Repression of the $\alpha 0$ Gene by ICP4 during a Productive Herpes Simplex Virus Infection

ERIK K. LIUM,¹ CHRISTOS A. PANAGIOTIDIS,² XIAOSHAN WEN,² AND SAUL SILVERSTEIN^{1,2*}

*Department of Microbiology² and Integrated Program in Cellular, Molecular and Biophysical Studies,¹
College of Physicians and Surgeons, Columbia University, New York, New York 10032*

Received 11 January 1996/Accepted 4 March 1996

During a productive infection by herpes simplex virus type 1 (HSV-1), ICP4, the major regulatory protein encoded by the $\alpha 4$ gene, binds to its transcription initiation site and represses the accumulation of $\alpha 4$ RNA. Evidence suggests that the degree of repression by ICP4 is a function of the absolute distance of an ICP4 binding site 3' from a TATA box. However, repression of HSV-1 gene expression by ICP4 through binding sites located 5' of TATA boxes, as in the case of the $\alpha 0$ gene, has not been adequately addressed. To this end, recombinant $\alpha 0$ promoters with various arrays of ICP4 binding sites flanking the $\alpha 0$ TATA box were constructed and recombined into the HSV-1 genome. Our results demonstrate the following. (i) Destruction of the endogenous $\alpha 0$ ICP4 binding site, located 5' of the TATA box, results in derepression of $\alpha 0$ protein and RNA accumulation in infected Vero cells. (ii) The degree of $\alpha 0$ derepression is equivalent to that reported for the $\alpha 4$ gene following destruction of the ICP4 binding site at the $\alpha 4$ mRNA cap site in HSV-1. (iii) Introduction of an ICP4 binding site at the $\alpha 0$ mRNA cap site represses the accumulation of $\alpha 0$ RNA greater than threefold relative to the wild type. (iv) Changes in the abundance of $\alpha 0$ protein and RNA in infected cells do not affect replication or growth of HSV-1 in tissue culture. Our findings are consistent with the conclusion that $\alpha 0$ transcription is repressed by ICP4. These results demonstrate that repression by ICP4 can occur through binding sites located 5' of virus gene TATA boxes in HSV-1. Thus, models addressing repression of HSV-1 gene expression by ICP4 should incorporate the role of binding sites located 5', as well as 3', of virus gene TATA boxes.

The genes of herpes simplex virus (HSV) type 1 (HSV-1) and HSV-2 are grouped into three kinetic classes (α , β , and γ) upon the basis of their temporal patterns of expression during productive infection (26, 27, 48). The α genes, $\alpha 0$, $\alpha 4$, $\alpha 27$, $\alpha 22$, $\alpha 47$, and αX , defined by their expression in the absence of de novo protein synthesis, are the first to be transcribed and, with the exception of αX , accumulate stable mRNAs upon initiation of infection (2, 48). Subsequent expression of β genes, whose products are largely involved in replication of the virus genome, and γ genes, which encode many of the structural components of the HSV virion, requires functional α gene products (7, 13, 25, 42). The α gene products, infected-cell proteins (ICP0, ICP4, ICP27, and ICP22), are nuclear phosphoproteins. ICP0, ICP4, and ICP27 regulate the expression of all kinetic classes of virus genes (14, 20, 32, 37, 42, 45, 49, 55, 57). Specifically, ICP4 is an essential protein that functions as both a repressor and an activator of transcription. Acting in concert with ICP0, another viral transactivator, ICP4 activates expression of β and γ genes while independently down-regulating its own expression (7, 19, 36, 42, 47). ICP27, another essential α protein with a critical role in the transition from β to γ gene expression, appears to act primarily at the posttranscriptional level (32, 46, 49). ICP22 has been demonstrated to affect the phosphorylation state of RNA polymerase II, and deletion mutants lacking ICP22 accumulate ICP0 to lower levels than does wild-type HSV-1 (43). ICP47, unlike the other α gene products, is a cytoplasmic protein that inhibits presentation of viral antigens on the surface of infected cells by major histocompatibility complex class I (24, 58).

Although genetic and biochemical studies have broadly defined the functional domains of ICP4 (8, 9, 40, 41, 50), the

mechanism(s) by which ICP4 affects virus gene expression is not understood. ICP4 binds DNA as a dimer to both high-affinity (A sites) and low-affinity (B sites) sequences (33). High-affinity binding sites conforming to the consensus sequence ATCGTCN₄YCGRC, as defined by Faber and Wilcox (17), have been identified in all kinetic classes of HSV-1 genes (11). For example, A-type sites in the regulatory regions of the $\alpha 0$, $\alpha 4$, αX (OrfP), glycoprotein D, alpha *trans*-inducing factor (α TIF), and $\gamma 42$ genes have been described (2, 17, 29, 35). B sites, which fail to conform to an identifiable consensus sequence, have been mapped in several genes (18, 35). Sequence-specific DNA binding by ICP4 is not required for activation of transcription by ICP4; however, binding is essential, but not sufficient, for repression (23, 47, 50).

Regulation of $\alpha 4$ gene expression is the best-studied example of repression by ICP4. ICP4 was shown to autoregulate in transient expression studies and in productive infection (20, 21, 37, 47). The degree of $\alpha 4$ repression by ICP4 is dependent upon the orientation and distance of its ICP4 binding site 3' from the TATA box. Formation of tripartite complexes among ICP4, TATA-binding protein, and transcription factor IIB may also play a role in repression (23, 31, 34, 53). However, the fundamental relationship between the position and function of ICP4 binding sites relative to most virus genes, such as the $\alpha 0$ gene, remains undefined.

Gelman and Silverstein demonstrated that in the absence of functional ICP4, $\alpha 0$ RNA levels increase (20). Subsequent transient expression studies implied that repression was dependent upon both the endogenous $\alpha 0$ ICP4 binding site and functional ICP4 (16, 20, 21, 44). However, in the context of the virus genome, destruction of the ICP4 binding site did not significantly affect the accumulation of ICP0 (16). Upon the basis of this result, Everett and Orr concluded that $\alpha 0$ expression is not regulated by ICP4 during a productive infection (16).

* Corresponding author. Phone: (212) 305-8149. Fax: (212) 305-5106. Electronic mail address: sjs@cuccfa.ccc.columbia.edu.

In this report, we present a series of experiments designed to address repression of $\alpha 0$ expression by ICP4 in the context of the HSV-1 genome. We demonstrate that the native ICP4 binding site, located 5' of the $\alpha 0$ TATA box, mediates repression of $\alpha 0$ expression by ICP4. The degree of repression is equivalent to that reported for the $\alpha 4$ gene (34). Our results establish that ICP4-mediated repression occurs through binding sites located 5', as well as 3', of HSV-1 TATA boxes.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, N.Y.) containing 5% bovine calf serum (Hyclone Laboratories Inc., Logan, Utah), 100 U of penicillin per ml, and 100 μ g of streptomycin per ml (Gibco BRL) unless otherwise noted. The parental viruses used in this study were wild-type HSV-1 (Glasgow strain 17) and the IE-0: LacZ transplacement virus previously described (4).

Plasmids and phage (ϕ). Plasmid pJC7 contains the *SacI*-*HpaI* fragment of the $\alpha 0$ gene (−810 to +3780) from HSV-1 strain 17 in pUC19 (5). Phage ϕ XW5 contains an *SmaI*-*RsaI* fragment spanning the $\alpha 0$ mRNA cap site (−133 to +167) cloned into M13mp19. ϕ MXW1 was derived from ϕ XW5 by oligonucleotide-directed mutagenesis (1) of the $\alpha 0$ mRNA cap site as described below. pXW7 is a derivative of pUC19 in which the *DrdI* sites were destroyed. pXW8 contains the *SacI*-*HindIII* fragment of $\alpha 0$ (−810 to +3780) from pJC7 inserted into pXW7. Plasmid pXW9 was constructed by replacing the *DrdI*-*NcoI* fragment of the $\alpha 0$ gene (−11 to +143) in pXW8 with the corresponding fragment from ϕ MXW1. Plasmids pTAE4, pTAE5, and pTAH5 contain PCR amplimers of the $\alpha 0$ promoter (−436 to +1) cloned into pGEM-T (Promega, Madison, Wis.) following sequential PCR mutagenesis (1) of the endogenous $\alpha 0$ ICP4 binding site. pEL11E and pEL11H were constructed by replacing the *BstXI*-*DrdI* fragment (−430 to −11) of pXW8 with either the *BstXI*-*EcoRI* and *EcoRI*-*DrdI* fragments from pTAE4 and pTAE5 or the *BstXI*-*DrdI* fragment from pTAH5, respectively. Plasmids pEL12E and pEL12H were constructed by replacing the *BstXI*-*DrdI* fragment of pXW9 with either the *BstXI*-*EcoRI* and *EcoRI*-*DrdI* fragments from pTAE4 and pTAE5 or the *BstXI*-*DrdI* fragment from pTAH5, respectively.

Construction of mutants. (i) **Creation of an ICP4 binding site at the $\alpha 0$ transcription initiation site.** An ICP4 binding site (11, 17) was created at the $\alpha 0$ mRNA cap site by oligonucleotide-directed mutagenesis (1) of ϕ XW5. The mutagenic primer (P.0-4BSC) sequence was 5'-ACAGTCTGGTCGATCGT CACCTCGGCACTCGG-3'. The mutated sequence was introduced into pXW8 to yield plasmid pXW9 as described above. The sequence of the recombinant ICP4 binding site was confirmed by cycle sequencing with Amplicycle (Perkin Elmer, Foster City, Calif.).

(ii) **Destruction of the wild-type ICP4 binding site in the $\alpha 0$ promoter.** Sequential PCR mutagenesis was performed as previously described (1), with minor modifications. The flanking primer sequences (Genset Corp., La Jolla, Calif.) were as follows: JC7-780-5'3', 5'-GCGTAACCACTCCCCCTGGGGT-3'; JC7-1284-3'5', 5'-TAGGCGGGGCTTCTGTGGTGAT-3'. The mutagenic primer sequences were as follows: JC7-1133E-5'3', 5'-GCCATTGGGGGAATTCTCACTGCC-3'; JC7-1133E-3'5', 5'-GGCAGTGAGAAATCCCCCAATGGC-3'; JC7-1136H-5'3', 5'-ATTGGGGGAAGCTTCACTGCCGC-3'; JC7-1136-3'5', 5'-GCGGCACTGAAGCTTCCCCCAAT-3'. Primary PCR amplification mixtures contained 1 ng of pXW8, 400 nM mutagenic primer, 400 nM flanking primer, 200 μ M deoxynucleoside triphosphates (Pharmacia Biotech, Uppsala, Sweden), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 1% Triton X-100, 7.5% glycerol, 1 mM spermidine (pH 7.0), and 2 U of *Taq* DNA polymerase (Promega). Primary PCR amplification products were purified on Qiaquick Spin columns (Qiagen, Chatsworth, Calif.). Secondary PCR amplifications to generate full-length fragments were performed as described above, with minor modifications. Reactions contained both flanking primers (JC7-780-5'3' and JC7-1284-3'5') at 800 nM and 5% of each sibling primary amplification mixture (50 to 100 ng). Secondary amplimers were ligated into pGEM-T (Promega). The DNA sequence of each mutant (pTAE4, pTAE5, and pTAH5) was determined by cycle sequencing, and the mutated ICP4 binding sites were introduced into either pXW8 or pXW9 as described above. The resulting $\alpha 0$ genomic clones contained (i) the endogenous $\alpha 0$ ICP4 binding site mutated to create either an *EcoRI* (E) or a *HindIII* (H) restriction site 5' of the TATA box (Fig. 1, pEL11H and pEL11E), (ii) the mutant ICP4 binding site from either pEL11E or pEL11H and a recombinant ICP4 binding site at the $\alpha 0$ mRNA cap site (Fig. 1, pEL12H and pEL12E), (iii) two ICP4 binding sites, the endogenous and the recombinant, located 5' of the $\alpha 0$ TATA box and at the mRNA cap site, respectively (Fig. 1, pXW9).

Construction of $\alpha 0$ mutant viruses. (i) **Transfections.** Vero cells were seeded at 5×10^5 cells per 60-mm-diameter plate. Cells were transfected by using the calcium phosphate precipitation method as previously described (56). Transfections contained 12.5 μ g of plasmid (pEL11E, pEL11H, pEL12E, pEL12H, or pXW9) DNA linearized with *SacI* and 100 PFU of IE-0: LacZ nucleocapsids (52).

(ii) **Purification of recombinant viruses.** The primary recombinant viruses resulting from cotransfection of cells with IE-0: LacZ nucleocapsids and linear-

FIG. 1. Schematic presentation of wild-type and mutant $\alpha 0$ promoters and Southern blot analysis of wild-type and recombinant virus DNAs. (A) $\alpha 0$ promoters were constructed with wild-type ($\square$), recombinant ($\blacksquare$), and mutant (X) ICP4 binding sites flanking the $\alpha 0$ TATA box as described in Materials and Methods. The wild-type ICP4 binding site in the $\alpha 0$ promoter (−71 to −54) was mutated in EL11E and EL12E to create an *EcoRI* (E) site and in EL11H and EL12H to create a *HindIII* (H) site. The DNA sequences of wild-type and mutant binding sites and the recombinant ICP4 binding site at the mRNA cap are given. Mutations are shown in boldface capital letters, and the ICP4 binding site core sequences are underlined. (B) Southern blot analysis of the $\alpha 0$ genomic loci was performed as described in Materials and Methods. Recombinant virus DNAs were digested with *SacI* and *PstI* in either the absence or the presence of *EcoRI* or *HindIII*. Electrophoretically separated restriction enzyme digests were transferred to nylon membranes and hybridized with a random primed $\alpha 0$ probe (+790 to +3350).

ized plasmids were passaged at low multiplicities of infection (MOI) on Vero cells, harvested, and used to infect Vero cell monolayers at 25 PFU/60-mm plate. Infected cells were overlaid with Dulbecco's modified Eagle's medium containing 1.5% methyl cellulose (Sigma Chemical, St. Louis, Mo.), 5% bovine calf serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Individual plaques were picked and used directly to infect Vero cell monolayers in 96-well plates. Infected-cell DNAs harvested from the 96-well plates were analyzed by dot blot hybridization and PCR as described below. Recombinant viruses were plaque purified three times, and final stocks were verified by PCR for the presence of $\alpha 0$ and the absence of *lacZ* sequences as described below. The appropriate sequence arrangement of the $\alpha 0$ loci and mutagenesis of the endogenous ICP4 binding site were confirmed by Southern blot and sequence analyses. The presence of the recombinant ICP4 binding site in vEL12E, vEL12H, and vXW9 was also confirmed by sequence analysis (Fig. 1).

Preparation and analysis of virus DNA. (i) **DNA probes.** The probes used consisted of the 2.5-kb *BamHI*-*SacI* fragment (+790 to +3350) of the $\alpha 0$ gene and a 2.2-kb *EcoRV* fragment of pBlueBacHisB (Invitrogen, San Diego, Calif.) containing 1.95 kb of the *lacZ* gene. Probes were labeled with [α -³²P]dCTP (DuPont NEN, Boston, Mass.) by random priming (Boehringer Mannheim, Indianapolis, Ind.).

(ii) **Dot blot hybridization.** Aliquots of infected cells in media were combined 1:1 with 200 mM Tris-HCl (pH 7.4), 10 mM EDTA, 300 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), and 160 μ g of proteinase K per ml and incubated for 3 h at 40°C. Samples were denatured by addition of NaOH and NaCl to 0.25 and 0.5 M, respectively. DNAs were blotted to GeneScreen Plus (DuPont NEN) with an SRC-96 dot blot apparatus (Schleicher & Schuell, Keene, N.H.), cross-linked by UV irradiation with a Stratalinker 2400 (Stratagene, La Jolla, Calif.), and hy-

bridized by following the manufacturer's (DuPont NEN) instructions. Blots were washed as recommended by the manufacturer and exposed to X-Omat AR film (Eastman Kodak, Rochester, N.Y.).

(iii) **DNA preparation for Southern blot and PCR analyses.** Infected cells were collected by centrifugation at $12,000 \times g$ for 5 min. Pellets were resuspended in 50 mM Tris-HCl (pH 7.4)–100 mM NaCl–5 mM EDTA–200 μ g of proteinase K per ml–0.5% SDS and incubated at 50°C for 3 h. Following digestion, 85 μ l of a saturated NaCl solution was added per 250- μ l sample and mixed thoroughly for 15 min at room temperature. The resulting precipitate was removed by centrifugation at $12,000 \times g$ for 15 min, and the DNA was precipitated with 0.7 volume of isopropanol and washed twice with 70% ethanol.

(iv) **PCR screening of recombinant viruses.** PCRs contained 10 ng of total infected-cell DNA, 1 μ M forward primer, 1 μ M reverse primer, 400 μ M deoxynucleoside triphosphates 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 7.5% glycerol, and 2 U of *Taq* DNA polymerase. $\alpha 0$ primers, which result in amplification of a 137-bp fragment of the $\alpha 0$ gene (+1246 to +1383), were 116-5'3' (5'-GGCGACGTGTGCGCGGTGTG-3') and 156-3'5' (5'-GGCGTTGCACAGCGGGCAGG-3'). LacZ primers, which result in amplification of a 237-bp fragment, were LacZ-5'3' (5'-CTCTATCGTGCG GTGGTTGA-3') and LacZ-3'5' (5'-CGGGCTTAAAGTTGTTCTGC-3'). Amplification products were visualized by ethidium bromide staining after separation on 6% native polyacrylamide gels.

(v) **Amplification and sequencing of virus IE-0 promoter regions.** The desired mutations in the recombinant viruses (Fig. 1) were verified by cycle sequencing of the 408-bp PCR amplicons of the $\alpha 0$ gene (–324 to +85). The amplification primers used were 1794 (5'-GGGTTCCGGGTATGGTAATG-3') and XW8Seq (5'-CGGGGCTTCTGTGGTGAT-3'). The DNA sequences of the amplicons were determined by cycle sequencing with the primer XW8Seq.

(vi) **Southern blot analysis.** Infected-cell DNA was digested with the enzymes indicated in Fig. 1, separated by agarose gel electrophoresis, and transferred to SS-Nytran (Schleicher & Schuell). Southern blot hybridization with the $\alpha 0$ probe was performed in accordance with the manufacturer's instructions. Blots were washed as recommended by the manufacturer and exposed to Biomar Blue film (Marsh Biochemical, Rochester, N.Y.).

Nuclear extract preparation and electrophoretic mobility shift analysis (EMSA). HeLa cells were propagated in monolayers as previously described (39). Cells were infected with HSV-1 strain 17 at an MOI of 5 PFU per cell, and nuclear extracts were prepared at 6 h postinfection as described by Dignam et al. (12). The protein concentrations were determined by the Bradford method (3), and all extracts were stored frozen in aliquots at –80°C.

EMSA of ICP4 complex formation with the 180-bp *Ava*I fragments from the wild-type and recombinant $\alpha 0$ promoters (–133 to +47) was performed by incubating 3 μ g of nuclear extracts from mock- or HSV-1-infected cells with 32 P-end-labeled restriction fragments in the absence or presence of anti-ICP4 monoclonal antibody 58S (51), followed by native polyacrylamide gel electrophoresis (PAGE) (39). The DNA-binding reactions were performed for 20 min at room temperature in 10 mM Tris-HCl (pH 7.5)–50 mM NaCl–5% (vol/vol) glycerol–1 mM EDTA–4 mM dithiothreitol–0.2 mg of bovine serum albumin per ml in a final volume of 20 μ l. Sonicated salmon sperm DNA (2 μ g) was included as a nonspecific competitor. One microliter (1 μ g) of purified antibody 58S was added to the samples indicated in Fig. 2, which were incubated at room temperature for an additional 30 min. Following separation by native PAGE, the DNA-protein complexes were visualized by autoradiography. Monoclonal antibody 58S was purified from ascites fluid by affinity chromatography on a protein G-agarose column (Pharmacia), dialyzed against binding buffer, and stored in aliquots at –80°C. The ascites fluids were obtained from mice inoculated intraperitoneally with HB8183 hybridoma cells (American Type Culture Collection) (51).

Virus growth assays. (i) Growth curves. Vero cells were seeded at 10^6 per well on six-well plates. On the following day, cells were infected with the viruses indicated in Fig. 3 in absorption medium (Dulbecco's modified Eagle's medium containing 1% bovine calf serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml) at either 0.1 or 5 PFU per cell (Fig. 3). Infections were halted by freezing (–80°C) at the times indicated in Fig. 3. Virus pools were subjected to five cycles of freezing and thawing, and virus yields were titrated on Vero cells. Growth curves represent the average of three independent infections, each titrated in duplicate.

(ii) **Burst size.** Vero cells were seeded at 10^6 per well on six-well plates. On the following day, the cells were infected at an MOI of either 0.1 or 5 (Fig. 4). Following 60 min for virus absorption, monolayers were washed twice with phosphate-buffered saline (PBS) to remove free virus particles and overlaid with fresh medium. Infections were stopped at 4 and 24 h by freezing (–80°C). Virus pools were subjected to five cycles of freezing and thawing, and virus yields were titrated on Vero cells. Burst size calculations represent the average of three independent infections, each titrated in duplicate.

Preparation and Western blot (immunoblot) analysis of virus proteins. Vero cells were seeded at 3×10^6 per 60-mm plate. On the following day, the cells were infected at an MOI of 1 (Fig. 5). Infected cells were scraped into 5 ml of ice-cold PBS at 3, 6, 9, and 12 h postinfection and collected by centrifugation at $2,000 \times g$ for 5 min at 4°C. Infected-cell pellets were resuspended in 300 μ l of $1.5 \times$ SDS-PAGE sample buffer (30) and boiled for 10 min. The proteins were separated by SDS-PAGE as previously described (30) and electrophoretically

FIG. 2. EMSA of ICP4 complex formation with the wild-type and recombinant $\alpha 0$ promoters. Nuclear extracts (3 μ g) from mock-infected (M) or HSV-1-infected (I) cells were incubated with 32 P-end-labeled *Ava*I fragments (–133 to +47) from the wild-type and mutant $\alpha 0$ promoters in the absence (–) or presence (+) of 1 μ g of anti-ICP4 monoclonal antibody (Ab) 58S. The ICP4-DNA complexes were analyzed by native PAGE as described in Materials and Methods.

transferred to nitrocellulose membranes (54). Immunodetection of virus proteins was performed as previously described (38). Antibodies against virus proteins are as follows: ICP0, rabbit polyclonal antibody CLU7 raised against a glutathione S-transferase-ICP0 fusion protein containing amino acids 312 to 400 of ICP0; ICP4, mouse monoclonal antibody H1114 (Goodwin Institute for Cancer Research, Plantation, Fla.); ICP27, rabbit polyclonal antibody CLU38 raised against a glutathione S-transferase-ICP27 fusion protein containing amino acids 1 to 252 of ICP27; glycoprotein B, rabbit polyclonal antibody R69 (provided by G. Cohen, Department of Microbiology, University of Pennsylvania, Philadelphia); α TIF, rabbit anti- α TIF (59) (provided by T. Kristie, National Institute of Allergy and Infectious Diseases). The secondary antibodies, conjugated to horseradish peroxidase, used in this study were goat anti-rabbit immunoglobulin G and goat anti-mouse immunoglobulin G. Immunoblots were developed by using the chemiluminescent substrate LumiGLO (Kirkegaard & Perry, Gaithersburg, Md.) and exposed to Biomar Blue film. To determine the changes in levels of the virus-specified proteins as a function of time postinfection and the levels of expression of virus-specified proteins elaborated by the mutant viruses, a series of X-ray film exposures of each Western blot was analyzed with a Molecular Dynamics scanning densitometer and peak heights were integrated and quantified with ImageQuant software.

RNA extraction and primer extension analysis. Total cellular RNAs were prepared from uninfected or HSV-1-infected Vero cells by a modification of the method of Chomczynski and Sacchi (6), with the commercially available TRIzol reagent (Gibco BRL). Specifically, Vero cell monolayers were infected at an MOI of 10, and RNA was prepared at 3 and 7 h postinfection in accordance with the manufacturer's instructions. The RNA pellets were dissolved in 100 μ l of RNase-free diethylpyrocarbonate-treated water.

To prepare RNA from cells infected in the absence of protein synthesis, Vero cells were preincubated for 1 h in the presence of 100 μ g of cycloheximide per ml and then infected at an MOI of 10 in the presence of cycloheximide. Total cellular RNA was prepared as described above at 6 h postinfection.

Primer extension analyses were performed by standard techniques (1). The following primers, synthesized by the DNA facility of the Columbia-Presbyterian Cancer Center, were used: 0-PrimExt, 5'-GTGATGCGGAGAGGGGGCGGC CCGAGTCTG-3'; 4-PrimExt, 5'-CGTCTGACGGTCTGTCTCTGCGCGGTCC CGC-3'; 27-PrimExt, 5'-GTAGCGAGCGACCGGGCCGAATCGGGAG-

FIG. 3. Analysis of virus growth in low- and high-MOI infections. Vero cells were infected with wild-type HSV-1 (●), vEL11E (□), vEL11H (△), vEL12E (▲), vEL12H (▼), vXW9 (◇), and IE-Ø:LacZ (○) at either 0.1 (A) or 5 (B) PFU per cell, and the infections were stopped at the indicated times postinfection. The resulting virus pools were titrated on Vero cell monolayers. Growth curves represent the average of three independent infections, each titrated in duplicate.

3'. The primers were designed so that their 3' ends would be approximately 40 bases from the transcription initiation sites of the $\alpha 0$, $\alpha 4$, and $\alpha 27$ genes, respectively. The primer extension products were separated on 6% sequencing gels, and the dried gels were autoradiographed. Quantitation of the primer extension products was performed by analysis with a Molecular Dynamics 445SI PhosphorImager and ImageQuaNT software.

FIG. 4. Analysis of burst size in low- and high-MOI infections. Vero cells were infected with the wild-type or recombinant viruses at an MOI of either 0.1 (A) or 5 (B) for 60 min. Free virus particles were removed, and the infections were overlaid with fresh medium. The infections were harvested at 4 and 24 h postinfection, and the burst sizes were determined. Burst size calculations represent the average of three independent infections, each titrated in duplicate.

RESULTS

Experimental design. The primary objective of this study was to clarify the role of ICP4 as a negative regulator of $\alpha 0$ gene expression in HSV-1. To this end, the endogenous $\alpha 0$ ICP4 binding site, located 5' of the TATA box, was mutated to create either an *EcoRI* or a *HindIII* site and introduced into the virus genome by homologous recombination. Destruction of the $\alpha 0$ ICP4 binding site was verified by EMSA, and the effects of the mutations on the abundance of ICP0 and $\alpha 0$ RNAs in infected cells were assessed by Western blot and

FIG. 5. Western blot analysis of virus proteins from total infected-cell extracts. Vero cells were infected at an MOI of 1, and total infected-cell extracts were prepared at 3, 6, 9, and 12 h postinfection (p.i.) as described in Materials and Methods. A 10- μ l sample (10^5 cell equivalents) of each infected-cell extract was separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Blots were probed with either a mouse monoclonal antibody (ICP4, H1114) or rabbit polyclonal antibodies (ICP0, CLU7; ICP27, CLU38; glycoprotein B [gB], R69; α TIF, anti- α TIF). Secondary antibodies were conjugated to horseradish peroxidase, and immunoblots were developed with the chemiluminescent substrate LumiGLO.

primer extension analyses. Concurrently, an ICP4 binding site was introduced into the $\alpha 0$ gene at the mRNA cap site in the presence or absence of the endogenous ICP4 binding site. EMSA confirmed the ability of ICP4 to bind to this site, and the effects of this mutation on $\alpha 0$ expression were ascertained by measuring $\alpha 0$ protein and RNA accumulation relative to the accumulation of other viral proteins and RNAs. Finally, to verify that the intrinsic strengths of the recombinant $\alpha 0$ promoters were not altered by the ICP4 binding site mutations, RNA levels from cells infected in the absence of protein synthesis were quantitated.

Mutagenesis and construction of recombinant viruses. Sequential PCR mutagenesis was performed to alter the sequence of the endogenous ICP4 binding site located 5' of the $\alpha 0$ TATA box to create either an *Eco*RI (E) or a *Hind*III (H) restriction site. These mutations were incorporated into the $\alpha 0$ promoters in plasmids pEL11E and pEL11H, respectively (Fig. 1). Oligonucleotide-directed mutagenesis of a single-stranded M13 template was used to create an ICP4 binding site at the $\alpha 0$ mRNA cap site in plasmid pXW9. This mutation was also incorporated into plasmids pEL11E and pEL11H to create pEL12E and pEL12H, respectively (Fig. 1). These recombinant promoters were introduced into the virus genome by homologous recombination, resulting in recombinant viruses vEL11E, vEL11H, vEL12E, vEL12H, and vXW9 (Fig. 1). The appropriate sequence arrangements of the $\alpha 0$ genomic loci and mutagenesis of the endogenous $\alpha 0$ ICP4 binding site were confirmed by Southern blot analysis (Fig. 1). The predicted sequences of the wild-type and recombinant $\alpha 0$ promoters (Fig. 1) were verified by cycle sequencing.

Analysis of ICP4 complex formation on wild-type and mutant $\alpha 0$ promoter fragments. The effects of the mutations on the ability of ICP4 to bind to the $\alpha 0$ promoter were analyzed by EMSA. The 180-bp *Ava*I fragments spanning the $\alpha 0$ mRNA cap site and upstream sequences (−133 to +47) from each of the plasmid constructs were used as probes. The presence of ICP4 in DNA-protein complexes was verified by supershift analysis with ICP4-specific monoclonal antibody 58S (51) (Fig. 2). The results of this analysis demonstrate that (i) mutagenesis of the endogenous ICP4 binding site abolished ICP4 complex formation (Fig. 2, pEL11E and pEL11H); (ii) introduction of a recombinant binding site at the mRNA cap site, in the absence of the endogenous binding site, resulted in formation of a single complex that was supershifted by 58S (Fig. 2, pEL12E and pEL12H); and (iii) introduction of the recombinant binding site into the wild-type $\alpha 0$ promoter at the mRNA cap site

resulted in the formation of two complexes, both of which were super-shifted by 58S (Fig. 2, pXW9). Thus, (i) mutagenesis of the endogenous ICP4 binding site abolished binding of ICP4, (ii) ICP4 bound to the recombinant binding site created at the $\alpha 0$ mRNA cap site, and (iii) ICP4 bound to both the endogenous and recombinant sites in pXW9.

Analysis of recombinant virus growth and burst size in low- and high-MOI infections. Growth curves were determined to assess the effects of the ICP4 binding site mutations on virus growth in tissue culture (Fig. 3). Vero cells were infected at an MOI of either 0.1 or 5 and harvested at 4, 8, 12, 16, and 24 h postinfection. The virus yields were determined by titration on Vero cell monolayers. All viruses, with the exception of the previously described $\alpha 0$ null virus IE-0:LacZ (4), exhibited indistinguishable growth kinetics and yielded similar titers from low- and high-MOI infections (Fig. 3). To confirm the lack of a growth phenotype, experiments were performed to determine the virus yield from a single round of replication in low- and high-MOI infections (Fig. 4). Vero cells were infected with 0.1 or 5 PFU per cell, and unabsorbed virus was removed by washing twice with PBS. Cells were harvested at 4 and 24 h postinfection, and the virus yields were titrated on Vero cell monolayers. The burst sizes were nearly identical for all viruses, indicating that the replication and growth of the recombinant viruses and wild-type HSV-1 were equivalent under these conditions (Fig. 4). Therefore, destruction of the endogenous ICP4 binding site or introduction of a novel binding site at the $\alpha 0$ transcription initiation site does not significantly affect the growth of HSV-1 in tissue culture.

Accumulation of virus-specified proteins in cells infected with wild-type or recombinant viruses. Western blot analysis was performed to determine the effects of the ICP4 binding site mutations on the accumulation of ICP0 (Fig. 5). Briefly, infected-cell extracts separated by denaturing PAGE were transferred to nitrocellulose membranes and probed with an anti-ICP0 polyclonal antibody, and polypeptide abundance was determined as described in Materials and Methods. The results of this analysis reveal that (i) destruction of the endogenous $\alpha 0$ ICP4 binding site consistently resulted in a two- to threefold increase in the accumulation of ICP0 relative to wild-type HSV-1 (Fig. 5, vEL11E and vEL11H), (ii) introduction of the ICP4 binding site at the $\alpha 0$ mRNA cap site resulted in a four- to fivefold decrease in the abundance of ICP0 relative to wild-type HSV-1 (Fig. 5, vEL12E, vEL12H, and vXW9), and (iii) the presence of two ICP4 binding sites in the $\alpha 0$ promoter did

not further decrease the abundance of ICP0 relative to the recombinant binding site at the mRNA cap alone (Fig. 5, vXW9).

Western blot analysis was also performed to determine whether the accumulation of representative viral proteins from each of the three major kinetic classes of virus genes was altered. Accumulation of immediate-early proteins ICP4 and ICP27, early protein glycoprotein B, and late protein α TIF was essentially the same for all viruses (Fig. 5). These results demonstrate that the endogenous $\alpha 0$ ICP4 binding site affects the accumulation of ICP0 in the context of an infection in tissue culture. Furthermore, the replication and growth of HSV-1 in tissue culture was not affected by greater-than-10-fold fluctuations in the abundance of ICP0 (Fig. 3, 4, and 5).

Accumulation of $\alpha 0$ RNA in cells infected with wild-type or recombinant viruses. To determine whether the endogenous $\alpha 0$ ICP4 binding site affects the accumulation of $\alpha 0$ RNA, total RNA was isolated at 3 and 7 h postinfection from cells infected with wild-type and mutant viruses, and the accumulation of $\alpha 0$ RNA was quantitated by primer extension analysis (Fig. 6). The accumulation of $\alpha 4$ and $\alpha 27$ RNAs was also determined. This experiment demonstrated that (i) destruction of the endogenous ICP4 binding site resulted in a ninefold increase in the abundance of $\alpha 0$ RNA, relative to $\alpha 4$ RNA, at 3 h postinfection (Fig. 6, vEL11E and vEL11H). At 7 h postinfection, $\alpha 0$ RNA levels were sixfold greater than wild-type levels (Fig. 6, vEL11E and vEL11H). Thus, the abundance of $\alpha 0$ RNA in infected cells is consistent with our observations regarding ICP0 accumulation (Fig. 5). (ii) Introduction of an ICP4 binding site at the $\alpha 0$ transcription initiation site resulted in a threefold decrease in the abundance of $\alpha 0$ RNA at 3 and 7 h postinfection relative to wild-type HSV-1 (Fig. 6, vEL12E, vEL12H, and vXW9). (iii) The presence of an additional binding site in the $\alpha 0$ promoter did not repress $\alpha 0$ RNA levels relative to those found when the binding site was only at the mRNA cap site (Fig. 6, vXW9). Introduction of the ICP4 binding site mutations did not affect the abundance of $\alpha 4$ and $\alpha 27$ RNAs.

These results demonstrate that (i) the accumulation of $\alpha 0$ RNA in infected cells correlates strongly with the presence and/or absence of ICP4 binding sites located 5' or 3' of the $\alpha 0$ TATA box (Fig. 6). (ii) The $\alpha 0$ transcription initiation sites within the recombinant promoters were not altered because all of the viruses used the same two major initiation sites (Fig. 6). The only detectable effect, caused by introduction of the binding site at the $\alpha 0$ mRNA cap site was an increase in the relative abundance of a minor transcript initiating at +4 (Fig. 6 and 7). We conclude that the native ICP4 binding site 5' of the $\alpha 0$ TATA box mediates repression of $\alpha 0$ RNA accumulation. Additionally, introduction of an ICP4 binding site at the mRNA cap site mediates a greater reduction in the accumulation of $\alpha 0$ RNAs than does the endogenous binding site.

Accumulation of $\alpha 0$ RNA in infected cells in the absence of ICP4. To eliminate the possibility that the reductions in the abundance of $\alpha 0$ RNA resulted from alterations in the intrinsic strength of the recombinant promoters, $\alpha 0$, $\alpha 4$, and $\alpha 27$ RNA levels were determined in the absence of protein synthesis. Total RNAs were isolated 6 h postinfection in the presence of cycloheximide, and RNA levels were quantitated by primer extension analysis. In the absence of protein synthesis, the abundance of $\alpha 0$ RNA, relative to $\alpha 4$ RNA, was nearly the same for the wild-type and recombinant viruses (Fig. 7). As previously noted, introduction of an ICP4 binding site at the $\alpha 0$ mRNA cap site resulted in increased representation of an RNA initiating from +4, without affecting $\alpha 0$ RNA levels overall (Fig. 7). Therefore, the intrinsic strengths of the recombinant $\alpha 0$ promoters were not altered.

FIG. 6. Quantitation of $\alpha 0$, $\alpha 4$, and $\alpha 27$ RNAs. Vero cells were infected at an MOI of 10, and total cellular RNA was prepared at 3 and 7 h postinfection (p.i.) as described in Materials and Methods. (A) Primer extensions were performed by using standard techniques, and the products were separated on 6% sequencing gels. (B) Quantitation of the primer extension products was performed by analysis with a Molecular Dynamics 445SI PhosphorImager and ImageQuaNT software, and the results are expressed as $\alpha 0/\alpha 4$ RNA ratios.

DISCUSSION

In this report, we demonstrate that ICP4 is a negative regulator of $\alpha 0$ gene expression in HSV-1. Furthermore, our findings resolve the contradictory conclusions in the literature regarding repression of $\alpha 0$ expression by ICP4 (16, 20, 22, 44). Our findings demonstrate that an ICP4 binding site 5' of a virus gene TATA box can repress gene expression. Therefore, models addressing repression of HSV-1 gene expression by ICP4 should incorporate the potential for binding sites located 5' or 3' of virus gene TATA boxes to act in a repressive fashion.

Upon the basis of the observation that viruses with temper-

FIG. 7. Quantitation of $\alpha 0$, $\alpha 4$, and $\alpha 27$ RNAs in the absence of protein synthesis. Vero cells were infected at an MOI of 10 in the presence of 100 μ g of cycloheximide per ml. Total cellular RNAs were prepared at 6 h postinfection as described in Materials and Methods. (A) The abundance of $\alpha 0$, $\alpha 4$, and $\alpha 27$ RNAs was determined by primer extension analysis. Lane M contained mock-infected cell RNA. (B) RNA levels were quantitated by analysis with a Molecular Dynamics 445SI PhosphorImager and ImageQuaNT software, and the results are expressed as $\alpha 0/\alpha 4$ RNA ratios.

ature-sensitive lesions in the $\alpha 4$ gene accumulated high levels of α gene products at the nonpermissive temperature, early studies suggested that ICP4 represses immediate-early gene expression (13, 42). Through the use of cotransfection assays and staggered coinfection experiments with wild-type HSV-1 and a temperature-sensitive $\alpha 4$ virus, Gelman and Silverstein (20, 21) provided evidence that ICP4 negatively regulates $\alpha 0$ expression. Their data also implied that repression occurs through an element in the $\alpha 0$ promoter. Subsequent transient expression studies in which the endogenous $\alpha 0$ ICP4 binding

site was destroyed supported the above-described conclusions that ICP4 represses $\alpha 0$ expression (16, 44). However, in the context of the virus genome, destruction of the $\alpha 0$ ICP4 binding site did not significantly affect the accumulation of ICP0 in infected cells (16).

In this study, a series of recombinant viruses were constructed with various arrays of ICP4 binding sites flanking the $\alpha 0$ TATA box (Fig. 1). The effects of these mutations on the accumulation of ICP0 and $\alpha 0$ RNA in infected cells were analyzed. We demonstrate that destruction of the endogenous ICP4 binding site located 5' of the TATA box results in a two- to threefold increase in the abundance of ICP0 (Fig. 5). $\alpha 0$ RNA levels increased six- to ninefold compared with wild-type levels (Fig. 6). The accumulation of other virus proteins (ICP4, ICP27, glycoprotein B, and α TIF) and RNAs ($\alpha 4$ and $\alpha 27$) was similar for the viruses examined in this study (Fig. 5 and 6). To verify that the intrinsic strengths of the recombinant $\alpha 0$ promoters were not altered by the ICP4 binding site mutations, $\alpha 0$ RNA levels were quantitated and found to be equivalent in cells infected in the absence of protein synthesis (Fig. 7). Concurrent with the above-described studies, recombinant viruses were constructed with an ICP4 binding site located at the $\alpha 0$ mRNA cap site in the presence or absence of the endogenous binding site (Fig. 1). Introduction of this ICP4 binding site resulted in a four- to fivefold reduction in the abundance of ICP0 (Fig. 5) and a threefold reduction in $\alpha 0$ RNA levels (Fig. 6) relative to the wild type. Strikingly, these changes did not affect the replication or growth of HSV-1 in tissue culture (Fig. 3 and 4).

Our conclusion that $\alpha 0$ expression is negatively regulated by ICP4 in HSV-1 is consistent with other studies (20, 21, 28, 44). However, these findings are contrary to the published conclusions of Everett and Orr (16). Specifically, these studies differ in our finding that destruction of the endogenous binding site resulted in increased accumulation of ICP0 in infected cells. We consistently observed increased accumulation of ICP0 when it was normalized to the levels of several other viral proteins (Fig. 5). More importantly, we demonstrated that the abundance of $\alpha 0$ RNA increased in cells infected with viruses containing either of two different promoter mutations that abolish binding of ICP4 (Fig. 6).

The repressive function of ICP4 is best demonstrated by its ability to autoregulate (34, 37). Destruction of the high-affinity binding site in the $\alpha 4$ promoter results in 6.6- to 11.7-fold derepression of $\alpha 4$ RNA accumulation in HSV-1-infected cells (34). Similarly, we demonstrated that destruction of the endogenous $\alpha 0$ ICP4 binding site resulted in a six- to ninefold increase in the abundance of $\alpha 0$ RNA. Therefore, the degrees to which $\alpha 4$ and $\alpha 0$ expression is repressed by ICP4 in HSV-1 are equivalent.

The mechanism of ICP4-mediated repression of HSV gene expression is not understood. However, current evidence does provide some insights. Studies suggest that repression is dependent predominantly upon binding of ICP4 to DNA in an orientation- and position-dependent manner (20, 23, 28, 31, 44, 47). The degree of repression is speculated to be a function of the absolute distance of the ICP4 binding site downstream of respective TATA boxes and the stereoaxial position of ICP4 binding relative to the TATA box (23, 31). Evidence also suggests that the repressive function of ICP4 correlates with its ability to form tripartite complexes with the general transcription factors TATA-binding protein and transcription factor IIB. However, the molecular mechanism of this phenomenon is unclear (23, 53).

Our findings provide compelling evidence that an ICP4 binding site located 5' of an HSV-1 TATA box mediates re-

pression by ICP4. In agreement with these findings, Koop et al. (28) previously demonstrated that introduction of an ICP4 binding site 5' of the TATA box in minimal HSV and non-HSV promoters can mediate repression. Michael et al. (35) also provided evidence of a repressive role of ICP4 binding sites positioned 5' of the $\alpha 4$ TATA box. Thus, ICP4 binding sites positioned either 5' or 3' of virus gene TATA boxes can mediate repression. These results suggest that repression is dependent on inhibition of transcription initiation. Furthermore, the relative distance of the ICP4 binding site from the TATA box in the $\alpha 0$ gene suggests that if repression is dependent upon interactions between ICP4, TATA-binding protein, and transcription factor IIB, then structural alterations (bending) of promoter DNA could be required (15). Parenthetically, DiDonato and Muller observed that binding of ICP4 to the $\alpha 0$ and $\alpha 4$ promoters induces an alteration in the helical geometry of the minor groove of the TATA box (10), whereas binding of ICP4 to the glycoprotein D promoter, which is not repressed by ICP4, fails to produce such an alteration.

Although ICP4 has been characterized as a repressor of α gene expression, its direct involvement in repression has been shown convincingly only in the case of the $\alpha 4$ gene (31, 34). As discussed above, previous studies using ICP4 mutants demonstrated that in the absence of functional ICP4 the abundance of α gene products increases. Although the study of viruses with mutated $\alpha 4$ alleles provided evidence that ICP4 acts as a negative regulator of α gene expression, the interpretation of these data was complicated by the absence of β gene expression and DNA replication. Therefore, it is difficult to determine whether the ICP4 effect on α gene repression is direct or indirect. The strength of the data presented here relies on the fact that only the presumed site of action of ICP4 has been altered in the context of an otherwise wild-type virus. Thus, the effects are limited to the expression of $\alpha 0$, whose promoter has been altered, and are not pleiotropic because of the lack of functional ICP4. Furthermore, the interpretation of our data is not complicated by variations in the abundance of ICP0, as these did not alter the yield or rate of growth of the recombinant viruses or the expression of other viral proteins. It is reasonable to conclude that ICP4 represses $\alpha 0$ expression and that ICP4 binding 5' of a virus gene TATA box can mediate repression in HSV-1. In this respect, the ability of ICP4 to regulate other α genes that either contain ICP4 binding sites within their coding sequences, i.e., $\alpha 27$ and $\alpha 22$ (11), or 5' as well as 3' of their TATA boxes, i.e., $\alpha 22$ and $\alpha 47$ (11), should be further explored.

ACKNOWLEDGMENTS

We thank Roy Bohenzky for help in the initial stages of this work and Elaine Hanson for preparation of antibody 58S. We also thank Gary Cohen, Roselyn J. Eisenberg, and Thomas Kristie for providing several of the antibodies against HSV proteins that were used during this study.

This study was supported by Public Health Service grant AI-33952 to Saul J. Silverstein.

REFERENCES

1. Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl. 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. Bohenzky, R. A., A. G. Papavassiliou, I. H. Gelman, and S. Silverstein. 1993. Identification of a promoter mapping within the reiterated sequences that flank the herpes simplex virus type 1 U_L region. *J. Virol.* **67**:632-642.
3. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
4. Chen, J., and S. Silverstein. 1992. Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. *J. Virol.* **66**:2916-2927.
5. 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.
6. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
7. DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer. 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J. Virol.* **56**:558-570.
8. 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.
9. 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.
10. DiDonato, J. A., and M. T. Muller. 1989. DNA binding and gene regulation by the herpes simplex virus type 1 protein ICP4 and involvement of the TATA element. *J. Virol.* **63**:3737-3747.
11. DiDonato, J. A., J. R. Spitzner, and M. T. Muller. 1991. A predictive model for DNA recognition by the herpes simplex virus protein ICP4. *J. Mol. Biol.* **219**:451-470.
12. Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
13. 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.
14. 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.
15. Everett, R. D., J. DiDonato, M. Elliott, and M. Muller. 1992. Herpes simplex virus type 1 polypeptide ICP4 bends DNA. *Nucleic Acids Res.* **20**:1229-1233.
16. Everett, R. D., and A. Orr. 1991. The VMW175 binding site in the IE-1 promoter has no apparent role in the expression of VMW110 during herpes simplex virus type 1 infection. *Virology* **180**:509-517.
17. Faber, S. W., and K. W. Wilcox. 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucleic Acids Res.* **14**:6067-6083.
18. 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.
19. 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.
20. 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.
21. 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.
22. Gelman, I. H., and S. Silverstein. 1987. Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. *J. Virol.* **61**:3167-3172.
23. Gu, B., R. Kuddus, and N. DeLuca. 1995. Repression of activator-mediated transcription by herpes simplex virus via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. *Mol. Cell. Biol.* **15**:3618-3626.
24. Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature (London)* **375**:411-415.
25. Holland, L. E., K. P. Anderson, C. Shipman, and E. K. Wagner. 1980. Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. *Virology* **101**:10-24.
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. Koop, K. E., J. Duncan, and J. R. Smiley. 1993. Binding sites for the herpes simplex virus immediate-early protein ICP4 impose an increased dependence on viral DNA replication on simple model promoters located in the viral genome. *J. Virol.* **67**:7254-7263.
29. Kristie, T. M., and B. Roizman. 1986. $\alpha 4$, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of alpha genes and of selected other viral genes. *Proc. Natl. Acad. Sci. USA* **83**:3218-3222.
30. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.

31. **Leopardi, R., N. Michael, and B. Roizman.** 1995. Repression of the herpes simplex virus 1 $\alpha 4$ gene by its gene product (ICP4) within the context of the viral genome is conditioned by the distance and stereoaxial alignment of the ICP4 DNA binding site relative to the TATA box. *J. Virol.* **69**:3042–3048.
32. **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.
33. **Michael, N., and B. Roizman.** 1989. Binding of the herpes simplex virus major regulatory protein to viral DNA. *Proc. Natl. Acad. Sci. USA* **86**:9808–9812.
34. **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.
35. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein $\alpha 4$ of herpes simplex viruses. *Science* **239**:1531–1534.
36. **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.
37. **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.
38. **Panagiotidis, C. A., S. C. Huang, and E. S. Canellakis.** 1995. Relationship of the expression of the S20 and L34 ribosomal proteins to polyamine biosynthesis in *Escherichia coli*. *Int. J. Biochem. Cell. Biol.* **27**:157–168.
39. **Papavassiliou, A. G., and S. J. Silverstein.** 1990. Characterization of DNA-protein complex formation in nuclear extracts with a sequence from the herpes simplex virus thymidine kinase gene. *J. Biol. Chem.* **265**:1648–1657.
40. **Paterson, T., and R. D. Everett.** 1988. Mutational dissection of the HSV-1 immediate early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* **166**:186–196.
41. **Paterson, T., and R. D. Everett.** 1988. The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site-specific DNA binding closely correspond to those involved in transcriptional regulation. *Nucleic Acids Res.* **16**:11005–11025.
42. **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.
43. **Purves, F. C., W. O. Ogle, and B. Roizman.** 1993. Processing of the herpes simplex virus regulatory protein alpha22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and proteins in infected cells. *Proc. Natl. Acad. Sci. USA* **90**:6701–6705.
44. **Resnick, J., B. A. Boyd, and M. L. Haffey.** 1989. DNA binding by the herpes simplex virus type 1 ICP4 protein is necessary for efficient down regulation of the ICP0 promoter. *J. Virol.* **63**:2497–2503.
45. **Rice, S. A., and D. M. Knipe.** 1988. Gene-specific transactivation by the herpes simplex virus type 1 alpha protein ICP27. *J. Virol.* **62**:3814–3823.
46. **Rice, S. A., M. C. Long, V. Lam, and C. A. Spencer.** 1994. RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. *J. Virol.* **68**:988–1001.
47. **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.
48. **Roizman, B., and A. Sears.** 1993. The human herpes viruses. Raven Press, New York.
49. **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.
50. **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.
51. **Showalter, S. D., M. Zwieg, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP4. *Infect. Immun.* **34**:684–692.
52. **Smiley, J.** 1980. Construction in vitro and rescue of a thymidine kinase deficient deletion mutant of herpes simplex virus. *Nature (London)* **285**:333–335.
53. **Smith, C. A., P. Bates, R. Rivera-Gonzalez, 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.
54. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
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. **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.
57. **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.
58. **York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson.** 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8⁺ T lymphocytes. *Cell* **77**:525–535.
59. **Zhang, Y., D. A. Sirko, and J. L. C. McKnight.** 1991. Role of herpes simplex virus type 1 UL46 and UL47 in α TIF-mediated transcriptional induction: characterization of three viral deletion mutants. *J. Virol.* **65**:829–841.