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The Cellular Transcription Factor USF Cooperates with Varicella-Zoster Virus Immediate-Early Protein 62 To Symmetrically Activate a Bidirectional Viral Promoter

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The mechanisms governing the function of cellular USF and herpesvirus immediate-early transcription factors are subjects of considerable interest. In this regard, we identified a novel form of coordinate gene regulation involving a cooperative interplay between cellular USF and the varicella-zoster virus immediate-early protein 62 (IE 62). A single USF-binding site defines the potential level of IE 62-dependent activation of a bidirectional viral early promoter of the DNA polymerase and major DNA-binding protein genes. We also report a dominant negative USF-2 mutant lacking the DNA-binding domain that permits the delineation of the biological role of both USF-1 and USF-2 in this activation process. The symmetrical stimulation of the bidirectional viral promoter by IE 62 is achieved at concentrations of USF-1 (43 kDa) or USF-2 (44 kDa) already existing in cells. Our observations support the notion that cellular USF can intervene in and possibly target promoters for activation by a herpesvirus immediate-early protein.

The cellular transcription factor USF belongs to the class of basic–helix-loop-helix–leucine zipper (bHLH) proteins. USF readily binds the consensus DNA sequence 5′-CACGTG-3′ (61). Members of several other families of bHLH proteins, viz., Myc (9, 31, 55), Max/Myn (10, 54), Mad/Mxi (5, 76), and TFE3/TFEB (8, 14), bind this motif as well. What determines the preferential binding of USF in vivo to sites that ostensibly may also bind other families of bHLH proteins is not fully understood. Putative USF recognition sites are found in a wide variety of cellular and viral transcriptional regulatory regions (reviewed in reference 65).

Two USF proteins were originally identified in HeLa cells (60, 62). Although differing slightly in their apparent molecular masses of 43 and 44 kDa, their abilities to bind the 12-bp prototypic USF motif of the adenovirus major late promoter (AdMLP) are identical (62). Together, the two USFs stimulate transcription from the AdMLP in vitro (60). The observations that purified USF interacts with TFIID (60) and can facilitate and/or stabilize (13, 60) preinitiation complex formation provide a mechanistic basis for USF's ability to stimulate transcription in vitro.

Cloning of the 43-kDa form of human USF (USF 1) (28) permitted its classification as a bHLH protein, as well as the characterization of its dimerization, DNA-binding (28), and transcriptional activation (37) domains, which are all necessary for the protein's biological activity. Partial cDNA clones for human USF 2 (also called hUSF 2 [66] and FIP [11]) were later isolated. More recently, a full-length 44-kDa form of murine USF (USF 2) was cloned (65) and shown to possess nearly 99% amino acid identity to a partial human USF 2 clone lacking its amino-terminal end. While USF 1 and USF 2 have highly conserved DNA-binding and dimerization domains, their amino-terminal portions are considerably divergent in amino acid sequence (65).

Both USF 1 and USF 2 are ubiquitous proteins that readily form homo- and heterodimers (65). Dimerization with members of other families of bHLH proteins has not been observed (5, 7, 10). The ratios of USF homo- and heterodimers vary with cell type (65). In HeLa cells, for instance, the heterodimers predominate, while USF 1 homodimers are in relatively low abundance, and those of USF 2 are virtually undetectable (65).

The biological role of USF 1 has been addressed by both in vivo transfection and in vitro transcription studies. Bacterially expressed recombinant USF 1 stimulates transcription via an AdMLP USF motif in a crude reconstituted system in vitro, in a manner indistinguishable from that of endogenous USF (53). In transfections, the ectopic addition of USF 1 (likely raising the amount of USF 1 homodimers) modestly stimulates promoters containing USF-binding sites (21, 56). Recombinant USF 1 was also shown, both in vitro and in vivo, to stimulate promoters by interacting with initiator elements (21). This type of stimulation may also involve transcription factor TFII-I (58). Whether USF 2, in its homo- or heterodimeric form, regulates promoter activity in a manner different from or similar to that of USF 1 has not been clarified.

Herpesviruses are no exception with regard to the use of cellular USF as one means by which to regulate their own promoters. Putative USF-binding motifs are found in promoters of several genes expressed in various phases of productive infection (27, 38). The immediate-early regulatory proteins of these viruses are essential, not only for the coordinate activation of the early genes but also for the stimulation of an array of cellular and other viral genes. In the case of the human herpesvirus varicella-zoster virus (VZV), one protein that functions in this way is the immediate-early protein 62 (IE 62) (32, 33, 64). VZV IE 62, a large 175-kDa phosphoprotein (23), is homologous to the immediate-early proteins ICP4 and IE 180 of the closely related herpes simplex virus (HSV) and pseudorabies virus, respectively (15, 20, 22, 74).

The mechanism by which VZV IE 62 or its homologs fully

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activate genes is not entirely understood but appears to include an interaction with components of the basal transcription complex (1, 67, 72) and with DNA, but in a relatively weak and sequence-nonspecific fashion (17, 63, 70). The activation domains of IE 62-like proteins, while diverging in amino acid sequence, resemble those of other acidic activators (e.g., VP16). When these immediate-early activation domains are tethered by a GAL4 DNA-binding domain to a site upstream and near a TATA box, they stimulate expression greatly, even more so than the acidic activation domain of VP16 (16, 48, 52).

We showed previously that IE 62 activates the transcriptionally divergent VZV early genes encoding the DNA polymerase (POL) and major DNA-binding protein (MDBP). The promoters of these genes have closely spaced (39 bp) back-to-back TATA boxes, cannot be readily separated, and have highly concordant patterns of responsiveness to transactivation by IE 62 or the combination of IE 62 and VZV IE 4 (32, 64). These findings suggested that the POL and MDBP promoters have a common *cis*-acting regulatory mechanism in their coordinate regulation (49).

Here, we show a novel form of coordinate gene regulation involving a cooperative interplay between cellular USF and viral IE 62. A single USF-binding site defines the potential levels of bidirectional and symmetrical activation of both the VZV POL and MDBP genes by IE 62. Pronounced activation is achieved at concentrations of USF 1 or USF 2 already existing within cells. These results provide insight into the mechanisms by which both cellular USF and herpesviral immediate-early transcription factors regulate genes in vivo. Our results support the notion that cellular USF can intervene in and modulate gene activation by VZV IE 62.

MATERIALS AND METHODS

Cells and virus. HeLa (American Type Culture Collection, Rockville, Md.) cells were grown in Dulbecco's modified Eagle's medium (Quality Biologicals, Inc., Rockville, Md.) containing 10% fetal bovine serum and GSP (glutamine [2 mM], streptomycin [100 μg/ml], and penicillin [100 U/ml]). The continuous human schwannoma cell line ST88-14 (hereafter referred to as ST88) (57) was kindly provided by Cynthia Morton. ST88 cells were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum and GSP. VZV strain Ellen (ATCC VR-586) was propagated by serial passage of infected cells onto either uninfected whole human fibroblast (WHF) (Flow Biologics, McLean, Va.), ST88, or MEWO (29) monolayers as described previously (68). WHF and MEWO cells were grown in a 1:1 mixture of minimal essential medium 199 (Quality Biologicals) containing GSP and 10% fetal bovine serum. The efficiency with which cell-associated VZV strain Ellen forms infectious centers on ST88 4 cells was comparable to that of infectious center formation on WHF cells and somewhat better than the efficiency of viral growth on MEWO cells (67a).

Plasmids. The derivation of LCJM-0, LCJM-1, pCMV4, pCMV62, pCMVCAT, pCMVβgal, and E1bCAT has been detailed previously (49, 51, 59). LCJM-1 contains the VZV open reading frame 28-29 intergenic domain (VZV nucleotide positions [n.p.] 50644 to 50845) (18) placed between the luciferase (LUX) and chloramphenicol acetyltransferase (CAT) reporter genes; LCJM-0 is the promoterless LUX-CAT reporter construct. LCJM-10 was derived from LCJM-1 by replacing the *Pst*I-*Nde*I segment (VZV n.p. 50644 to 50746) of LCJM-1 with a fragment differing in only a three-base substitution (VZV n.p. 50726 to 50728) at the USF-binding site; the mutated fragment was generated by the oligonucleotide overlap and extension technique (4).

Synthetic 20-bp duplex oligonucleotides (VZV n.p. 50644 to 50746), spanning the putative USF-binding site and bearing *Sal*I protruding ends, were cloned individually into the *Xho*I site of E1bCAT. Insertion of the 20-bp wild-type (Wt) oligonucleotide (5′-AGTGTAATCACGTGATTTGT-3′) upstream of the CAT gene produced plasmid Wt_{29}CAT, while cloning this same oligonucleotide in the reverse orientation yielded Wt_{28}CAT. Insertion of oligonucleotides with selected base substitutions (underlined in the sequences below) produced Wt_{ad}CAT, 28GCAT, LF_{29}CAT, RF_{29}CAT, and LRFCAT, respectively. The sequences of the oligonucleotides used were as follows:

Wt_{ad}, 5′-AGTGTGGCCACGTGACCTGT-3′
28G, 5′-ACAAATCACGCTCTTACACT-3′
LF, 5′-AGCGAAATCACGTGATTTGT-3′
RF, 5′-AGTGTAATCACGTGATTCGA-3′
LRF, 5′-AGCGAAATCACGTGATTCGA-3′

E_{28}CAT was constructed by replacing the E1b TATA-containing *Xba*I-*Bam*HI fragment of E1bCAT with the duplex oligonucleotide 5′-CTAGACAAATCACGTGATTACACTG AGGGTATATAATG-3′.

The 50-bp latency associated transcript (LAT)-CAT construct was created by inserting a 50-bp duplex oligonucleotide possessing the HSV-1 LAT promoter sequence (bases −52 to −3 relative to the RNA start site) into the *Hin*dIII site of pCATbasic (Promega Biotec, Madison, Wis.).

Plasmids psvUSF1 and psvUSF2 contain full-length cDNA sequences encoding human USF 1 (43-kDa protein) and murine USF 2 (44-kDa protein), respectively. These constructs were made by subcloning the USF-containing *Eco*RI-*Nsi*I fragments of dI2 and M2-2 (65), respectively, into the *Bam*HI site of PsG5 (Stratagene, La Jolla, Calif.); the *Eco*RI-*Nsi*I fragments and the *Bam*HI sites were end filled. psvUSF2ΔB was derived from psvUSF2 by deletion of amino acid residues 228 to 247, using BAL 31 digestion of the *Esp*I-cut plasmid followed by religation.

All DNA insertions or mutations in reporter plasmids were sequenced (Sequenase; U.S. Biochemical Corp.) to verify intended changes.

In vitro transcriptions. In vitro transcription-grade HeLa nuclear extract was purchased from Promega. Transcription reaction mixtures included 6 mM MgCl, 0.4 mM ribonucleotides, 40 U of RNasin, and supercoiled DNA templates in the amounts indicated. Reactions were carried out at 30°C for 60 min after a 5-min preincubation (30°C) in the absence of ribonucleotides. Reactions were terminated and processed as instructed by Promega except that an additional 10 μg of tRNA was added per sample. DNA concentrations were determined spectrophotometrically and assessed by ethidium staining after gel electrophoresis. Primer extension analysis was performed as described previously (49) except that the RNA was not subjected to denaturation by methylmercury or heat at 90°C. The CAT primer used was 5′-GATGCCATT GGGATATATCAACGGT-3′.

EMSAs. Electrophoretic mobility shift assays (EMSAs) using the ^{32}P-labeled duplex oligonucleotides indicated were performed as described previously (41); all oligonucleotides were gel purified. HeLa nuclear extracts were prepared as described by Dignam et al. (19) and also purchased from Promega. Binding of nuclear extract to 1 ng (40,000 to 70,000 cpm) of probe was performed at 30°C for 20 min in 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–65 mM KCl–5% glycerol–0.1 mM dithiothreitol–1.0

μg of poly(dI-dC) in a total volume of 20 μl. Antibody reactions were carried out at a 1:40 dilution for 30 min at room temperature. The polyclonal antibody to USF 2 reacts on Western blots (immunoblots) specifically to this protein and not USF 1 (66), while the polyclonal antibody to USF 1 cross-reacts with USF 2 (unpublished observation). Ficoll (final concentration, 1.7%) was added to each sample immediately prior to analysis on a 4% polyacrylamide–0.25× Tris-borate-EDTA gel at room temperature.

Transfections and reporter assays. HeLa and ST88 cells grown to 60 to 70% confluency in 60-mm-diameter dishes were transfected by the DNA-calcium phosphate coprecipitation method of Graham and van der Eb (26); the coprecipitate was added directly to the medium and incubated under growth conditions for 12 and 8 h, after which this altered medium was removed and replaced with fresh medium. Reporter plasmids tested in parallel were also first quantitated in parallel by three independent spectrophotometric measurements and analyzed by ethidium staining after agarose gel electrophoresis. CAT, LUX, and β-galactosidase activities were quantitated 48 h after transfection as described previously (49). CAT and LUX activities were standardized to either protein concentration or cotransfected β-galactosidase activity, when indicated. Notably, the VZV transactivators IE 4 and IE 62 variably induce heterologous promoters (33, 49, 51), thus limiting the use of reporter coexpression as an internal control by which to normalize for the modest differences (typically less than twofold) in transfection efficiencies. LUX activity represents the difference in measured activity minus the background LUX activity produced by LCJM-0; for each series of experiments, three plates of LCJM-0-transfected cells were assayed to obtain a measurement of background luminescence.

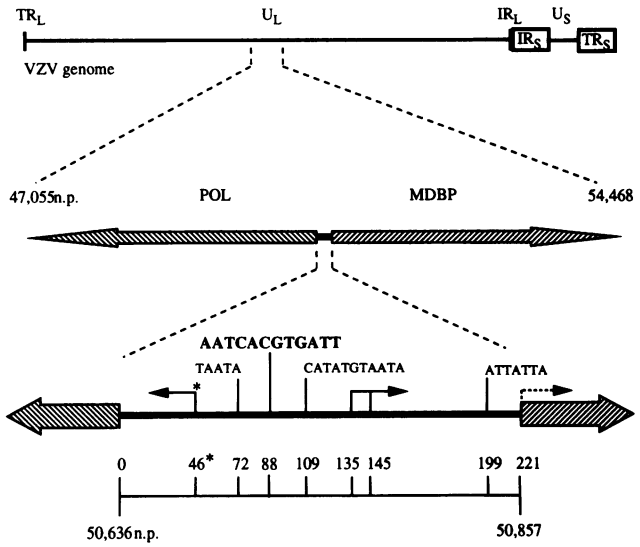


FIG. 1. Structural organization of the transcriptionally divergent promoters of VZV DNA POL and MDBP genes. The POL and MDBP open reading frames (striped arrows) are located in the unique long (U$_L$) segment of the VZV genome (n.p. 47,055 to 50,636 and 50,857 to 54,468, respectively). Their promoters are arranged back-to-back in the 221-bp intergenic region (thick black bar). Depicted are RNA start sites (solid arrows) and their accompanying TATA boxes, an equivocal minor MDBP RNA start site (interrupted arrow), and possible TATA. An asterisk denotes the center of the triplet of POL RNA 5′ termini. Located between the POL and MDBP TATA boxes is the 12-bp palindromic HLH$_{28/29}$ motif (nucleotide sequence in boldface). Numerical (base) positions of RNA start sites, TATA boxes, and the center of the HLH$_{28/29}$ motif are shown in reference to the origin of POL open reading frame (thin black line).

RESULTS

Requirement of a single consensus bHLH recognition site in bidirectional transactivation. A search of the transcriptionally divergent VZV POL (gene 28) and MDBP (gene 29) promoters for a *cis*-acting element that would serve as a bidirectional mediator of transactivation by VZV IE 62 focused our attention on a 12-bp palindrome, 5′-AATCACGTGATT-3′ (Fig. 1). The hexameric core of this element is the consensus 5′-CACGTG-3′ sequence recognized by members of several families of bHLH transcription factors, such as USF (61). In addition to having perfect dyad symmetry, this 12-bp VZV bHLH-binding element (HLH$_{28/29}$ motif) was suitably positioned upstream and nearby both POL and MDBP putative TATA boxes; only 16 and 20 bp separated the center of the dyad from the POL and MDBP TATA elements, respectively. To explore the role of the HLH$_{28/29}$ motif in the regulation of the POL and MDBP promoters, we first used transient transfections with reporter constructs that had both promoters situated en bloc between opposing CAT and LUX genes.

A mutation targeting the hexameric core of the HLH$_{28/29}$ motif revealed its importance in rendering both promoters amply responsive to stimulation by IE 62 as well as to their further augmentation by the ancillary VZV immediate-early protein, IE 4 (Fig. 2). Two cell lines differing in the ability to support virus replication were studied. ST88 cells, derived from a human schwannoma (57), support VZV growth well (unpublished observation; see Materials and Methods), whereas HeLa cells are relatively nonpermissive for VZV growth. In both of these cell types, the activation profile of reporter LCJM-1, which possesses the native POL and MDBP promoters (49), was dramatically different than that of LCJM-10, a comparable reporter plasmid altered only by a three-base substitution of the consensus bHLH recognition half-site. LCJM-1 was markedly stimulated in both directions by IE 62 that was expressed from a cotransfected plasmid; 135- to 165-fold and 40-fold activations were observed in HeLa and ST88 cells, respectively. Coexpression of IE 4 served to further increase IE 62-mediated activation.

In sharp contrast to the strong activation of LCJM-1 by IE 62 (alone or together with IE 4), LCJM-10 proved nearly unresponsive to these transactivators. In HeLa cells, for instance, IE 62 activated LCJM-10 bidirectionally only three- to fivefold. The poor responsiveness of LCJM-10 seems not due to differences in its basal activity relative to LCJM-1. In the absence of viral transactivators, both constructs showed extremely low basal activities (i.e., 0.27 to 0.4% acetylation), and no differences could be distinguished when the assays were corrected for protein concentration or cotransfected β-galactosidase activity (data not shown), albeit these assays may be insensitive to modest differences in basal activity.

These data indicate that the bidirectional activation of the transcriptionally divergent VZV POL and MDBP promoters by IE 62, whether present alone or together with IE 4, is dependent upon a single consensus bHLH recognition element and is abrogated symmetrically by a half-site mutation within this element.

Responsiveness of a minimal promoter construct to the addition of an HLH$_{28/29}$ motif. We next asked whether the HLH$_{28/29}$ motif by itself was sufficient to impart responsiveness of minimal promoters to VZV transactivators in a manner resembling that shown for the POL and MDBP promoters. To address this issue, we placed a single HLH$_{28/29}$ motif upstream

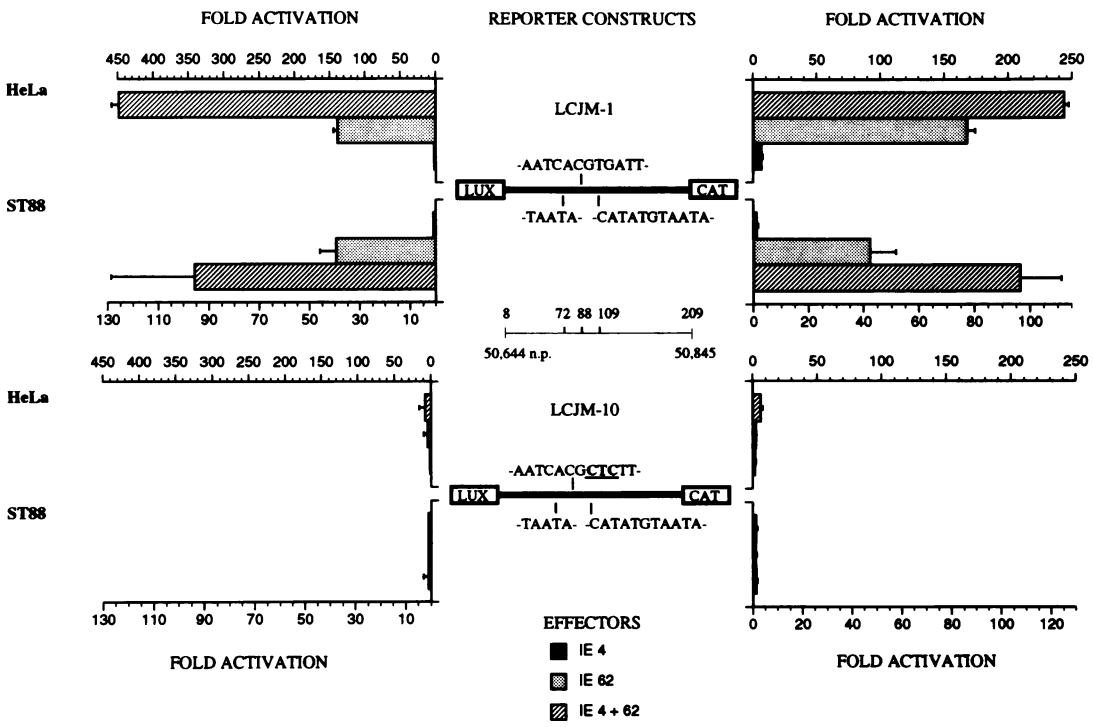


FIG. 2. Effect of $HLH_{28/29}$ motif mutation on IE 62-dependent activation. The divergent POL and MDBP promoters were placed between opposing CAT and LUX genes (LCJM-1) (49). A three-base substitution (underlined) was introduced in half of the consensus 5′-CACGTG-3′ site (LCJM-10). Transfections were performed in either HeLa (nonpermissive) or ST88 (permissive) cells (see Materials and Methods), using reporter plasmids (2 μg) either alone or combined with effector plasmids (0.5 μg of each) pCMV4 (VZV IE 4 driven by the CMV promoter), pCMV62 (VZV IE 62 driven by the CMV promoter), or both pCMV4 and pCMV62. Total amount of CMV promoter was kept constant by adding pCMV accordingly; pGEM2z (3 μg) was used as carrier DNA. Fold induction of CAT and LUX activity was determined as described in Materials and Methods and standardized for protein concentration. No differences in basal CAT (0.27 to 0.4% acetylation) or LUX activities of LCJM-1 and LCJM-10 were apparent. Bar graphs represent fold activation of the indicated reporter constructs by IE 4, IE 62, and the combination of IE 4 and IE 62. Error bars represent standard deviations of three and two independent HeLa and ST88 cell experiments, respectively; each set of experiments evaluated all constructs in parallel.

of an E1b TATA cassette lacking any other known *cis*-acting or initiator elements. In the absence of any a priori knowledge as to whether sequences flanking the consensus bHLH recognition site (5′-CACGTG-3′) would influence this site's function, we first studied the larger 20-bp VZV sequence bearing the 12-bp palindromic $HLH_{28/29}$ motif within it (Fig. 3). This larger 20-bp Wt element possesses imperfect dyad symmetry (12 of 20 residues are symmetrical), allowing its evaluation in both orientations. One construct, Wt_{28}CAT, simulated its orientation relative to POL (gene 28) expression, and conversely, Wt_{29}CAT simulated its orientation relative to MDBP (gene 29) expression. The activation of these reporter constructs, and additional reporter constructs described below, by VZV IE 62 and/or IE 4 was evaluated in HeLa cells, using the methods applied previously in studying LCJM-1 and LCJM-10.

As shown in Fig. 3, the parent E1bCAT plasmid (lacking an HLH-binding site) was only weakly activated by either IE 62 (fourfold) or the combination of IE 4 and IE 62 (sixfold); IE 4 alone was ineffective. Insertion of a single 20-bp Wt element in either orientation 53 bp upstream of the E1b TATA box (i.e., Wt_{28}CAT and Wt_{29}CAT) yielded promoters that were markedly stimulated by IE 62 (40- to 45-fold), and this stimulation was further augmented by concomitant IE 4 expression. Positioning the 20-bp Wt element only 16 bp upstream of the E1b TATA (i.e., Wt_{E28}CAT), exactly its distance from the POL TATA box, increased responsiveness to the viral transactivators an additional fourfold. In fact, stimulation of Wt_{E28}CAT by IE 62 approached 165-fold, the level of activation observed in separate studies of the authentic POL or MDBP promoter (Fig. 2). As with the native promoters, IE 4 alone was unable to activate Wt_{28}CAT, Wt_{29}CAT, or Wt_{E28}CAT. Again, there was no apparent difference in the very low (0.2 to 0.4% conversion) basal CAT expression between the E1bCAT and Wt element-containing plasmids when values were normalized for protein concentration or coexpressed β-galactosidase activity (data not shown).

We also evaluated the ability of the 12-bp USF motif (5′-GGCCACGTGACC-3′) of the AdMLP, known to stimulate transcription through its interaction with USF (61), to supplant the function of the 12-bp $HLH_{28/29}$ (5′-AATCACGT GATT-3′) motif. Accordingly, plasmid Wt_{ad}CAT, in which the 12-bp $HLH_{28/29}$ motif of the 20-bp Wt element was replaced with the adenovirus sequence (Wt_{ad}), was compared with VZV Wt element-containing constructs. As shown in Fig. 3, the activation profiles of Wt_{ad}CAT, Wt_{28}CAT, and Wt_{29}CAT were remarkably similar. This finding establishes USF as a candidate transcription factor operating at this site in vivo. That AdMLP USF and $HLH_{28/29}$ motifs share only the central sequence, 5′-CACGTGA-3′, implies that the function of the $HLH_{28/29}$ site does not rely on preservation of nucleotide sequence immediately flanking this conserved sequence.

To further validate the core bHLH-binding site as the sequence critical for transactivation, base substitutions of the core bHLH half-site in the parent Wt element were generated

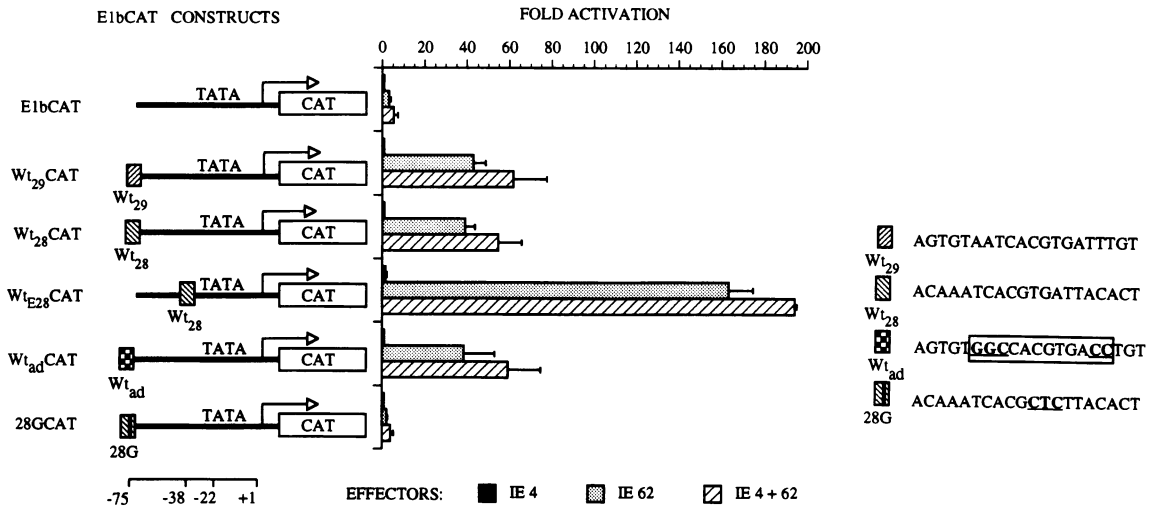


FIG. 3. Effect of the $HLH_{28/29}$ motif on IE 62-dependent activation of the E1b TATA box. HeLa cells were transfected with the indicated reporter constructs (2 μg) and effector plasmids (0.5 μg) as described for Fig. 2. A 20-bp Wt element containing the 12-bp $HLH_{28/29}$ motif plus flanking viral sequence was evaluated in both orientations. Wt_{28}CAT and Wt_{29}CAT simulate direction of POL and MDBP expression, respectively; the center of the Wt element is 53 bp away from the TATA box. Wt_{E28}CAT simulates the natural distance (16 bp) of Wt_{28} from POL TATA. The 12-bp $HLH_{28/29}$ motif of Wt_{29}CAT was replaced with the 12-bp AdMLP USF motif of the adenovirus promoter, producing Wt_{ad}CAT. Mutation of half of the consensus CACGTG motif of Wt_{28}CAT produced 28GCAT. Bases differing from the Wt sequence are in boldface and underlined. The bar graph represents fold activation of the reporter constructs by transactivators IE4, IE 62, and the combination of IE 4 and IE 62, as standardized for protein concentration. No difference in basal CAT activities (0.2 to 0.4% conversion) was apparent. Error bars represent standard deviations of three independent experiments.

to construct 28GCAT. In contrast to Wt_{28}CAT, the response of 28GCAT to IE 62 or combined IE 4 and IE 62 was very low and comparable to that of E1bCAT (Fig. 3).

Taken together, the data to this point indicate that an $HLH_{28/29}$ motif nested within a 20-bp VZV sequence functions in an orientation-independent but position-dependent fashion. The simple presence of a single 20-bp Wt element, appropriately positioned upstream of a heterologous TATA box, is sufficient to yield profiles of responsiveness to VZV IE proteins that closely resemble those exhibited by the transcriptionally divergent native viral POL and MDBP promoters.

$HLH_{28/29}$ binding stimulates basal transcription from the E1b TATA in vitro. Others reported that USF binding to the USF motif of the AdMLP serves to stimulate transcription in vitro (13, 53, 60). Appreciating that our transient transfection expression assays may be insensitive to meaningful differences in the basal transcriptional activities of the promoters tested thus far, we used an in vitro transcription assay to ascertain whether the $HLH_{28/29}$ motif also influences basal transcription. Constructs E1bCAT and Wt_{E28}CAT were first studied as examples of templates that lack and possess the $HLH_{28/29}$ motif, respectively (Fig. 4A). Supercoiled templates were incubated with HeLa nuclear extracts, and the transcription products were characterized and quantitated by primer extension. The addition of pCMVCAT template to all reactions provided an internal control by which to judge the relative levels of E1bCAT and Wt_{E28}CAT transcription. To assess the fidelity of transcriptional initiation, the CAT RNA start sites were compared with the dideoxy sequencing products generated from an E1bCAT template, using the same oligonucleotide applied in primer extensions.

Figure 4A verifies that for Wt_{E28}CAT, in vitro transcription initiates appropriately, while no correct transcriptional initiation is detectable for E1bCAT. Comparable levels of transcription from the cytomegalovirus (CMV)-CAT controls were noted in each in vitro reaction.

To show that $HLH_{28/29}$-mediated transcriptional stimulation is not affected by residual VZV flanking sequence, we compare in Fig. 4B the in vitro basal activity of Wt_{29}CAT with that of constructs bearing base substitutions in nucleotides flanking either one or both sides of the 12-bp $HLH_{28/29}$ motif but residing within the 20-bp Wt domain. Regardless of whether these three-base substitutions were introduced into the leftward (29LFCAT), rightward (29RFCAT), or both (RLFCAT) flanking regions, the mutations were inconsequential with regard to basal transcriptional activity. These results indicate that it is the 12-bp $HLH_{28/29}$ motif itself that is required to fully maintain transcription in vitro from the E1b TATA box. Again, there was no correct transcriptional initiation detectable from E1bCAT, although the level of concomitant CMV-CAT transcription was comparable to that in the other reactions.

Using this assay, we also examined the importance of the $HLH_{28/29}$ motif in directing transcription in vitro from the MDBP promoter. In a study with LCJM-1 as the template (Fig. 4C), appropriate transcriptional initiation/elongation was readily detected from the MDBP promoter, while plasmid LCJM-10, possessing an $HLH_{28/29}$-binding half-site mutation, produced no such transcripts. The two MDBP RNA start sites, separated by nine bases, corresponded to the two sites from which polyadenylated [poly(A)$^+$] RNA of active viral infection was shown to originate (49). Notably, the 3′-most RNA start site lies within a sequence that resembles a potential initiator element recently described by Javahery et al. (35); the presence of such an element may explain why mutation of the MDBP TATA-like box(es) failed to abolish this promoter's activity (49).

Unfortunately, transcriptional initiation from the opposing POL promoter could not be satisfactorily demonstrated in vitro. Nonetheless, these data indicate that the presence of an $HLH_{28/29}$ motif does indeed augment the basal levels of transcription initiation/elongation in vitro.

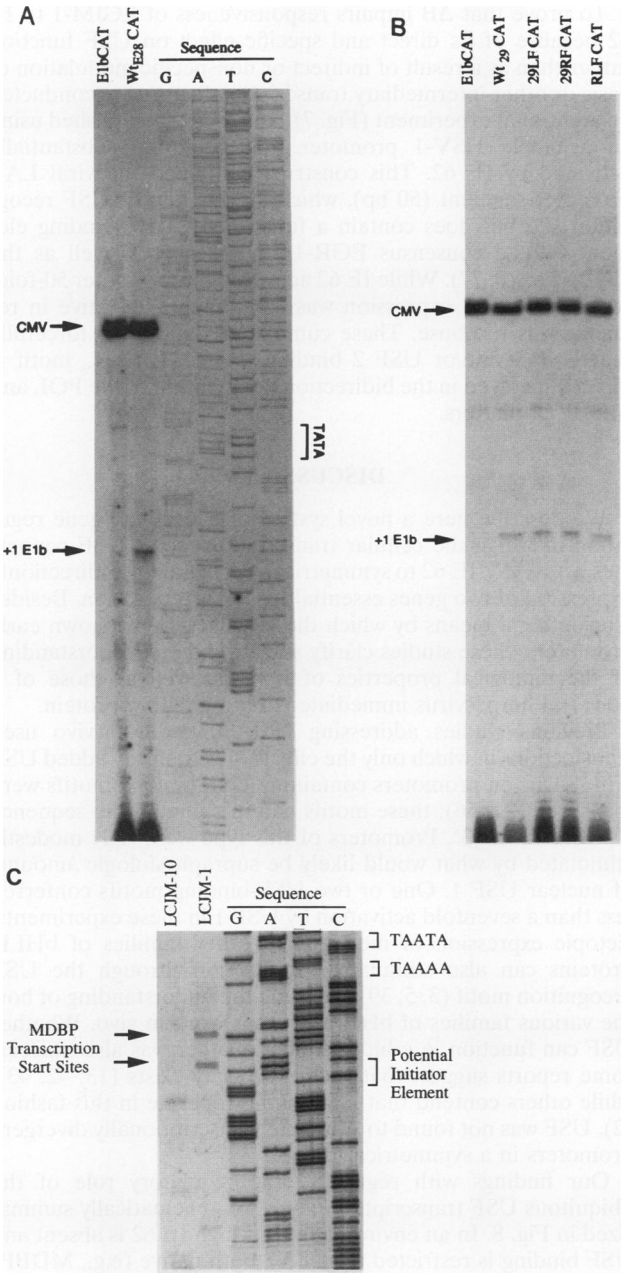


FIG. 4. Role of the $HLH_{28/29}$ motif in transcription in vitro. (A) Supercoiled E1bCAT and Wt_{E28}CAT plasmids (250 ng) were individually transcribed in vitro, using HeLa nuclear extract (see Materials and Methods). pCMVCAT (50 ng) was an internal control. Shown are CAT RNAs analyzed by primer extension and compared with the dideoxy sequencing products generated from an E1bCAT template, using the same oligonucleotide applied in primer extensions. Arrows denote positions of expected RNA start sites of pCMVCAT (CMV) and E1bCAT or Wt_{E28}CAT (+1 E1b); the E1b RNA start site has been described previously (50, 75). The position of the E1b TATA box (TATA) is depicted. (B) Comparison of Wt_{29}CAT with constructs bearing mutations in nucleotides flanking the 12-bp $HLH_{28/29}$ motif. Three-base substitutions were introduced into the leftward (29LFCAT), rightward (29RFCAT), and bilateral (RLFCAT) flanking regions of the 20-bp Wt domain (see Materials and Methods). In vitro transcriptions were performed as described above. Ratios of levels of E1b reporter to pCMVCAT transcription were 1, 0.13, 0.18, 0.19, and 0.16 for E1bCAT, Wt_{29}CAT, 29LFCAT, 29RFCAT, and RLFCAT, respectively. (C) Comparison of the MDBP promoter possessing the $HLH_{28/29}$ motif (LCJM-1) with the same promoter differing only by a mutation of the $HLH_{28/29}$-binding half-site (LCJM-10). In vitro transcriptions were performed and analyzed as described above.

USF binds the 12-bp $HLH_{28/29}$ motif. From the information provided so far, the binding of the ubiquitous nuclear USF proteins to the $HLH_{28/29}$ motif in vitro was anticipated. This was verified formally in a series of EMSA studies. Using a 20-bp Wt sequence oligonucleotide as the probe, two abundant, closely migrating complexes were formed with HeLa nuclear extract (Fig. 5A, lane 4; Fig. 5B). Mutation of one side of the symmetrical bHLH consensus site (H probe; half-site mutation) markedly impaired formation of both of these complexes (Fig. 5A, lane 1). Addition of 20- to 40-fold excess of unlabeled Wt probe completely abolished their formation (Fig. 5A, lane 7; Fig. 5B). These two complexes formed unabated on probes in which sequence flanking the 12-bp $HLH_{28/29}$ motif to the left (LF) or right (RF) had been mutated (Fig. 5A, lanes 2 and 3, respectively). Moreover, competition EMSAs comparing the binding properties of these two complexes on the Wt probe with those forming on a probe bearing alterations in both flanking sequences (RLF; Fig. 5B) more clearly revealed that the 12-bp $HLH_{28/29}$ motif itself is sufficient to dictate formation of both of these complexes.

Addition of polyclonal antibodies to USF 1 (43 kDa) or USF 2 (44 kDa) to the reaction mixtures further retarded mobility of both of the complexes specifically binding the $HLH_{28/29}$ motif (Fig. 5A, lanes 5 and 6, respectively). The supershifts conferred with USF-specific antibodies further demonstrate the interaction of both USF 1 and USF 2 with the $HLH_{28/29}$ site.

The most slowly migrating and most abundant of the two complexes (upper arrows in Fig. 5A and B) may be composed of USF 1:2 heterodimers, while the slightly faster migrating and less abundant complex (lower arrows in Fig. 5A and B) may be composed of USF 1 homodimers, which cross-react with polyclonal antibodies to USF 2. This assumption is consistent with previous work showing that in HeLa nuclear extract, the heteromeric form of USF predominates and USF 1 homodimers are present in relatively low concentrations (61).

A dominant negative USF 2 mutant defines the role of USF in IE 62-dependent activation. The next series of experiments was designed to clarify USF's role in the IE 62-mediated activation of the POL and MDBP promoters in in vivo transfections, since the potential remains that other classes of bHLH proteins more directly influence the stimulation of these promoters. The strategy employed relied on the use of a plasmid (psvUSF2ΔB) expressing a murine USF 2 protein that lacks the basic region (amino acids 228 to 247) required for DNA binding (ΔB). Like the full-length mouse USF 2 (USF2), which is nearly identical (~99% identity) to its human counterpart (65), the ΔB product readily forms dimers with either human USF 1 or human USF 2 or with itself (unpublished data). Because USF binding to cognate sites requires dimers possessing two functional DNA-binding domains (28, 65, 66), the sequestration of endogenous USF by ectopically expressed ΔB facilitates the identification of transcriptional processes that are directly dependent upon USF.

As shown in Fig. 6A, plasmid LCJM-1 was again used as a moiety sensitive to bidirectional activation by IE 62. The extent of activation of the POL and MDBP promoters by IE 62 was not appreciably augmented by additional USF 1 and was only modestly increased by additional USF 2. However, IE 62-

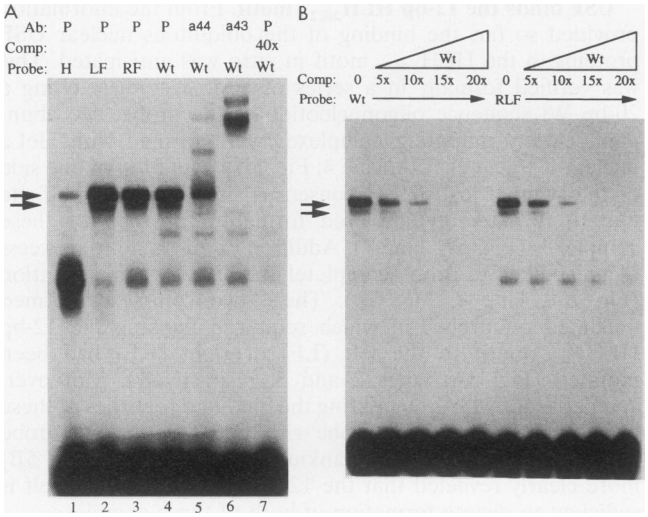


FIG. 5. Analysis of USF binding to the $HLH_{28/29}$ motif. HeLa nuclear extract (4 μg) was bound to a ^{32}P-labeled, 20-bp duplex probe (1 ng) (see Materials and Methods). All binding reaction mixtures included 50-fold molar excess of extraneous 24-bp duplex DNA. The 20-bp Wt probe contained the 12-bp $HLH_{28/29}$ motif plus flanking viral sequence. Alteration of Wt sequences flanking the left, right, and both sides of the $HLH_{28/29}$ motif yielded LF, RF, and RLF probes, respectively. Probe H (AGTGTAATCACGCTCTTTGT) contains a consensus bHLH half-site mutation (underlined). Unlabeled Wt duplex at the indicated fold molar excess relative to labeled probe was used in competition (Comp) studies. Polyclonal rabbit preimmune (P), anti-44-kDa USF2 (a44), and anti-43 kDa USF1 (a43) antibodies (Ab) were used in supershifts. Arrows denote positions of USF complexes. The faster-migrating complexes appearing in lane 1 represent nonspecific protein binding; these complexes fail to react to anti-USF 1 or anti-USF 2 antibodies, and they compete poorly with the H probe (data not shown).

mediated activation of both these promoters was reduced six- to eightfold by concomitant ΔB expression. Furthermore, ΔB's repressive effect was negated by addition of ectopically expressed USF 2 and was partially reversed by addition of ectopic USF 1. The basal activity of LCJM-1 was not appreciably altered by the expression of ΔB or the combination of ΔB and USF 1 or USF 2 in the absence of IE 62 (data not shown).

The relative abilities of additional USF 1 and USF 2 to relieve ΔB-mediated repression seem to correspond to the level of USF proteins competent to bind the $HLH_{28/29}$ motif (Fig. 6B). A difference in efficiencies of ectopic expression of USF 1 and USF 2 likely accounts for their discordant levels, as both proteins are equivalent in the ability to bind the same cognate sites (62, 65) and to form dimers with ΔB (data not shown).

In the absence of IE 62, ectopic expression of neither USF 1 nor USF 2 was able to stimulate expression from the MDBP promoter (Fig. 6A). On the other hand, the hyperexpression of USF did possess some capacity to stimulate the POL promoter, but at lower levels relative to those attained by IE 62. What stimulation was apparent from the POL promoter seems not dependent on binding to the $HLH_{28/29}$ core element because its mutation in construct LCJM-10 (lacking any consensus 5′-CACGTG-3′ sequence) yielded no substantial difference in promoter responsiveness. The mechanism for this residual activity is unknown, but it is inadequate to mediate IE 62-dependent activation of the POL promoter and does not confound the interpretation of USF's role in the regulation of the contralateral MDBP promoter.

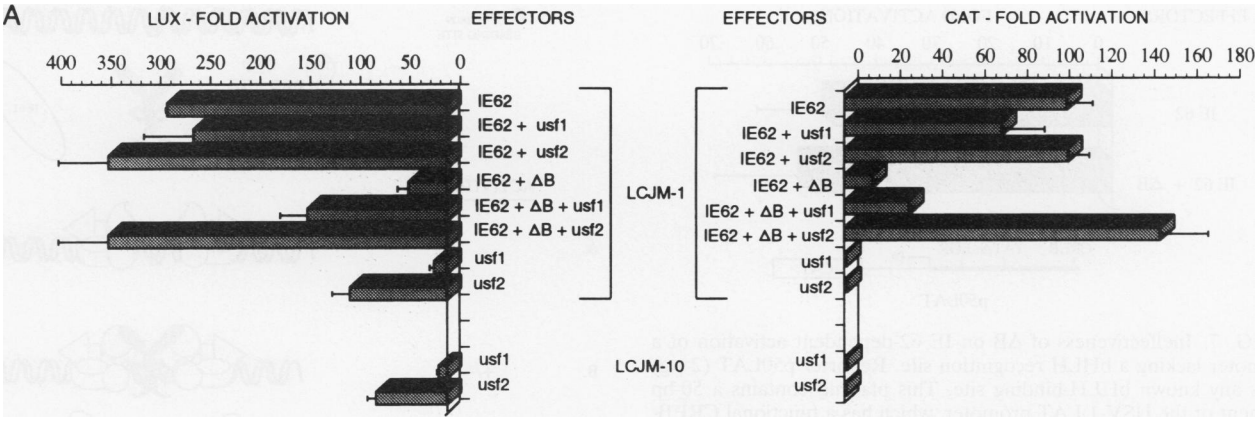
To prove that ΔB impairs responsiveness of LCJM-1 to IE 62 because of its direct and specific effect on USF function rather than as a result of indirect or nonspecific modulation of basic or other intermediary transcription factors, we conducted an additional experiment (Fig. 7). This was accomplished using an available HSV-1 promoter that could be substantially activated by IE 62. This construct contained the viral LAT promoter segment (50 bp), which lacks a known USF recognition site but does contain a functional CREB-binding element (40), a consensus EGR-1-binding site, as well as the TATA box (6, 77). While IE 62 activated this promoter 50-fold, concomitant ΔB expression was completely ineffective in reducing this response. These cumulative data argue forcefully that USF 1 and/or USF 2 binding to the $HLH_{28/29}$ motif is directly involved in the bidirectional regulation of the POL and MDBP promoters.

DISCUSSION

We describe here a novel system of coordinate gene regulation in which the cellular transcription factor USF cooperates with VZV IE 62 to symmetrically activate the bidirectional expression of two genes essential for VZV replication. Besides elucidating a means by which the virus activates its own early promoters, these studies clarify and extend our understanding of the biological properties of USF, as well as those of a powerful herpesvirus immediate-early regulatory protein.

Previous studies addressing USF function in vivo used transfections in which only the effects of ectopically added USF 1 (43 kDa) on promoters containing USF-binding motifs were measured (21, 56); these motifs had in common the sequence 5′-CCACGTG-3′. Promoters of this type were only modestly stimulated by what would likely be supraphysiologic amounts of nuclear USF 1. One or two USF-binding motifs conferred less than a sevenfold activation by USF 1 in these experiments. Ectopic expression of members of other families of bHLH proteins can also stimulate transcription through the USF recognition motif (3, 5, 39), clouding the understanding of how the various families of bHLH proteins work in vivo. Whether USF can function in a bidirectional manner was also unclear; some reports suggest that such a capacity exists (13, 42, 43), while others contend that USF cannot operate in this fashion (2). USF was not found to stimulate transcriptionally divergent promoters in a symmetrical fashion.

Our findings with regard to the regulatory role of the ubiquitous USF transcription factor are schematically summarized in Fig. 8. In an environment in which IE 62 is absent and USF binding is restricted (Fig. 8A), both native (e.g., MDBP) and heterologous (e.g., E1b TATA) viral promoters are inactive in both the in vivo transfection and in vitro transcription systems. Providing a single $HLH_{28/29}$ motif to endogenous USF also fails to appreciably stimulate these same promoters in the in vivo transfection setting (Fig. 8B). Even when the concentrations of USF 1 or USF 2 were clearly augmented through ectopic expression, they failed to substantially stimulate the MDBP promoter (Fig. 6A). The POL promoter was stimulated modestly by ectopic USF 1 or USF 2 despite absence of the $HLH_{28/29}$ motif. This activity may result from specific binding of USF to an uncharacterized initiator-like element (21, 58) or because of its adventitious interaction with a low-affinity site. That increases in USF concentration were ineffective in stimulating the MDBP promoter would argue against the possibility that IE 62-mediated activation depends upon heightened USF concentrations. In fact, neither VZV infection itself nor IE 62 expressed in transient transfections grossly alters nuclear USF concentrations (unpublished observation).



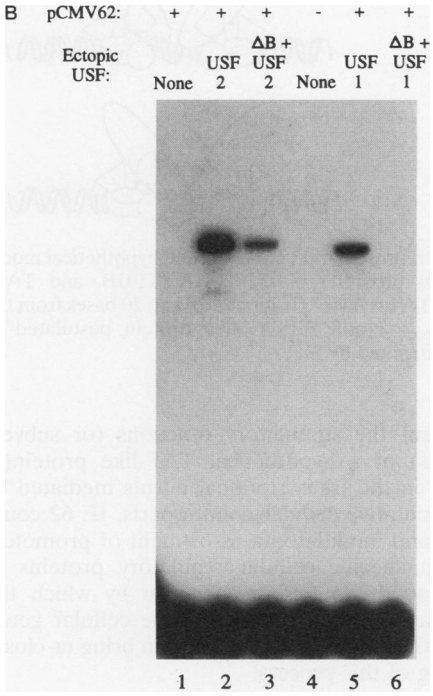


FIG. 6. Role of USF proteins in IE 62-dependent bidirectional activation of POL and MDBP promoters in vivo. (A) HeLa cells were transfected as described for Fig. 2. The bidirectional POL and MDBP promoter constructs (2 μg of each), in which the $HLH_{28/29}$ motif is either intact (LCJM-1) or mutated (LCJM-10), were the target reporters. Effector constructs (2 μg) comprise plasmids expressing IE 62 (pCMV62 [500 ng]), human USF 1 (psvUSF1 [10 μg], murine USF 2 (psvUSF2 [10 μg]), or/and murine USF 2 minus its DNA-binding domain (ΔB and pΔB [10 μg]). When used together, 5 μg of psvUSF1 or psvUSF2 was combined with 10 μg of pΔB. The total DNA concentration was held constant by adding pCMV and psG5, accordingly. Bar graphs represent fold activation, as standardized for protein concentration. All constructs were tested in parallel with duplicate transfections; several other experiments in which these constructs were tested yielded comparable results. (B) USF protein capable of binding the $HLH_{28/29}$ motif is shown for selected transfections depicted in panel A. The same protein extracts used in the CAT assays described above were analyzed by EMSA as detailed in the legend to Fig. 4. The 20-bp probe used contains the 12-bp $HLH_{28/29}$ motif and random flanking sequence.

In contrast to the transfection studies in which promoters containing a single 12-bp $HLH_{28/29}$ motif appeared unresponsive to endogenous nuclear USF, a single $HLH_{28/29}$ motif in these same promoters could stimulate basal transcription in vitro. Activity in the in vitro assays (Fig. 4) is concordant with previous reports (13, 53, 60) directly linking USF to the in vitro stimulation of the related AdMLP USF motif. The reason for the disparate in vivo and in vitro results is unknown but could reflect differences in the sensitivities of the techniques or the fact that rates of transcription are dependent on whether USF-mediated preinitiation complex formation (13, 60, 73) has to compete with a full panopoly (in vivo) or limited arrangement (in vitro) of histones and nonhistone proteins.

Viral IE 62 transactivates the VZV bidirectional and E1b promoter constructs very poorly when binding of USF is prevented by alteration of the consensus 5′-CACGTG-3′ sequence (Fig. 8C). The residual level of activation is commensurate with the magnitude of IE 62-mediated activation of the isolated E1b TATA box. By analogy to data regarding its homologs HSV ICP 4 (67) and pseudorabies virus IE 180 (1, 72), we presume that IE 62 can directly stimulate transcription through its interactions with components of the basal transcription complex. In the case of a more complex promoter, this is not the sole mechanism by which a IE 62-like protein stimulates transcription (67); ablation of the protein domain responsible for this interaction reduces but does not abolish the ability of the transactivator to function.

When present together, cellular USF and VZV IE 62 activate the bidirectional viral promoter in a synergistic and symmetrical fashion (Fig. 8D). Maximal activation can be achieved by concentrations of USF already present within cells. Sequestration of endogenous USF by ectopic expression of a dominant negative USF 2 mutant lacking the DNA-binding domain abrogates the IE 62-dependent stimulation specifically governed by the $HLH_{28/29}$ motif (ΔB; Fig. 6A). This inhibition can be overcome by an excess of ectopically expressed USF 1 or USF 2. In addition to validating the role of USF proteins in the coordinate regulation of the VZV POL and MDBP genes, these experiments establish that the 44-kDa species of USF 2 is comparable to USF 1 in its ability to

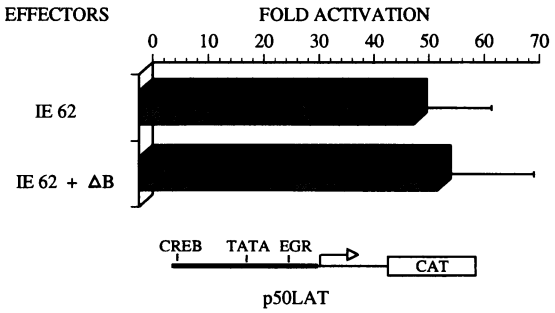


FIG. 7. Ineffectiveness of ΔB on IE 62-dependent activation of a promoter lacking a bHLH recognition site. Reporter p50LAT (2 μg) lacks any known bHLH-binding site. This plasmid contains a 50-bp segment of the HSV-1 LAT promoter, which has a functional CREB-binding element (40), a consensus EGR-1-binding site, and a TATA box (6, 77). Transfections were performed and CAT activity was analyzed as described for Fig. 6A; the same amounts of effector plasmids were used. Shown are the standard deviations of four independent experiments.

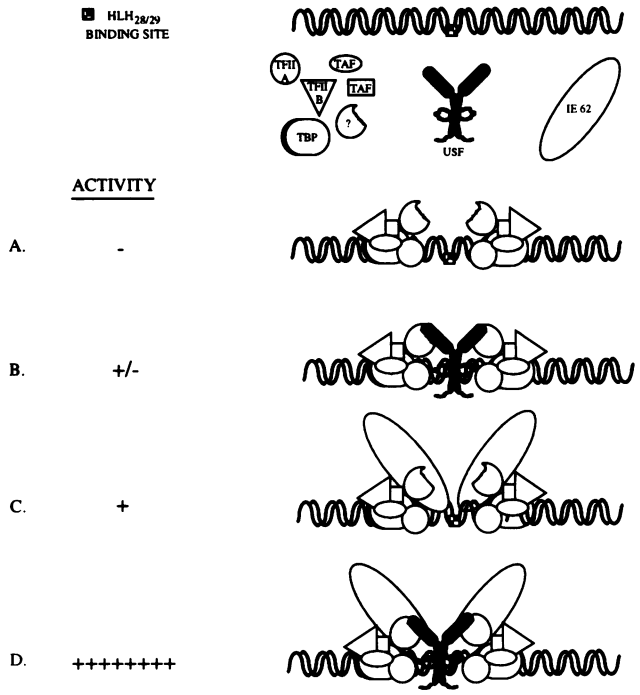


FIG. 8. Schematic representation of results and hypothetical model of VZV bidirectional promoter. TBP, TFIIA, TFIIB, and TAFs assemble on the viral TATA boxes positioned 16 and 20 bases from the center of the $HLH_{28/29}$ motif. A TAF-like protein postulated to interact with USF is denoted by ?.

stimulate promoters in vivo. While it is possible that IE 62 renders USF proteins active by a posttranslational modification event or that USF proteins (e.g., ΔB) dimerize with other types of bHLH-like proteins, there are no published data supporting such mechanisms.

The present studies do not discern whether the cooperative effect produced by USF and IE 62 involves physical contact between these two transcription factors, but they do argue that USF prepares or targets promoters for activation by IE 62. In many regards, this process is functionally analogous to tethering of the activation domain of IE 62 to a nearby TATA box via a GAL4 DNA-binding moiety (16, 52). In both cases, the magnitude of IE 62-dependent activation is exemplary.

One possibility suggested by the present data is that IE 62 serves as a molecular bridge between USF and a component(s) of the preinitiation complex. The reduction in IE 62-dependent activation observed when the distance between the $HLH_{28/29}$ motif and TATA box is increased is consistent with this notion (Fig. 3). While there are no reports of physical interactions between USF and herpesvirus IE proteins, or evidence that these viral proteins interact with other specific cellular transcription factors, there are precedents for this idea. Indeed, some other viral regulatory proteins can bind both a common *cis*-acting transcription factor and a component(s) of the basal transcription complex, e.g., TEF1/simian virus 40 T antigen/TATA-binding protein (TBP) (30), Sp1/Tat/TFIID (36), CBP or ATF-2/E1A/TBP (46, 47), and Oct-1/VP16/TFIIB and TBP (24, 34, 44). In those instances, the interactions between all three elements correlate with the ability of the viral proteins to transactivate their respective targeted promoters. That viral proteins may alter gene regulation through interaction with USF is supported by the report that adenovirus E1A binds the myogenic bHLH proteins and, in so doing, represses muscle-specific gene expression (12, 69). Most relevant to the present hypothesis is the recent demonstration by Liu and Green (45) that cellular USF also interacts with E1A.

Cellular TATA-associated factors (TAFs) are believed to link together a series of TATA-binding and specific *cis*-acting regulatory factors (25). A single species of TAF may interact with different classes of *cis*-acting regulatory proteins (71). If the hypothesis that viral activators can form similar protein "bridges" stands correct, then it seems plausible that IE 62 may supplant or augment the stimulatory functions (or subvert inhibitory functions) of a hypothetical TAF-like protein(s) ordinarily involved in the transcriptional events mediated by USF. Also like its putative cellular counterparts, IE 62 could conceivably target and modulate an assortment of promoters by interacting with specific cellular regulatory proteins of diverse types. While the precise mechanism by which the ubiquitous USF transcription factors regulate cellular genes remains unknown, the studies described herein bring us closer to an understanding of this process.

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