

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

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We examined the ability of binding sites for the herpes simplex virus immediate-early protein ICP4 to alter the regulation of closely linked promoters by placing strong ICP4 binding sites upstream or downstream of simple TATA promoters in the intact viral genome. We found that binding sites strongly reduced the levels of expression at early times postinfection and that this effect was partially overcome after the onset of viral DNA replication. These data confirm that DNA-bound ICP4 can inhibit the activity of a closely linked promoter and raise the possibility that ICP4 binding sites contribute to temporal regulation during infection.

The 70 genes of herpes simplex virus type 1 (HSV-1) (37) are sequentially expressed in a three-tiered regulatory cascade during lytic infection (26, 27; see references 14, 53, and 71 for reviews). Five immediate-early (IE) genes are expressed first, and these are the only HSV genes that are transcribed in the absence of de novo viral protein synthesis. The IE proteins then induce expression of the early (E) and late (L) genes. E genes are activated prior to the onset of viral DNA replication, while L genes require DNA replication for maximal expression. L genes can be further subclassified as leaky L or true L, according to the stringency of the replication requirement. Considerable evidence indicates that IE proteins regulate viral gene expression at the both the transcriptional (22, 48, 60, 73) and the posttranscriptional (56) levels and that the kinetics of expression of individual HSV genes are greatly influenced by sequences in their promoter regions (24, 25, 29, 32, 35, 48, 60). However, the precise mechanisms underlying temporal regulation of gene expression remain unknown.

The IE protein ICP4 (infected-cell protein 4 [26]) plays a critical role during infection: it is essential for activation of E and L transcription and serves to downregulate IE gene expression. Thus, ICP4-deficient temperature-sensitive and deletion mutants fail to express most E and L genes and overproduce IE gene products (7, 12, 49, 72). These regulatory responses can be reproduced in transient cotransfection assays, in which ICP4 activates a number of E and L promoters (8, 13, 19, 41, 50) and represses the promoters of the IE ICP4 and ICP0 genes (8, 20, 21, 42, 51, 52). These results demonstrate that ICP4 acts directly as both a positive and a negative regulator of HSV gene expression.

ICP4 is a site-specific DNA-binding protein, and a binding consensus sequence, 5'-ATCGTCnnYnCCGRCnnCRYCR-3', has been described (16, 17, 31, 40), although binding to nonconsensus sites also occurs (28, 33, 39, 68). The results of extensive mutational studies of ICP4 suggest that DNA binding is required for its transregulatory activities (9, 10, 45, 46, 59; but see also references 47 and 58). However, the extent to

which specific ICP4 binding sites modulate the activity of nearby promoters is less clear. For example, ICP4 binding sites in the vicinity of the glycoprotein D (gD) promoter appear to contribute to the activation of gene expression *in vitro*, and multimerization of these sites increased induction by ICP4 in transient transfection assays (3, 68, 69); however, mutations that eliminate these sites have no detectable effect on gD expression during lytic virus infection (63). Similarly, mutations of ICP4 that eliminate binding to low-affinity sites in the thymidine kinase (*tk*) gene have no effect on TK expression (28). In contrast, mutation of an ICP4 binding site in the UL 49.5 gene was reported to reduce expression during lytic infection (54). There is stronger evidence for a role of individual ICP4 binding sites in the negative regulation of IE gene expression. High-affinity ICP4 binding sites are located over the ICP4 transcription start site and upstream of the ICP0 TATA element (17, 33, 40). In both cases, these sites serve as targets for ICP4-mediated repression in transient transfection assays (20, 51, 52). Consistent with these data, Michael and Roizman (38) found that the ICP4 binding site located at the ICP4 transcription start site serves as a target for negative regulation during lytic HSV infection. In contrast, Everett and Orr (15) found that a mutation that eliminates the ICP4 binding site in the ICP0 promoter had no detectable effect on ICP0 expression after transfer into the viral genome.

We reasoned that the contribution of individual ICP4 binding sites might be more readily apparent in the context of simple promoters lacking response elements for other regulators. To this end, we introduced ICP4 binding sites into model promoters consisting of an isolated TATA box, reproducing the same spacing as is found in the native ICP4 and ICP0 genes. These structures were then incorporated into the intact HSV genome and tested for activity during lytic infection of Vero cells. We found that a single ICP4 binding site placed upstream or downstream of the TATA box strongly reduced the levels of transcripts produced at early times postinfection. Furthermore, this inhibition was partially reversed after the onset of viral DNA replication, leading to a shift to later kinetics of expression. These results confirm that ICP4 binding sites in the viral genome can serve as targets for negative

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regulation and demonstrate that they can also influence the temporal regulation of a closely linked promoter.

MATERIALS AND METHODS

Viruses and cells. HSV-1 strain KOS PAA'5 (23) was used throughout this study. Virus stocks were grown and their titers were determined on monolayers of Vero cells. Vero cells were maintained in a minimal essential medium (GIBCO) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin G per ml, and 100 µg of streptomycin sulfate per ml.

Insertion of ICP4 binding site into model promoters. Synthetic oligonucleotides bearing ICP4 binding sites (purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University) were inserted into pTKSB derivatives containing previously described TATA promoters (32) by using standard methods (55). pTKSB (70) contains the HSV-1 *tk* gene bearing a 200-nucleotide (nt) deletion extending from +480 to +680; this deletion eliminates a promoter for the overlapping and diverging UL24 gene and is spanned by a *Bam*HI linker. Constructs 11T and MLPT (previously referred to as US11 TATA and MLP TATA [32]) bear the TATA box regions of the HSV-1 US11 and adenovirus type 2 (Ad2) major late promoter (MLP), respectively, inserted into the *Bam*HI site of pTKSB in the UL24 orientation (32). These insertions regenerate the *Bam*HI site downstream of the TATA box and introduce a unique *Xho*I site upstream of the TATA box.

Promoters bearing downstream ICP4 binding sites were constructed by inserting a double-stranded 35-mer, consisting of 31 nt spanning the ICP4 mRNA cap site and 5'-GATC protruding ends, into the unique *Bam*HI sites on constructs 11T and MLPT, regenerating the *Bam*HI site downstream of the insert. The sequence of the top strand of the oligonucleotide used was 5'-gatcACGCCCGATCGTCCACACGGAGCGCGGCTG-3'. The resulting constructs were designated 11T/4 and MLPT/4. Control promoters bearing a 2-nt deletion within the ICP4 binding site were also made (constructs 11T/mut4 and MLPT/mut4). The sequence of the top strand of the oligonucleotides used to insert the mutant site was 5'-gatcACGCCCGCGTCCACACGGAGCGCGGCTG-3'.

ICP4 binding sites were also introduced into the *Xho*I site upstream of the US11 TATA box by using double-stranded 40-mers consisting of sequences spanning the ICP4 mRNA cap site and 5'-TCGA protruding ends. The *Xho*I site was regenerated upstream of the insert. The sequences of the top strands of the oligonucleotides used were as follows: (i) distal site, 5'-tcgagGACGCCCGATCGTCCACACGGAGCGCGGCTGCCg-3', and (ii) proximal site, 5'-tcgagCCGAGGACGCCCGATCGTCCACACGGAGCGCGGg-3'. The resulting constructs were designated D4/11T and P4/11T.

Control promoters bearing a 2-nt deletion within the upstream ICP4 binding sites were also constructed in a similar manner (Dmut4/11T and Pmut4/11T). The sequences of the top strands of the oligonucleotides used were as follows: (i) distal site, 5'-tcgagGACGCCCGCGTCCACACGGAGCGCGGCTGCCg-3', and (ii) proximal site, 5'-tcgagCCGAGGACGCCCGCGTCCACACGGAGCGCGGg-3'. The sequences of all insertions were verified by the method of Sanger et al. (57).

Transfer of mutations into the viral genome. Viral recombinants bearing inserts of the test promoters were generated by *in vivo* recombination following cotransfection of infectious HSV-1 PAA'5 DNA and linearized plasmids containing the appropriate sequences (61, 62). TK-deficient progeny were isolated by plaque purification in the presence of 100 µg of

5-bromodeoxycytidine per ml, and recombinants bearing the desired insert were identified by Southern blot hybridization (66).

RNA extraction. Vero cells were infected at a multiplicity of 10 PFU per cell; where indicated, aphidicolin was added at 10 µg/ml at the time of infection. Total cellular RNA was extracted by using a modified version of the RNazol B method (TEL-TEST, Inc., Friendswood, Tex.), based on the method of Chomczynski and Sacchi (6). Briefly, 1 ml of RNazol B was added to each 10-cm-diameter culture dish containing approximately 5×10^6 cells. The lysate was passed through a pipette several times to solubilize RNA and transferred to a microcentrifuge tube. After the addition of 200 µl of chloroform, the suspension was incubated for 5 min on ice. Samples were then centrifuged at $12,000 \times g$ for 15 min (4°C). Following an extraction with chloroform, the RNA was precipitated from the aqueous phase with isopropanol. After a second precipitation with ethanol, the pellet was washed three times with 1:3 diethylpyrocarbonate-treated 0.4 M sodium acetate-ethanol, once with 70% ethanol, and once with 95% ethanol. Pellets were dried under vacuum for 10 to 15 min and resuspended in diethylpyrocarbonate-treated water.

Primer extension analysis. Primer extension analysis was performed as previously described (64). The following synthetic primers were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University: (i) UL24b, 5'-ACACAACACCGCCTCGACCA GGGTG-3'; (ii) gD, 5'-CCCATACCGGAACGCACCACA CAA-3' (43); and (iii) 7SL RNA, 5'-AACTTAGTGC GGACA CCCGATCGGC-3' (44).

Preparation of an ICP4-GST fusion protein. A fusion protein containing the DNA-binding domain of ICP4 (74) fused to glutathione S-transferase (GST) from *Schistosoma japonicum* (65) was prepared as follows. A *Sac*II fragment of pGX58 (67) containing codons 262 to 490 of the ICP4 gene was inserted into the *Bam*HI site in the multiple cloning site of pGEX-2T (Pharmacia) by using a *Bam*HI-*Sac*II adaptor (5'-GATCCTC CGCGGAG-3', purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University) to maintain the proper reading frame; the termination codon was provided by vector sequences downstream of the insert. The sequence spanning the junction between the GST and ICP4 coding sequences was confirmed by Sanger sequencing (57). The resulting plasmid was designated pIGF-17.

Purified ICP4-GST fusion protein was prepared as follows. *Escherichia coli* HB101 transformed with pIGF-17 was grown to an optical density at 600 nm of 0.9 in 1 liter of TB (55) at 37°C. Cultures were then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and incubated for 2 h at 37°C. All subsequent steps were performed at 4°C. Cells were harvested by centrifugation at $11,000 \times g$ for 15 min, washed in 50 mM Tris hydrochloride (pH 8.0), and then lysed in 25 ml of lysis buffer [13% sucrose, 100 mM EDTA (pH 8.0), 20 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; pH 8.0), 50 mM Tris hydrochloride (pH 8.0), 250 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, and 20 mg of lysozyme per ml]. After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.1%, and the lysate was incubated for a further 15 min. Cellular debris was then pelleted by centrifugation at $150,000 \times g$ for 1 h in a Beckman Ti50.2 rotor, and the fusion protein was purified from the supernatant by affinity chromatography on glutathione-Sepharose 4B (Pharmacia) per the manufacturer's instructions. Eluted material was then dialyzed against 2 liters of dialysis buffer (100 mM KCl, 20 mM

N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol [DTT]).

DNA affinity chromatography. DNA affinity chromatography was performed by the method of Kadonaga and Tjian (30), with the exception that the column was constructed by using commercially prepared CNBr-activated Sepharose 4B (Pharmacia). Oligomers of ICP4 binding sites were constructed by using double-stranded 30-mers prepared from the following oligonucleotides: 5'-GATCCCGATCGTCCACACGGAGC GCGGCTA-3' and 5'-GATCTAGCCGCGCTCCGTGTGGA CGATCGG-3' (purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University). GST-Sepharose-purified ICP4-GST was subjected to dialysis (see above) prior to application to the DNA affinity column. DNA-bound protein was eluted from the column with buffer Z (25 mM HEPES [pH 7.8], 12.5 mM MgCl₂, 1 mM DTT, 20% glycerol, 0.1% Nonidet P-40) containing 1 M KCl and dialyzed against 2 liters of dialysis buffer.

Southwestern (DNA-protein) blotting. Protein samples were subjected to electrophoresis through a 13% *N,N'*-diallyltartardiamide-cross-linked sodium dodecyl sulfate (SDS)-polyacrylamide gel (34) and transferred to a nitrocellulose membrane by electroblotting at 15 V for 12 min. The gel, which retained some of the protein, was stained with Coomassie brilliant blue. The protein affixed to the nitrocellulose was renatured and probed with the duplex ICP4 binding site used to generate constructs 11T/4 and MLPT/4, by the method of Michael et al. (39). The DNA probe was 3' end labelled with the Klenow fragment of *E. coli* DNA polymerase I and [α -³²P]dCTP.

DNase I footprinting assays. DNase I footprinting assays were conducted by using *Xho*I-*Pvu*II fragments from the plasmids used to construct viral recombinants MLPT/4 and MLPT/mut4, which were 3' end labelled at the *Xho*I site with the Klenow fragment of *E. coli* DNA polymerase I and [α -³²P]dCTP. The footprinting procedure used was based on that of Galas and Schmitz (18) with some of the modifications of Brenowitz et al. (4). Binding reactions were performed in 50- μ l mixtures containing 25 mM Tris hydrochloride (pH 7.9), 2 mM MgCl₂, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 0.5 μ g of sheared herring sperm DNA, and 0.5 to 20 μ g of the ICP4-GST fusion protein. After preincubation at room temperature for 5 min, 20,000 cpm of labelled DNA was added and was incubated at 30°C for 20 min. The protein-bound DNA was then digested for 60 s at room temperature with 0.2 U of DNase I (Pharmacia) freshly diluted in DNase I dilution buffer (50 mM Tris hydrochloride [pH 7.2], 10 mM MgSO₄, 1 mM DTT, 50% glycerol, 25 mM CaCl₂). DNase I digestion was terminated by the addition of 100 μ l of DNase I stop buffer (1% SDS, 100 μ g of tRNA per ml, 200 mM NaCl, 20 mM EDTA, 200 μ g of proteinase K per ml. After incubation for 10 min at 50°C, the DNA was ethanol precipitated and then loaded on a 7 M urea-8% polyacrylamide sequencing gel.

RESULTS

Experimental design. We wished to determine the effects of a strong ICP4 binding site on the activity and temporal regulation of a simple model promoter located in the HSV genome. To this end, we generated two sets of artificial promoters in which the ICP4 binding site from the ICP4 gene transcription start site was linked to a TATA box (Fig. 1); as controls, we also generated corresponding constructs containing a mutant ICP4 binding site lacking the first two residues of the ATCGTC consensus (52). One set of constructs contained the ICP4 binding site downstream of the HSV-1 US11 or Ad2

MLP TATA boxes, reproducing the arrangement found in the native ICP4 promoter (constructs 11T/4 and MLPT/4 and their mutant derivatives 11T/mut4 and MLPT/mut4, respectively). The other set contained the binding site at either of two positions upstream of the US11 TATA element; in one case, the site was placed to mimic the spacing in the ICP0 promoter (D4/11T and Dmut4/11T), while in the other case, the site was located 5 nt closer to the TATA box (P4/11T and Pmut4/11T). We then used a previously described system to link these artificial promoters to the UL24 gene in the HSV genome (32). Each promoter was inserted across the endpoints of a 200-nt deletion (Δ SB) that extends from -172 to +24 relative to the transcription start site of the largest UL24 transcript (UL24b), thereby placing UL24b sequences under the control of the inserted promoter (32). The resulting structures were then transferred into the UL24 locus of HSV-1 strain KOS PAA⁵ by DNA-mediated marker rescue and then selected for TK-deficient recombinants (Materials and Methods).

Interaction with ICP4. The oligonucleotides used to assemble the ICP4 binding sites were chosen to span the minimal region required for ICP4 binding (52). We confirmed the ability of ICP4 to bind to the site present in construct MLPT/4 by DNase I footprinting. In this assay, we used an ICP4-GST fusion protein bearing the DNA-binding domain of ICP4 (residues 262 to 490 [74]) linked to GST from *S. japonicum* (65). Gel electrophoretic analysis of fusion protein isolated by affinity chromatography on glutathione-Sepharose demonstrated a major product with an apparent molecular mass of ca. 50 kDa, the predicted size of the full-length fusion protein (Fig. 2A). In addition, two less-abundant larger products and a prominent 25-kDa band (corresponding to the expected size of GST) were observed. Only the 50-kDa protein bound a labelled DNA probe bearing an ICP4 binding site in a Southwestern blotting assay (Fig. 2B) and was selectively retained on a DNA affinity column bearing tandem arrays of ICP4 binding sites (Fig. 2). In DNase I footprinting assays using a DNA fragment spanning the MLPT/4 site, the ICP4-GST fusion protein protected a ca. 23-nt region (Fig. 3); the position of the protected region was similar to that produced by intact ICP4 on the native ICP4 promoter (17), although the size of the footprint was marginally smaller (ca. 23 versus ca. 28 nt). As expected (52), no footprint was observed on the corresponding construct bearing the mutant ICP4 binding site (MLPT/mut4 [Fig. 3]).

Effects of an ICP4 binding site located downstream of the TATA box. Vero cells were infected with recombinant viruses bearing inserts of the US11 TATA element (11T), the US11 TATA box linked to a downstream ICP4 binding site (11T/4), or the US11 TATA box linked to a mutant ICP4 binding site (11T/mut4). Where indicated, viral DNA replication was blocked by the addition of aphidicolin at the time of infection. Total cellular RNA was then extracted at 6 and 12 h postinfection and examined by primer extension with a primer complementary to residues +91 to +67 of the native UL24b transcript (Fig. 4). As controls, the RNA samples were also scored for HSV gD mRNA and cellular 7SL RNA by primer extension. In agreement with previous work (32), construct 11T gave rise to a ca. 53-nt primer extension product, the predicted size for transcripts initiating 30 nt downstream of the inserted TATA element. As expected, constructs 11T/4 and 11T/mut4 gave rise to larger extension products (ca. 85 nt), reflecting the insertion of oligonucleotides bearing the ICP4 binding sites. Comparison of constructs 11T/4 and 11T/mut4 indicated that the functional ICP4 binding site present in 11T/4 altered UL24b expression in three ways. First, the levels of UL24b transcripts in construct 11T/4 were reduced more than

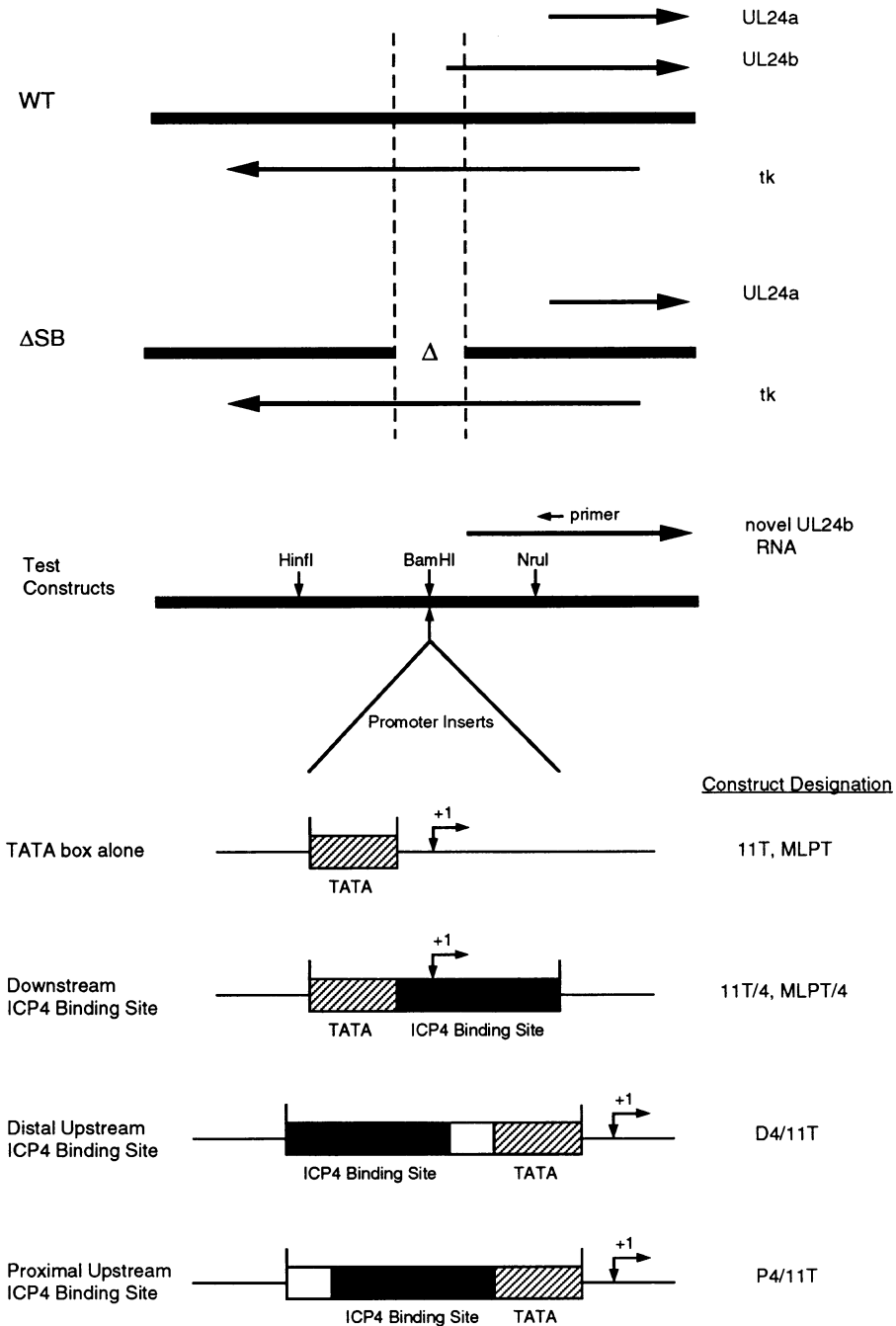


FIG. 1. Experimental design. The ΔSB deletion removes 200 nt of *tk* coding sequences and the promoter for the larger UL24 transcript (UL24b) and is spanned by a *Bam*HI linker. Oligonucleotides bearing test promoter sequences consisting of an ICP4 binding site located upstream or downstream of a TATA box were inserted into the intact HSV-1 genome across the ΔSB deletion, oriented to drive the UL24 gene. Corresponding constructs bearing mutant binding sites were also made. The activity of each test promoter was then scored by assaying the levels of novel UL24b transcripts by primer extension. The diagrams of test promoters shown in the bottom portion are not drawn precisely to scale.

100-fold at 6 h postinfection relative to 11T/mut4 (Fig. 4 and Table 1). Second, this negative effect was largely overcome by 12 h postinfection, such that 11T/4 displayed only a three to fourfold reduction relative to 11T/mut4. Thus, the ICP4 binding site shifted the kinetics of transcript accumulation to later times postinfection. Third, the binding site conferred a greatly increased dependence on viral DNA replication. Thus, at 12 h postinfection, transcripts arising from construct 11T/4 were

reduced more than 100-fold when infections were carried out in the presence of aphidicolin; in contrast, 11T/mut4 transcripts were reduced only 4 to 7-fold (Table 2). Taken in combination, these findings demonstrate that addition of the ICP4 binding site changed the kinetics of UL24b expression from leaky late to true late.

To determine whether these effects depended on the nature of the TATA element, we analyzed analogous constructs

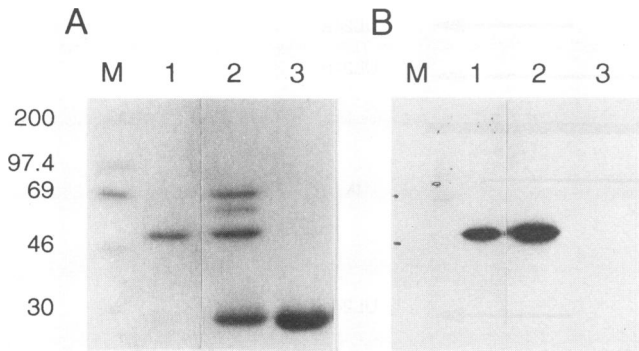


FIG. 2. Analysis of the ICP4-GST fusion protein produced from plasmid pIGF-17. Protein samples were subjected to electrophoresis through an SDS-13% polyacrylamide gel and then transferred to nitrocellulose. (A) Coomassie brilliant blue staining pattern of proteins retained in the gel after transfer; (B) proteins bound to nitrocellulose reacted with a ³²P-labelled oligonucleotide duplex bearing an ICP4 binding site. Lanes 1, ICP4-GST purified by DNA affinity chromatography; lanes 2, ICP4-GST purified by chromatography on glutathione-Sepharose; lanes 3, GST purified by chromatography on glutathione-Sepharose; lanes M, protein markers (Amersham); sizes in kilodaltons are shown on the left.

bearing the much stronger Ad2 MLP TATA box (constructs MLPT, MLPT/4, and MLPT/mut4). In this case as well, addition of the ICP4 binding site led to greatly reduced transcript levels at 6 h postinfection, and the imposition of an increased dependence on viral DNA replication (Fig. 5; Tables 1 and 2). Therefore, we conclude that the observed effects observed are independent of the sequence of TATA box. In particular, these data establish that the effects observed with construct 11T/4 are not due to the weak promoter activity of the US11 TATA box (construct MLPT is at least as active as the native UL24b promoter [32]).

Effects of upstream ICP4 binding sites. The preceding results demonstrated a negative effect of an ICP4 binding site placed over the transcription start site—the arrangement found in the native ICP4 promoter. We next examined constructs bearing the binding site located upstream of the TATA box to mimic the spacing found in the ICP0 promoter (constructs D4/11T and its mutant derivative, Dmut4/11T [33]). The results demonstrated that the upstream binding site had a similar, but less pronounced, effect (Fig. 6). Thus, levels of UL24b RNA arising from D4/11T were reduced more than sixfold at 6 h postinfection, and two- to threefold at 12 h postinfection, relative to the construct bearing the mutated binding site (Table 1). In addition, at 12 h postinfection,

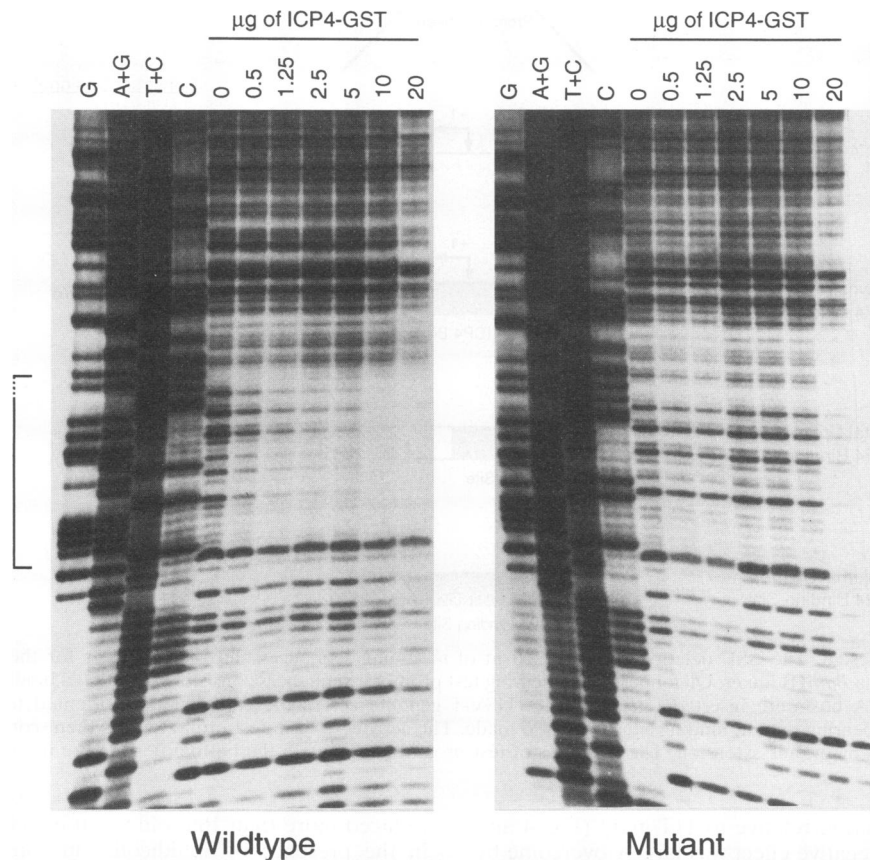


FIG. 3. DNase I footprinting of the ICP4 binding site in construct MLPT/4. *XhoI-PvuII* fragments spanning the ICP4 binding site in constructs MLPT/4 and MLPT/mut4 (3' end labelled at the *XhoI* end) were incubated with 0, 0.5, 1.25, 2.5, 5, 10, or 20 µg of ICP4-GST (purified by chromatography on glutathione-Sepharose) and then treated briefly with DNase I. Digestion products were separated by electrophoresis through a 7 M urea-8% polyacrylamide gel. The region of the fragment protected by the ICP4-GST fusion product is indicated by the bracket on the left. Wildtype, MLPT/4; mutant, MLPT/mut4. Lanes G, A+G, T+C, and C, base-specific chemical cleavage products of the same end-labelled fragments (36).

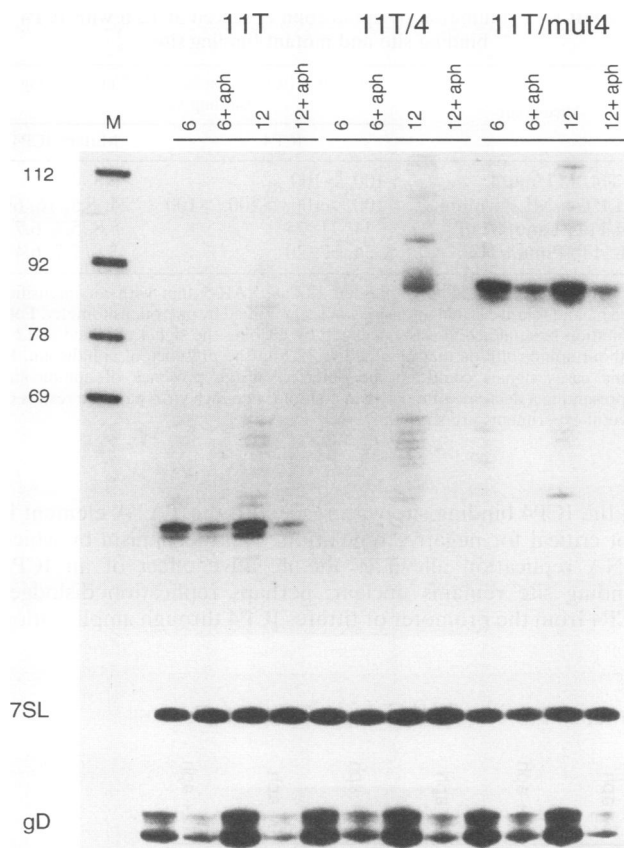


FIG. 4. Effects of an ICP4 binding site placed downstream of the US11 TATA element. Vero cells were infected with 10 PFU per cell of the indicated viral recombinants, and total cellular RNA was harvested at 6 and 12 h postinfection. Samples (20 μ g) were then assayed for UL24 transcripts by primer extension with a primer complementary to residues +91 to +67 of the native UL24b transcript. Samples were also scored for 7SL and gD RNAs by primer extension, by using 0.5 and 5 μ g of RNA, respectively. Where indicated, 10 μ g of aphidicolin (aph) per ml was added at the time of infection and maintained continuously. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

transcripts arising from D4/11T were reduced over 20-fold by blocking DNA replication, while those arising from Dmut4/11T were reduced only 5- to 7-fold (Table 2).

DiDonato and Muller (1) found that ICP4 and TFIID are located on opposite helical faces of the DNA when bound at their respective sites in both the ICP4 and the ICP0 promoters; furthermore, chemical probing suggested that ICP4 binding altered the helical geometry of the TATA region. On the basis of these observations, these investigators proposed that the stereospecific orientation of ICP4 and TFIID might be important to ICP4-induced repression of IE gene expression. To examine the importance of the helical orientation of bound ICP4 with respect to TFIID, we constructed an additional set of viral recombinants in which the ICP4 binding site was positioned 5 bp (approximately 1/2 helical turn) closer to the TATA box (P4/11T and Pmut4/11T). We found that this shift did not alter the effect of the ICP4 binding site relative to construct D4/11T (Fig. 6; Tables 1 and 2), suggesting that the helical orientation of ICP4 with respect to the TATA binding protein is not critical for the effects that we have observed.

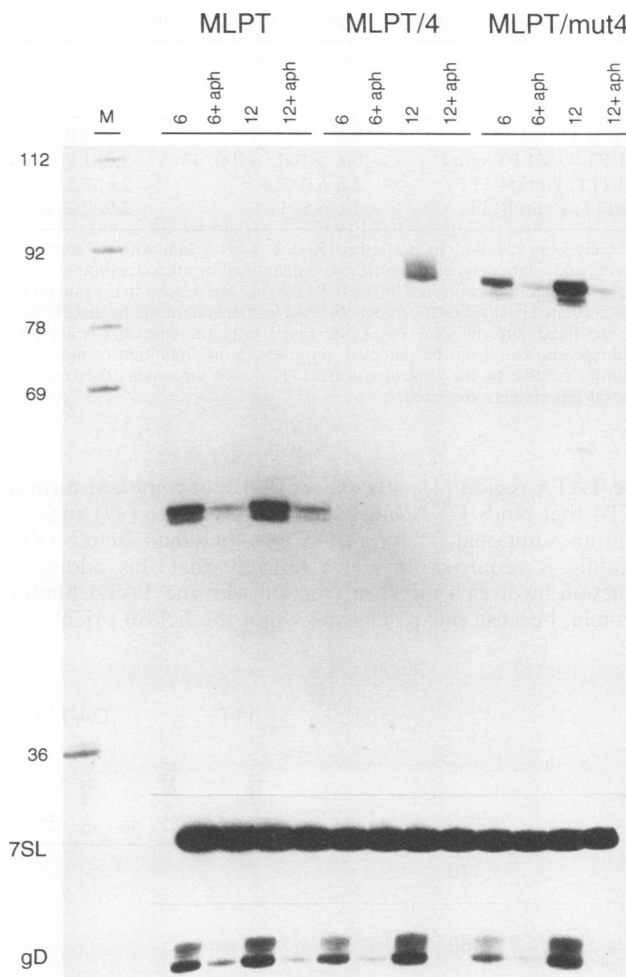


FIG. 5. Effects of an ICP4 binding site placed downstream of the Ad2 MLP TATA element. Vero cells were infected with 10 PFU per cell of the indicated viral recombinants, and RNA samples extracted 6 and 12 h postinfection were scored for UL24b, 7SL, and gD RNAs as described in the legend to Fig. 4. Where indicated, 10 μ g of aphidicolin (aph) per ml was added at the time of infection and maintained continuously. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

DISCUSSION

The experiments described in this paper demonstrate that an ICP4 binding site placed either upstream or downstream of a simple TATA box promoter can alter both the levels and the kinetics of gene expression during HSV infection. Thus, addition of an intact binding site reduced expression relative to control constructs, and this inhibitory effect was substantially more pronounced at early times postinfection. In addition, inhibition was exaggerated when viral DNA replication was blocked with aphidicolin. We take these results to indicate that ICP4 bound in close proximity to the TATA box blocks gene expression by interfering with some aspect of promoter function and that DNA replication partially bypasses this effect; the net outcome is to shift the kinetics of expression to later times postinfection. How does DNA-bound ICP4 inhibit promoter function? Although it has been proposed that repression may involve steric hindrance or conformational changes induced in

TABLE 1. Inhibition conferred by ICP4 binding site

Virus pair	Inhibition (fold) relative to mutant site ^a	
	6 h	12 h
11T/4, 11T/mut4	>100, >100	3.8, 3.0
MLPT/4, MLPT/mut4	>100, >100, >100, 43	3.7, 1.9, 4.4, 8
D4/11T, Dmut4/11T	22, 6.0, 7.6	2.6, 2.2, 2.3
P4/11T, Pmut4/11T	10, 6.5, 11	2.9, 2.2, 2.8

^a Gels were exposed to preflashed Kodak XAR-5 film with an intensifier screen, and relative signal intensities were quantified by microdensitometry. The degree of inhibition conferred by the ICP4 binding site relative to the mutant site was calculated by dividing the signal obtained for the mutant site by that obtained for the intact site. In some cases, the signal from the construct bearing the wild-type site could not be detected, representing an inhibition of more than 100-fold relative to the control construct. For each virus pair, the results of several experiments are listed.

the TATA region (11, 40), the existence of a mutant form of ICP4 that binds DNA but fails to autoregulate (47) suggests that an additional function of ICP4 other than simple DNA binding is required. It seems unlikely that this additional function involves a direct interaction with the TATA binding protein, because our results show that the helical orientation

TABLE 2. Inhibition by aphidicolin observed at 12 h with ICP4 binding site and mutant binding site

Virus pair	Inhibition (fold) by aphidicolin ^a at following binding site:	
	ICP4	Mutant ICP4
11T/4, 11T/mut4	>100, >100	6.8, 4.1
MLPT/4, MLPT/mut4	>100, >100, >100, >100	24, 8.5, 16, 69
D4/11T, Dmut4/11T	44, 21, 24	5.8, 5.3, 6.7
P4/11T, Pmut4/11T	34, 57, 20	3.8, 7.7, 6.4

^a Gels were exposed to preflashed Kodak XAR-5 film with an intensifier screen, and relative signal intensities were quantified by microdensitometry. Fold inhibition by aphidicolin was calculated by dividing the signal obtained at 12 h without aphidicolin by that obtained at 12 h in the presence of aphidicolin. In some cases signals could not be detected in the presence of aphidicolin, representing a decrease of more than 100-fold. For each virus pair, the results of several experiments are shown.

of the ICP4 binding site with respect to the TATA element is not critical for negative regulation. The mechanism by which DNA replication alleviates the negative effect of an ICP4 binding site remains unclear: perhaps replication dislodges ICP4 from the promoter or titrates ICP4 through amplification

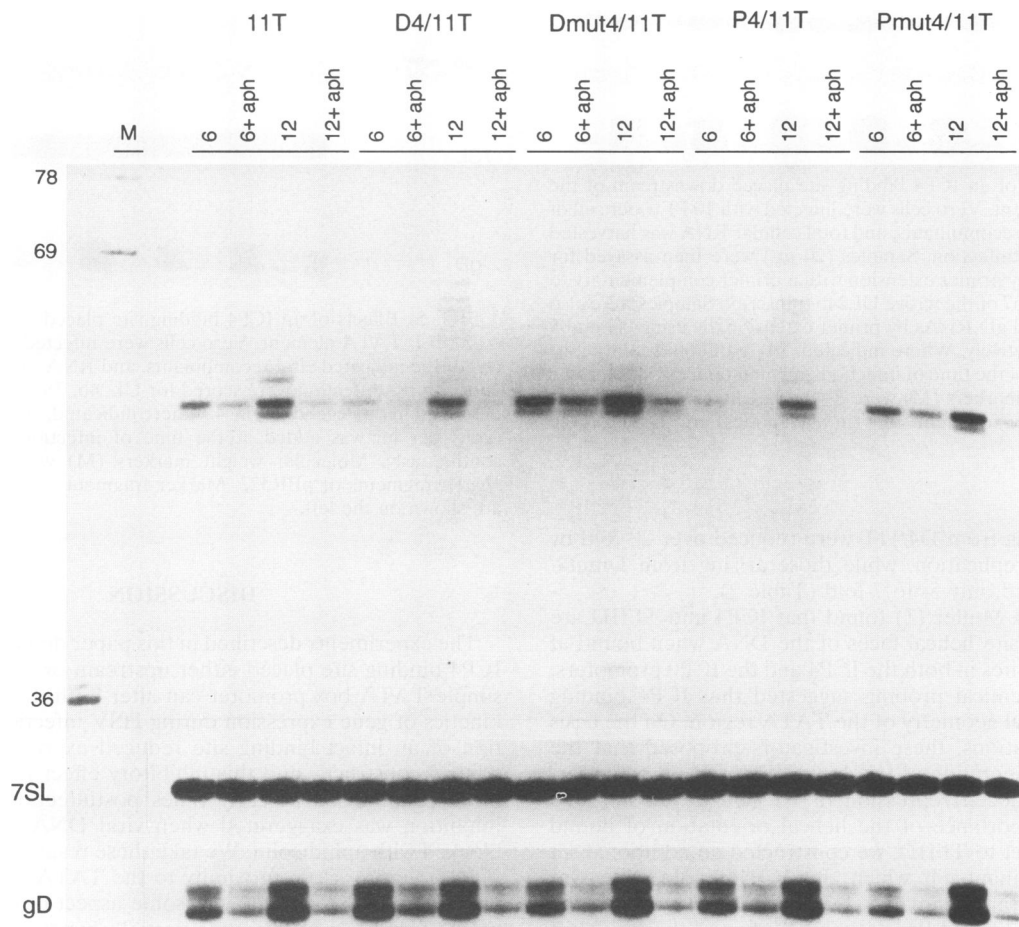


FIG. 6. Effects of ICP4 binding sites placed upstream of the US11 TATA element. Vero cells were infected with 10 PFU per cell of the indicated viral recombinants, and RNA samples extracted 6 and 12 h postinfection were scored for UL24b, 7SL, and gD RNAs as described in the legend to Fig. 4. Where indicated, 10 μ g of aphidicolin (aph) per ml was added at the time of infection and maintained continuously. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

of binding sites; alternatively, replication-induced conformational changes in the promoter might allow transcription even in the presence of bound ICP4. Further experiments are required to distinguish between these possibilities.

The ability of an ICP4 binding site to strongly reduce expression from a simple TATA promoter at early times postinfection is consistent with findings that the ICP4 binding sites in the native ICP4 and ICP0 promoters are required for ICP4-mediated repression in transfection assays (20, 51, 52) and the observation that the site at the ICP4 gene transcription start site serves as a target for negative regulation during infection (38). However, Everett and Orr (15) found that deleting the ICP4 binding site from the native ICP0 promoter had no effect on ICP0 expression during lytic HSV infection. We suggest that three factors might contribute to the minimal effect of ICP4 binding to the native ICP0 promoter. First, we found that a binding site placed upstream of the TATA box to mimic the arrangement in the ICP0 promoter had a less pronounced inhibitory effect than a site placed over the transcription start site, as in the ICP4 promoter. Second, transactivation by Vmw65 strongly boosts IE gene transcription (2, 5, 48) before the accumulation of ICP4, and it is possible that these preexisting transcription complexes partially inhibit binding of ICP4 to native IE promoters. Third, our data demonstrate that the negative effects of ICP4 binding sites are partially reversed after the onset of viral DNA replication. These considerations suggest that downregulation of IE promoters through ICP4 binding sites might assume greater importance under conditions of limiting Vmw65, particularly prior to the onset of viral DNA replication, for example, during the early stages of infection at low multiplicity or following reactivation from latency. A similar argument might also explain why mutating the ICP4 binding sites in the gD promoter had no obvious effect on the levels of gD expression during infection (63). Thus, the ICP4 binding site most proximal to the gD transcription start site begins at ca. -90, perhaps too far from the core promoter to exert a strong inhibitory effect in the presence of viral activators and DNA replication.

Our finding that ICP4 binding sites can confer a greatly increased requirement for viral DNA replication on simple TATA promoters raises the possibility that this mechanism contributes to the replication dependence of at least some bona fide HSV L promoters. According to this hypothesis, ICP4 binding sites located in the vicinity of L promoters might act as negative control elements that restrict expression at early times postinfection. In this context, it is interesting to note that our data demonstrate that the magnitude of the replication requirement imposed by an ICP4 binding site can vary with the position of the site, illustrating that this relatively simple mechanism could give rise to the wide range of replication requirements that is observed among HSV L promoters. The hypothesis that DNA-bound ICP4 serves as a negative regulator of nearby L promoters is consistent with the observation of Arsenakis et al. (1) that ICP4 can inhibit expression of the E/L gD gene in stably transfected cell lines; in addition, earlier work from our laboratory has shown the true L US11 promoter contains sequences that are able to impose an increased requirement for DNA replication on a simple TATA promoter (32). However, this hypothesis leads to an interesting paradox: ICP4 is also a required activator of L gene expression, and the available evidence indicates that it must bind DNA in order to serve this function (9, 10, 45, 46, 59). Perhaps DNA replication converts DNA-bound ICP4 from a local repressor into a local activator; alternatively, it is possible that L promoters can be induced only by ICP4 molecules that are bound to distal sites.

According to the latter hypothesis, DNA binding may serve to concentrate ICP4 in the vicinity of the viral genome, rather than directly targeting individual L promoters for activation. We currently seek to directly assess the regulatory role of ICP4 binding sites in HSV L promoters.

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