Repression of the α0 Gene by ICP4 during a Productive Herpes Simplex Virus Infection

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During a productive infection by herpes simplex virus type 1 (HSV-1), ICP4, the major regulatory protein encoded by the α0 gene, binds to its transcription initiation site and represses the accumulation of α4 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 α0 gene, has not been adequately addressed. To this end, recombinant α0 promoters with various arrays of ICP4 binding sites flanking a TATA box were constructed and recombined into the HSV-1 genome. Our results demonstrate the following. (i) Destruction of the endogenous α0 ICP4 binding site, located 5′ of the TATA box, results in derepression of α0 protein and RNA accumulation in infected Vero cells. (ii) The degree of α0 derepression is equivalent to that reported for the α4 gene following destruction of the ICP4 binding site at the α4 mRNA cap site in HSV-1. (iii) Introduction of an ICP4 binding site at the α0 mRNA cap site represses the accumulation of α0 RNA greater than threefold relative to the wild type. (iv) Changes in the abundance of α0 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 α0 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, α0, α4, α27, α22, α47, 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). ICP4, 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 ATCGTNC, 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 α0, α4, αX (OrfP), glycoprotein D, alpha trans-inducing factor (αTIF), and γ2 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 α4 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 α4 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 α0 gene, remains undefined.

Gelman and Silverstein demonstrated that in the absence of functional ICP4, α0 RNA levels increase (20). Subsequent transient expression studies implied that repression was dependent upon both the endogenous α0 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 α0 expression is not regulated by ICP4 during a productive infection (16).
In this report, we present a series of experiments designed to address the repression of α expression by ICP4 in the context of the HSV-1 genome. Our data demonstrate that the native ICP4 binding site, located 5' of the α TATA box, mediates repression of α expression by ICP4. The degree of repression is equivalent to that reported for the α 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% horse call serum (Hy-line Laboratories Inc., Logan, Utah), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Gibco BRL) unless otherwise noted. The parental viruses used in this study were wild-type HSV-1 (Giemson Corp., La Jolla, Calif.) and the IE-0LaCZ transplacental virus previously described (4).

Plasmids and phage. Plasmids pC7 contains the SacI-BamHI fragment of the α gene (−810 to +3780) from HSV-1 strain 17 in pUC19 (5). Phage XW5 contains a SacI-BamHI fragment spanning the α mRNA cap site (−133 to +167) cloned into M13mp8-5XMW1. Plasmids pXW8 was derived from pXW7 by amplification with mutagenic primers in pJC7 inserted into pXW7. Plasmid pXW9 was constructed by replacing the DrI-NcoI fragment of the α gene (−11 to +143) in pXW8 with the corresponding fragment from 3XMW1. Plasmids pTAE4, pTAE5, and pTAH5 contain PCR amplifiers of the α promoter (−436 to +1) cloned into pGEM-T (Promega, Madison, Wis.) following sequential PCR mutagenesis (1) of the endogenous α ICP4 binding site. pEL11E and pEL11H were constructed by replacing the SacI-HindIII fragment of the α gene (−810 to +3780) from phage XW5 inserted into pXW7 with the corresponding fragment from 3XMW1. Plasmids pTAE4, pTAE5, and pTAH5 contain PCR amplifiers of the α promoter (−436 to +1) cloned into pGEM-T (Promega, Madison, Wis.). The sequence of the recombinant JC7-1284-3 was confirmed by cycle sequencing with Amplicycle (Perkin Elmer, Foster City, Calif.).

Construction of mutants. (i) Construction of an ICP4 binding site in the α transcription initiation site. An ICP4 binding site (α1, 17) was created at the α mRNA cap site by oligonucleotide-directed mutagenesis (1) of αXW5. The mutagenic primer (P-HSBC) sequence was 5′-AAGATGTCGAGCTGTCCATCGGGCACCCCCCTGCTGG-3′. The sequence of the recombinant JC7-1284-3 was confirmed by cycle sequencing with Ampliplyase (Perkin Elmer, Foster City, Calif.).

(ii) Destruction of the wild-type ICP4 binding site in the α promoter. Sequential PCR mutagenesis was performed as previously described (1), with minor modifications. The flanking primers sequences were 5′-CCCTCGGACATCTCGG-3′ and 5′-GGGTACCATCTCATTGGG-3′.

(iii) Purification and analysis of virus DNA. (A) DNA probes. Probes were labeled with [γ-32P]dCTP (DuPont NEN, Boston, Mass.) by random priming (Boehringer Mannheim, Indianapolis, Ind.).

Fig. 1. Schematic presentation of wild-type and mutant α promoters and Southern blot analysis of wild-type and recombinant virus DNAs. (A) α promoters were constructed with wild-type (C), recombinant (R), and mutant (X) ICP4 binding sites flanking the α TATA box as described in Materials and Methods. The wild-type ICP4 binding site in the α promoter (−71 to −54) was mutated in EL11E and EL12E to create an EcoRI (E) site and in EL11H and EL13H 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 α 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 α probe (−790 to +3350).

Preparation and analysis of virus DNA. (i) DNA probes. The probes used consisted of the 2.5-kb BamHI-Sall fragment (−790 to +3350) of the α gene and a 2.2-kb EcoRI fragment of pBbuelHabII (Intergen, San Diego, Calif.) containing 1.95 kb of the lacZ gene. Probes were labeled with [γ-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 nm Tris-HCl (pH 7.4), 10 mM EDTA, 300 mM NaCl, 0.2% sodium deoxycholate solution (SDS), and 160 μg 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, Boston, Mass.) containing 2.25 μg of α DNA per ml (pEL11E, pEL11H, pEL12E, and pEL12H, respectively). Plasmid pXW9 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-Sall fragment (−790 to +3350) of the α gene and a 2.2-kb EcoRI fragment of pBbuelHabII (Intergen, San Diego, Calif.) containing 1.95 kb of the lacZ gene. Probes were labeled with [γ-32P]dCTP (DuPont NEN, Boston, Mass.) by random priming (Boehringer Mannheim, Indianapolis, Ind.).

(iii) Dot blot hybridization. Aliquots of infected cells in media were combined 1:1 with 200 nm Tris-HCl (pH 7.4), 10 mM EDTA, 300 mM NaCl, 0.2% sodium deoxycholate solution (SDS), and 160 μg 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, Boston, Mass.) containing 2.25 μg of α DNA per ml (pEL11E, pEL11H, pEL12E, and pEL12H, respectively). Plasmid pXW9 was also confirmed by sequence analysis (Fig. 1).
briedized 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 × g for 5 min. Pellets were resuspended in 50 mM Tris-HCl (pH 7.4)–100 mM NaCl–5 mM EDTA–200 μg of protease K per ml at 65°C. SDS and incubated at 50°C for 3 h. Following digestion, 85 μl of a saturated NaCl solution was added per 250-μl sample and mixed thoroughly for 15 min at room temperature. The resulting precipitate was removed by centrifugation at 12,000 × 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 μM forward primers, and 1 μM reverse primers, which result in amplification of a 137-bp fragment of the wild-type genome. The primers were synthesized by the DNA facility of the Columbia-Presbyterian Medical Center, were used: 0-PrimExt, 5’-AGCTGAGGCTGTTTCCTTGATGAGT-3’ and 0-PrimExt, 5’-CCGGGCTCTTCTGTGGATG-3’. The DNA sequences of the amplifiers were determined by cycle sequencing with the primer 0PrimSeq.

(v) Southern blot analysis. Infected-cell DNA was digested with the enzymes indicated in Fig. 1, separated by agarose gel electrophoresis, and transferred to SS-NTanon (Schleicher & Schuell). Southern blot hybridization with the α-labelled 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). (i) Virus IE-0 promoter regions. The desired mutations in the recombinant viruses (Fig. 1) were verified by cycle sequencing of the 408-bp PCR amplimers of the α gene (–324 to +85). The amplification primers result in amplification of a 137-bp fragment of the wild-type genome (+1246 to +1383), were 5’-GGGCAAGCTTGGGGGTGTTGTTGAT-3’ and 5’-GGGGTTCGTCAAGCGGGCGGAGG-3’. LacZ primers, which result in amplification of a 237-bp fragment, were LacZ-5’-5’-CTCTATCGTGCGGGTGGTGATGGAATCTATGC-3’ and LacZ-3’-5’-CCGGGCTCTTCTGTGGATGATG-3’. The DNA sequences of the amplimers were determined by cycle sequencing with the primer LacZSeq.

(ii) Southern blot hybridization. Infected-cell DNA was digested with the enzymes indicated in Fig. 1, separated by agarose gel electrophoresis, and transferred to SS-NTanon (Schleicher & Schuell). Southern blot hybridization with the α-labelled 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.).

Transferred to nitrocellulose membranes (54). Immunodetection of virus proteins was performed as previously described (38). Antibodies against virus proteins are as follows: ICPO, rabbit polyclonal antibody CLU7 raised against a glutathione S-transferase–ICPO fusion protein containing amino acids 312 to 400 of ICPO; ICPO, mouse monoclonal antibody H1114 (Goodwin Institute for Cancer Research, Philadelphia, Pa.). rabbit polyclonal antibody CLU139 raised against a glutathione S-transferase–ICPO fusion protein containing amino acids 2 to 252 of ICPO; glycophorin B, rabbit polyclonal antibody R6 (provided by G. Cohen, Department of Microbiology, University of Pennsylvania, Philadelphia, Pa.); STIF, rabbit anti-α-tIF (J9) (provided by T. Kirste, 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 enhanced chemiluminescent substrate Lumiglo (Kirkgaard & 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 μl of RNAase-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 μg 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’-GTGATGAGGCTGTTTCCTTGATGAGT-3’; 5’-CCGGGCTCTTCTGTGGATG-3’. The DNA sequences of the amplifiers were determined by cycle sequencing with the primer 0PrimSeq.

To prepare RNA from cells infected in the absence of protein synthesis, vero cells were preincubated for 1 h in the presence of 100 μg 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’-GTGATGAGGCTGTTTCCTTGATGAGT-3’; 5’-CCGGGCTCTTCTGTGGATG-3’. The DNA sequences of the amplifiers were determined by cycle sequencing with the primer 0PrimSeq.
The primers were designed so that their 3' ends would be approximately 40 bases from the transcription initiation sites of the 0, 4, and 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.

**RESULTS**

**Experimental design.** The primary objective of this study was to clarify the role of ICP4 as a negative regulator of 0 gene expression in HSV-1. To this end, the endogenous 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 0 ICP4 binding site was verified by EMSA, and the effects of the mutations on the abundance of ICP0 and 0 RNAs in infected cells were assessed by Western blot and...
immunoblots were developed with the chemiluminescent substrate LumiGLO. Polyclonal antibodies (ICP0, CLU7; ICP27, CLU38; glycoprotein B [gB], R69; αTIF, anti-αTIF). Secondary antibodies were conjugated to horseradish peroxidase, and immunoblots were developed with the chemiluminescent substrate LumiGLO.

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EMSA. The 180-bp /H9251 fragment spanning the α0 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 presence of this mutation on α0 expression was ascertained by measuring α0 protein and RNA accumulation relative to the accumulation of other viral proteins and RNAs. Finally, to verify that the intrinsic strengths of the recombinant α0 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 α0 TATA box to create either an EcoRI (E) or a HindIII (H) restriction site. These mutations were incorporated into the α0 promoters in plasmid 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 α0 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 α0 genomic loci and mutagenesis of the endogenous α0 ICP4 binding site were confirmed by Southern blot analysis (Fig. 1). The predicted wild-type and recombinant α0 promoters (Fig. 1) were verified by cycle sequencing.

Analysis of ICP4 complex formation on wild-type and mutant α0 promoter fragments. The effects of the mutations on the ability of ICP4 to bind to the α0 promoter were analyzed by EMSA. The 180-bp AvaI fragments spanning the α0 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 (S1) (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 α0 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 α0 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 α0 null virus IE-0:β-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 α0 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. The results of this analysis reveal that (i) destruction of the endogenous α0 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 α0 mRNA cap site resulted in a fourfold 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 α0 promoter did not affect the growth of HSV-1 in tissue culture.

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 10μl sample (106 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; αTIF, anti-αTIF). Secondary antibodies were conjugated to horseradish peroxidase, and immunoblots were developed with the chemiluminescent substrate LumiGLO.
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 α27 was essentially the same for all viruses (Fig. 5). These results demonstrate that the endogenous α0 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 α0 RNA in cells infected with wild-type or recombinant viruses. To determine whether the endogenous α0 ICP4 binding site affects the accumulation of α0 RNA, total RNA was isolated at 3 and 7 h postinfection from cells infected with wild-type and mutant viruses, and the accumulation of α0 RNA was quantitated by primer extension analysis (Fig. 6). The accumulation of α4 and α27 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 α0 RNA, relative to α4 RNA, at 3 h postinfection (Fig. 6, vEL11E and vEL11H). At 7 h postinfection, α0 RNA levels were sixfold greater than wild-type levels (Fig. 6, vEL11E and vEL11H). Thus, the abundance of α0 RNA in infected cells is consistent with our observations regarding repression of ICP0 accumulation (Fig. 5). (ii) Introduction of an ICP4 binding site at the α0 transcription initiation site resulted in a threefold decrease in the abundance of α0 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 α0 promoter did not repress α0 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 α4 and α27 RNAs.

These results demonstrate that (i) the accumulation of α0 RNA in infected cells correlates strongly with the presence and/or absence of ICP4 binding sites located 5′ or 3′ of the α0 TATA box (Fig. 6). (ii) The α0 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 α0 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 α0 TATA box mediates repression of α0 RNA accumulation. Additionally, introduction of an ICP4 binding site at the mRNA cap site mediates a greater reduction in the accumulation of α0 RNAs than does the endogenous binding site.

Accumulation of α0 RNA in infected cells in the absence of ICP4. To eliminate the possibility that the reductions in the abundance of α0 RNA resulted from alterations in the intrinsic strength of the recombinant promoters, α0, α4, and α27 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 α0 RNA, relative to α4 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 α0 mRNA cap site resulted in increased representation of an RNA initiating from +4, without affecting α0 RNA levels overall (Fig. 7). Therefore, the intrinsic strengths of the recombinant α0 promoters were not altered.

DISCUSSION

In this report, we demonstrate that ICP4 is a negative regulator of α0 gene expression in HSV-1. Furthermore, our findings resolve the contradictory conclusions in the literature regarding repression of α0 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-
expression studies in which the endogenous and a temperature-sensitive and staggered coinfection experiments with wild-type HSV-1 expression (13, 42). Through the use of cotransfection assays studies suggested that ICP4 represses immediate-early gene/H9251 (20, 21) provided evidence that ICP4 negatively regulates through an element in the promoter. Subsequent transient expression studies in which the endogenous α0 ICP4 binding site was destroyed supported the above-described conclusions that ICP4 represses α0 expression (16, 44). However, in the context of the virus genome, destruction of the α0 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 α0 TATA box (Fig. 1). The effects of these mutations on the accumulation of ICP0 and α0 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), α0 RNA levels increased six- to ninefold compared with wild-type levels (Fig. 6). The accumulation of other virus proteins (ICP4, ICP27, glycoprotein B, and αTIF) and RNAs (α4 and α27) was similar for the viruses examined in this study (Fig. 5 and 6). To verify that the intrinsic strengths of the recombinant α0 promoters were not altered by the ICP4 binding site mutations, α0 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 α0 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 α0 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 α0 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 α0 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 α4 promoter results in 6.6- to 11.7-fold derepression of α4 RNA accumulation in HSV-1-infected cells (34). Similarly, we demonstrated that destruction of the endogenous α0 ICP4 binding site resulted in a six- to ninefold increase in the abundance of α0 RNA. Therefore, the degrees to which α4 and α0 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 IIIB. 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-

FIG. 5. Quantitation of α0, α4, and α27 RNAs in the absence of protein synthesis. Vero cells were infected at an MOI of 10 in the presence of 100 μg of cycloheximide per ml. Total cellular RNAs were prepared at 6 h postinfection as described in Materials and Methods. (A) The abundance of α0, α4, and α27 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 α0/α4 RNA ratios.
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 α4 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 α0 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 α0 and α4 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 a gene expression, its direct involvement in repression has been shown convincingly only in the case of the α4 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 α4 alleles provided evidence that ICP4 acts as a negative regulator of a 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 α0, 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 ICP9, 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 α0 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., α27 and α22 (11), or 5′ as well as 3′ of their TATA boxes, i.e., α22 and α47 (11), should be further explored.

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