferative or differentiation state of the cell (23, 47, 96). ICP27 also appears to be multifunctional. ICP27 has been shown to regulate viral and cellular mRNA processing events (11, 39, 40, 59, 60, 78–80, 85). Several studies have also shown that it may modulate the activity of ICP4 and ICP0 (61, 71, 81), as well as the modification state of ICP4 (61, 71, 89). The combined activities of ICP27 contribute to efficient DNA replication and late gene expression (58, 73). Recent studies have shown that ICP27 also significantly contributes to elevated levels of early gene expression (76, 92). Therefore, ICP4, ICP0, and ICP27 have been postulated to affect gene expression by a variety of mechanisms. They do so by modulating different host cell metabolic pathways and must collectively result in the alteration of a wide range of host cell processes.

In cells infected with mutant viruses that do not specify ICP4 activity, the remaining IE proteins are overexpressed relative

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to the levels produced in the presence of ICP4 (17). As a consequence, and despite the fact that most of the HSV genome is not expressed, ICP4 mutants are very toxic to cells (46). There are presumably several underlying reasons for the toxicity, and published reports support the involvement of both necrotic and apoptotic modes of cell death (46, 52). The observed toxicity of mutants that lack ICP4 can be markedly reduced through the additional inactivation of VP16, which results in the reduced expression of the remaining IE genes (45). We have also previously shown that the simultaneous inactivation of ICP4, ICP22, and ICP27 results in prolonged cell survival and gene expression relative to other previously existing IE mutant backgrounds (98). Presumably, the high level of gene expression observed in the mutant lacking ICP4, ICP22, and ICP27 activities is in part related to the overexpression of ICP0 in this background. The current study examines the contribution of ICP0 to gene expression and cell survival in a background that is already deleted for the ICP4 and ICP27 genes. The results of these experiments are relevant to the function of ICP0 and to the use of virus-based vectors for gene transfer.

MATERIALS AND METHODS

Cells and viruses. Vero cells and the complementing cell lines E26, L7, and FO6 were maintained by standard cell culture procedures as previously described (16, 22). The E26 cell line has been previously described (76), and construction of the L7 and FO6 cell lines is described below. Viruses carrying mutations in either ICP4 or ICP27 or both, including d120 (17), 5dl1.2 (58), and d92 (76), were propagated, and titers were determined on E26 cells. The ICP0 mutant virus n212 (6) was grown on L7 cells, and the d97 virus was propagated on FO6 cells.

The transfection procedures used to construct the cell lines have been described before (76). The DNA-calcium phosphate precipitates were prepared essentially as described by Graham and van der Eb (35). To construct the L7 cell line, approximately 10^6 Vero cells plated in two 60-mm dishes were transfected with 5 μg of pW3ΔHS8 (19) and 1 μg of pSV2neo for G418 selection (87). Two days after transfection, the cells were trypsinized and seeded into 20 100-mm petri dishes, and the dishes were incubated in medium supplemented with 700-μg/ml G418. This concentration of G418 was lowered to 400 μg/ml 5 days after transfection. G418-resistant colonies were expanded and screened for their abilities to complement n212.

The FO6 cell line was constructed by transfecting approximately 10^6 E26 cells in two 60-mm petri dishes with 5 μg of pW3ΔHS8 and 1 μg of plasmid pSV2Hyg, which encodes the hygromycin resistance gene from *Escherichia coli* under the control of the simian virus 40 early promoter (obtained from Paul Robbins, University of Pittsburgh). Two days after transfection, the cells were trypsinized and seeded into 20 100-mm petri dishes, and the dishes were incubated in media containing 500-μg/ml G418 and 400-μg/ml hygromycin for 6 days, after which the antibiotic concentrations were lowered to 400 μg/ml for G418 and 300 μg/ml for hygromycin. Individual colonies resistant to G418 and hygromycin were expanded and screened for their abilities to complement d92 and n212.

Plasmid constructions. Plasmid p0β was derived from pW3ΔHS8 (19), which contains the 4.5-kb *Sac*I-to-*Pst*I insert encoding the ICP0 gene. A unique *Nco*I site (CCATGG) in pW3ΔHS8 contains the initiator methionine codon for ICP0, and 0.7 kb into the protein-coding sequence is a unique *Bam*HI site. p0β was constructed by replacing the 0.7-kb *Nco*I-*Bam*HI fragment in pW3ΔHS8 with the 3-kb *Bam*HI fragment from plasmid pSC8 (10), which encodes the *E. coli* β-galactosidase (β-gal) gene. This was done by first modifying with the oligonucleotide linkers 5′-CATGGAGCCCCCG-3′ and 5′-GATCCGGGGGCTC-3′ the *Nco*I site in pW3ΔHS8, which has *Bam*HI ends, thereby putting the ATG in frame with the β-gal coding sequence. This construction puts β-gal expression under the control of the ICP0 promoter. Plasmid pucBN was constructed by cloning the wild-type *Bam*HI-N fragment into the unique *Bam*HI site in puc19. The orientation of the insert fragment was such that digestion of the plasmid with *Hin*dIII produced a 2-kb fragment containing ICP22 sequence. The plasmids M13-ICP6 and M13-βtub were used to produce the single-stranded probes to detect ICP6 and β-tubulin mRNAs, respectively, in the nuclear runoff transcription assays. M13-ICP6 contained the 0.8-kb *Bam*HI fragment derived from pKX2-βG3 (34), and M13-βtub contained the 1.5-kb *Pst*I fragment isolated from pRβT.3 (4). Both insert fragments were cloned into the appropriate restriction sites in the polylinker region of the M13mp19 vector.

Preparation of viral DNA and Southern blot analysis. Small-scale viral DNA preparations were done as described previously (76). DNAs isolated from the wild-type and IE mutant viruses were cleaved with *Hpa*I to detect the 5dl1.2 mutation in ICP27 (58), with *Pst*I and *Sac*I to detect the 0β substitution in ICP0, and with *Bam*HI to detect the d120 mutation in ICP4 (17). Digested viral DNAs were fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to nick-translated probes as previously described (77, 86). Gel-purified DNA fragments used as probes included the 2.4-kb *Bam*HI-*Sac*I fragment derived from pKHX-BH (5) for detecting ICP27, the 3-kb *Bam*HI-*Pst*I fragment from pW3ΔHS8 (19) for detecting ICP0, and the 1.8-kb *Bam*HI-Y fragment from pKBY (76) for detecting ICP4.

Isolation of infected-cell RNA and Northern blot analysis. Total infected-cell RNA was prepared as previously described (76). Fractionation of the RNA samples in 1.3% agarose formaldehyde gels and the conditions for blotting, hybridization, and washing have also been described previously (44, 77). Gel-purified DNA fragments that were nick-translated and used for probes included the 2-kb *Hin*dIII fragment from pucBN for detecting the ICP22 mRNA, the 1.6-kb *Sca*I fragment from pKX2-βG3 (34) for detecting ICP6, and the 0.7-kb *Sac*I-*Sma*I fragment from the thymidine kinase (TK)-coding region for detecting TK (76).

Analysis of viral proteins. Viral polypeptides were radiolabeled by incubating approximately 6×10^5 cells infected at a multiplicity of infection (MOI) of 10 PFU per cell with 100 μCi of [^{35}S]methionine per ml of medium at 6 to 9 h postinfection (hpi). For cycloheximide reversal experiments, the medium was supplemented with 100 μg of cycloheximide per ml 1 h prior to and during infection. The infected cells were incubated in the presence of cycloheximide for 6 h. The monolayers were then washed twice with Tris-buffered saline and further incubated for 3 h in the presence of actinomycin D (10 μg/ml) and [^{35}S]methionine (100 μCi per plate). The labeled viral proteins were extracted from the infected cells and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (50, 55).

Nuclear runoff transcription. The nuclear runoff transcription assay was performed as described previously (20, 82, 95). The M13 probes used for detecting ICP22 and TK have been described previously (32, 58, 83); construction of M13-ICP6 and M13-βtub was described above. Typically, 3 μg of single-stranded M13 DNA containing a portion of the HSV type 1 (HSV-1) DNA sequences complementary to the mRNA species being detected was immobilized on nitrocellulose membranes. The ^{32}P-labeled runoff RNA was generated from infected-cell nuclei isolated at 6 hpi. For the infections done at a MOI of 10, the infected nuclei for each sample were derived from 5×10^6 cells. For the infections done at a MOI of 1, the infected nuclei were derived from 10^7 cells. All of the RNAs isolated from each sample (approximately 10^8 cpm/5×10^6 cells) was used to probe the filters above. The conditions for hybridization, washing, and RNase treatment of the probed filters were exactly as described previously (95). The dried filters were then exposed to film for autoradiography and quantitated with the AMBIS 4000 radioanalytic imaging system (AMBIS, Inc., San Diego, Calif.).

Colony-forming inhibition assays. Inhibition of colony formation by the different viral mutants was assayed as previously described (98). Briefly, Vero cells infected at different MOIs and control uninfected monolayers were trypsinized at 6 hpi to prepare single-cell suspensions that were then serially diluted and plated in medium supplemented with 20% serum. Two weeks after plating, the colonies were stained with crystal violet and counted. The fractions of surviving cells were represented relative to those obtained with the uninfected monolayers.

Measurement of β-gal expression. β-Gal was assayed in situ by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as previously described (2). The *o*-nitrophenyl-β-D-galactopyranoside (ONPG) cleavage assay was used for quantification of β-gal activity in cells. Cell lysates of infected Vero cell cultures on 60-mm petri dishes were prepared at the indicated times as previously described (2). The ONPG assay for β-gal was performed as previously described (2). The β-gal activity (in arbitrary units) was normalized to the protein concentration determined by using the Bio-Rad reagent.

RESULTS

In the absence of ICP4, the remaining IE proteins are overexpressed relative to the levels seen in cells infected with wild-type virus. In addition, low levels of RNA (about 1 to 5% of wild-type levels) of certain early genes can also be detected (44, 76). We have previously described mutants deficient in combinations of ICP4, ICP27, and ICP22 (98). These studies revealed that the simultaneous inactivation of ICP4, -27, and -22 results in the prolonged high-level expression of ICP6 and ICP0 and residual *tk* expression (98). Expression was prolonged due to the decreased toxicity associated with the inactivation of ICP22 in an ICP4- and ICP27-deficient background. It was hypothesized that expression remained at a high level due to the continued expression and accumulation of ICP0. However, the infected cells stopped dividing and eventually died. It was not possible to directly assess the contribution of ICP0 to the observed levels of gene expression. In the present study, we addressed the ability of ICP0 to affect gene expression and transcription, independent of the regulatory functions

TABLE 1. Growth of HSV-1 IE mutant strains in Vero, E26, and FO6 cells

Expt	Cell line (passage no.)	Virus yield (PFU/ml)		
		KOS	d92	n212
1	Vero	9.1×10^9	0	3.3×10^7
	E26	8.8×10^9	2.8×10^8	9.0×10^6
	FO6 (p11)	9.2×10^9	3.5×10^8	2.3×10^8
2	Vero	ND[a]	ND	2.1×10^7
	FO6 (p7)	ND	ND	8.3×10^8

[a] ND, not done.

of ICP4 and ICP27. The effect of ICP0 on survival of the infected cells was also determined. These studies were undertaken following the construction and analysis of a mutant simultaneously deficient in ICP4, ICP27, and ICP0.

Construction of an ICP4-, ICP27-, and ICP0-complementing cell line and an ICP4$^-$ ICP27$^-$ ICP0$^-$ virus. In contrast to mutants in ICP4 and ICP27, mutants in which ICP0 is inactivated can be propagated in cells that do not supply ICP0. However, the plating efficiency of ICP0 mutants on noncomplementing cells is markedly reduced (74, 88). We have previously constructed mutants from which ICP4 and ICP27 were simultaneously deleted and a complementing cell line (E26) in which to propagate and assay such viruses (76). Not surprisingly, E26 cells will inefficiently host mutants in ICP0 in a manner similar to that for nontransformed Vero cells. However, despite considerable effort, we were not able to isolate a virus using E26 cells that had both copies of the ICP0 gene mutated in the background of a genome from which ICP4 and ICP27 were deleted. Therefore, E26 cells were used to construct a cell line that simultaneously complemented ICP4, ICP27, and ICP0 mutants. These cells, designated FO6, were constructed as described in Materials and Methods and resulted in a 10- to 40-fold increase in the plating efficiency (Table 1) of an ICP0 mutant, n212 (6). This does not represent full complementation of an ICP0 mutant, since it was possible to obtain a cell line that resulted in 80-fold more plaques than those seen on Vero cells (data not shown). This cell line, L7, contains the genes for ICP0 and G418 resistance and was constructed as described in Materials and Methods. The plating efficiency of ICP0 mutants on FO6 cells also tended to decrease with passage (Table 1); therefore, FO6 cells were not used for plaque assays past passage 15. Because of the difficulty in obtaining full and consistent complementation of ICP0 deficiencies, stocks of viruses that have mutations in ICP0 often contain more infectious units than PFU. For this reason, the titer of ICP0 mutants used in this study was normalized with respect to the titer of the ICP0$^+$ viruses by determining the relative numbers of genomes in infected-cell nuclei at 6 hpi, the amount of a particular IE mRNA synthesized in the presence of cycloheximide, and the numbers of cells containing antigen expressed from the viral genome as measured by immunofluorescence. The number of infectious units of an ICP0 mutant was typically 3-fold higher than the titer on FO6 cells at passage 7 (data not shown). Table 1 also shows that FO6 cells were as efficient hosts for d92 (ICP4$^-$ ICP27$^-$) as E26 cells.

The isolation of FO6 cells enabled the construction of a mutant virus deficient in ICP4, -0, and -27. This was carried out in two steps. First, the gene for β-gal was inserted into the ICP0 locus of KOS, deleting the first exon and intron of ICP0, such that the initiator ATG of ICP0 would direct the translation of β-gal (Fig. 1B). This was performed by cotransfection of

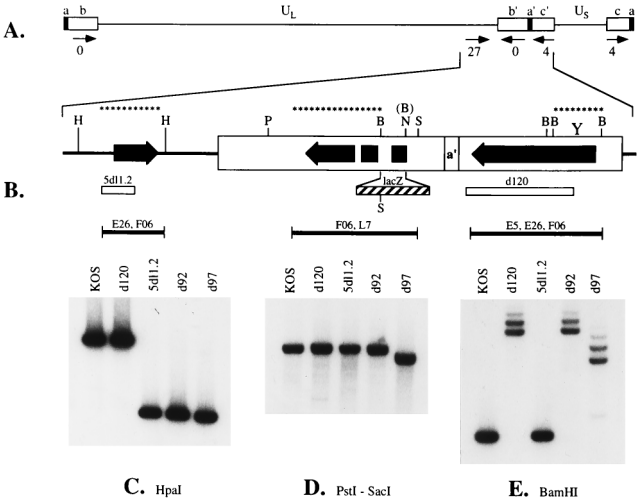

FIG. 1. Structure of the d97 virus with mutations in ICP4, ICP0, and ICP27. (A) The general structure of the genome; small arrows, locations of the IE genes. U_L and U_S, unique long and unique short sequences, respectively. (B) Expanded maps of ICP4, -0, and -27; boldface arrows, exon structures. The relevant restriction sites (B, *Bam*HI; H, *Hpa*I; N, *Nco*I; P, *Pst*I; and S, *Sac*I) are indicated. White bars, d120 and 5dl1.2 deletions in ICP4 and ICP27, respectively; striped bar, 0β-gal substitution in ICP0. Insertion of the *lacZ* sequence introduced a *Bam*HI restriction site (indicated in parentheses) next to the *Nco*I site. The bracketed boldface lines indicate the sequences present in the different complementing cell lines: E5 (4^+), E26 (4^+ 27^+), FO6 (4^+ 27^+ 0^+), and L7 (0^+). Viral DNAs from cells infected with wild-type KOS, d120 (4^-), 5dl1.2 (27^-), d92 (4^- 27^-), and d97 (4^- 27^- 0^-) were isolated; cleaved with *Hpa*I (C), *Pst*I-*Sac*I (D), or *Bam*HI (E); and analyzed by Southern blotting. The following nick-translated IE fragments used to probe the corresponding blots are indicated by lines of asterisks: a 2.4-kb *Bam*HI-*Sac*I fragment derived from plasmid pKHX-BH (5) for the ICP27 probe, a 3-kb *Bam*HI-*Hpa*I fragment derived from plasmid pW3ΔHS8 (19) (where the *Hpa*I site was converted to a *Pst*I site) for the ICP0 probe, and the 1.8-kb *Bam*HI-Y fragment from plasmid pKBY (76) for the ICP4 probe.

L7 cells with KOS DNA and plasmid p0β. Progeny that stained blue with X-Gal were further plaque purified and analyzed by Southern blot hybridization to obtain an isolate in which both ICP0 loci were modified by insertion of the β-gal coding sequence. This virus was referred to as 0β. Second, the virus 0β was used to coinfect FO6 cells with the virus d92 (ICP4$^-$ ICP27$^-$ [76]). The progeny of this infection that produced blue plaques on FO6 cells and failed to form plaques on L7 cells were analyzed by Southern blot analysis for incorporation of the d120 (ICP4), 5dl1.2 (ICP27), and 0β (ICP0) mutant alleles. One isolate, d97, contained all three mutant alleles and was further plaque purified on FO6 cells.

Figure 1 shows a Southern blot in which restriction digests of KOS, d120, 5dl1.2, d92, and d97 were probed to demonstrate the structures of the ICP4, ICP27, and ICP0 genes in d97. Figure 1A shows the locations of the ICP0, ICP4, and ICP27 genes relative to the HSV genome. Figure 1B shows an expanded map of a section of the genome, highlighting the sequences used to probe the Southern blots (asterisks); the locations of the coding sequences for ICP4, -0, and -27; relevant restriction sites; the mutant alleles; and the sequences present in complementing cell lines. To visualize the ICP27 sequences in the viruses, electrophoretically separated *Hpa*I fragments of the indicated viruses were probed with the indicated fragments. The viruses 5dl1.2, d92, and d97 all contained the shortened *Hpa*I fragment as a consequence of the 1.2-kb deletion in ICP27 (Fig. 1C). Therefore, d97 contained the ICP27 mutant allele from 5dl1.2 (58). To visualize the ICP0 sequences in the viruses, electrophoretically separated *Pst*I-*Sac*I fragments of

the indicated viruses were probed with the indicated fragments. The insertion of β-gal sequences into the ICP0 locus provides an additional *Sac*I site (Fig. 1B). Digestion of d97 DNA with *Sac*I and *Pst*I produced a smaller version of the *Sac*I-*Pst*I fragment relative to the other viruses (Fig. 1D). Moreover, there were no detectable fragments in the d97 digests characteristic of the wild-type ICP0 allele. This demonstrates that d97 contained the β-gal insertion at both ICP0 loci. To visualize the ICP4 sequences in the viruses, electrophoretically separated *Bam*HI fragments of the indicated viruses were probed with the indicated fragments. d120 and d92 contained the d120 allele (17), as indicated by the fusion of *Bam*HI Y sequences to the *Bam*HI fragment spanning the joint, by virtue of the deletion in ICP4. This fragment extended into ICP0 (Fig. 1B). d97 did not contain *Bam*HI Y and possessed a shortened version of the fragment characteristic of d120 (Fig. 1E) due to the incorporation of a *Bam*HI site adjacent to the *Nco*I site at the beginning of ICP0 (Fig. 1B). Moreover, there were no detectable fragments in the d97 digests characteristic of the wild-type ICP4 allele, demonstrating that d97 contained the ICP4 mutant allele from d120 at both genomic loci. Therefore, d97 is mutant in both copies of ICP4, both copies of ICP0, and ICP27. In addition, it possesses an insertion of the β-gal gene in both copies of ICP0.

Figure 1 shows that d97 contains the intended mutations in the genes for ICP4, ICP0, and ICP27 and thus should not express these proteins. To determine if this is the case, a cycloheximide reversal experiment (42) was performed on cells infected with the same viruses used in the experiment depicted in Fig. 1. The autoradiogram of the SDS-PAGE gel from this experiment is depicted in Fig. 2. ICP4 was not synthesized in d120-, d92-, and d97-infected cells. ICP27 was not synthesized in 5dl1.2-, d92-, and 97-infected cells. As expected, ICP22 was synthesized in all of the infected cell cultures. In addition, ICP0 was not synthesized in d97-infected cells; instead, a 115-kDa protein corresponding to β-gal was observed. Furthermore, ICP0 was not detected by Western blot analysis of extracts from d97-infected Vero cells (data not shown). The expression of β-gal as an IE protein is consistent with the engineering of d97 to express β-gal from the ICP0 promoter. Therefore, the d97 genome does not express ICP0, ICP27, and ICP4 and expresses β-gal as an IE gene.

Expression of the viral genome in the absence of ICP4, ICP27, and ICP0. Of the IE proteins, ICP4, ICP0, and ICP27 have the most-profound effects on the expression of viral genes. The mutant d97 provides an opportunity to view viral gene expression in the absence of the activities of these gene products, possibly more closely resembling the situation in the absence of prior viral protein synthesis. Therefore, expression from the d97 genome is likely to be a sole function of cellular factors and virion components.

The experiment described in the legend to Fig. 2 examined gene expression in the absence of prior viral protein synthesis. This type of experiment predominantly limits viral gene expression to that of the IE genes. The experiment depicted in Fig. 3 was performed in parallel to that of Fig. 2, except that the macromolecular synthesis inhibitors were omitted in order to observe the effects of the expressed IE genes on subsequent gene expression. Figure 3 shows the typical permissive pattern of viral protein synthesis from 6 to 9 hpi in cells infected with wild-type virus (KOS). In the absence of ICP4 (d120), ICP6 was abundantly expressed and was expressed to somewhat reduced levels in the absence of ICP27, regardless of whether ICP4 was expressed. However in the d97 background, ICP6 was undetectable. In fact, the polypeptide profile of d97 in Fig. 3 is very similar to that observed in the absence of prior viral protein synthesis (Fig. 2). This is the only mutant background thus far examined to produce this result. From the standpoint of viral vectors, this may be desirable, since the expression of genes inserted into the HSV background will most likely be expressed and possibly regulated as a function of cellular factors without the involvement of viral regulatory proteins.

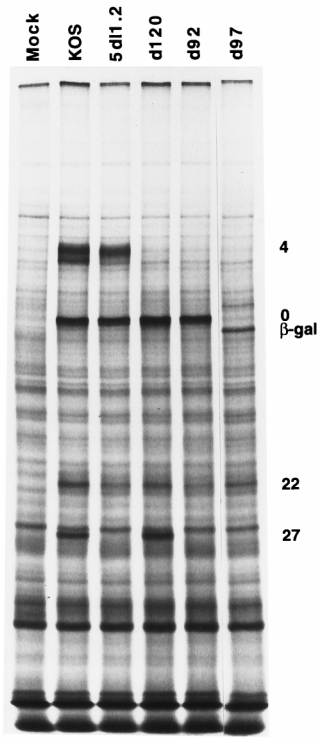

FIG. 2. Synthesis of IE proteins in cells infected with the d97 virus. A cycloheximide reversal experiment was conducted in Vero cells infected with the indicated viruses at a MOI of 10 PFU per cell as described in Materials and Methods. The infected cultures were labeled with 100 μCi of [^{35}S]methionine per 35-mm plate at 6 to 9 hpi and were processed for SDS-PAGE. The positions of the IE polypeptides and the β-gal protein expressed from the ICP0 loci are indicated on the right.

It is accepted that the expression of ICP6 is different from that of other classical early genes in that it is abundantly expressed in infected cells in the absence of ICP4 (17). In addition, it is expressed to a much greater level in the absence of ICP4 than in the absence of prior viral protein synthesis (17). This led to the suggestion that other IE proteins mediate the elevated levels of ICP6 expression in the absence of ICP4 (17). We have previously shown that the deletion of ICP27 from the background of an ICP4 mutant reduced the levels of ICP6 protein and RNA at 6 hpi relative to those seen in the ICP4 mutant-infected cells, suggestive of the involvement of ICP27 in determining the expression levels of ICP6 (76). Other investigators have shown that ICP0 also contributes to the level of ICP6 expression in infected cells (21, 90). Figure 4 is a Northern blot of electrophoretically separated RNAs isolated from infected cells at 6 hpi and probed for ICP6 mRNA. This experiment was performed to determine the relative contributions of ICP4, ICP0, and ICP27 to the accumulation of ICP6 mRNA. Deletion of ICP4, ICP27, or ICP0 resulted in reduction in accumulation of ICP6 mRNA. This is consistent with previous findings (21, 90), although the contribution of ICP4 to the level of ICP6 RNA was substantial. This has been largely overlooked due to the high level of expression of ICP6 in the

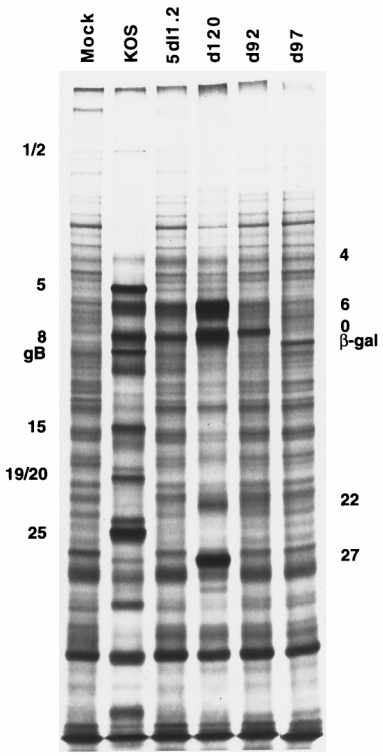

FIG. 3. Synthesis of viral polypeptides in cells infected with the d97 virus. Vero cells were infected with the indicated viruses at a MOI of 10 PFU per cell, pulse-labeled with 100 μCi of [^{35}S]methionine per 35-mm plate at 6 to 9 hpi, and processed for SDS-PAGE. The positions of the IE polypeptides and the β-gal protein expressed from the ICP0 loci are indicated on the right. Some of the early and late viral proteins are also indicated on the left.

absence of ICP4. However, it is consistent with the general ability of ICP4 to activate polymerase II promoters. Consistent with previous results (76), deletion of ICP27 in addition to ICP4 further reduced the accumulation of ICP6 mRNA, and the additional deletion of ICP0 resulted in no detectable ICP6 RNA. Despite these findings, the levels at which ICP27 and ICP0 function to elicit these effects have not been established. The following experiments address the effect of ICP0 on the expression of viral genes in the absence of ICP4 and ICP27 and determine if the observed effects are due to ICP0-dependent changes in the transcription rates of the analyzed genes.

In the first experiment, the levels of ICP6, ICP22, and *tk* accumulating in Vero cells infected with d92 (ICP4$^-$ ICP27$^-$) and d97 (ICP4$^-$ ICP27$^-$ ICP0$^-$) were compared. In addition,

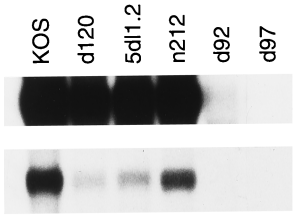

FIG. 4. Accumulation of ICP6 mRNA in cells infected with d97. Vero cells were infected with the indicated viruses at a MOI of 10 PFU per cell. Ten micrograms of total infected cellular RNA prepared from cells harvested at 6 hpi per sample was used in Northern blot analysis. The 1.6-kb *Sca*I fragment isolated from plasmid pKX2-βG3 (34) was used as a probe. A long exposure and a short exposure of the gel are shown.

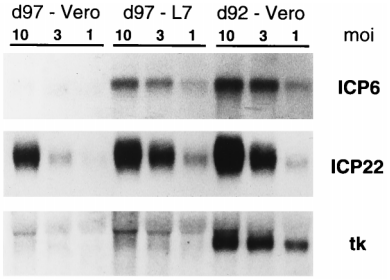

FIG. 5. Accumulation of IE and early mRNAs as a function of ICP0 at high and low MOIs. Vero and L7 cells were infected with d97 or d92 at a MOI of 10, 3, or 1 as indicated. Total RNA was prepared from cells harvested at 6 hpi and analyzed by Northern blot hybridization. For the blots probed for ICP22 and ICP6, 10 μg of total cell RNA per sample was used. For the blot probed for *tk*, 40 μg of total RNA was loaded per lane, and the blot was exposed considerably longer to increase the sensitivity of detection.

the levels of these transcripts were also compared to those in d97-infected L7 cells. L7 cells supply complementing levels of ICP0. Therefore, this experiment observes the contribution of ICP0 to the levels of transcript accumulation by providing ICP0 from two different sources. The second set of experiments was performed to determine if the levels of these transcripts were affected by ICP0 at the level of transcription rate.

ICP6 mRNA was clearly detectable in d92-infected Vero cells at a MOI of 1 PFU/cell and increased as a function of MOI (Fig. 5). Consistent with the results shown in Fig. 4, ICP6 RNA was not detected in d97-infected Vero cells at any of the MOIs tested. However, the accumulation of ICP6 RNA was readily seen in d97-infected L7 cells. Therefore, ICP0 clearly results in elevated levels of ICP6 RNA. *tk* is another early gene that differs from ICP6 in that its expression is more markedly reduced in the absence of ICP4 (or ICP4 and ICP27). The level of *tk* RNA accumulating in d92-infected Vero cells is detectable (Fig. 5) and has been estimated to be less than 1% of that seen in wild-type-virus-infected cells (76). Like ICP6, *tk* RNA was clearly detectable in d92-infected Vero cells but was not detected at any MOI in d97-infected Vero cells. Therefore, ICP0 contributes to the levels of *tk* RNA in the absence of ICP4. The inability to detect *tk* mRNA in d97-infected L7 cells may reflect the limiting quantity of ICP0 provided by L7 cells and the fact that the detection of *tk* mRNA is at the assay's limit of sensitivity. ICP22 is an IE gene and, therefore, is transcribed in the absence of viral protein synthesis by virtue of the activity of VP16 in the virion. ICP22 RNA was abundantly expressed as a function of MOI in d92-infected Vero cells. Lower levels of ICP22 RNA accumulated in d97-infected Vero cells at comparable MOI. Again, the levels of ICP22 RNA were greater in d97-infected L7 cells than in d97-infected Vero cells and were similar to the levels observed in d92-infected Vero cells. Therefore, the accumulation of ICP22 RNA is a function of ICP0 in the absence of ICP4. This demonstrates that ICP0 functions in the absence of ICP4, and the general increase in the abundance of mRNA from different promoters is consistent with previous cotransfection experiments demonstrating that ICP0 is a promiscuous activator of gene expression (12, 26, 28, 66, 67, 70).

The approach applied above was also used to determine if ICP0 elevated the expression of the three different types of HSV genes (ICP22, ICP6, and *tk*) at the level of transcription rate. Instead of harvesting the infected cells and isolating total RNA, nuclei were isolated and used in in vitro transcription runoff experiments. Nascent transcripts were extended by incubation of the nuclei with nucleoside triphosphates, including

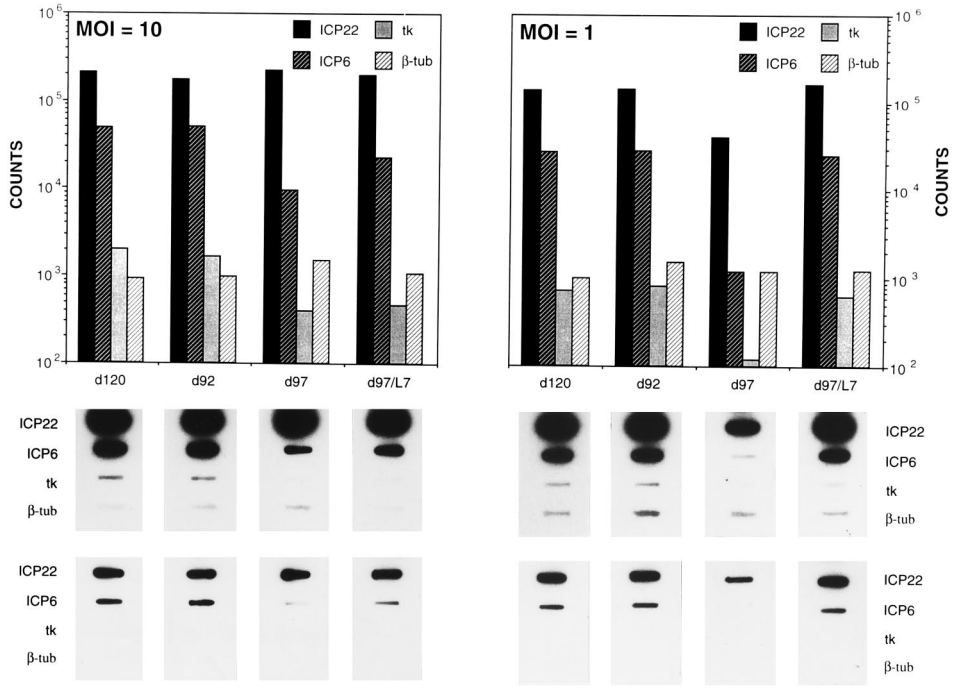

FIG. 6. Transcription rates of viral and cellular genes as a function of ICP0 at high and low MOIs. Vero and L7 cells were infected with the indicated viruses at a MOI of 10 or 1 PFU per cell. Nuclei were isolated from cells infected at 6 hpi and used for transcription runoff reactions as described in Materials and Methods. The in vitro-labeled RNA samples were hybridized to filters on which single-stranded DNA probes containing ICP22, ICP6, *tk*, and β-tubulin sequences complementary to the mRNA were immobilized. No hybridization to the strand homologous to the mRNA was detected, with the exception of a very low level of hybridization to the ICP22 gene (data not shown). The blots were quantitated as described in Materials and Methods, and the results are summarized at the top in graph form. Autoradiographs of the blots are also shown, including long (top row) and short (bottom row) exposures.

[α-^{32}P]CTP. The radioactively labeled RNA was then used as a probe to hybridize to filters containing single-stranded DNA probes representative of the ICP22, ICP6, and *tk* genes. β-tubulin was used as an example of a cellular gene. Previous results from others (1) and those of Fig. 5 suggest that the relative contributions of VP16 and ICP0 to the levels of expression of IE genes may be dependent on MOI. Therefore, the runoff experiments were performed at MOIs of 1 and 10 PFU per cell.

Figure 6 shows two exposures of the runoff signals representing transcription of the sense strands of the indicated genes. Above the autoradiographic images is a quantitative analysis of the radioactivity present in each of the signals. The patterns of transcription rates of the ICP22, ICP6, and *tk* genes in d120-infected cells (Fig. 6) were consistent with previously published results (20). The transcription rate of the ICP22 gene was approximately 2 orders of magnitude greater than those of the *tk* gene and the cellular β-tubulin gene. ICP6 was transcribed at a rate intermediate between ICP22 and *tk*. The rates of transcription of these genes in d92-infected cells were not significantly different from those observed with d120-infected cells, suggesting that under these conditions, ICP27 had little effect on transcription. However, the additional inactivation of ICP0 resulted in reduction in the rates of transcription of all of the viral genes tested. This effect was most pronounced at the lower MOI. At a MOI of 1 PFU per cell, the rates of transcription of the ICP22, ICP6, and *tk* genes in the d97 genome in Vero cells were 4-, 30-, and 7-fold lower, respectively, than those in the d92 genome. d97 infection of L7 cells restored transcription to the levels seen in d92-infected Vero cells. The same trend, albeit to a lesser extent, was observed at a MOI of 10 PFU per cell. Rates of ICP6 and *tk* transcription were five- and fourfold lower, respectively, in d97-infected Vero cells than those in d97-infected Vero cells. ICP22 transcription was unaffected. Additionally, complementation by L7 cells was not complete at this MOI, probably reflecting the limiting quantity of ICP0 expressed from L7 cells. Lastly, the rates of transcription of the cellular β-tubulin gene were similar in all of the genetic backgrounds, indicating that ICP27 and ICP0 had no effect on the transcription of this cellular gene.

From the above studies, it can be concluded that ICP0 affects gene expression in the absence of ICP4 and that it does so by increasing the transcription rates of genes. Without ICP0, the transcription rates of viral genes such as ICP6 and *tk* are very low and are similar to the rate of transcription of a cellular gene.

Transgene expression and cell survival. Viruses lacking ICP4 have been pursued as potential vehicles for transgene delivery (13). However, the high level of IE protein expression from these viruses is toxic to cells. Johnson and colleagues have shown that ICP4, -0, -22, and -27 are individually toxic to cells (46). We have provided the means to delete sets of the genes encoding these proteins and to assess the appropriateness of the resulting viral constructs as potential vectors (98). Previously, we described a virus that does not express ICP4, ICP27, and ICP22 (98). Infection of cells with this virus results in prolonged cell survival and gene expression from the viral genome (98). Thus far, the results of the present study that may have implications for transgene expression and cell survival in d97-infected cells are the following. (i) In the absence of ICP4, ICP27, and ICP0, expression from the viral genome most closely approximates the expression of genes as a function of cell factors alone. Exceptions to this are the HSV IE genes, which are activated by the virion component, VP16. (ii)

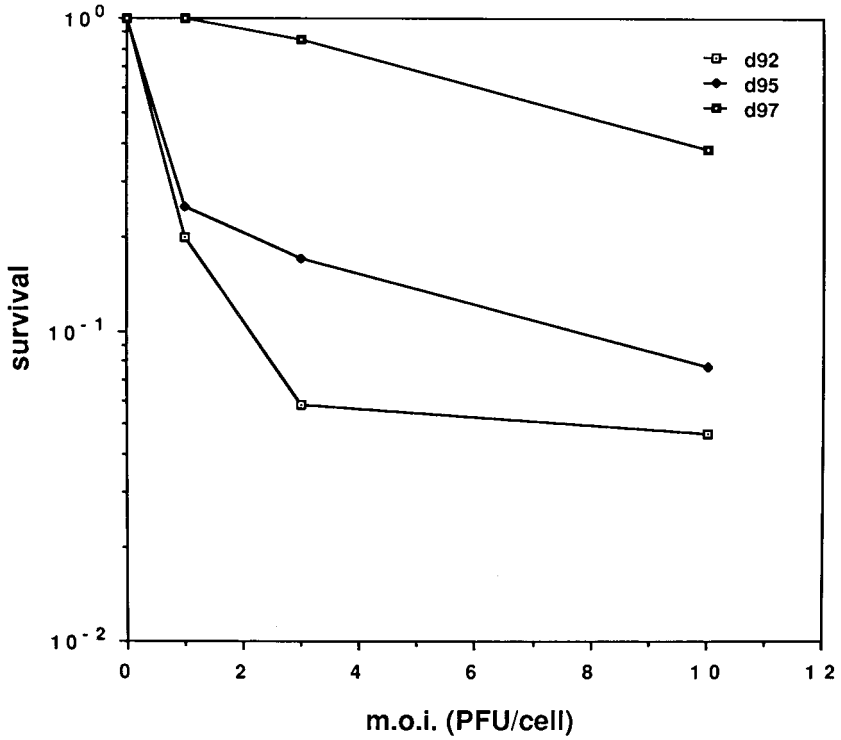

FIG. 7. Survival of cells infected with d97. Monolayers of Vero cells were infected with the viruses d92 (ICP4$^-$ ICP27$^-$), d95 (ICP4$^-$ ICP27$^-$ ICP22$^-$), and d97 (ICP4$^-$ ICP27$^-$ ICP0$^-$) at the indicated MOIs. At 6 hpi, the monolayers were harvested and plated out for CFU as described in Materials and Methods. Colonies were counted at 14 days postplating. Shown are the surviving fractions relative to uninfected cells. It was not possible to observe infected L7 cells at 14 days postinfection due to the destruction of the monolayer.

The simultaneous abrogation of ICP4, ICP27, and ICP0 activity results in an extremely low level of early gene expression. Therefore, the potential contribution of viral early proteins to virus-mediated toxicity would be reduced. (iii) ICP4, ICP0, and ICP27 have all been shown to be involved in virus-mediated toxicity (46). The elimination of these activities should improve long-term cell survival. (iv) Relatively high levels of IE gene transcription still occur in the d97 background. d97 contains the gene for β-gal under the control of the HSV ICP0 promoter, and it is expressed as an IE gene. Therefore, it can be used as a convenient model for transgene expression in the d97 background. The following experiments address cell survival and transgene expression in d97-infected cells.

To assess the toxicity of d97 in Vero cells, monolayers were infected with d97 and d92 at different MOIs. Six hours later, the monolayers were trypsinized to generate single-cell suspensions, and dilutions of these suspensions were plated for CFU. Enhanced cell survival was observed for d97 relative to d92 (Fig. 7). However, at the highest MOI, the survival of d97-infected cells was reduced to 40%. This most probably reflects the levels of expression of ICP22 at the higher MOI. We have previously shown that the inactivation of ICP22 in an ICP4$^-$ ICP27$^-$ background results in prolonged cell survival (98). Cells infected with an ICP4$^-$ ICP27$^-$ ICP22$^-$ virus (d95) do not demonstrate the characteristic rounding up and detachment from the monolayer seen with mutants blocked early in the HSV life cycle. A survival curve for d95-infected cells was included in the experiment shown in Fig. 7 to demonstrate that in this assay, d97 was less toxic to cells than d95.

Given that there was a substantial fraction of cells surviving infection with 3 PFU of d97 per cell, an experiment was then performed to determine the extent of transgene (β-gal) expression as a function of time postinfection with a MOI of 3 PFU per cell. d97-infected Vero and L7 cells stained with X-Gal at 3 and 14 days postinfection are shown in Fig. 8. Approximately 50% of the d97-infected Vero cells stained blue at 3 days postinfection. They are uniformly blue at 1 day postinfection (data not shown). The reduction in the number of blue cells at 3 days probably reflects the fact that the Vero cells double at least once in this time. An important point is that the β-gal-expressing cells at 3 days postinfection were morphologically normal. By contrast, the d97-infected L7 monolayer was virtually destroyed at 3 days postinfection, and the remaining cells were intensely blue. This observation is again indicative of both the activation function of ICP0 and its contribution to toxicity. The increased toxicity of d97 in L7 cells may also be due to an increase in the level of ICP22 expression as a consequence of ICP0. The histogram in Fig. 9 shows the amount of β-gal activity as a function of time postinfection for the 2-week period. The amount of β-gal enzyme activity is represented as units per microgram of cell extract. The units of β-gal enzyme activity in d97-infected Vero cells was greatest at 1 day postinfection, decreasing to 5% of this level by 14 days postinfection. In L7 cells, the level of β-gal was 2-fold greater than that in Vero cells at 1 day postinfection. However, by 3 days postinfection, the level of β-gal in L7 cells was 20-fold greater than that in Vero cells. It was not possible to measure β-gal activity in infected L7 cells beyond 3 days postinfection due to the destruction of the monolayer. The increased accumulation of β-gal over time in L7 cells is most probably due to complementation by ICP0 from L7 cells. The difference between the relative accumulation of β-gal in Vero cells and that in L7 cells

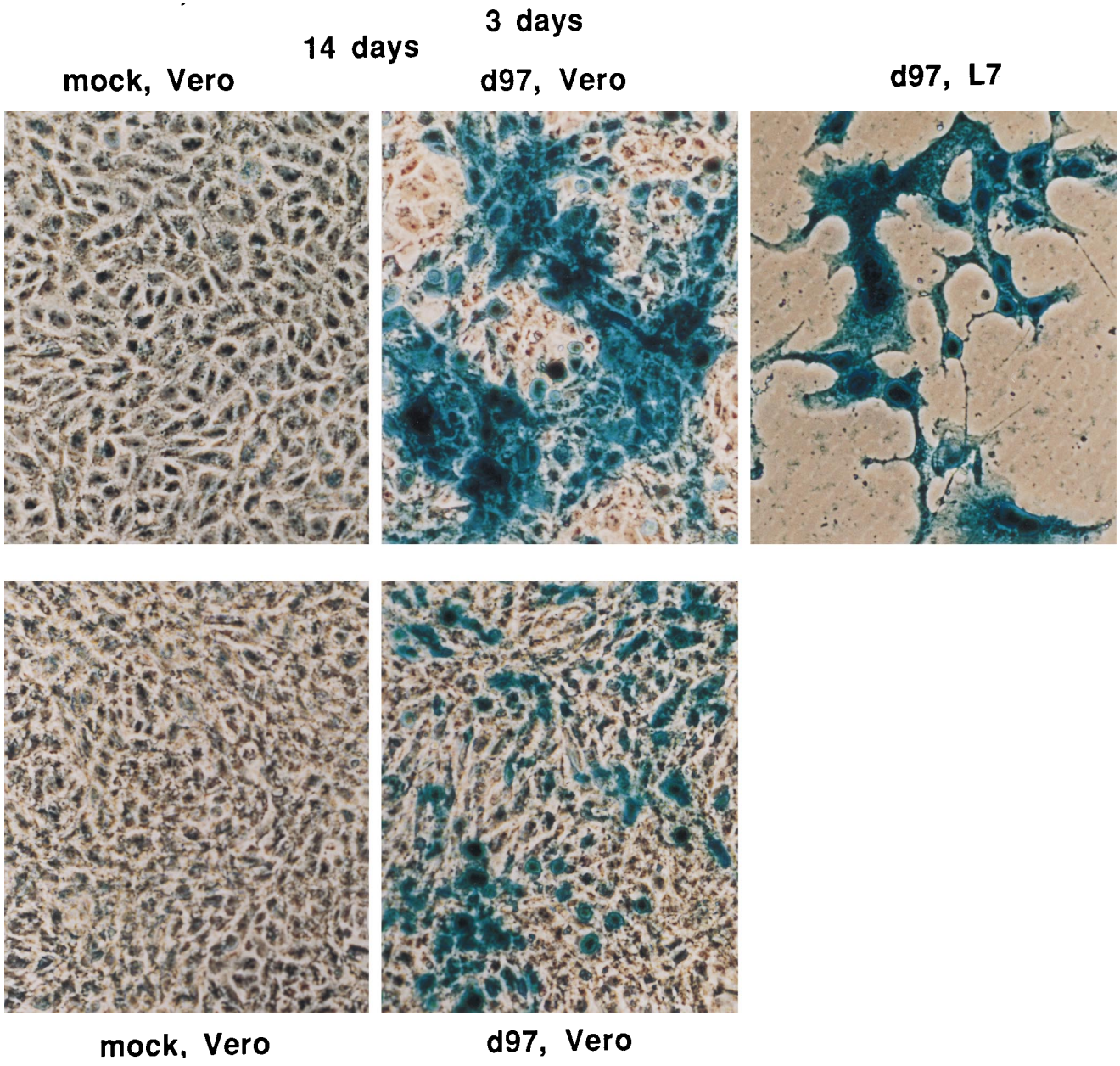

FIG. 8. Transduction of Vero cells by d97. Vero and L7 cells were infected with d97 at a MOI of 3 PFU per cell. At 3 and 14 days postinfection, the monolayers were fixed and stained with X-Gal for detection of β-gal. The samples were viewed and photographed through a 40× phase-contrast objective. Also shown are fixed and stained uninfected Vero cells 3 and 14 days after the duplicate cultures were infected.

at 1 and 3 days postinfection may be due to the turnover of VP16, which is supplied by the virion and is not expressed following infection.

DISCUSSION

Many studies have shown that ICP0 will induce the expression of most test genes in transient assays (12, 26, 28, 66, 67, 70). Its function is required during viral infection for the expression of wild-type levels of all classes of HSV genes, particularly at low MOIs (74, 88). We have shown that ICP0 contributes to elevated levels of viral gene expression and transgene expression in the absence of ICP4. The increase in gene expression as a function of ICP0 is largely due to the elevation of transcription. Without ICP0, in an ICP4-deficient background, gene expression is dramatically reduced, as is cellular toxicity. The results of this study pertain to how ICP0 potentiates gene expression and provide important insights into the use of HSV as a gene delivery vehicle.

Stimulation of transcription in the absence of ICP4 and ICP27. In previous studies, the effects of ICP0 have been more difficult to examine because of the simultaneous expression of

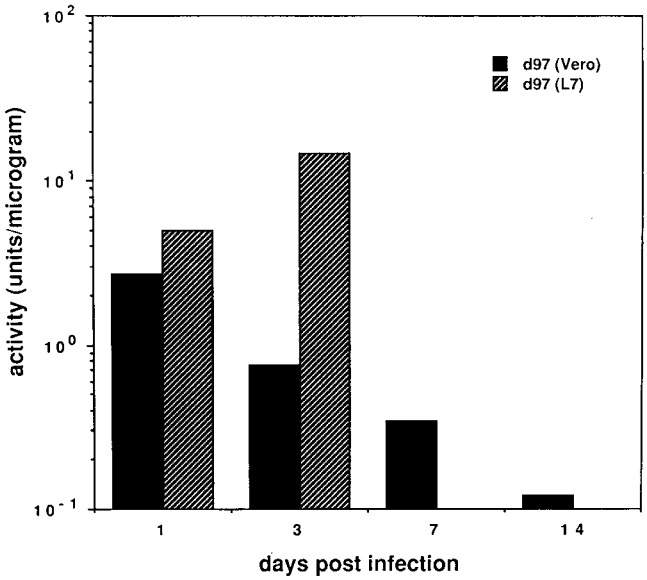

FIG. 9. Quantitation of β-gal activity in d97-infected cells as a function of time postinfection. Vero and L7 cells were infected with d97 at a MOI of 3 PFU per cell. These infections were performed in parallel to those performed to generate Fig. 8. At the indicated times postinfection, the monolayers were harvested, and extracts were prepared for the quantitation of β-gal activity. The assay for β-gal as well as the procedures for preparing the extracts is described in Materials and Methods. Shown are the values of β-gal activities in arbitrary units per microgram of extract. This experiment was performed on Vero and L7 cells at 1 and 3 days postinfection. Only Vero cells were analyzed at days 7 and 14, because L7 cells would be completely destroyed by the virus at these times.

ICP4. To some degree, the activities of these two proteins are redundant, because they both broadly activate gene expression. Also, in the context of virus infection, it appears that ICP4 activates gene expression to a greater extent. This presumably accounts for the facts that ICP4 is absolutely required for viral growth and that ICP0 is not. The present study examines the activity of ICP0 in the absence of ICP4. This was done to determine if ICP0 influences gene expression from the viral genome in the absence of ICP4 and to more clearly establish if ICP0 can influence the transcription rates of viral genes. It has previously been shown that in the absence of ICP4, the levels of some of the expressed genes are influenced by the presence of ICP27 (76). Consistent with previous studies of ICP27 (40, 59, 60, 80), we have subsequently found that the effect of ICP27 in the ICP4$^-$ background is predominantly posttranscriptional (75). Consequently, ICP27 was also deleted from the viral genome in order to more clearly examine the effects of ICP0 on gene expression.

In the present study, genes under the control of HSV IE promoters were abundantly expressed in the absence of ICP4, ICP27, and ICP0, relative to the other genes. In d97-infected cells, the transcription rate of the ICP22 gene was approximately 3 orders of magnitude greater than that of the *tk* gene (Fig. 6). While the presence of ICP0 had a more substantial effect on the transcription of ICP6 and *tk*, it only marginally increased the transcription of ICP22 at a MOI of 1 PFU per cell. The signals in runoff transcription experiments reflect the numbers of RNA polymerase molecules on the gene when the nuclei were isolated and thus reflect the frequency of transcription initiation (95). It may be that by virtue of the activation function of VP16, the loading of polymerase II on the ICP22 gene approaches saturation. In this case, ICP0 is expected to have a minimal effect on transcription.

Interestingly, Ace et al. also found that mutations that affect the VP16 activation function significantly affect IE transcription only at low MOI and that the reduction in IE gene expression as a consequence of the VP16 mutation can be partially overcome by ICP0 (1). Their study, in combination with our results, suggests that VP16 and ICP0 are indeed redundant and that they increase the initiation of transcription of HSV IE genes by two different mechanisms. The possession by HSV of an activity that stimulates HSV IE transcription by a mechanism independent of virus structural components is advantageous for activating the genome in situations in which the structural components are not present, as in reactivation from latency. Along these lines, the work of Schaffer and colleagues (6, 51) has shown that ICP0 helps the virus reactivate from latency. Additionally, ICP0 was shown to be sufficient to reactivate HSV from an in vitro latency model (41, 100).

VP16 and ICP0 are redundant with respect to promoting the elevated rate of transcription of IE genes and viral growth. ICP4 and ICP0 independently increase the rates of transcription of viral early genes. In our studies, both the *tk* and the ICP6 genes were activated as a consequence of ICP0 in the absence of ICP4. These results, taken in combination with previous results showing that ICP0 increases the expression of all classes of HSV genes, suggest that ICP0 is probably required for the optimal transcription of most HSV genes. While these studies do not address the potential synergy between ICP0 and other IE proteins (24, 25, 29, 70), they do demonstrate that ICP0 can increase the transcription rates of HSV genes independently of ICP4 and ICP27. In addition, while there are no examples at present of early or late genes that are solely activated by ICP4 or ICP0 in virus infection, it is likely that the relative contributions of these proteins to the level of activation of a particular gene may differ. The ICP6 and *tk* genes provide an example of this. Both are activated by ICP0, albeit to much different extents.

ICP6 is different from most early genes in that its expression can be detected in the absence of ICP4. Several studies examining the effects of HSV regulatory proteins on the expression of ICP6 have been published (21, 90, 99). The results of our studies are consistent with previous work which shows that ICP6 is substantially activated by ICP0 (21, 90, 99). The runoff experiments (Fig. 6) show that ICP0 increases the transcription rate of the ICP6 gene. However, our results also show that the level of ICP6 mRNA is decreased in the absence of either ICP4, ICP27, or ICP0 (Fig. 4). Due to the extremely low level of ICP6 mRNA in the absence of ICP4, ICP27, and ICP0, our results appear to conflict with those suggesting that VP16 activates the ICP6 gene (99). Therefore, if VP16 induces transcription of the ICP6 gene, it does not act on the ICP6 promoter to the same extent as it acts on the IE promoters. It should also be noted that ICP6 is not readily visible in cycloheximide reversal experiments (16) (Fig. 2). The difference in observations may in part be explained by the fact that the previous study was performed with transient assays in the HSV-2 system (99).

The nuclear runoff studies and the experiments examining the levels of RNA and protein indicate that ICP0 generally increases the transcription rates and expression of different types of HSV genes. At present, it is difficult to reconcile the stimulation of transcription with other observations regarding ICP0. ICP0 has been shown to interact with a 135-kDa cellular protein (62, 63) and localizes to ND10 complexes and dissociates them (27, 56, 57). Additionally, when ICP0 is overproduced in the absence of ICP4, ICP27, and ICP22, extremely high levels of gene expression are accompanied by the inhibition of cell division (98). That some of these phenomena are

connected is suggested by studies describing mutants unable to bind the 135-kDa cellular protein or to disperse ND10 structures that are also defective for transactivation and virus replication (27, 56, 62). Therefore, the effect of ICP0 on transcription may be indirect. It may be that ICP0 stimulates transcription not by interacting with the basal transcription machinery (as does ICP4 and VP16) but instead by altering the physical state or compartmentalization of the transcription machinery or the template or some other event upstream of the formation of transcription initiation complexes. Alternatively, ICP0 may be affecting these apparently diverse phenomena by independent mechanisms. Also, given that ICP0 may be influencing a number of host cell processes, it is possible that it increases gene expression by mechanisms in addition to increasing transcription initiation.

ICP4-, ICP27-, and ICP0-defective viruses as gene transfer vectors. We have previously shown that a virus lacking ICP4, ICP27, and ICP22 is greatly reduced in toxicity relative to other viruses from which subsets of these genes have been deleted (98). However, the colony-forming ability of cells infected with an ICP4, ICP27, and ICP22 mutant (d95) is impaired due to the inhibition of cell division (98). d97 is significantly less toxic to cells than d95 from the standpoint of colony-forming ability (Fig. 7). Therefore, as speculated in the previous study (98), expression of ICP0 may influence the division potential of the cells or cell cycle progression. The toxicity of d97 at high MOI (Fig. 7) may reflect the increased accumulation of ICP22 (Fig. 5). This hypothesis is consistent with the previous observation that the inactivation of ICP22 in an ICP4- and ICP27-deficient background resulted in prolonged cell survival (98). If true, the further inactivation of ICP22 from the d97 background may result in even more-reduced toxicity.

While reduced toxicity is one consequence of the deletion of ICP0 in an ICP4- and ICP27-deficient background, reduced gene expression is another. Genes in the viral genome are transcribed at a reduced level in the absence of ICP0. The model transgene used in this study, ICP0–β-gal, was maximally expressed in Vero cells at 1 day postinfection. This early high level of expression is due to the activity of VP16 in the virion. Presumably, with time this protein turns over and expression decreases. This is consistent with the lower β-gal activity at 3 days postinfection (Fig. 9). However in L7 cells, which supply ICP0, the expression of β-gal remains high at 3 days postinfection. This is similar to the situation seen with the ICP4, ICP27, and ICP22 mutant (d95), in which expression remained at a high level for the life span of the infected cells (98). Therefore, in the absence of ICP0, transgene expression is expected to be solely a consequence of cellular factors. This may be a low level of expression, depending on the promoter, but it may also provide a situation in which genes inserted into the virus may be more faithfully controlled by cellular mechanisms.

The abrogation of ICP0 may also provide a background that will not induce other agents that may be undesirable. ICP0 is required for the efficient reactivation of HSV from latency (6, 14, 51), and when expressed from a recombinant adenovirus, it is sufficient to reactivate HSV in a model latency system (100). Additionally, ICP0 is required to potentiate the growth of or to reactivate human immunodeficiency virus (HIV) under certain circumstances (33). In the latter experiments, it was also found that ICP4 mutants were able to turn on HIV. Therefore, the elimination of ICP0 from IE-defective mutants will reduce the toxicity of the vector and provide a backbone with a reduced potential to activate other pre-existing agents in vivo.

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REFERENCES

1. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J. Virol. **63:**2260–2269.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1991. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J. Virol. **46:**371–377.
4. **Bond, J. F., G. S. Robinson, and S. R. Farmer.** 1984. Differential expression of two neural cell-specific β-tubulin mRNAs during rat brain development. Mol. Cell. Biol. **4:**1313–1319.
5. **Bond, V. C., and S. Person.** 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology **132:**368–376.
6. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J. Virol. **66:**2904–2915.
7. **Cai, W. H., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J. Virol. **67:**7501–7512.
8. **Campbell, M. E., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate-early transcription. J. Mol. Biol. **180:**1–19.
9. **Carrozza, M. J., and N. A. DeLuca.** 1996. Interaction of the viral activator protein ICP4 with TFIID through TAF250. Mol. Cell. Biol. **16:**3085–3093.
10. **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.
11. **Chapman, C. J., J. D. Harris, M. A. Hardwicke, R. M. Sandri-Goldin, M. K. Collins, and D. S. Latchman.** 1992. Promoter independent activation of heterologous virus gene expression by the herpes simplex virus immediate-early protein ICP27. Virology **186:**573–578.
12. **Chen, J., X. Zhu, and S. Silverstein.** 1991. Mutational analysis of the sequence encoding ICP0 from herpes simplex virus type I. Virology **180:** 207–220.
13. **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.
14. **Clements, G. B., and N. D. Stow.** 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate-early gene 1 is latency competent in mice. J. Gen. Virol. **70:**2501–2506.
15. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12:**275–285.
16. **DeLuca, N. A., M. A. Courtney, and P. A. Schaffer.** 1984. Temperature-sensitive mutants in HSV-1 ICP4 permissive for early gene expression. J. Virol. **52:**767–776.
17. **DeLuca, N. A., A. 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.
18. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5:**1997–2008.
19. **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.
20. **DeLuca, N. A., and P. A. Schaffer.** 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **62:**732–743.
21. **Desai, P., R. Ramakrishnan, Z. W. Lin, B. Osak, J. C. Glorioso, and M. Levine.** 1993. The RR1 gene of herpes simplex virus type 1 is uniquely transactivated by ICP0 during infection. J. Virol. **67:**6125–6135.
22. **Dixon, R. A. F., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36:**189–203.
23. **Dyck, J. A., G. G. Maul, W. H. Miller, Jr., J. D. Chen, A. Kakizuka, and R. M. Evans.** 1994. A novel macromolecular structure is a target of the promyelocytic-retinoic acid receptor oncoprotein. Cell **76:**333–343.
24. **Everett, R. D.** 1984. Transactivation of transcription by herpesvirus products: requirements of two HSV-1 immediate-early polypeptides for maxi-

mum activity. EMBO J. **3:**3135–3141.
25. **Everett, R. D.** 1987. A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J. **6:**2069–2076.
26. **Everett, R. D.** 1988. Promoter sequence and cell type can dramatically affect the efficiency of transcriptional activation induced by herpes simplex virus type 1 and its immediate-early gene products Vmw175 and Vmw110. J. Mol. Biol. **203:**739–751.
27. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. EMBO J. **13:**5062–5069.
28. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82:**5265–5269.
29. **Gelman, I. H., and S. Silverstein.** 1986. Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate-early gene products. J. Mol. Biol. **191:**395–409.
30. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus transactivating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85:**6347–6351.
31. **Goding, C. R., and P. O'Hare.** 1989. Herpes simplex virus Vmw65-octamer binding protein interaction: a paradigm for combinatorial control of transcription. Virology **173:**363–367.
32. **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.
33. **Golden, M. P., S. Kim, S. M. Hammer, E. A. Ladd, P. A. Schaffer, N. DeLuca, and M. A. Albrecht.** 1992. Activation of human immunodeficiency virus by herpes simplex virus. J. Infect. Dis. **166:**494–499.
34. **Goldstein, D. J., and S. K. Weller.** 1988. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 *lacZ* insertion mutant. J. Virol. **62:**196–205.
35. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus type 5 DNA. Virology **52:**456–467.
36. **Greaves, R., and P. O'Hare.** 1989. Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. J. Virol. **63:**1641–1650.
37. **Gu, B., and N. A. DeLuca.** 1994. Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4. J. Virol. **68:**7953–7965.
38. **Gu, B., R. Rivera-Gonzalez, C. A. Smith, and N. A. DeLuca.** 1993. Herpes simplex virus infected cell polypeptide 4 preferentially represses Sp1-activated over basal transcription from its own promoter. Proc. Natl. Acad. Sci. USA **90:**9528–9532.
39. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 can cause a decrease in cellular mRNA levels during infection. J. Virol. **68:**4797–4810.
40. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J. Virol. **68:**7790–7799.
41. **Harris, R. A., R. D. Everett, X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J. Virol. **63:**3513–3515.
42. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpes virus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14:**8–19.
43. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpes virus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72:**1276–1280.
44. **Imbalzano, A. N., D. Coen, and N. A. DeLuca.** 1991. Herpes simplex virus transactivator ICP4 operationally substitutes for the cellular transcription factor Sp1 for efficient expression of the viral thymidine kinase gene. J. Virol. **65:**565–574.
45. **Johnson, P. A., M. J. Wang, and T. Friedman.** 1994. Improved cell survival by the reduction of immediate-early gene expression in replication-defective mutants of herpes simplex virus type 1 but not by mutation of the virion host shutoff function. J. Virol. **68:**6347–6362.
46. **Johnson, P. A., A. Miyanohara, F. Levine, T. Cahill, and T. Friedman.** 1992. Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. J. Virol. **66:**2952–2965.
47. **Koken, M. H. M., F. Puvion-Dutilleul, M. Guillemin, A. Viron, G. Linares-Cruz, N. Stuurman, L. De Jong, C. Szostecki, F. Calvo, C. Chomienne, L. Degos, E. Puvion, and H. De The.** 1994. The t(15;17) translocation alters a nuclear body in a retinoic acid reversible fashion. EMBO J. **13:**1073–1083.
48. **Kristie, T. M., J. H. LeBowitz, and P. A. Sharp.** 1989. The octamer-binding proteins form multi-protein-DNA complexes with the HSV alpha TIF regulatory protein. EMBO J. **8:**4229–4238.
49. **Kristie, T. M., and B. Roizman.** 1984. Host cell proteins bind to the cis-acting site required for virion mediated induction of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **84:**71–75.
50. **Laemmli, U. K.** 1979. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227:**680–684.
51. **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.
52. **Leopardi, R., and B. Roizman.** 1996. The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. Proc. Natl. Acad. Sci. USA **93:**9583–9587.
53. **Mackem, S., and B. Roizman.** 1980. Regulation of herpesvirus macromolecular synthesis: transcription initiation sites and domains of α genes. Proc. Natl. Acad. Sci. USA **79:**4917–4921.
54. **Mackem, S., and B. Roizman.** 1982. Structural features of the α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44:**939–949.
55. **Manservigi, R., P. Spear, and A. Buchan.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74:**3913–3917.
56. **Maul, G. G., and R. D. Everett.** 1994. The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J. Gen. Virol. **75:**1223–1233.
57. **Maul, G. G., H. H. Guldner, and J. G. Spivack.** 1993. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate-early gene 1 product (ICP0). J. Gen. Virol. **74:**2679–2690.
58. **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.
59. **McLauchlan, J., A. Phelan, C. Loney, R. M. Sandri-Goldin, and J. B. Clements.** 1992. Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3′ processing. J. Virol. **66:**6939–6945.
60. **McLauchlan, J., S. Simpson, and J. B. Clements.** 1989. Herpes simplex virus induces a processing factor that stimulates poly(A) site usage. Cell **59:**1093–1105.
61. **McMahan, L., and P. A. Schaffer.** 1990. The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection. J. Virol. **64:**3471–3485.
62. **Meredith, M. R., A. Orr, M. Elliot, and R. D. Everett.** 1995. Separation of the sequence requirements for HSV1 Vmw110 multimerisation and interaction with a 135kD cellular protein. Virology **209:**174–187.
63. **Meredith, M. R., A. Orr, and R. D. Everett.** 1994. Herpes simplex virus type 1 immediate-early protein Vmw110 binds strongly and specifically to a 135kD cellular protein. Virology **200:**457–469.
64. **Michael, N., and B. Roizman.** 1993. Repression of the herpes simplex virus 1 alpha 4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. Proc. Natl. Acad. Sci. USA **90:**2286–2290.
65. **O'Hare, P., C. R. Goding, and A. Haigh.** 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. EMBO J. **7:**4231–4238.
66. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53:**751–760.
67. **O'Hare, P., and G. S. Hayward.** 1985. Three transactivating regulatory proteins of herpes simplex virus modulate immediate early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56:**723–733.
68. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpes virus macromolecular synthesis. V. Properties of polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77:**733–749.
69. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsK*. J. Virol. **29:**275–284.
70. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5:**957–963.
71. **Rice, S. A., and D. M. Knipe.** 1988. Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. J. Virol. **62:**3814–3823.
72. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62:**4307–4320.
73. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. **55:**796–805.
74. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. **61:**829–839.
75. **Samaniego, L. A., and N. A. DeLuca.** Unpublished observations.

76. **Samaniego, L. A., A. L. Webb, and N. A. DeLuca.** 1995. Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. J. Virol. **69:**5705–5715.
77. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
78. **Sandri-Goldin, R. M.** 1994. Properties of an HSV-1 regulatory protein that appears to impair host cell splicing. Infect. Agents Dis. **3:**59–67.
79. **Sandri-Goldin, R. M., M. K. Hibbard, and M. A. Hardwicke.** 1995. The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. J. Virol. **69:**6063–6076.
80. **Sandri-Goldin, R. M., and G. E. Mendoza.** 1992. A herpes virus regulatory protein appears to act posttranscriptionally by affecting mRNA processing. Genes Dev. **6:**848–863.
81. **Sekulovich, R. E., K. Leary, and R. M. Sandri-Goldin.** 1988. The herpes simplex virus type 1 alpha protein ICP27 can act as a *trans*-repressor or a *trans*-activator in combination with ICP4 and ICP0. J. Virol. **62:**4510–4522.
82. **Shepard, A. A., and N. A. DeLuca.** 1989. Intragenic complementation among partial peptides of herpes simplex regulatory protein ICP4. J. Virol. **63:**1203–1211.
83. **Shepard, A. A., A. N. Imbalzano, and N. A. DeLuca.** 1989. Separation of primary structural components conferring autoregulation, transactivation, and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **63:**3714–3728.
84. **Smith, C. A., P. Bates, R. Rivera-Gonzales, B. Gu, and N. A. DeLuca.** 1993. ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. J. Virol. **67:**4676–4687.
85. **Smith, I. L., M. A. Hardwicke, and R. M. Sandri-Goldin.** 1992. Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. Virology **186:**74–86.
86. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
87. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1:**327–341.
88. **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.
89. **Su, L., and D. M. Knipe.** 1989. Herpes simplex virus alpha protein ICP27 can inhibit or augment viral gene transactivation. Virology **170:**496–504.
90. **Sze, P., and R. C. Herman.** 1992. The herpes simplex virus type 1 ICP6 gene is regulated by a 'leaky' early promoter. Virus Res. **26:**141–152.
91. **Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight.** 1988. Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. **2:**730–742.
92. **Uprichard, S. L., and D. M. Knipe.** 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J. Virol. **70:**1969–1980.
93. **Watson, R. J., C. M. Preston, and J. B. Clements.** 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNAs. J. Virol. **31:**42–52.
94. **Watson, R. J., and J. B. Clements.** 1978. Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. Virology **91:**364–379.
95. **Weinheimer, S. P., and S. L. McKnight.** 1987. Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. **195:**819–833.
96. **Weis, K., S. Rambaud, C. Lavau, J. Jansen, T. Carvalho, M. Carmo-Fonseca, A. Lamond, and A. DeJean.** 1994. Retinoic acid regulates aberrant nuclear localization of PML-RARα in acute promyelocytic leukaemia cells. Cell **76:**345–356.
97. **Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33:**167–182.
98. **Wu, N., S. C. Watkins, P. A. Schaffer, and N. A. DeLuca.** 1996. Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. J. Virol. **70:**6358–6369.
99. **Wymer, J. P., T. D. Chung, Y.-N. Chang, G. S. Hayward, and L. Aurelian.** 1989. Identification of immediate-early-type *cis*-response elements in the promoter for the ribonucleotide reductase large subunit from herpes simplex virus type 2. J. Virol. **63:**2773–2784.
100. **Zhu, X. X., J. Chen, C. S. H. Young, and S. Silverstein.** 1990. Reactivation of latent herpes simplex virus by adenovirus recombinants encoding mutant IE-0 gene products. J. Virol. **64:**4489–4498.