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Herpes Simplex Viruses with Mutations in the Gene Encoding ICP0 Are Defective in Gene Expression

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Herpes simplex virus type 1 (HSV-1) mutants with codon insertions and deletions in IE-0, the gene encoding ICP0, were constructed. The HSV-1 deletion mutant *dl*1403 (N. D. Stow and E. C. Stow, J. Gen. Virol. 67:2571–2585, 1986) and an *IE-0:lacZ* transplacement vector isolated in this study were used to facilitate the construction of mutant viruses. Mutant viruses, all of which produced stable ICP0, were examined for their ability to plaque and grow on both Vero and HeLa cells because previous results showed that HSV-1 immediate-early (IE) gene promoters and their products are differentially expressed in these cells (J. Chen, X. Zhu, and S. Silverstein, Virology 180:207–220, 1991; I. H. Gelman and S. Silverstein, J. Virol. 61:2286–2296, 1987). Viruses with IE-0 genes that only poorly activated reporter genes in transient expression assays plaqued less efficiently on Vero cells and consistently accumulated decreased levels of late proteins. These mutants were also examined in single-step growth curve experiments and for the dependence of virus yield on multiplicity of infection (MOI). At low MOIs, their yields were less in Vero cells than in HeLa cells; by contrast, at high MOIs, there was no apparent difference in yield in either cell type, although each virus produced considerably fewer progeny than wild-type virus. Analysis of steady-state levels of RNA from genes representing each of the three major kinetic classes demonstrated that lower levels of RNAs accumulate in these mutants. We conclude from these studies that while ICP0 is not essential for virus growth in tissue culture, defects in this gene result in impairment of virus replication and delay the expression of early and late gene transcripts.

Gene expression in cells infected with herpes simplex virus type 1 (HSV-1) is coordinately regulated and sequentially ordered in a cascade fashion (26, 27). Three major kinetic classes of genes termed immediate early α, early (β), and late (γ) are temporally expressed during a productive infection (27). The immediate-early (IE) genes are the first group of genes expressed, and their products are involved in activation of early (β), early-late (β-γ), and late (γ) virus genes (2, 38, 55) and regulation of IE genes (8, 18, 31, 34, 42, 46, 51).

(In the abbreviations used to describe the HSV-1 genes, IE refers to the immediate-early gene, and the number afterward refers to the infected cell protein it encodes, e.g., IE-0 is the gene encoding ICP0, and IE-4 encodes ICP4.)

The IE-0 gene is present in the repeated sequences that flank the unique long region of the virus genome and is therefore diploid. Nucleotide sequence analysis coupled with S1 nuclease mapping and cDNA analysis revealed that IE-0 is 3,587 bp long and composed of three exons (37, 60). Based on the sequence, ICP0 is predicted to be composed of 775 amino acids (37). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), however, the apparent molecular weight of ICP0 is 110,000 to 124,000, depending on the cross-linking agent used and on the acrylamide concentration (26, 36). ICP0 is phosphorylated and found in the nucleus of infected cells (36). As deduced from the predicted amino acid sequence, the amino terminus of ICP0 is highly acidic. Acidic motifs have been correlated with transactivation domains in some transcriptional activators (for reviews, see references 39 and 50). Immediately adjacent to the acidic region is a putative zinc-finger domain that contains a structural motif found in many proteins that bind to DNA and participate in the regulation of transcription (3, 9). There are, in the middle of ICP0, two proline-rich regions. Transcription factors such as CTF/NF-1, OTF-2, and AP-2 contain proline-rich regions that act as transcription activation domains (19, 32, 58). There is also a serine-rich region from amino acid residues 554 to 591 that might serve as a phosphate acceptor site. Computer analysis of the hydropathy and secondary structure of ICP0, using GCG's Sequence Analysis Software Package, revealed that ICP0 is hydrophilic with many α-helices (6).

In transient expression assays, in which cloned HSV-1 genes were cotransfected with appropriate indicator genes, plasmids encoding either ICP0 or ICP4 were able to activate IE, early, and late gene expression (16, 18, 30, 34, 35, 40, 47). For early gene targets, when both IE-0 and IE-4 DNAs were present, the level of activation was much greater than that found with either effector alone (10, 17, 40).

In-frame insertion and deletion mutations and nonsense mutations were made in plasmids encoding ICP0, and the mutant plasmids were assayed for their ability to transactivate HSV-1 IE, early, and late gene promoters and for their ability to activate cooperatively early promoters after cotransfection with a plasmid encoding ICP4 (4, 6, 11, 12). These studies revealed the presence of several domains within ICP0 that differentially affected transactivation of HSV-1 gene expression. The major determinant of transactivation maps to a region overlapping a cysteine-rich region in exon 2, and this region was consistently required to activate each promoter. When these insertion and/or nonsense mutations were returned to the virus genome and characterized, there was a correlation between the transactivation levels seen in transient expression assays and impairment of virus replication (4, 14).

HSVs deleted of IE-0 have been constructed and characterized (45, 49). These viruses did not grow as efficiently in

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cell culture as wild-type virus, and their replication and efficiency of plaquing appeared to be host-range and multiplicity dependent. These studies show that ICP0 plays an important, although not essential, role during productive infection in cell culture.

ICP0 has also been suggested to play a role in the efficient establishment and reactivation of latency (28), in which virus gene expression is limited to transcription of the latency-associated transcript (LAT) and no infectious virus can be found. In an in vitro latency system, the product of the IE-0 gene, in the context of an adenovirus expression vector, was shown to be sufficient to reactivate latent virus (63). When mutant IE-0 genes were examined, the transactivation domains of ICP0 were also shown to be required for reactivation (61).

In the present study, we inserted two copies of many of the IE-0 mutants we constructed into the HSV-1 genome (6). These mutations were previously shown to affect the ability of ICP0 to transactivate reporter genes in a transient expression assay system. We asked whether defects in the various activities of ICP0 affect the ability of mutant viruses to grow. Previously, we and others demonstrated a cell-type-dependent specificity of ICP0 expression (6, 13). Because we are interested in understanding how virus regulatory proteins interact with cell factors, the mutants isolated in this analysis were examined in both Vero and HeLa cells. Several mutants exhibited cell-type specificity that was multiplicity dependent. These studies showed that when transactivation-defective IE-0 genes are placed into the virus genome, expression of virus-specified RNAs from the three major classes of temporally regulated genes is delayed.

MATERIALS AND METHODS

Cells and viruses. Vero and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. The parental viruses were wild-type HSV-1 (Glasgow strain 17) and *dl*1403, an IE-0 deletion mutant that has been described previously (49).

Plasmids. Plasmid pEC2 was constructed by inserting a 450-bp *Sal*I-*Hpa*I fragment from IE-0 (+3349 to +3785 relative to the transcription initiation site) into the *Bam*HI site of pEC1 (6) in the same orientation that is found in the original gene. pJC18 was a derivative of pEC2; a *Bgl*II linker was inserted into the *Eco*RI site in the polylinker region of the vector next to the IE-0 promoter and regulatory sequences. The pXQ1 and pCM plasmid series were described previously (6).

Transfection. Vero cells were seeded at 10^6/60-mm dish in DMEM containing 10% calf serum and transfected the next day with a calcium phosphate-DNA precipitate containing 1 μg of virus and 0.5 μg of linearized plasmid DNA as previously described (57); this was followed by a shock with 1 ml of 15% glycerin in phosphate-buffered saline (PBS; 0.137 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4) for 2 min and three washes with PBS before being overlaid with fresh medium.

Virus titration. The titers of the virus preparations were determined by infecting Vero or HeLa cell monolayers with serially diluted virus in PBS-ABC-ICS-glucose (PBS supplemented with 5 mM $MgCl_2 \cdot 6H_2O$, 7 mM $CaCl_2$, 1% heat-inactivated calf serum, and 0.1% glucose). When using Vero cells, infected cells were overlaid with DMEM containing 1% calf serum and 1.5% methylcellulose and incubated at 37°C for 4 days, and plaques were counted directly. When using HeLa cells, the infected cells were overlaid with DMEM containing 1% calf serum and 0.75% agarose and incubated for 5 days. The cells were then overlaid with DMEM containing 1% calf serum, 0.75% agarose, and 0.02% neutral red. Plaques were counted 24 h after the neutral red overlay. Titers of viruses were also determined by indirect immunofluorescence. Confluent Vero or HeLa cell monolayers in 35-mm plastic dishes were infected with appropriate dilutions of virus. Following 1 h of adsorption, the cells were overlaid with 3 ml of DMEM containing 1% calf serum and incubated for 7 h at 37°C. The plates were then washed three times with PBS and fixed for 30 min with 70% ethanol. The fixed cells were subsequently incubated for 1 h with 100 μl of mouse monoclonal antibody (diluted 1:1,000 with PBS) specific for ICP4 (H640), provided by Lenore Pereira. Excess antibody was removed by washing three times in PBS, and the cells were then incubated with fluorescein isothiocyanate-conjugated goat antibody directed against mouse immunoglobulin (Organon Teknika-Cappel, West Chester, Pa.) for 30 min, washed three times in PBS, and overlaid with a coverslip. The fluorescein-stained cells were visualized by using a Leitz Dialux microscope with vertical illumination and optical systems for the selective visualization of fluorescein. The foci were quantitated by using a grid in one ocular.

Virus growth assays. Virus yield and growth curve experiments were performed with 7×10^5 cells in 35-mm petri dishes infected at the stated multiplicity of infection (MOI). One hour after addition of virus, the inoculum was removed and replaced with DMEM containing 1% calf serum. Progeny virus was harvested by scraping the cells into the medium and subjecting them to three cycles of freeze and thaw. Titration of the virus stocks was performed on confluent monolayers of Vero cells.

Preparation and analysis of polypeptides. Vero or HeLa cells (7×10^5) in a 35-mm dish were infected approximately 20 h after seeding at various MOIs as described in the text and were labeled with 50 μCi of [^{35}S]methionine (>800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) at the times indicated. Labeling was in 1 ml of DMEM without L-methionine. After labeling, the cells were washed and harvested as described previously (61). Briefly, cellular extracts were prepared by lysing the cells in 100 μl of RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and 10 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma). Labeled proteins were electrophoresed at constant voltage (5 V/cm) on SDS-9% polyacrylamide gels (acrylamide: bisacrylamide weight ratio of 29:1) that were fixed, dried, and autoradiographed.

IE polypeptides were generated by growth of infected cells in 100 μg of cycloheximide per ml as described by Preston et al. (38). Cell extracts were then prepared and analyzed by SDS-PAGE as described above.

Marker rescue. Vero cell monolayers in 60-mm plastic petri dishes were cotransfected with 1 μg of intact VCM2/7 DNA and 1 μg of *Hin*dIII-linearized pXQ1. Two days after transfection, the plates were harvested by scraping infected cells into the medium, and the stock was used to infect fresh monolayers of Vero cells at an MOI of 0.01. Virus was harvested 2 days after infection, and the infection process was repeated two times. The final stocks were vigorously mixed and used to infect 24-well plates. Plaques were picked from wells that contained only a single plaque. DNA was prepared and analyzed by Southern blot hybridization.

Isolation of mutant viruses. (i) Isolation of an *IE-0:lacZ*

transplacement vector. Confluent monolayers of Vero cells in 50-mm petri dishes were transfected with 1 μg of infectious *dl*1403 DNA and 1 μg of pJC18 linearized with *Bgl*II. Three days posttransfection, the progeny were harvested. Virion particles were separated by vigorous mixing, and viruses were titrated on Vero cell monolayers and overlaid with medium containing 1.5% methylcellulose. After incubation at 37°C for 4 days, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added at a final concentration of 100 μg/ml. Blue plaques were picked and purified.

(ii) Isolation of mutant viruses. Vero cells were cotransfected with DNA from *dl*1403 or the *IE-0:lacZ* transplacement virus with linearized plasmids containing mutant IE-0 genes. Three days later, cultures were harvested, virus progeny were passed three times at low MOI, and individual plaques were picked. When using *IE-0:lacZ* as the target for transfection, white plaques in the presence of X-Gal were picked. Southern blot analysis was performed on DNA from each isolate to verify the insertion of a mutated IE-0 sequence and the loss of the *lacZ* sequences.

Preparation and analysis of virus DNA. Infectious virus DNA was prepared from infected cells by centrifugation through NaI equilibrium density gradients as described by Walboomers and Ter Schegget (54). Virus DNA was prepared as total cellular DNA from infected cell monolayers and analyzed by Southern blot hybridization with DNA probes labeled by random priming. The Nytran filters (Schleicher & Schuell) were washed successively at 68°C in 2×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2×SSC and then exposed to Kodak XAR film at −70°C.

Isolation and characterization of cytoplasmic RNA. Five million cells were washed three times with ice-cold PBSA and then scraped and centrifuged at 4°C. The pellet was resuspended in 230 μl of lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.6], 0.5% Nonidet P-40), and incubated on ice for 5 min. After the nuclei were pelleted, the supernatant was carefully transferred to another tube containing 300 μl of 200 mM Tris-HCl (pH 7.5), 25 mM EDTA, 300 mM NaCl, 2% SDS, and 200 μg of proteinase K per ml and incubated at 65°C for 30 min. This was followed by phenol-chloroform extraction and precipitation with ethanol. Total cytoplasmic RNAs were electrophoresed on agarose gels containing formaldehyde as previously described (63). The integrity of the RNA in each sample was monitored by ethidium bromide staining of 28S and 18S rRNA. After hybridization, the filter was stained with methylene blue to quantitate the RNA loaded and transferred (24). For Northern (RNA) blot analysis, if the virus DNA was in M13, fragments from the replicative-form DNA were used to make probes. The probes for ICP8 were from D. M. Knipe, those for ICP0 and ICP4 were from S. L. McKnight, and ICP27, TK, VP5, VP16, and gC were kindly provided by E. K. Wagner.

DNA probes. The following HSV-1 probes in M13 were used: ICP0, a 1.6-kb *Sst*I-*Bam*HI fragment in mp10 and mp11 (29); ICP4, a 1.85-kb *Bam*HI Y fragment (map coordinates 0.853 to 0.865) in mp10 and mp11 (33); ICP27, a 1.2-kb *Bam*HI-*Sal*I fragment (map coordinates 0.742 to 0.750) in mp8 and mp9 (59); TK, a 0.82-kb *Pst*I-*Hin*cII fragment (map coordinates 0.309 to 0.314) in mp18 and mp19 (59); ICP8, a 1.4-kb *Kpn*I-*Bam*HI fragment (map coordinates 0.398 to 0.407) in mp18 and mp19 (20); VP5, a 1-kb *Sal*I-*Bam*HI fragment (map coordinates 0.261 to 0.268) in mp8 and mp9 (59); and gC, a 0.96-kb *Eco*RI-*Xba*I fragment (map coordinates 0.633 to 0.639) in mp8 and mp9 (59). The α-TIF probe, a 1.2-kb *Sal*I fragment (map coordinates 0.674 to 0.681) from a 6-kb *Hin*dIII-*Xho*I fragment (map coordinates 0.647 to 0.689) was in pBR322 (21).

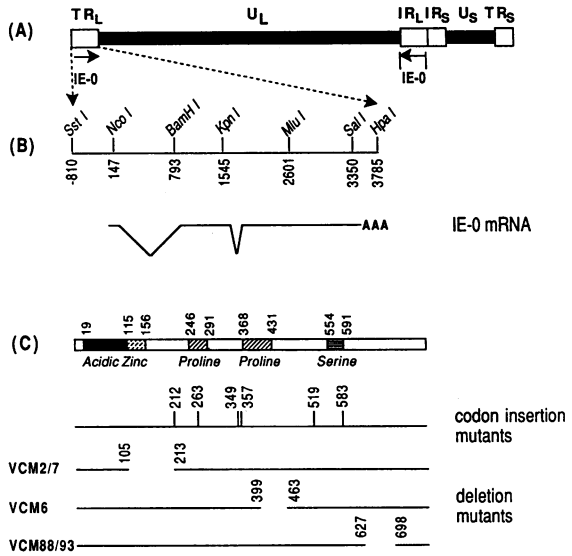

FIG. 1. Genome structure and location of insertion and deletion mutations. (A) Structure of the HSV-1 genome showing the prototype arrangement. The long unique region (U_L) and short unique region (U_S) are flanked by internal and terminal repeats (IR and TR). The sequences encoding ICP0 are contained entirely within TR_L and IR_L. (B) Restriction endonuclease map of the IE-0 gene and structure of the mRNA encoding ICP0. The structure of the mRNA encoding ICP0 is shown beneath the map. The numbering is with respect to the transcription initiation site at +1 (37). (C) The diagram below indicates the positions of the various domains within ICP0 as deduced from the predicted amino acid sequence. These include an acidic region, a potential zinc-binding domain, two proline-rich regions, and a serine-rich region as indicated. The acidic, zinc finger-like motifs, and serine-rich regions have been described previously (6, 11, 12, 37). The proline-rich regions were deduced from the sequence. The positions of insertion and deletion mutations used in this study are shown. The amino acid position of each insertion mutant is indicated by a vertical line. The boundaries of each deletion mutant are shown.

RESULTS

Isolation of recombinant viruses with mutations in IE-0. The construction and transactivation properties of in-frame codon insertion and deletion mutants in a plasmid containing the sequence that encodes ICP0 were previously described (6). The location of each mutation and the phenotypic properties of the plasmids are summarized in Fig. 1 and Table 1. The following results from our previous study are pertinent to this present analysis: the region between amino acid residues 106 and 212, which contains a putative zinc finger, was required for activation of promoters from each of the three major regulatory classes of virus genes independent of cell type or the presence of a plasmid encoding ICP4. pCM88/93, a mutant with a mutation in the carboxy terminus, was more efficient at activating IE and late promoters in HeLa cells than in Vero cells; however, its ability to cooperate with ICP4 was defective in HeLa cells but not in Vero cells. Mutant pCM6 could efficiently activate the TK reporter; however, the ICP0 encoded by this mutant did not cooperate with ICP4 to activate this promoter, and it was defective in efficient transactivation of the IE-0 promoter. To

TABLE 1. Location of mutations and transactivation activities of IE-0 plasmid mutants

Plasmid	Site[a]	Amino acid	Activity[b]							
			TK, 0[c]		TK, 0 + 4[d]		IE-0, 0[e]		gC, 0[f]	
			Vero	HeLa	Vero	HeLa	Vero	HeLa	Vero	HeLa
pCM2/7	1230–1547	Δ106–212	Low	Low	Low	Low	Low	Low	Low	Low
pCM7	1546	212	Low	Low	Low	Medium	Low	Low	Low	Low
pCM84	1835	263	High	ND[g]	High	ND	High	ND	High	ND
pCM5	2094	349/350	High	High	High	High	High	High	High	High
pCM149	2177	357	High	ND	High	ND	High	ND	High	ND
pCM6	2249–2433	Δ400–462	High	ND	Low	ND	Medium	ND	Medium	ND
pCM10	2601	519	Medium	ND	High	ND	Medium	ND	High	ND
pCM136	2795	583	High	ND	High	ND	High	ND	High	ND
pCM88/93	2932–3138	Δ628–697	Low	Low	High	Low	Medium	High	Medium	High

[a] The position relative to the transcription initiation site at +1.
[b] The percent activity of each mutant was normalized to the level of activity of pXQ1, and mutants with activities of ≤35% of the wild-type level are termed low, those that are ≥35% but ≤65% are medium, and those that are ≥65% are high.
[c] Activation of TK–β-galactosidase (pJC13) by the mutants.
[d] Transactivation in the presence of ICP4 with a TK–β-galactosidase cassette as the target.
[e] Activation of an IE-0–β-galactosidase (peC1) target.
[f] Activation of a gC-chloramphenicol acetyltransferase (pDS9) target in Vero cells and a gC–β-galactosidase (pEJ1) target in HeLa cells.
[g] ND, not done.

determine whether these mutations affected the virus life cycle and whether the cell-type specificity seen in transient expression assays was mimicked when these mutations were incorporated into virus, we returned some of the cloned IE-0 mutants to the virus genome. Transfer was achieved by homologous recombination between infectious virus DNA and individual mutant plasmid DNAs after transfection. To do this, two approaches were taken. First, we used the HSV-1 mutant *dl*1403 that is deleted of 2 kb within both copies of IE-0. This virus exhibits impaired growth in cell culture (49). Mutant plasmid DNAs were linearized with *Hin*dIII, and the resulting DNAs were cotransfected with intact *dl*1403 DNA into Vero cells. Progeny viruses were passaged at low MOI in Vero cells three times before individual plaques were picked. At least two independent isolates of each virus were investigated for their growth properties. The rationale behind this strategy was that many of the mutations to be introduced retained some activity in a transfection assay; therefore, recombinant viruses containing these insertions or deletions would have a growth advantage over *dl*1403 (14). This approach was vindicated when, after three passages, most plaques picked contained recombinant viruses with the expected lesions in IE-0. The insertion of the IE-0 mutants was verified by Southern blot hybridization (Fig. 2). Recombinant viruses containing IE-0 sequences from pCM6, -149, -136, and -88/93 as well as pXQ1, the plasmid DNA containing a wild-type IE-0 gene, were isolated by this approach.

In the second approach, the return of the cloned IE-0 mutants into the virus genome was facilitated by creating a transplacement virus with both copies of IE-0 replaced by the *Escherichia coli lacZ* gene driven by the IE-0 promoter. This transplacement vector gave rise to blue plaques in the presence of X-Gal. Replacement of the *lacZ* gene by recombination resulted in viruses that generated clear plaques in the presence of X-Gal. This provided for rapid identification of recombinants containing IE-0 inserts without selecting for viruses with a growth advantage. The transplacement vector was constructed by cotransfecting linearized pJC18, which contained the *lacZ* gene flanked by IE-0 sequences, with infectious *dl*1403 DNA onto Vero cells. Progeny viruses were grown in the presence of X-Gal, and blue plaques were picked and purified. The replacement of IE-0 by *lacZ* was verified by Southern blot analysis (Fig. 3). This transplacement vector was designated *IE-0:lacZ*. In cycloheximide reversal experiments designed to detect the expression of IE polypeptide synthesis, no protein that migrated as wild-type ICP0 was detected from cells infected with the *IE-0:lacZ* construct. Instead, a novel IE peptide of slower mobility was detected (Fig. 4). This polypeptide migrated with an apparent molecular weight of 135,000.

The mutants pCM2/7, -7, -84, -5, and -10 were then transferred to the virus genome by cotransfection of each linearized plasmid with *IE-0:lacZ* DNA. The resulting recombinant viruses, designated VCM2/7, -7, -84, -5, and

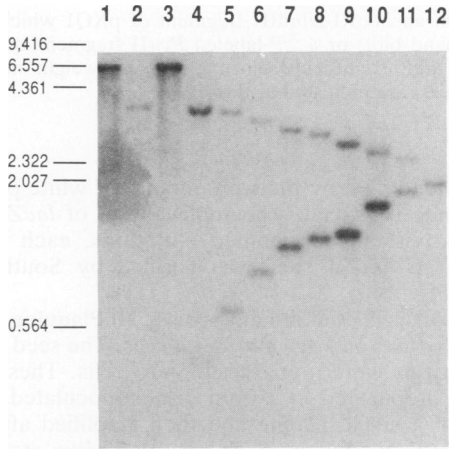

FIG. 2. Southern blot analysis of HSV-1 mutants in IE-0. Total DNA (1 μg) from virus-infected cells was digested with *Pst*I, *Sac*I, *Eco*RI, and *Bam*HI and analyzed by Southern blot hybridization with a ^{32}P-labeled *Bam*HI-*Sal*I fragment of pXQ1 as a probe. Molecular weight markers (in kilobase pairs) are indicated at the left. Lanes 1 through 12 are the DNAs from cells infected with the respective following viruses: 17, *dl*1403, VXQ1, VCM2/7, VCM7, VCM84, VCM5, VCM149, VCM6, VCM10, VCM136, and VCM88/93. All the mutant viruses have an *Eco*RI site in each copy of the IE-0 gene at the position indicated in Table 1.

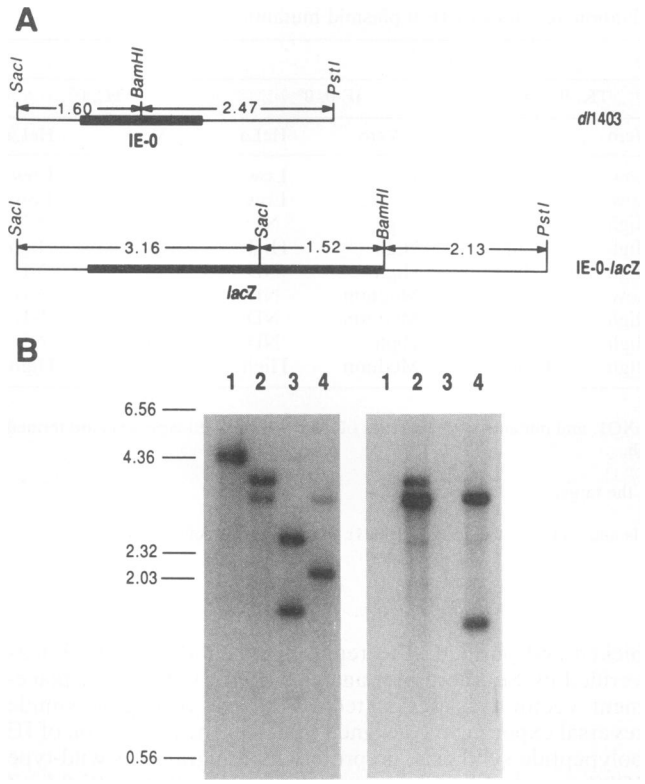

FIG. 3. Construction of the *IE-0:lacZ* transplacement vector. (A) Partial genomic structure of *dl*1403 and predicted genomic structure of *IE-0:lacZ*. This diagram shows a *Sac*I-*Bam*HI-*Pst*I restriction map of the sequences containing IE-0 and *lacZ*. The numbers show the size (in kilobase pairs) of relevant restriction fragments. (B) Southern blot analysis of *IE-0:lacZ*. Virus DNA was digested with specific restriction enzymes, separated by agarose gel electrophoresis, and analyzed by Southern blot hybridization. Lanes 1 and 3, *dl*1403 DNA; lanes 2 and 4, *IE-0:lacZ*. Lanes 1 and 2 were digested with *Pst*I and *Sac*I; lanes 3 and 4 were digested with *Pst*I, *Sac*I, and *Bam*HI. Duplicate filters were hybridized to either ^{32}P-labeled probes from an *Eco*RI-*Hin*dIII fragment of pXQ1 which contains IE-0 (left-hand blot) or a ^{32}P-labeled *Pvu*II fragment containing a portion of *lacZ* (right-hand blot). Molecular weight markers (in kilobase pairs) are indicated at the left.

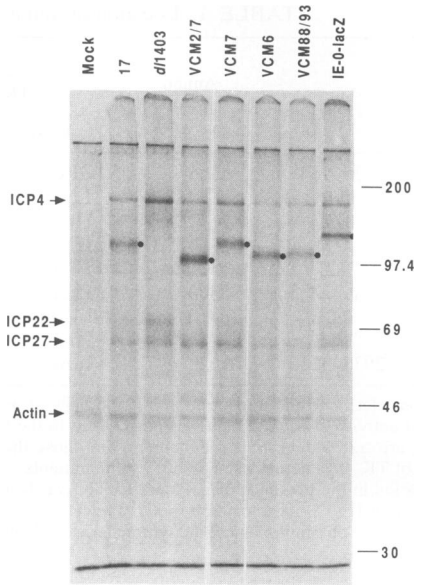

FIG. 4. IE polypeptide expression by mutant viruses. Cells were mock infected or infected with 5 PFU of HSV-1 17, *dl*1403, VCM2/7, VCM7, VCM6, VCM88/93, or *IE-0:lacZ* per cell. Polypeptides were labeled with [^{35}S]methionine for 1 h following release from a cycloheximide block and analyzed by SDS-PAGE. The major polypeptides expressed under these conditions (ICP4, ICP22, ICP27, and actin) are indicated to the left, and molecular weight markers ($\times 10^3$) are indicated on the right. Wild-type and mutant forms of ICP0 as well as β-galactosidase are indicated by dots.

-10, were identified by their production of white plaques in the presence of X-Gal. The replacement of *lacZ* by IE-0 sequences with the designated mutations, each of which contained an *Eco*RI site, was verified by Southern blot analysis (Fig. 2).

Growth properties of mutant viruses. (i) Plaquing efficiency of mutant viruses on Vero and HeLa cells. The seed stocks of mutant viruses were prepared in Vero cells. These viruses were first propagated in 35-mm dishes inoculated with the progeny of a single plaque and then amplified after infection of Vero monolayers. The resulting virus stocks were titrated on Vero and HeLa cells. Their titers are shown in Table 2. Although there is variation in the absolute titer of each stock, only *dl*1403, VCM2/7, and the transplacement vector gave consistently low yields. The wild-type virus, VCMXQ1, and mutants that showed wild-type activity in transfection assays plaqued equally well in both cell lines, whereas *dl*1403, *IE-0:lacZ*, and VCM2/7 plaqued 10-fold more efficiently in HeLa cells than in Vero cells. We note that the carboxy-terminus deletion mutant pCM88/93 plaqued slightly more efficiently in Vero cells than in HeLa cells.

The fluorescent-focus unit (FFU) titers of mutant viruses were also determined in Vero and HeLa cells at 7 h postinfection (p.i.). Cai and Schaffer (5) showed that an HSV-1 mutant in IE-0 and the wild-type HSV-1 virus were equally efficient at entering cells and inducing expression of ICP4. Therefore, the FFU of virus stocks should be proportional to their particle numbers, and the difference between FFU and

TABLE 2. Plaquing efficiency of mutant viruses on Vero and HeLa cells[a]

Virus	Titer		Ratio[b]
	Vero	HeLa	
HSV-1 17	6.0×10^7	5.0×10^7	1.20
VCMXQ1	6.0×10^7	5.2×10^7	1.15
*dl*1403	3.4×10^5	3.4×10^6	0.10
IE-0:lacZ	2.7×10^5	2.8×10^6	0.10
VCM2/7	9.5×10^5	7.7×10^6	0.12
VCM7	2.5×10^7	2.8×10^7	0.89
VCM84	6.4×10^7	4.1×10^7	1.56
VCM5	7.5×10^7	6.2×10^7	1.21
VCM149	1.2×10^8	6.4×10^7	1.88
VCM6	5.4×10^7	2.8×10^7	1.93
VCM10	1.0×10^8	5.7×10^7	1.75
VCM136	2.5×10^8	2.4×10^8	1.04
VCM88/93	1.0×10^7	4.2×10^6	2.38

[a] Infected cells were overlaid with DMEM, 1% calf serum, and 0.75% agarose. Four days p.i., plaques formed in Vero cells were counted, while those in HeLa cells were stained with neutral red and counted the following day.

[b] Ratio = titer of virus in Vero cells/titer of virus in HeLa cells.

TABLE 3. Fluorescent-focus assay

Virus	Vero			HeLa		
	PFU	FFU[a]	Ratio[b]	PFU	FFU[a]	Ratio[b]
HSV-1 17	1.4×10^6	5.9×10^5	0.4	7.0×10^5	5.3×10^5	0.8
*dl*1403	5.2×10^4	1.2×10^6	23.7	3.4×10^5	1.3×10^6	3.8
VCM2/7	3.1×10^5	1.2×10^6	3.7	1.3×10^6	1.1×10^6	0.8
VCM7	6.7×10^7	1.3×10^8	1.9	7.5×10^7	1.1×10^8	1.5
VCM6	9.2×10^4	7.8×10^4	0.8	8.0×10^4	3.3×10^4	0.4
VCM88/93	5.6×10^5	6.4×10^5	1.2	5.5×10^5	5.7×10^5	1.0

[a] FFU as detected by an antiserum specific for ICP4.
[b] Ratio = FFU/PFU.

PFU reflects the intrinsic ability of the various viruses to initiate a lytic infection. Virus stocks were also titrated for PFU in a parallel experiment. The results of the fluorescent-focus assay were summarized, and the ratio of FFU/PFU was calculated, and these data are presented in Table 3. In Vero cells, that ratio for *dl*1403 is roughly 50-fold higher than that for the wild-type virus, indicating that in *dl*1403-infected cells, many genomes enter but do not cause plaques. Viruses with mutations in the major transactivation domain (VCM2/7 and -7) also revealed a slightly elevated FFU/PFU ratio, but not to the same extent as *dl*1403. However, there is only a severalfold difference in the ratio between *dl*1403 and the wild-type virus in HeLa cells, and no significant differences were found for other mutants. This is probably a result of the higher plaquing efficiency of these mutants in HeLa cells (Tables 2 and 3).

The plaque size of some mutants differed from that of wild-type HSV-1 on Vero cells. Mutant viruses with wild-type levels of transactivation activity had plaque sizes similar to that of the wild-type virus, while plaques formed by VCM2/7, -7, -6, and -88/93 were smaller (data not shown). Nevertheless, the plaques formed by all the mutants constructed in this study are larger than those of *dl*1403 or *IE-0:lacZ*. On Vero cells, the development of a visible plaque for some mutant viruses was slower than that for wild-type virus. For example, while the number of plaques formed by HSV-1 17 remained the same between 2 and 4 days; for VCM7, some increase was seen. For VCM2/7, the number of plaques nearly doubled. On HeLa cells, on which plaques formed by wild-type viruses are visible within 3 days, it takes 5 days for plaques from VCM2/7, -7, -6, and -88/93 to be detected.

(ii) Growth curves of mutant viruses. The phenotype of these mutant viruses was analyzed by observing their yield as a function of time p.i. Vero cells were infected at an MOI of 1. Most mutants did not show a significant difference in the kinetics of virus production from the wild-type virus. Specifically, mutants that had wild-type levels of transactivation activity in the transfection assay grew almost as efficiently as the wild-type virus. In contrast, mutants that showed a reduced level of transactivation grew poorly. Figure 5 shows the growth curves of mutants that differed from wild-type virus. Consistent with a previous report (49), the yield for *dl*1403 was reduced approximately 300-fold. VCM2/7, which lacks the major transactivation domain in ICP0, also grew poorly. The growth defect of other "down" mutants varied between a 36-fold reduction for VCM7 to just a 2-fold reduction for VCM6. When the mutants that grew less efficiently in Vero cells were tested in HeLa cells, a similar result was seen, but the yield was not reduced to the same extent as in Vero cells (Fig. 5). The experiments that follow were confined to examining the properties of down mutant viruses.

(iii) Yield of mutant viruses at low MOI. The virus yield in Vero and HeLa cells at low MOI (0.01 PFU per cell, as determined by titration on Vero cells) was examined. At 24 h p.i., the progeny were harvested and then titrated on Vero cells. Table 4 shows that in Vero cells, wild-type virus

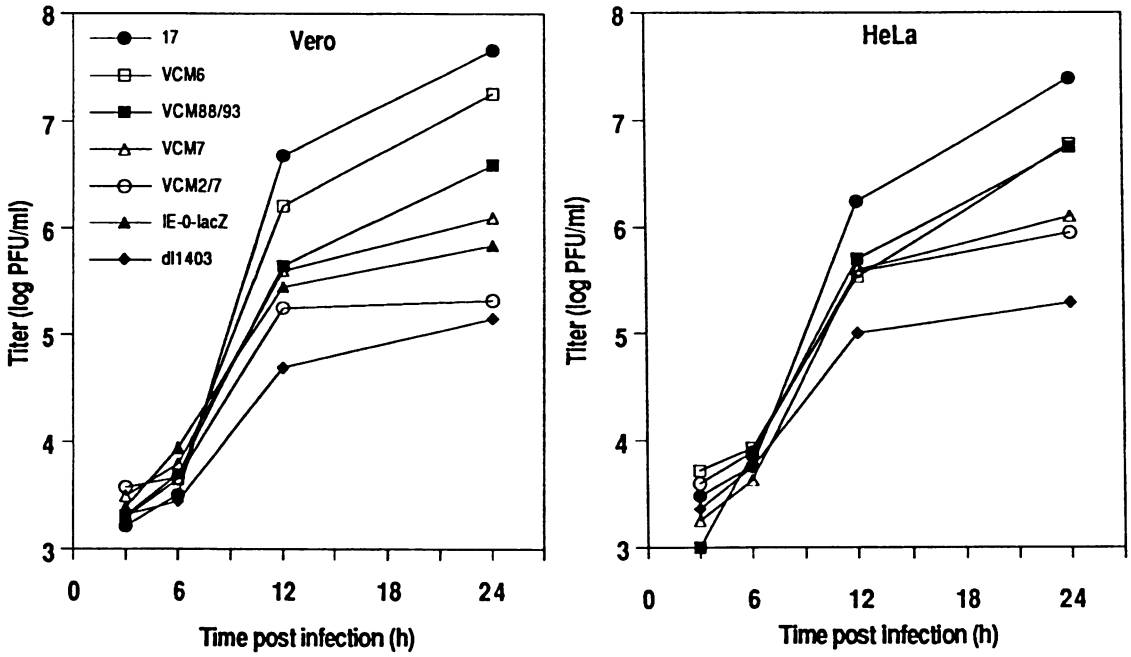

FIG. 5. Growth curves of the mutant viruses in Vero and HeLa cells. Cells were infected at an MOI of 1 and incubated at 37°C. At 3, 6, 12, and 24 h after infection, the cultures were harvested and titrated in Vero cells.

TABLE 4. Yield of mutant viruses on Vero and HeLa cells at low MOI[a]

Virus	Titer		Ratio[b]	Wild type/mutant[c]	
	Vero	HeLa		Vero	HeLa
HSV-1 17	1.7×10^7	5.4×10^6	3.2	1	1
*dl*1403	6.3×10^4	2.9×10^5	0.2	270	19
VCM2/7	5.5×10^4	7.2×10^5	0.1	309	8
VCM7	1.4×10^5	5.2×10^5	0.3	121	10
VCM6	9.8×10^5	5.6×10^5	1.8	17	10
VCM88/93	1.5×10^5	1.6×10^5	0.9	113	34

[a] Cells were infected with viruses at an MOI of 0.01 and harvested at 24 h. The virus titers were then determined on Vero cells.
[b] Ratio = titer of virus stock grown in Vero cells/titer of virus stock grown in HeLa cells.
[c] Wild type/mutant = titer of wild-type virus/titer of mutant virus.

yielded more than 2,000 PFU per infectious unit of inoculum, while *dl*1403 produced no more than 10 PFU per infectious unit of inoculum. VCM2/7 replicated as poorly as *dl*1403, and VCM7 and VCM88/93 also showed a reduction in yield of more than 100-fold. By contrast, VCM6 showed a reduction of less than 20-fold relative to the wild-type control. Therefore, the extent of reduction in virus replication correlates with decreased activity in transfection assays. While wild-type and VCM6 replicated less well in HeLa cells, the other mutants had higher yields, varying between approximately 10- and 30-fold less than wild-type. This is consistent with their efficiency of plaquing on Vero and HeLa cells.

(iv) Yield of mutant viruses at high MOI. Virus yield was also tested at high MOI by infecting monolayers of Vero and HeLa cells at 5 PFU per cell. The progeny were harvested at 24 h p.i. and titrated on Vero cells. In Vero cells, as seen at low MOI, the yield of the mutant viruses was reduced relative to wild-type, although not to the same extent (Table 5). An extreme example of this is VCM7, which showed a reduction of more than 100-fold at low MOI relative to wild type, but was reduced only 9-fold at high MOI. At high MOI in HeLa cells, *dl*1403 and VCM2/7 still showed greatly reduced yields of 273- and 181-fold, respectively. This is largely because of the increased yield in HeLa cells infected with wild-type virus at high MOI, as the titers for these two viruses at low and high MOI were essentially the same.

Expression of ICP0 and other IE polypeptides by mutant viruses. A comparison of IE polypeptide synthesis by the mutants in Vero cells is shown in Fig. 4. Cells were infected with 5 PFU of each virus per cell and labeled with [^{35}S]methionine in the presence of actinomycin D following a 5-h incubation in the presence of cycloheximide. Except for *dl*1403, all the mutants expressed stable ICP0 with an apparent molecular weight that was commensurate with the nature of the mutation. Specifically, VCM7, a 5-amino-acid-insertion mutant, produced a peptide with a mobility indistinguishable from that of wild-type ICP0. The IE-0 deletion mutants VCM2/7, -6, and -88/93 produced peptides that migrated more rapidly than wild-type ICP0. ICP4 and -27 were also produced by the mutant viruses, while ICP22 was not readily detected in this experiment. Slightly more IE polypeptides were produced by *dl*1403, VCM2/7, and VCM7. This is not surprising because, although the cells were infected with the same number of PFU, their FFU/PFU ratios are high. Therefore, more genomes and α-TIF enter cells infected with these virus stocks. Accordingly, more IE gene products would be expected to be expressed. The abundance of IE polypeptides produced from VCM6- and VCM88/93-infected cells was slightly lower. These viruses showed a similar FFU/PFU ratio, suggesting that under the conditions of this experiment, equivalent numbers of virus genomes and α-TIF reach the nuclei of the infected cells. Because they specify mutant forms of ICP0, these proteins may not be as efficient as wild-type ICP0 in augmenting activation of the IE gene promoters.

TABLE 5. Yield of mutant viruses on Vero and HeLa cells at high MOI[a]

Virus	Titer		Ratio	Wild type/mutant	
	Vero	HeLa		Vero	HeLa
HSV-1 17	8.2×10^7	1.5×10^8	0.55	1	1
*dl*1403	3.8×10^5	5.5×10^5	0.69	216	273
VCM2/7	5.8×10^5	8.3×10^5	0.70	141	181
VCM7	9.3×10^6	1.0×10^7	0.93	9	15
VCM6	1.6×10^7	3.4×10^7	0.47	5	4
VCM88/93	2.0×10^6	3.6×10^6	0.56	41	42

[a] This experiment is the same as that shown in Table 4 except that the cells were infected at an MOI of 5.

Expression of virus polypeptides. When cells were infected at the same MOI with *dl*1403 or wild-type virus, the patterns of polypeptide synthesis were almost indistinguishable (49). But FFU/PFU ratios reveal that under that condition, far more mutant virus would enter cells, generating polypeptide synthesis profiles similar to that of wild-type virus. To control for the difference in particle/PFU ratios, we infected cells with the same numbers of FFUs, assuming that using this criterion, identical numbers of virus genomes enter and express IE proteins. Vero cells were infected at 2 FFU per cell and labeled from 4 to 12 h p.i., and cell extracts were subjected to SDS-PAGE analysis. For wild-type virus, the 2 FFU per cell is roughly equivalent to 5 PFU per cell; for *dl*1403, this represents only 0.1 PFU. Quantitative differences in the abundance of specific polypeptides synthesized were evident (data not shown). For example, except VCM6, all mutants produced less of the late proteins VP5 and VP16, ranging from two- to sixfold less in abundance, compared with wild-type virus. Again, except for VCM6, the least defective of the mutants, all IE-0 mutants accumulated lower amounts of ICP27, an essential IE gene product that is required for quantitative expression of late gene products (31, 41, 44).

RNA accumulation. To analyze the effect of mutations in IE-0 on the accumulation of specific virus RNAs, we prepared total cytoplasmic RNA from infected cells after 2 h and at 2-h intervals until 8 h p.i. RNAs were separated by gel electrophoresis, transferred to filters, and probed with radiolabeled DNA fragments representing seven virus genes. The patterns of RNA accumulation from these genes (Fig. 6) are consistent with previous findings (23, 52, 56, 59).

The pattern of RNA accumulation from IE genes was examined with probes specific for IE-4 and IE-27. As expected, high levels of IE-27 RNA appeared at 2 h p.i., peaked at 4 h, and declined steadily to low levels at 8 h. However, although high levels of IE-4 RNA are found after 2 h, that level was maintained until 8 h p.i., a pattern not observed for the synthesis of the ICP4 polypeptide (25, 62).

The pattern of RNA accumulation from early genes was examined with probes specific for TK and ICP8. Similar patterns of RNA accumulation were observed with each

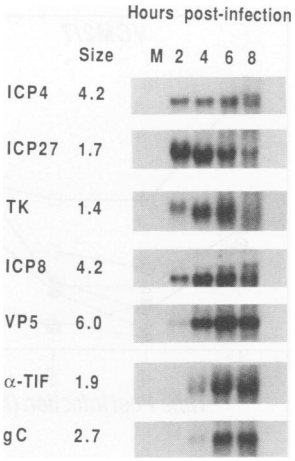

FIG. 6. Temporal pattern of HSV-1 mRNA accumulation. Vero cells were mock infected (lane M) or infected with 5 PFU of wild-type virus per cell. Cells were harvested at 2, 4, 6, and 8 h p.i., and total cytoplasmic RNAs were isolated and analyzed by Northern blot analysis with ^{32}P-labeled probes as described in Materials and Methods. The identity of the probe and the approximate size of each mRNA (in kilobases) are indicated at the left.

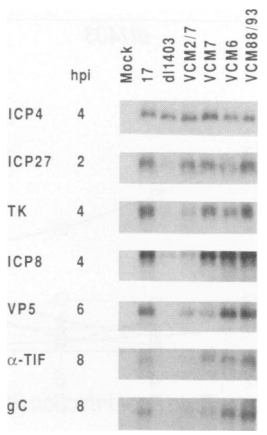

FIG. 7. mRNA accumulation by mutant viruses. Vero cells were mock infected or infected with 2 FFU of the indicated virus per cell. Cells were harvested at 2, 4, 6, and 8 h p.i., and total cytoplasmic RNAs were isolated and analyzed by Northern blot analysis with ^{32}P-labeled probes as described in Materials and Methods. The identity of the probe and the hours p.i. are indicated at the left.

probe. The early RNAs were present at low abundance at 2 h, accumulated to maximal levels between 4 and 6 h, and declined at 8 h p.i. This is consistent with the pattern of polypeptide synthesis from early genes (26).

The temporal pattern of RNA accumulation from early/late and true late genes (52) was examined with probes specific for VP5, α-TIF, and gC. Substantial levels of VP5 RNA appeared after 4 h, peaked at 6 h, and declined at 8 h p.i. The α-TIF and gC RNAs were barely detectable at 2 h, accumulated in increasing amounts after that, peaking at 6 h, and stayed at this high level until 10 h (10-h RNA samples were also prepared in a separate experiment).

We next examined RNA synthesized in Vero cells infected with 2 FFU of wild-type or mutant virus per cell. RNA was isolated at 2, 4, 6, and 8 h p.i. and analyzed by Northern blot hybridization with the seven virus probes described above. The amount of virus gene-specific RNA was determined by densitometric analysis of autoradiograms. Representative samples of the autoradiograms are shown in Fig. 7, and the complete data set from these analyses is presented in graphic form in Fig. 8.

Because the fluorescent-focus titer was based on ICP4 accumulation in the infected cell and the same number of FFUs from each mutant virus was used for infection, the mutant viruses were not defective in accumulation of ICP4 RNA. *dl*1403 accumulated significantly less RNAs than wild-type virus at all time points for all genes tested except IE-4. VCM2/7 accumulated lower amounts of IE-27 RNA at 2 h p.i., and this was reflected in the abundance of this protein as measured after long-term labeling. For any given time point during the productive infection with wild-type virus, the abundance of early and late RNAs was lower and they accumulated at later times p.i. Unlike wild-type virus, the abundance of early RNAs increased at late times p.i., and late gene transcripts were extremely scarce in the time intervals examined. The RNA accumulation profile in cells infected with VCM7 was very similar to that of VCM2/7. Cells infected with VCM6 accumulated decreased levels of IE-27 at very early times. The amount of IE-27 RNA increased to wild-type levels at later times. Just as with IE-27 RNA, accumulation of early and late mRNAs was also delayed. Cells infected with VCM88/93 accumulated higher levels of IE and early mRNAs at 2 and 8 h p.i., and accumulation of late RNAs was delayed.

Marker rescue of mutant virus VCM2/7. To verify that the altered phenotype of VCM2/7 resulted from the deletion in IE-0 and not a double mutation, we did a marker rescue experiment. Vero cells were cotransfected with VCM2/7 DNA and linearized pXQ1, and rapidly growing large plaques were isolated. Southern blot analysis of the DNA from cells infected with these viruses revealed that the resulting recombinant virus (VCM2/7R) had both copies of the IE-0 gene restored (data not shown).

Vero cells were infected with 1 PFU of VCM2/7R and of wild-type HSV-1 per cell, and 24 h later, virus was harvested and titrated on Vero cells. The yield from cells infected with VCM 2/7R was 6.0×10^6 PFU/ml and the yield from cells infected with wild-type HSV-1 was 1.5×10^7 PFU/ml. In addition, the plaque morphology exhibited by the rescued virus was indistinguishable from that of wild-type HSV-1, indicating that the deletion of IE-0 is responsible for impaired growth of VCM2/7 in tissue culture. These viruses were also titrated in HeLa cells, in which the yields were 1.6×10^7 PFU/ml for wild-type virus and 1.0×10^7 PFU/ml for the recombinant.

DISCUSSION

In this report, we describe the construction and characterization of HSV-1 mutants with insertions or deletions in IE-0 sequences. The aim of this work was to examine the role of ICP0 in the lytic cycle of HSV-1-infected cells and to correlate this with the description of its activities in transfection assays.

A difficulty in analyzing IE-0 mutants is that the gene is located completely within the region of the genome that encodes the LAT (48, 53). Therefore, any of the mutations we introduced into the virus would have the potential to affect any LAT gene product. Although it is not possible to unequivocally show that some defects described result from LAT IE-0 double mutants, we (15) and Cai and Schaffer (5a) have constructed LAT$^-$ viruses that exhibit no obvious

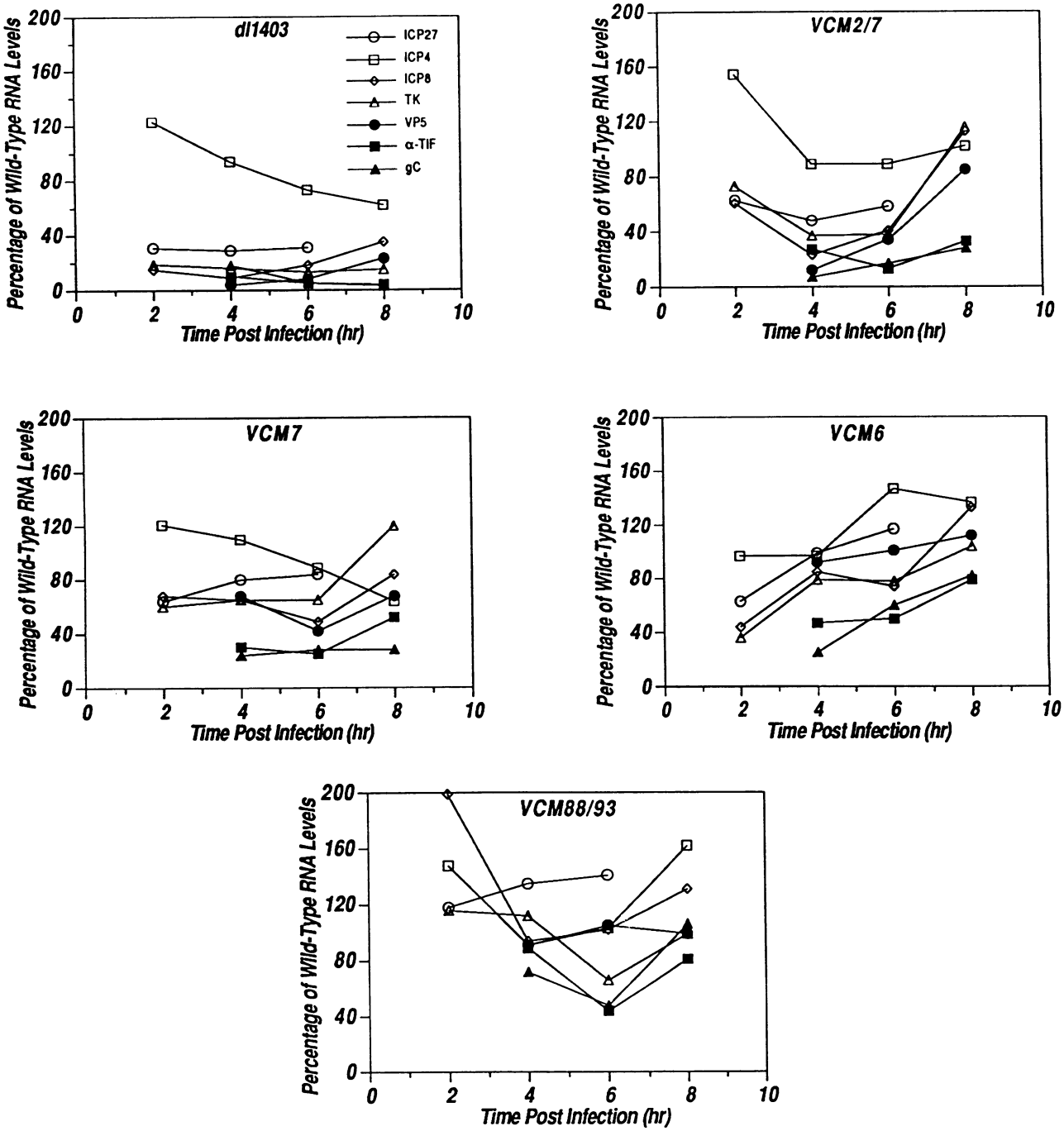

FIG. 8. Kinetics of mRNA accumulation from representative HSV-1 genes by mutant viruses. The abundance of RNAs extracted from cells infected with the indicated mutant viruses at the times stated in each panel was examined by Northern blot analysis. The resulting autoradiograms were subjected to densitometric analysis. The results of this analysis are plotted as percentage of wild-type RNA levels that accumulate in cells infected by each of the mutant viruses as a function of time p.i.

defects in virus growth or yield. These observations coupled with the vanishingly small amount of LAT transcript suggest that the contribution of any LAT gene product to productive infection in tissue culture is small. Moreover, because all the mutants were isolated on more than one occasion, using two different approaches, it is unlikely that their phenotypes reflect acquisition of additional mutations which may have arisen during the generation of the recombinants.

Mutant viruses were first analyzed for their plaquing efficiency in Vero and HeLa cells. It was previously demonstrated that cell type affected the efficiency of transcriptional activation induced by ICP4 and -0 (6, 13, 18) and that *dl*1403, the parental virus used in this study to construct the mutants, exhibited greatly reduced efficiency of plaquing in Vero and human fetal lung cells compared with BHK and HeLa cells (49). Our findings were compatible with these

observations and those of Everett (14). We showed that *dl*1403, *IE-0:lacZ*, and VCM2/7 plaqued more efficiently in HeLa cells than in Vero cells. VCM2/7 is deleted of the major transactivation domain, whose activity is required for high levels of activity in both Vero and HeLa cells (6). pCM88/93, a deletion mutant with a mutation in the carboxy terminus, plaqued slightly better in Vero cells than in HeLa cells. In transfection assays, although its activity on IE and late promoters was higher in HeLa cells than in Vero cells, its ability to cooperate with ICP4 to induce high levels of activity from an early gene promoter target was defective in HeLa cells but not in Vero cells. This synergism between ICP0 and ICP4 may play an important role in the virus life cycle.

Despite the differences in the plaquing efficiencies of some mutant viruses in Vero and HeLa cells, their FFU titers are almost the same in these two cell lines. This shows that these mutant viruses enter both cell types with equal efficiency. Similar findings were recently described by Cai and Schaffer (5), and other ICP0-defective viruses produced similar numbers of particles (5, 14, 49). In a recent study of HSV-1 mutants with defects in IE-0, Cai and Schaffer (5b) correlated particle number with ICP4-inducing units, PFUs, and DNA content and concluded that ICP4-inducing units are a valid measurement of virus delivery. Thus, the difference between mutant and wild-type virus lies in the ability of virus genomes to form plaques in different cell lines rather than in their ability to enter cells. We do not know the fate of the virus genomes that fail to give rise to plaques, but they may be present in a latentlike state as is found in their natural host.

Consistent with their plaquing efficiency on Vero and HeLa cells, the yield of the down mutant viruses was lower than that of wild-type virus and higher in HeLa cells than in Vero cells following infection at low MOI. A reduction in yield following low-MOI infection with HSV-1 mutants that were defective in ICP0 activity was also observed in BHK cells (49) and in Vero cells (5). In the present study, when infections were performed at high MOI, more PFU were produced in infected Vero cells for all the viruses examined. While some viruses, including the wild-type and mutants with partial transactivation activities, had higher yields of infectious particles in HeLa cells than they did at low MOI, the yields from *dl*1403 and VCM2/7 were the same as they were at low MOI. Thus, for these two mutants, there was no difference in yield between Vero and HeLa cells when infections were performed at higher MOI. However, the yields of these mutant viruses were still low compared with that of the wild-type virus. This result is consistent with the findings of Sacks and Schaffer (45) but differs from what was described by Stow and Stow (49) when different cell lines were infected with *dl*1403 at high MOI.

Differences in polypeptide synthesis were observed between mutant and wild-type viruses, and in particular, the accumulation of early and late gene products appeared to be reduced when cells were infected with the same number of FFU. Similar findings were observed by Everett (14) when infection was performed at very low MOI using the same number of particles per cell. When the kinetics of polypeptide synthesis in cells infected with the same number of PFU of mutant viruses was analyzed, there was no apparent difference between mutant and wild-type viruses (data not shown). This difference between what occurs at low versus high MOI could result solely from the introduction of larger amounts of α-TIF that might substitute for ICP0 under these conditions.

Because the cascade of macromolecular synthesis in cells infected by HSV-1 is established as a consequence of mechanisms that regulate both transcription and accumulation of virus RNA (56), we also analyzed the effect of mutations to the ICP0-coding sequence on the accumulation of specific virus RNAs. Our results showed that *dl*1403 was defective in accumulation of virus RNAs from each kinetic class examined. Although none of the other viruses examined in this study, except for VCM11/93, was as defective in accumulation of IE-27 RNA at 2 h p.i., the other mutants were defective in accumulating early (TK and ICP8) or early-late (VP5) RNAs, and the peak levels of accumulation were delayed. Cells infected with the mutant viruses were most notably impaired for accumulation of late (gC and α-TIF) RNAs. The accumulation of early and late RNAs from mutant viruses correlates with their efficiencies of plaquing, yields, and polypeptide synthesis. However, steady-state RNA measurements do not address all the issues, because the rates of virus gene transcription in cells infected with the various mutants remain unknown. One of the novel results from this study is the observation that there is an apparent defect in accumulation of IE-27 RNA synthesis in cells infected with transactivation-defective IE-0 mutants. The lag in synthesis of late gene products correlates with the temporal delay in IE-27 RNA accumulation and supports the findings of McCarthy et al. (31) and Sacks et al. (44), who first suggested that ICP27 played an important role in control of late gene expression. Thus, it may be that the abundance of ICP27 is critical in temporal regulation of virus gene expression.

The target of ICP0 action is not clear. Both analysis of virus polypeptide expression and RNA accumulation suggest that expression from all three classes of temporally regulated genes is affected by mutations in the IE-0 gene. Cai and Schaffer (5) immunoprecipitated polypeptides synthesized at different times p.i. by a different *IE-0:lacZ* virus and reached a similar conclusion. The decrease in the yield of virus-specified RNAs and proteins may reflect a defect in one of the steps, which is enhanced by ICP0, that controls the cascade regulating HSV-1 protein synthesis.

Correlations between transactivation activity in transfection assays and growth of virus mutants with defects in ICP0 were observed. Mutant viruses that exhibited wild-type levels of activity in transfection assays did not show any defect in plaquing efficiency or growth, while those with low levels of transactivation activity had low efficiencies of plaquing and grew poorly in Vero cells. However, in transfection assays, ICP0 transactivates the expression of all three classes of HSV-1 genes. In cells infected with mutant viruses, IE-4 gene expression was normal, while IE-27 and early and late gene expression was reduced. The lack of requirement for ICP0 to induce IE-4 might be specific to tissue culture cells, in which the role of ICP0 is overshadowed by α-TIF, the virion-associated transcription factor (1). However, during the reactivation of virus from latency α-TIF is probably unavailable, and here the activating function of ICP0 on IE genes might be critical. In support of this idea, we note that viruses deleted of the gene encoding ICP0 reactivate only poorly from latency (7, 28, 43) and that adenoviruses that express functional but not mutant forms of ICP0 reactivate latent HSV-2 in an in vitro latency system (22, 62, 63). Thus, the domains of ICP0 that are important for transactivation and augmentation of virus production also have a role in reactivation.

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REFERENCES

1. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46:**371–377.
2. **Beard, P., S. Faber, K. W. Wilcox, and L. I. Pizer.** 1986. Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA **83:**4016–4020.
3. **Berg, J.** 1986. Potential metal binding domains in nucleic acid binding proteins. Science **232:**485–487.
4. **Cai, W., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J. Virol. **63:**4579–4589.
5. **Cai, W., and P. A. Schaffer.** 1991. A cellular function can enhance gene expression and plating efficiency of a mutant defective in the gene for ICP0, a transactivating protein of herpes simplex virus type 1. J. Virol. **65:**4078–4090.

5a. **Cai, W., and P. A. Schaffer.** Personal communication.

5b. **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.

6. **Chen, J., X. Zhu, and S. Silverstein.** 1991. Mutational analysis of the sequence encoding ICP0 from herpes simplex virus type-1. Virology **180:**207–220.
7. **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.
8. **Dixon, R. 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.
9. **Evans, R. M., and S. M. Hollenberg.** 1988. Zinc fingers: gilt by association. Cell **52:**1–3.
10. **Everett, R. D.** 1984. Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3:**3135–3141.
11. **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.
12. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J. Mol. Biol. **202:**87–96.
13. **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.
14. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J. Gen. Virol. **70:**1185–1202.
15. **Flanagan, W. M., A. G. Papavassiliou, M. Rice, L. B. Hecht, S. Silverstein, and E. K. Wagner.** 1991. Analysis of the herpes simplex virus type 1 promoter controlling the expression of U_L38, a true late gene involved in capsid assembly. J. Virol. **65:**769–786.
16. **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.
17. **Gelman, I. H., and S. Silverstein.** 1986. Coordinate 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.
18. **Gelman, I. H., and S. Silverstein.** 1987. Herpes simplex virus immediate-early promoters are responsive to virus and cell *trans*-acting factors. J. Virol. **61:**2286–2296.
19. **Gerster, T., C.-G. Balmaceda, and R. G. Roeder.** 1990. The cell type-specific octamer transcription factor OTF-2 has two domains required for the activation of transcription. EMBO J. **9:**1635–1643.
20. **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.
21. **Hall, L. M., K. G. Draper, R. J. Frink, R. H. Costa, and E. K. Wagner.** 1982. Herpes simplex virus mRNA species mapping in *Eco*RI fragment. J. Virol. **43:**594–609.
22. **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.
23. **Harris-Hamilton, E., and S. L. Bachenheimer.** 1985. Accumulation of herpes simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. J. Virol. **53:**144–151.
24. **Herrin, K. L., and G. W. Schmidt.** 1988. Rapid, reversible staining of Northern blots prior to hybridization. BioTechniques **6:**196–198.
25. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpesvirus polypeptides in infected cells. J. Virol. **12:**1347–1365.
26. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14:**8–19.
27. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72:**1276–1280.
28. **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.
29. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus alpha-gene 4, 0, and 27 promoter-regulatory sequences which confer alpha-regulation on chimeric thymidine kinase genes. J. Virol. **44:**939–946.
30. **Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J. C. McKnight, and B. Roizman.** 1986. Regulation of herpes simplex virus 1 genes: α gene sequence requirements for transient induction of indicator genes regulated by β or late (γ2) promoters. Virology **149:**152–164.
31. **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.
32. **Mermod, N., E. A. O'Neil, T. J. Kelly, and R. Tjian.** 1989. The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. Cell **58:**741–753.
33. **Murchie, M. J., and D. McGeoch.** 1982. DNA sequence analysis of an immediate early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 to 0.978). J. Gen. Virol. **62:**1–15.
34. **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.
35. **O'Hare, P., and G. S. Hayward.** 1985. Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56:**723–733.
36. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77:**733–749.
37. **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.

38. **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.
39. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335:**683–689.
40. **Quinlan, M. P., and D. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral factors. Mol. Cell. Biol. **5:**957–963.
41. **Rice, S. A., and D. M. Knipe.** 1988. Gene-specific *trans*-activation by the herpes simplex virus type 1 alpha protein ICP27. J. Virol. **62:**3814–3823.
42. **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 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62:**4307–4320.
43. **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.
44. **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.
45. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the HSV-1 immediate-early protein, ICP0, exhibit impaired growth in cell culture. J. Virol. **61:**829–839.
46. **Sekulovich, R. E., K. Leary, and R. M. Sandri-Goldin.** 1988. The herpes simplex virus type 1 α protein ICP27 can act as a *trans*-repressor or a *trans*-activator in combination with ICP4 and ICP0. J. Virol. **62:**4510–4522.
47. **Shapira, M., F. L. Homa, J. C. Glorioso, and M. Levine.** 1987. Regulation of the herpes simplex virus type 1 late (γ2) glycoprotein C gene: sequences between base pairs −34 to +29 control transient expression and responsiveness to transactivation by the products of the immediate early (α) 4 and 0 genes. Nucleic Acids Res. **15:**3097–3111.
48. **Steiner, I., J. G. Spivak, D. R. O'Boyle II, E. Lavi, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virol. **62:**3493–3496.
49. **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.
50. **Struhl, K.** 1987. Promoters, activator proteins and the mechanism of transcriptional initiation in yeast. Cell **49:**295–297.
51. **Su, L., and D. M. Knipe.** 1989. Herpes simplex virus α protein ICP27 can inhibit or augment viral gene transactivation. Virology **170:**496–504.
52. **Wagner, E. K.** 1985. Individual HSV transcripts: characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), The herpesviruses. Plenum Press, New York.
53. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y. Zhang, W. M. Flanagan, and J. G. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62:**1194–1202.
54. **Walboomers, J. M., and J. Ter Schegget.** 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology **74:**256–258.
55. **Watson, R. J., and J. B. Clements.** 1980. A herpes simplex type 1 function continuously required for early and late virus RNA synthesis. Nature. (London) **285:**329–330.
56. **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.
57. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76:**1373–1376.
58. **Williams, T., and R. Tjian.** 1991. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev. **5:**670–682.
59. **Zhang, Y.-F., and E. K. Wagner.** 1987. The kinetics of expression of individual herpes simplex virus type 1 transcripts. Virus Genes **1:**49–60.
60. **Zhu, X., J. Chen, and S. Silverstein.** 1991. Isolation and characterization of a functional cDNA encoding ICP0 from herpes simplex virus type 1. J. Virol. **65:**957–960.
61. **Zhu, 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.
62. **Zhu, X., A. G. Papavassiliou, H. G. Stunnenburg, and S. Silverstein.** 1991. Transactivation by herpes simplex virus proteins ICP4 and ICP0 in vaccina virus infected cells. Virology **184:**67–78.
63. **Zhu, X., C. S. H. Young, and S. Silverstein.** 1988. Adenovirus vector expressing functional herpes simplex virus ICP0. J. Virol. **62:**4544–4553.