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Transactivation of the Major Capsid Protein Gene of Herpes Simplex Virus Type 1 Requires a Cellular Transcription Factor

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The purpose of this investigation was to identify and characterize the regulatory elements involved in the transcriptional activation of the βγ (leaky-late or γ_1) genes of herpes simplex virus type 1 (HSV-1) by using the major capsid protein (VP5 or ICP5) gene as model. Gel mobility shift assays with nuclear extracts from uninfected and infected HeLa cells enabled us to identify two major protein-DNA complexes involving the VP5 promoter. The mobilities of these two complexes remained unaltered, and no unique complexes were observed when infected cell nuclear extracts were used. DNase I and orthophenanthroline-Cu^+ footprint analyses revealed that the two complexes involve a single binding site, GGCCATCTTGAA, located between −64 and −75 bp relative to the VP5 cap site. To determine the function of this leaky-late binding site (LBS) in VP5 gene activation, we tested the effect of mutations in this region by using transient expression of a *cis*-linked chloramphenicol acetyltransferase gene. Deletion of the above sequence resulted in a seven- to eightfold reduction in the level of transactivation of the chloramphenicol acetyltransferase gene by superinfection with HSV-1 or by cotransfection of HSV-1 immediate-early genes. From these results, we conclude that the LBS sequence and a cellular factor(s) are involved in the transactivation of the VP5 gene. A search of published gene sequences revealed that sequences related to the LBS exist in a number of other HSV-1, cytomegalovirus, retrovirus, and cellular promoters. Sequence homologies of binding sites and results of unpublished competition binding studies suggest that this leaky-late binding factor may be related to, or the same as, a ubiquitous cellular transcriptional factor called YY1 or common factor-1 (also known as NF-E1, δ, and UCRBP).

During productive infection of cells in culture, the genes of herpes simplex virus type 1 (HSV-1) are expressed as three major classes that are coordinately and sequentially regulated. On the basis of the kinetics of their expression and sensitivity to metabolic inhibitors, these classes have been referred to as α or immediate-early (IE), β or delayed-early, and γ or late (26; for a review, see references 53 and 62). This differential regulation of gene expression is mediated largely at the transcriptional level (20, 24, 30, 62, 65), although posttranscriptional processes apparently also play a role (24, 30, 58, 65). The late genes have been further subdivided into two classes on the basis of the stringency of their requirement for viral DNA replication (53, 62). Thus, the βγ genes (also called γ_1 or leaky-late) are first transcribed and expressed at relatively low levels prior to viral DNA replication; following replication they are expressed at more abundant levels. This contrasts with the true late, or γ_2, genes whose expression exhibits a more stringent requirement for viral DNA replication (25, 53, 62). The studies described in this paper focus on the regulation of the βγ class by using primarily the VP5, or ICP5, gene as a model. This gene codes for a 155-kDa polypeptide which serves as the major capsid protein of the virus.

The molecular mechanisms involved in both the initial activation of expression of the βγ genes and the subsequent acceleration of their expression after DNA replication are not well understood. For the initial activation it is clear, however, that viral IE proteins are required. This was first suggested in studies using cycloheximide reversal (26, 66) and amino acid analogs (27) and subsequently by investigations with IE gene mutants of HSV-1 (4, 8, 11, 15, 20, 36, 46, 48, 50, 51, 54, 63, 66). The latter studies indicated that at least three of the five IE proteins, ICP0, ICP4, and ICP27, are involved.

More specific information on the requirements for viral IE proteins has come from various transient expression experiments. The initial findings from these were that promoters of genes frequently assigned to the βγ or leaky-late class (e.g., VP5, gD, and gB) exhibit low activity when transfected into uninfected cells but that they are strongly activated by HSV-1 superinfection or by cotransfection of HSV-1 IE genes (3, 6, 8, 9, 55, 59). The latter experiments confirmed that three IE proteins, ICP0, -4, and -27, are required for full transactivation of βγ genes such as VP5 (6, 16, 55, 59). Exactly how the three IE proteins function in this context is not clear, although at least part of the activity of ICP27 appears to be at the posttranscriptional level (58). Moreover, with the exception of ICP4 binding sites in the gD promoter (60), direct binding of IE proteins to regulatory regions of the βγ genes has not been demonstrated.

Studies using inhibitors of viral DNA synthesis have clearly demonstrated that the βγ genes require, in addition to IE gene function, viral DNA replication for their full expression (6, 20, 25, 45). Transcription from βγ promoters, such as those of VP5, gD, and gB, is initiated early during infection along with those of typical early, or β, genes such as the thymidine kinase (TK) gene, but in the absence of viral DNA synthesis, the usual postreplicative increase in their expression does not occur (20, 65, 66). Exactly how

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expression of both the βγ and γ genes is coupled to DNA replication is not clear. However, the structural requirements for this coupling appear to be a minimal TATA box promoter, some downstream sequences, and a replication function provided in *cis* (29). In addition, it seems that ongoing replication and not simply amplification of the template is the key ingredient provided by viral DNA synthesis (29).

Transfection studies have also provided insight concerning the *cis*-acting DNA sequences required for transactivation of βγ promoters. Transient expression assays with the VP5 promoter coupled to the bacterial chloramphenicol acetyltransferase (CAT) gene demonstrated that sequences located no more than −125 bp from the cap site are required for maximum transactivation either by superinfection or by cotransfection of IE genes. Sequences located between −4 and −75 were found to suffice for transactivation, but at a 2.5- to 5-fold-lower level of that obtained with the complete −125 sequence. In addition, these investigations identified a region located between −75 and −168 that exerted a silencer effect on transcription from a VP5 promoter placed downstream from a simian virus 40 enhancer (2, 3, 6). These studies, however, did not disclose the precise regulatory sequences or the proteins that interact with them.

A detailed analysis of the gD promoter revealed that sequences upstream to −33 (essentially only a TATA box and downstream regions) sufficed for true late gene type of activation by a *cis*-linked origin of replication, whereas DNA sequences located between −33 and −83 were essential for βγ type of transactivation by virus superinfection (13, 14, 29). Deletion analysis further localized the region required for transactivation to two G-rich sequences, one located between −63 and −73 and one of lesser importance located between −42 and −55 (14). However, in this work the proteins that interact with these sequences were not identified.

From the foregoing discussion, it is apparent that the general requirements for transactivation of certain βγ promoters have been established. However, we still do not know what cellular factors are involved, what specific promoter sites are required, how individual IE proteins function in the activation process, and why DNA replication is required for maximum βγ gene expression. The investigations we report here provide new insight into the first two questions. By using gel mobility shift and DNA footprinting analyses we have identified a regulatory sequence, present in the VP5 promoter and in several other HSV-1 genes, that binds one or more cellular factors. We further show, by deletion analysis and transient expression assays, that this binding site is essential for fully activated expression of the VP5 gene. From these studies we conclude that transcriptional activation of the VP5 gene, and probably several other βγ genes, requires not only the action of viral IE proteins, but also the binding of a cellular factor(s) to a specific promoter regulatory site.

MATERIALS AND METHODS

Cells and virus. HeLa cells (ATCC CCL2) and HEp-2 cells (ATCC CCL23) were propagated at 37°C under 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM; GIBCO Laboratories, Inc.) containing 10% heat-inactivated calf serum (Hyclone Labs, Inc.), penicillin (100 U/ml), and streptomycin (100 μg/ml). The *vhs-1* mutant of HSV-1 (KOS), defective in a virion-associated host shutoff function which maps in the UL41 gene (33, 49), was kindly provided by G. S. Read, Loyola University of Chicago. It was propagated in HEp-2 cell monolayers as previously described (40).

Plasmids and DNA fragments. Plasmid VP5(−168)CAT, containing the HSV-1 VP5 promoter fragment −4 to −168, relative to the cap site, linked to the reporter gene for bacterial chloramphenicol acetyltransferase (CAT), was kindly provided by E. Blair and E. Wagner, University of California, Irvine (3, 6). Plasmid pGTSa2, prepared in this laboratory and containing the 4.0-kbp *Sal*I D fragment of HSV-1 (KOS) cloned in pBR322, was used to prepare gB probes. Plasmids pP017 and pGR150B were generously provided by G. Hayward, Johns Hopkins University. The former, consisting of the 4.9-kbp *Hin*dIII N fragment of HSV-1 (MP) cloned in pCATB, was used to prepare gD promoter fragments. The latter contains the 23.7-kbp *Bgl*II HM fragment of HSV-2 cloned into pBR322 and encodes three IE proteins, ICP0, -4, and -27. Plasmid DNA was prepared by the alkaline lysis procedure (1) with modifications (34). Plasmids used for transfection were further purified by CsCl buoyant density centrifugation. DNA fragments from restricted plasmids were isolated by the DEAE paper electroelution method (12). Fragments were labeled at their 3′ ends by Klenow fill-in (34) using α-^{32}P-labeled deoxyribonucleoside triphosphates (NEN Research Products).

Oligonucleotides. A double-stranded oligonucleotide containing the VP5 leaky-late binding site (LBS) and having the sequence CCAGGATCCAGGGCCATCTTGAATGGATCC TGG was synthesized at the oligonucleotide facility of the Vollum Institute, Oregon Health Sciences University. A control double-stranded oligonucleotide (C2) having the sequence CCTTGCCACATGACCTGCTTCCT that lacks an LBS was kindly provided by K. Riggs, Columbia University.

Enzymes and coenzymes. Enzymes, coenzymes, and their sources are as follows: restriction endonucleases (Bethesda Research Laboratories, Inc. [BRL]; New England Biolabs, Inc.; and Boehringer Mannheim GmbH), used with either the 10× reaction mixes provided by BRL or the appropriate amounts of KGB buffer (34); exonuclease BAL 31 (U.S. Biochemical Corp.); Klenow fragment of DNA polymerase, T4 DNA ligase, and RNase A (BRL); and bacterial CAT, *S*-acetyl coenzyme A synthetase, and coenzyme A (Sigma Chemical Co.).

Nuclear extracts and mobility shift assays. Nuclear extracts were prepared from HeLa S monolayers that were either uninfected (mock infected) or infected with HSV-1, multiplicity of infection of 10 to 15, for 8 h (10). For mobility shift assays (18, 19), 0.1 to 1 ng of DNA probe, 0.7 to 3.5 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia), and nuclear extract (1 to 5 μg of protein) were mixed in a reaction buffer providing final concentrations of 6 mM Tris-HCl (pH 7.9), 40 mM KCl, 2 mM EDTA, 0.2 mM dithiothreitol, and 8% (vol/vol) glycerol in a total volume of 20 or 25 μl. Following a 30-min incubation at room temperature, 2 μl of 0.25% bromphenol blue was added and the samples were electrophoresed at 4°C through polyacrylamide gels made with 4% acrylamide–0.13% bisacrylamide in a Tris-borate buffer (25 mM Tris base, 25 mM boric acid, 1 mM EDTA). Gels were dried under vacuum and exposed to Fuji RX film with intensifying screens. For competition binding assays, the above conditions were used but with 100- to 300-fold-excess unlabeled competitor DNA added 10 min before the radiolabeled probe. The probe was then added, and the incubation was continued for an additional 20 min at room temperature. A high level of competitor was needed for effective competition because of the relatively high concentration of the

leaky-late binding factor (LBF) we and others (52) have found to be present in nuclear extracts.

DNA footprinting. Mobility shift assays were carried out as described above but scaled up 10- to 30-fold. After the protein binding reaction the samples were treated for 30 to 120 s with DNase I at 3.5 ng/μl in the presence of 3.5 mM $MgCl_2$. The reactions were stopped by addition of EDTA to 2.5 mM, and the samples were loaded onto a polyacrylamide gel and electrophoresed as described above for the mobility shift assay. Alternatively, following the mobility shift procedure, the gels were subjected to the orthophenanthroline-Cu^+ footprinting procedure (32). Protein-DNA complexes and free DNA probe were excised as gel strips and inserted into a 2% agarose slab gel. Following the addition of 5 μg of *Escherichia coli* tRNA per strip, the DNA fragments were electrophoresed onto DEAE paper strips and isolated as described above (12). Dried DNA samples, containing 5×10^3 to 10×10^3 cpm, were run on DNA sequencing gels (made with 8% acrylamide) along with the probe that had been subjected to Maxam and Gilbert cleavage reactions (35).

Construction of VP5 promoter mutations. Plasmid VP5(−168)CAT (90 μg) was linearized at the *Sal*I site at the 5′ end of the VP5 promoter and then digested with 2.25 U of BAL 31 exonuclease for 23 to 38 s (34). This produced deletions of various sizes that extended through the VP5 binding site region. The deleted ends were polished with a Klenow fill-in reaction, and *Bgl*II linkers (New England Biolabs, Inc.) were added by blunt-end ligation (34). Following cleavage with *Nco*I in the CAT gene, fragments of 617 to 642 bp containing the deletions (fragment 1) were isolated by agarose gel electrophoresis. Fragment 2 was prepared by partial digestion of VP5(−168)CAT with *Bst*NI to cleave at one of two sites located 5′ to the VP5 binding site. Following Klenow polishing, *Bgl*II linker attachment (34), and *Sal*I digestion, fragments of 78 and 94 bp were isolated by agarose gel electrophoresis (fragment 2). Fragment 3 was prepared by restriction of VP5(−168)CAT with *Nco*I and *Sal*I and isolating the 3.02-kbp *Nco*I-*Sal*I vector fragment. Finally, fragments 1, 2, and 3 were ligated together to produce VP5CAT plasmids with deletions in the VP5 promoter (see Fig. 4A). Competent *E. coli* HB101 cells (BRL) were transformed with the resulting mutated plasmids, and 40 ampicillin-resistant clones were picked. From restriction and Maxam and Gilbert (35) sequencing analyses, eight mutant clones that contained the desired range of deletions in the VP5 promoter were selected for further study.

Transfections and CAT assays. HeLa cells were seeded in 35-mm well cluster dishes 20 h prior to transfection at 3×10^5 cells per well in DMEM containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (DMEM–10% IFBS-P/S). The medium was changed after 17 h, and 0.5 ml of calcium phosphate-precipitated DNA was added 20 h after seeding (22). Each 0.5-ml sample contained either 5 μg of target plasmid and 11 μg of carrier pUC18 DNA or 8 μg of target plasmid DNA and 8 μg of cotransfected effector plasmid (pGR150B). Four hours after adding the DNA, the cells were shocked by adding to their medium 1 ml of 15% (vol/vol) glycerol in DMEM–10% ICFS-P/S (21). After 1 min, the cells were washed with PBS-A (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4), overlaid with DMEM–10% ICFS-P/S, and incubated further at 37°C. The transfected cells were either superinfected 20 h after transfection with the *vhs-1* mutant of HSV-1 (KOS) at a multiplicity of infection of 3 and harvested 26 h later for CAT assay or cotransfected and harvested for CAT assay 44 h after transfection. Cell washing, lysis, and CAT assay were performed by the method of Nordeen et al. (43). The CAT assay consisted of measuring incorporation of ^{3}H-Na-acetate (NEN Research Products, Inc.; 3.3 Ci/mmol, 10 mCi/ml) into acetyl-chloramphenicol by using a coupled reaction with acetyl coenzyme A synthetase. The assay was verified by thin-layer chromatography and autoradiography.

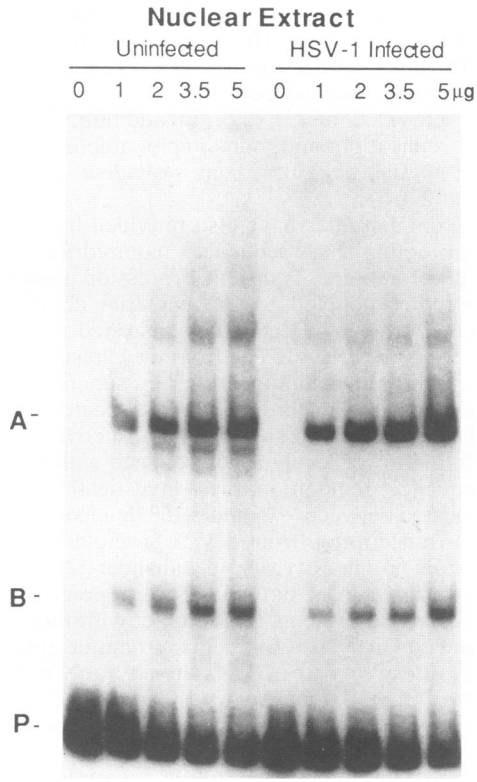

FIG. 1. Gel mobility shift analysis of the VP5 promoter. Various amounts of nuclear extracts from either uninfected (i.e., mock-infected) or HSV-1-infected HeLa cells were reacted with 0.11 ng of VP5 promoter fragment (−4 to −168 bp relative to the cap site, end labeled with [^{32}P]deoxynucleoside triphosphates) under conditions described in Materials and Methods. The complexes formed were analyzed by polyacrylamide gel electrophoresis. Amounts of nuclear extracts used, in micrograms of protein per reaction, and positions of the two major complexes formed (A and B) and free probe (P) are indicated.

RESULTS

Two major protein-DNA complexes are formed with the VP5 promoter. To identify and characterize potential regulatory complexes formed with the VP5 promoter, we carried out a series of mobility shift analyses by using a ^{32}P-labeled VP5 promoter fragment (−4 to −168 bp relative to the cap site) and various amounts of nuclear extracts from uninfected and HSV-1-infected HeLa cells. Complexes formed were analyzed by electrophoresis through polyacrylamide gels (Fig. 1). Two major complexes were observed, one of lower mobility (complex A) and one of higher mobility (complex B), when nuclear extracts from either uninfected or infected cells were used. Although viral proteins are known to transactivate the VP5 promoter, no complexes unique to the infected-cell extracts were observed, even at high concentrations of nuclear extract. In addition, the

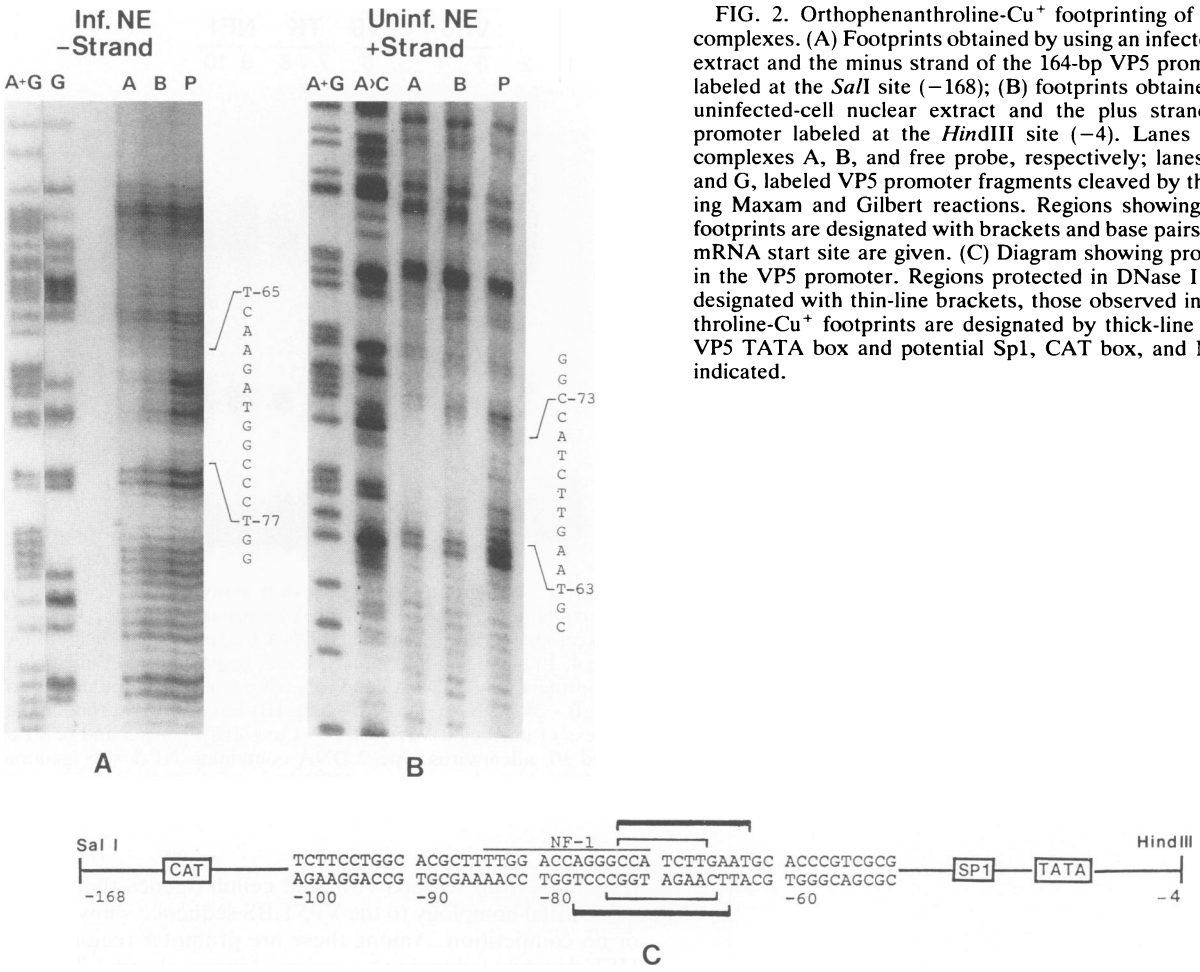

FIG. 2. Orthophenanthroline-Cu$^+$ footprinting of VP5 promoter complexes. (A) Footprints obtained by using an infected-cell nuclear extract and the minus strand of the 164-bp VP5 promoter fragment labeled at the *Sal*I site (−168); (B) footprints obtained by using an uninfected-cell nuclear extract and the plus strand of the VP5 promoter labeled at the *Hin*dIII site (−4). Lanes A, B, and P, complexes A, B, and free probe, respectively; lanes A+G, A>C, and G, labeled VP5 promoter fragments cleaved by the corresponding Maxam and Gilbert reactions. Regions showing the strongest footprints are designated with brackets and base pairs relative to the mRNA start site are given. (C) Diagram showing protected regions in the VP5 promoter. Regions protected in DNase I footprints are designated with thin-line brackets, those observed in orthophenanthroline-Cu$^+$ footprints are designated by thick-line brackets. The VP5 TATA box and potential Sp1, CAT box, and NF-1 sites are indicated.

relative mobilities of the two complexes remained unchanged when nuclear extract from infected cells was used. The only difference we have observed between uninfected and infected cell complexes is a six- to ninefold increase in the ratio of complex B to A with some nuclear extract preparations. As discussed below, this may simply reflect differing amounts of proteolytic activity in the extracts.

DNA footprinting analyses reveal that a common core sequence is involved in both VP5 promoter complexes. To define the DNA sequences involved in the formation of the two VP5 promoter complexes, we first carried out DNase I footprinting analyses on complexes A and B with nuclear extracts from uninfected and infected HeLa cells. The DNase-treated complexes were resolved on mobility shift gels and excised, and the DNA was extracted and analyzed on DNA-sequencing gels. With infected-cell nuclear extracts, both complexes were found to protect a similar region, nucleotides −66 to −74. With uninfected nuclear extracts, a similar footprint was obtained (data not shown). Since the footprints obtained by the DNase method were somewhat weak, probably because of the short half-life of the complexes (see Discussion and reference 64), we subsequently employed the orthophenanthroline-Cu$^+$ footprinting method (32). This more clearly showed, with either uninfected or infected nuclear extracts, that essentially the same promoter sequence was protected in both complexes (Fig. 2A and B). A summary of the footprint mapping is presented in Fig. 2C. These results indicate that a cellular protein(s) forms both complexes A and B and that these two complexes encompass a common sequence, GGCCATCTTGAA, which we call the LBS (the leaky-late, or βγ, binding site), located between −64 and −75, in the VP5 promoter.

The observed promoter complexes are unique to the VP5 gene and several other genes of the βγ class. To obtain information on the binding site specificity of the protein(s) involved in the VP5 complexes, we carried out a series of binding assays using as competitors unlabeled DNA fragments from a number of different HSV-1 genes as well as from several other viral and cellular genes. Some of the more significant competition binding results with the labeled 164-bp VP5 promoter fragment as probe are depicted in Fig. 3A, and all competition results are summarized in Table 1. Of the HSV-1 gene sequences that contained potential homologs to the VP5 LBS (Table 2), good competition was observed by using excess unlabeled promoter fragments from the VP5 and glycoprotein D (gD) genes and with a fragment from the nontranslated leader region of the glycoprotein B gene that contains an LBS homolog at +137 (gB_a) (Fig. 3A, lanes 3 to 7). A gB promoter fragment containing a partial match to the VP5 LBS at −249 (gB_b) showed little competition (lanes 8 and 9). Likewise, a fragment from the UL37 promoter containing a 9 of 11 bp match to the VP5 LBS at −137 showed negligible competition, whereas a sequence from an internal position (+750) in the UL37 gene that has a 10-bp perfect match to the VP5 site was found to compete well (data not shown; Tables 1 and 2). Promoter

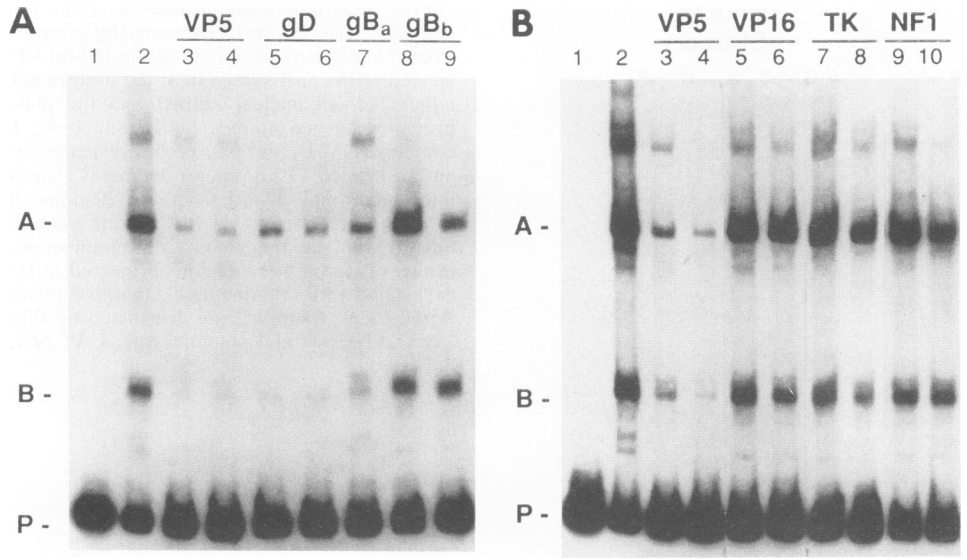

FIG. 3. Competition of VP5 promoter complex formation by unlabeled DNA fragments. Gel mobility shift assays were performed as described in the legend to Fig. 1 and Materials and Methods. All reactions contained ^{32}P-labeled 164-bp VP5 promoter probe, 3.6 μg of uninfected HeLa nuclear extract (except in lanes 1), and 100- and 300-fold excesses of various unlabeled DNA fragments as indicated. (A) Lane 1, VP5 probe alone; lane 2, probe without competitor DNA; lanes 3 and 4, 100- and 300-fold molar excess, respectively, of unlabeled, homologous VP5 promoter DNA (−4 to −168); lanes 5 and 6, unlabeled glycoprotein D (gD) DNA (+13 to −389); lane 7, 300-fold excess of glycoprotein B (gB-137) DNA (+17 to +187); lanes 8 and 9, glycoprotein B (gB - 249) DNA (−151 to −264). (B) Lane 1, VP5 probe alone; lane 2, probe without competitor; lanes 3 and 4, 100- and 300-fold molar excess of unlabeled VP5 DNA (−4 to −168); lanes 5 and 6, VP16 DNA (−280 to +150); lanes 7 and 8, TK DNA (+14 to −222); lanes 9 and 10, adenovirus type 2 DNA containing NF-1 site (genome coordinates, 0 to 189). All competitors except gB-137 were used in 100- and 300-fold molar excesses. HSV-1 gene coordinates are given in base pairs relative to mRNA start sites.

TABLE 1. Competition of VP5 promoter complex formation by unlabeled viral and cellular DNA fragments

Source of fragment	DNA fragments showing:	
	No competition	Competition
HSV-1 DNA	gH, gC (γ)	VP5, gD, gB+136 (βγ), UL37+750 (β or βγ)[a]
	UL14/15, UL24, UL37-137, UL46, VP16, (βγ)[b]	
	TK, UL40 (β)	
	ICP47 (α)	
Adenovirus type 2 DNA	Major late promoter	
	NF-1 site	
Cell DNA	ε-globin promoter	

[a] UL37, see references 17a and 56.

[b] Although these genes are considered in the βγ class, the UL24 promoter shows little homology and the UL14/15, UL46, and VP15 promoters have only limited homology to the LBS site of VP5. DNA fragments used in competitions not shown in Fig. 2 are the following: gH (glycoprotein H, −176 to +17), gC (glycoprotein C, −350 to +256), UL14/15 (2.7-kb βγ, −179 to +32), ICP47 (−300 to +11), UL40 (ribonucleotide reductase, small subunit, −187 to +156), adenovirus major late promoter (−240 to +175), and ε-globin (−207 to +67), in coordinates relative to cap site, and UL37-275 (−302 and −43), UL37+610 (+573 to +950), and UL46 (−525 to +417), in coordinates relative to ATG.

fragments from various viral and cellular genes that lacked substantial homology to the VP5 LBS sequence showed little or no competition. Among these are promoter regions from HSV-1 genes belonging to various kinetic classes: TK (DE or β) and VP16 (βγ), shown in Fig. 3B, and ICP47 (α or IE) and gC (γ_2), presented in Table 1. In other experiments not shown, promoter fragments from adenovirus type 2 major late and cellular epsilon globin promoters also failed to compete (Table 1). Since the VP5 LBS overlapped a potential NF-1 site (Fig. 2C), an adenovirus type 2 fragment containing an NF-1 site was used as a competitor. Since this DNA failed to compete significantly (Fig. 3B, lanes 9 and 10), it is unlikely that the observed complexes involve NF-1. These results suggest that the DNA sequences involved in the formation of VP5 promoter complexes A and B may be unique to a subset of HSV-1 genes that includes several βγ genes and that transcriptions factors common to other viral and cellular promoters, such as Sp1, CBF, and NF-1/CTF, are not likely to be involved in the formation of these complexes.

To demonstrate that the gD and gB fragments that competed for complex formation by the VP5 promoter (Fig. 3) bind the same (or very similar) protein(s), we ran ^{32}P-labeled VP5, gD, and gB DNA fragments in a gel shift assay with nuclear extracts from uninfected HeLa cells and a synthetic oligonucleotide containing an 11-bp match to the VP5 LBS as a competitor (Fig. 4). An oligonucleotide lacking the LBS sequence (C2) was used as negative control. First, it can be seen that the gD and gB fragments form two major complexes of relative mobilities similar to those formed by the VP5 probe (lanes 2, 7, and 12). Second, it is apparent that the LBS sequence (lanes 3, 4, 8, 9, 13, and 14), but not the control oligonucleotide, C2 (lanes 5, 10, and 15), competes

TABLE 2. Promoter sequences homologous to the VP5 LBS sequence

Gene	DNA sequence	Location (relative to cap site or ATG)	Competition against VP5	Sequence reference
Viral genes				
HSV-1				
VP5	GGGCCATCTTGAATG (LBS core sequence)	−70 (cap)	+	37, 38
gD	GGGCCATtTTacg	−63 (cap)	+	37, 38
gB	tGGCCATCgTcgA	+137 (cap)	+	37, 38
	GGcCCcTCTTtgAT	−249 (cap)	−	37, 38
UL46	GGGCCATCggG	−55 (ATG)	−	37, 38
UL37	GcGCCATCTTGA	+750 (cap)	+	17a, 56
UL37	GGGCCATtTgG	−137 (cap)	−	17a, 56
HSV-1 LBS consensus sequence	GGCCATNTT			
CMVs				
MCMV IE1	GCCAT$^{A}_{C}$TTG$^{A}_{G}$	1st exon, three sites +133 to +180 (cap)		31
	GCCATgTAct	−247 (cap)		
HCMV IE68 and SCMV IE94	GCCATNT	Enhancer/promoter, multiple copies		28
Retroviruses				
Murine leukemia viruses[a]	GGaCCATCT	−180 (cap)		61
SIV_{mac}	GGCCtTCTTaA	−131 (cap)		5
SRV-1	GCCATCTTGAATGC	−140 (TATA)		47
Adeno-associated virus				
P5 promoter	tctCCATtTTGA (YY1 site)	+1 (cap)		57
	GcGaCATtTTGc (YY1 site)	−60 (cap)		
Cellular genes				
Immunoglobulin heavy chain	GGCCATCTTGA (μE1 site)	Enhancer		44, 64
c-*myc* (murine)	GaCCTtTTct (CF1 site)	−260 (cap)		52
Skeletal α-actin	cGCCATgTacc	−90 (cap)		52

[a] For example, Akv, Gross, Seoule, and FBR murine leukemia viruses and murine osteosarcomavirus.

for the complexes formed by the VP5, gD, and gB promoter/leader DNA fragments. These results strongly suggest that the same or very similar protein(s) forms the A and B complexes with these three DNA probes.

When a nuclear extract from infected cells was used with the labeled gD promoter, the mobilities of complexes A and B remained unchanged. However, a new complex of higher molecular weight was observed (data not shown). Since this new complex had approximately the same mobility as a gD complex formed with a cloned ICP4 protein (a generous gift from K. Wilcox) and since the gD fragment used contains a known ICP4 binding site (60), it is likely that the new complex represents an ICP4 complex.

Mutational analysis of the VP5 binding site. To assess the role of the VP5 binding site, or LBS, in transcriptional regulation, a series of mutated VP5 promoter constructs were generated from plasmid VP5-CAT by progressive BAL 31 nuclease deletion from *Bst*NI sites located at approximately −76 and −93 and ligation of the resulting fragments with *Bgl*II linkers. The deletion strategy is described in Materials and Methods, and a summary of the deletion and insertion mutations used is presented in Fig. 5A.

To correlate complex A and B formation with promoter activity, we tested the mutated VP5 promoter constructs in a competition gel shift assay (Fig. 5B). The results showed that constructs 6 (12-bp insertion between −76 and −77) and 2 (deletion −83 to −93 [data not presented]) competed as well as the wild-type VP5 promoter, whereas deletion 10 (−70 to −93) showed weak competition and deletion 13 (−59 to −93) failed to compete significantly for protein binding (Fig. 5B). These results are compatible with the mapping of the LBS as determined by footprint analyses (Fig. 2).

The VP5 promoter binding site (LBS) is required for optimal transactivation by superinfecting HSV-1 or by co-transfected IE genes. To analyze the role of the A and B complexes in the regulation of VP5 gene expression, we tested the wild-type and mutated VP5 promoter constructs in transient expression assays for bacterial CAT expression. HeLa cells were transfected with the VP5-CAT plasmids; 20 h later they were superinfected with HSV-1 KOS (*vhs-1* mutant) and harvested 26 h after that for CAT enzyme assay (Fig. 6A). The host shutoff mutant, *vhs-1* (33, 49), was used to achieve greater CAT mRNA stability. In agreement with earlier findings from several laboratories (6, 8, 9, 16), the wild-type as well as mutated VP5 promoters were essentially inactive without viral superinfection (Fig. 6A, bars labeled U); CAT activities were approximately the same as those of the controls without transfected CAT plasmids. Virus superinfection, however, stimulated activity of the wild-type promoter 425-fold over control levels. A small deletion to the left of the NF-1 homology (construct 2, −83 to −93 [data not shown]) as well as disruption of the NF-1 sequence with a 12-bp linker insertion (construct 6) had only a small effect on CAT activity. However, a deletion that extended through the potential NF-1 site and half of the VP5 binding site (construct 10) reduced virus transactivation 4.6-fold. A more

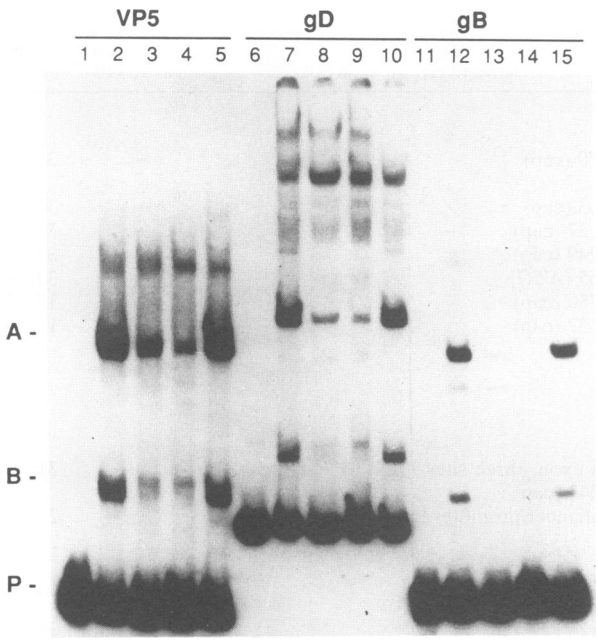

FIG. 4. Competition binding of labeled VP5, gD, and gB promoter fragments by oligonucleotides. Gel shift binding reactions were performed as described in the legend to Fig. 1 and in Materials and Methods by using labeled DNA fragments from either the VP5 (0.76 ng; −4 to −168), gD (0.58 ng; +13 to −260), or gB genes (0.7 ng; +17 to +187) and excess unlabeled oligonucleotide competitors. Lanes 1, 6, and 11, probe without nuclear extract; lanes 2, 7, and 12, probes with 2.34 μg of uninfected HeLa cell nuclear extract; lanes 3 and 4, 8 and 9, and 13 and 14 contained, in addition, 4 and 8 ng, respectively, of a 33-bp oligonucleotide containing the VP5 LBS; lanes 5, 10, and 15, 7.1 ng of a 23-bp control oligonucleotide, C2, lacking an LBS. See Materials and Methods for oligonucleotide sequences.

extensive deletion that removed all of the VP5 binding site (deletion 13) produced an 8.3-fold reduction in the level of virus-transactivated CAT gene expression.

Cotransfection experiments have very clearly demonstrated that at least three HSV-1 IE (α) genes are required to transactivate the VP5 promoter (6, 16, 55, 59). To determine the role of the LBS sequence in this process, cells were cotransfected with the VP5-CAT constructs and plasmid pGR150B that contain IE genes for ICP0, ICP4, and ICP27 (kindly provided by G. Hayward, Johns Hopkins University). CAT activity was determined 44 h later (Fig. 6B). Although the level of CAT expression in this case was not as great as that observed with virus superinfection, the results were qualitatively similar: (i) a 12-bp *Bgl*II linker insertion at −80 (construct 6) reduced CAT expression approximately twofold; (ii) deletion 10, which removed one-half of LBS, reduced CAT expression about fivefold; and (iii) deletion of all of the VP5 binding site (deletion 13) reduced CAT gene expression sevenfold relative to that of the wild-type promoter.

The results of the foregoing experiments using VP5 promoter mutations indicate that the VP5 LBS sequence is required for maximum levels of virus transactivation. Since disruption of the potential NF-1 sequence with a 12-bp insertion had only a small effect on transactivated VP5 promoter activity, it appears that this site plays a minor role, if any, in VP5 transactivation under the conditions used.

DISCUSSION

Two major complexes are formed on the VP5 promoter with cellular proteins. In this investigation we have examined DNA-protein interactions that occur on promoter/regulatory sequences of the major capsid protein (VP5) gene and several other genes of the βγ (leaky-late) class of HSV-1. Gel mobility shift assays revealed that two major complexes were formed on the VP5 promoter when either uninfected or infected-cell nuclear extracts were used. Similar complexes were also observed with gD and gB gene fragments. Since no significant changes in the mobilities of the complexes were observed when infected-cell extracts were used, we conclude that a cellular factor(s) is involved in both cases. We were surprised to find no evidence for virus-specific promoter complexes, since the region of the VP5 promoter used in this study contains the elements necessary for transactivation by viral proteins (Fig. 6) (3, 6). These observations suggest, therefore, that any viral regulatory proteins that interact with this region of the VP5 promoter do so either directly by binding weakly to the DNA or indirectly by binding weakly to cellular factors, by binding to cellular factors that weakly bind to the promoter, or by altering the activity of one or more cellular transcription factors.

From both our footprinting results and our oligonucleotide competitions it appears that the VP5 complexes A and B involve essentially the same base sequence. However, the reason for the different mobilities of the complexes is not known. It could be that two different cellular proteins bind the same sequence, the complex of lower mobility involves an additional protein, or a breakdown product of the protein forming complex A forms complex B. Evidence supporting the latter possibility has been presented to explain the formation of two complexes with the μE1 site of the immunoglobulin heavy chain enhancer (64). (As discussed below, the cellular factor that binds to the μE1 site, NF-μE1 or simply NF-E1, may be related to, or the same as, the VP5 binding factor, LBF). The formation of two complexes involving one binding site appears not to be restricted to the VP5 promoter, since DNA fragments that contain only one LBS from other βγ genes (gD and gB) also produce two complexes (Fig. 4; Table 2).

The VP5 promoter binding site, LBS, is required for transactivation by viral factors. We have shown by both chemical and enzyme footprint analyses that the two VP5 promoter complexes involve a unique sequence, GGCCAT CTTGAA, located between −64 and −75 bp relative to the mRNA start site. By using transient expression assays with plasmids containing mutations in the VP5 promoter, we further showed that this sequence, the LBS, is required for maximum levels of transactivation of the VP5 promoter by either superinfecting HSV-1 or cotransfected IE genes for ICP0, -4, and -27. When the LBS was completely deleted from the promoter, transactivation levels were reduced seven- to eightfold. But, since a promoter deleted in this sequence (deletion 13) could still be transactivated, albeit at levels considerably lower than those of the wild-type promoter (Fig. 6), it appears that other regions of the VP5 promoter may also be involved in the transactivation process. This observation suggests that a multiplicity of factor interactions may be required for maximum transactivation of the VP5 promoter.

Homologs of the LBS may be involved in the regulation of other viral genes. A search of published herpesvirus DNA sequences (7, 37, 38) revealed that sequences bearing close homologies to the VP5 LBS occur in promoters as well as in

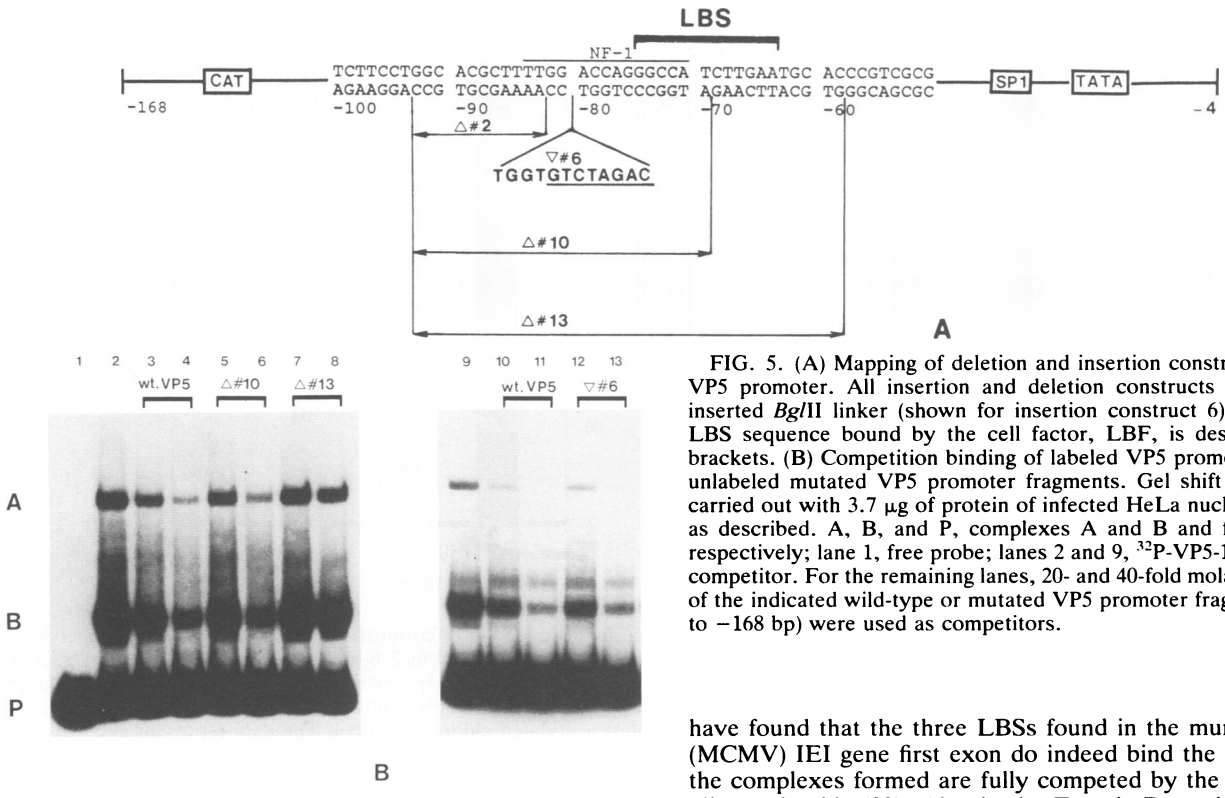

FIG. 5. (A) Mapping of deletion and insertion constructs in the VP5 promoter. All insertion and deletion constructs contain an inserted *Bgl*II linker (shown for insertion construct 6). The core LBS sequence bound by the cell factor, LBF, is designated by brackets. (B) Competition binding of labeled VP5 promoter versus unlabeled mutated VP5 promoter fragments. Gel shift assay was carried out with 3.7 μg of protein of infected HeLa nuclear extract as described. A, B, and P, complexes A and B and free probe, respectively; lane 1, free probe; lanes 2 and 9, ^{32}P-VP5-168 without competitor. For the remaining lanes, 20- and 40-fold molar excesses of the indicated wild-type or mutated VP5 promoter fragments (−4 to −168 bp) were used as competitors.

internal sites of a number of genes. Some of these are listed in Table 2. So far, we have identified sequences in three HSV-1 genes (gD, gB, and UL37), in addition to the homologous VP5 sequence, that compete for LBF binding, and we have shown that an oligonucleotide containing the VP5 LBS effectively competed with two of these sequences (gD and gB) for complex formation (Fig. 4). Of these three genes, gD and gB are frequently assigned to the βγ class, and UL37 appears to encode either a β (17a) or βγ protein (56). Although it is likely that the same cellular factor is also involved in the regulation of these genes, a transcriptional role for the LBS sequence in the gD, gB, and UL37 genes remains to be firmly established. In the case of gD, however, deletion analyses by Everett (14) indicated that the promoter region containing the LBF binding site is very critical for viral transactivation of the gene. Potential βγ promoters that failed to compete against the VP5 complexes, UL14/15, UL24, and VP16 (Table 1), lack a close match to the LBS sequence. Of further interest is the occurrence of 11 copies of an LBS homolog, GCCATRT, within the first intron of the ICP0 gene. The role of these sequences in ICP0 regulation is currently under investigation.

Of related interest is the existence of multiple copies of LBS homologs in the IE genes of the cytomegalovirus (CMV) genomes. For example, in the IE gene I of MCMV, one copy of the sequence GCCATGT is found at −247 bp, and three copies of the sequence GCCAT$^{C}_{A}$TTG are found between +133 and +180 in the first exon (Table 2). Even more bizarre is the occurrence of 9 copies of an LBS homolog, GCCATNT, in the enhancer/promoter region and first exon of human CMV (HCMV) genome, and 24 copies of this same sequence in the simian CMV (SCMV) enhancer/promoter and first intron (28). Although we don't yet know what role these sites may play in CMV gene regulation, we have found that the three LBSs found in the murine CMV (MCMV) IEI gene first exon do indeed bind the LBF, and the complexes formed are fully competed by the VP5 LBS oligonucleotide (39). Also in the Epstein-Barr virus BZLF promoter, two inverted copies of an LBS homolog exist and these have been shown to confer negative regulation on BZLF expression (42).

Computer search also showed that homologies to the VP5 LBS exist in promoters (LTRs) of a number of retroviruses, including simian immunodeficiency virus type 1 (SIV-1) and simian retrovirus type 1 (SRV-1) (Table 2). All of the LTRs containing homologies have a 7- to 14-bp match to the VP5 LBS located approximately −130 to −180 bp upstream from the mRNA start sites. Although we do not know as yet if these all bind the cellular factor, LBF, we found that a fragment of the SRV-1 LTR that contains a 14-bp perfect match to the VP5 LBS formed two complexes of similar mobility to the VP5 complexes, and an oligonucleotide containing the VP5 LBS effectively competed with the SRV-1 fragment for LBF binding (39). It will be of interest to learn to what extent the LBS homologs in these viruses play a role in regulation and if the LBS confers the ability to be transactivated by HSV-1.

The cellular factor, LTF, may be identical or closely related to a ubiquitous transcription factor. We noted that the VP5 promoter binding site, LBS, bears a close sequence homology to the binding sites reported for a cellular factor variously called YY1 (57), CF1 (52), NF-E1 (44), δ (23), or UCRBP (17). This factor appears to be somewhat unique in that it can exert either a positive or a negative control over transcription, depending on the particular gene and binding site involved. Calame's laboratory has presented evidence that common factor 1 (CF1), which binds to homologous sequences in the murine c-*myc* and skeletal α-actin promoters and acts as a transcriptional activator (Table 2), is the same as factor NF-μE1, or simply NF-E1 (52 and references therein).

Four groups have independently cloned a cDNA encoding this factor and characterized the protein. Shi et al. have

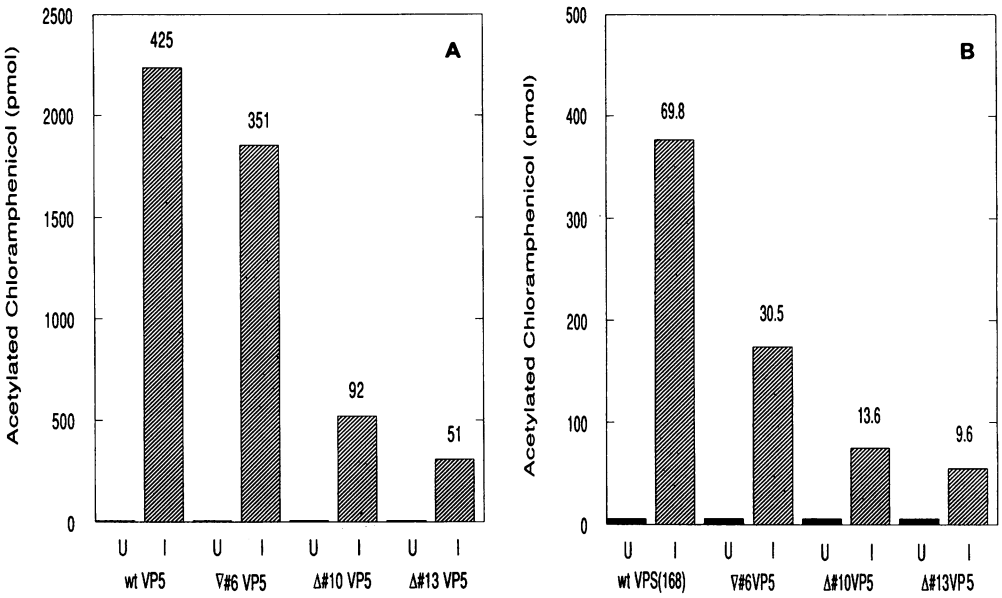

FIG. 6. Induction of transfected VP5 promoter-CAT constructs by superinfected HSV-1 or cotransfected IE genes. Amount of acetylated chloramphenicol produced is expressed as picomoles of the monoacetylated form produced in 2 h at 37°C with 25 μl of cell extract. U represents uninduced levels, and I represents induced levels. (A) Induction by HSV-1 (*vhs-1*) superinfection. Numbers on the bar graph indicate fold-induction over uninduced levels. (B) Induction by cotransfected plasmid, pGR150B, containing HSV IE genes for ICP0, -4, and -27. Average uninduced CAT levels for both experiments were 5.6 pmol of acetylated chloramphenicol. The data represent averages of a minimum of three separate transfection assays.

termed this factor YY1 and have shown that it binds to two sites in the adeno-associated virus P5 promoter and cap site regions where it acts as a transcriptional repressor; in the presence of adenovirus E1A protein, however, it acts as a transcriptional activator (57). Hariharan et al. called the factor δ and demonstrated that it positively regulates certain ribosomal protein genes (23). Park and Atchison termed it NF-E1, a factor that apparently both positively and negatively regulates immunoglobulin in gene transcription by binding to sites in the kappa and heavy chain enhancers (44 and references therein). Flanagan et al. called the factor UCRBP and demonstrated its role in repressing transcription from a site in the Moloney murine leukemia virus LTR (17).

As shown in Table 2, the VP5 LBS sequence matches the known binding sites for this factor (i.e., CF1, YY1, etc.) from 6 of 7 to 11 of 11 positions. This similarity of LBF to CF1 extends beyond its binding site homology, for we have found that oligonucleotides containing CF1, NF-μE1, and YY1 binding sites fully compete for complex formation by the VP5 LBS (41). In the case of VP5 (Fig. 6) and possibly also gD (14), it seems that the cellular factor by itself does not cause repression, since deletion of the LBS site did not activate gene expression from these promoters. However, the LBS sequence in the context of these two genes appears to function as a positive control element in the presence of HSV-1 IE proteins (Fig. 6B) (14). This may indeed be analogous to the activity of YY1 in the presence of adenovirus E1A on the adeno-associated virus P5 promoter (57).

In summary, the key findings presented in this paper are the following. (i) We have identified a regulatory sequence, GGCCATCTTGAA, which we call the LBS, in the promoter of the major capsid protein gene, VP5. This site binds a cellular factor (or factors), the leaky-late binding factor, LBF. (ii) By mutational analysis of the LBS we have demonstrated that it is required for maximum levels of VP5 transactivation either by superinfecting HSV-1 or by cotransfected IE HSV-1 genes, ICP0, -4, and -27. (iii) Homologs of the LBS consensus sequence, GCCATNT, occur throughout the HSV-1 genome, and we have shown that the LBS sequences found in the gD, gB, and UL37 genes also form complexes with the LBF. (iv) Finally, we have identified homologs of the VP5 LBS in regulatory regions of MCMV, SCMV, and HCMV genomes and in a number of retroviruses LTRs. From results to be presented elsewhere, we have evidence that the cellular factor, LBF, described in this paper is the same, or very similar to, a previously described cellular factor variously referred to as YY1 (57), CF1 (52), NF-E1 (44), δ (23), or UCRBP (17).

The results presented in this paper also provide new insight into the regulation of the HSV-1 major capsid protein, or VP5, gene, and our data suggest that this regulation is very likely relevant to several other HSV-1 genes of the βγ (leaky-late or γ_1) kinetic class. Finally, we have provided the first evidence that a cellular transcription factor is required for full activation of VP5 gene expression by either a superinfecting virus or by HSV-1 IE genes ICP0, -4, and -27. Exactly how the promoter complexes formed by the LBF interact, either directly or indirectly, with the viral IE proteins is being investigated in this laboratory.

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