JOURNAL OF VIROLOGY, Aug. 1995, p. 4693–4701 Vol. 69, No. 8
0022-538X/95/$04.00+0

Proteins and *cis*-Acting Elements Associated with Transactivation of the Varicella-Zoster Virus (VZV) Immediate-Early Gene 62 Promoter by VZV Open Reading Frame 10 Protein

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Received 16 February 1995/Accepted 28 April 1995

Varicella-zoster virus (VZV) open reading frame 10 (ORF10) protein, the homolog of herpes simplex virus type 1 (HSV-1) VP16, is a virion-associated transactivator of the VZV immediate-early (IE) gene 62 (IE62) promoter. VP16 forms a complex with cellular factors (Oct1 and host cell factor [HCF]) and TAATGARAT elements (found in all HSV-1 IE promoter/enhancer sequences) to mediate stimulation of IE transcription. The VZV IE62 promoter also contains three TAATGARAT-like elements. Mutagenesis studies of the VZV IE62 promoter indicated that TAATGARAT-like elements contribute to transactivation of the VZV IE62 promoter by ORF10 protein. Other *cis*-acting elements such as GA-rich and cyclic AMP-responsive elements were also needed for full transactivation by ORF10 protein. In mobility shift assays, ORF10 protein formed a complex with either of two TAATGARAT-like elements that lack an overlapping octamer-binding motif (octa−/TAAT GARAT) but not with a TAATGARAT element with an overlapping octamer-binding motif (octa+/TAATGA RAT). In contrast, VP16 formed a high-affinity ternary complex with an octa+/TAATGARAT element and a low-affinity complex with octa−/TAATGARAT elements. Addition of antibodies to ORF10 protein, Oct1, or HCF disrupted the complexes, demonstrating that ORF10 protein interacts with Oct1 and HCF. These results suggest that transactivation of the VZV IE62 gene by ORF10 protein and HSV IE genes by VP16 require similar cellular proteins but distinct *cis*-acting elements.

Varicella-zoster virus (VZV) is a member of the alphaherpesvirus subfamily. Viral genes of alphaherpesviruses are coordinately regulated and expressed in a cascade fashion and can be divided into three major kinetic classes, immediate-early (IE), early, and late genes (12). IE gene expression of herpes simplex virus type 1 (HSV-1), another member of the alphaherpesvirus subfamily, is induced by the virion-associated transactivator VP16 (also called Vmw65 or α-gene transinducing factor) (3, 10). VP16 is a late gene product that contains two distinct functional domains. The amino and middle portion of the protein is sufficient for promoter targeting through interaction with the cellular proteins Oct1 and host cell factor (HCF) (also called CFF or C1 factor) and the TAATGARAT (R = purine) element (1, 8, 19, 38, 42, 49, 51), while the carboxy-terminal acidic tail is required for transcriptional activation (5, 47) through interaction with basal transcriptional factors such as TFIIB (26), TATA-binding protein (14), and $TAF_{II}40$ (9).

Viral gene expression of VZV is less well defined than that of HSV-1 but is presumed to be regulated in a similar fashion. VZV open reading frame 4 (ORF4), ORF61, ORF62, and ORF63 encode proteins with homology to HSV-1 IE proteins ICP27, ICP0, ICP4, and ICP22, respectively (7). Each of these VZV proteins has been shown to regulate transcription from VZV promoters in transient expression assays (13, 15, 32, 35, 37, 40). The VZV ORF10 protein has considerable amino acid sequence homology to HSV-1 VP16 (7); it is also incorporated into virus particles (17). The VZV ORF10 protein transactivates VZV ORF62 and HSV-1 ICP4 and ICP0 promoters (34) but does not transactivate other putative VZV IE (ORF4, ORF61, or ORF63) promoters in transient expression assays (18, 36). Since both ORF10- and VP16-responsive promoter sequences (VZV ORF62, HSV-1 ICP4, and HSV-1 ICP0) contain TAATGARAT (or TAATGARAT-like) elements, transactivation by ORF10 protein, like that of its VP16 homolog, might also be mediated by formation of a multiprotein complex on a TAATGARAT-like element. Prior efforts to demonstrate the formation of a complex between ORF10 protein and cellular factors with one TAATGARAT-like element from the VZV ORF62 promoter sequence in mobility shift assays were not successful (27).

In this study, using TAATGARAT-like elements within the VZV ORF62 promoter that are different from the one previously tested, we show that ORF10 protein, like its HSV homolog VP16, interacts with Oct1 and HCF on these elements. Furthermore, we show that TAATGARAT-like elements contribute to ORF10 protein-mediated transactivation of the VZV ORF62 promoter and that several other *cis*-acting sequences such as cyclic AMP-responsive element (CRE)-like motifs and a GA-rich (or CGGAAR [R = purine]) element are also involved in transactivation by ORF10 protein.

MATERIALS AND METHODS

Cells. Human malignant melanoma (MeWo) cells were propagated as previously described (33). HeLa (human cervical carcinoma) cells and BJAB cells (an Epstein-Barr virus-negative B-cell line) were grown in Dulbecco's minimal essential medium and RPMI 1640, respectively, with 10% fetal bovine serum.

Plasmids. Plasmids pCMV10 and pCMV16 encode VZV ORF10 and HSV-1 VP16, respectively, under the control of the human cytomegalovirus (HCMV) major IE promoter (MIEP) (36). Plasmids p62CAT(−1440), p62CAT(−578), and p62CAT(−258) were kindly provided by L. P. Perera (41). To construct p62CAT(−408), p62CAT was digested with *Sal*I, and a 0.48-kb fragment was isolated and ligated into the *Sal*I site of plasmid pCATbasic (Promega). The resultant plasmid contains the VZV ORF62 promoter sequence (nucleotides −408 to +68 relative to the transcriptional start site) upstream of the chloramphenicol acetyltransferase (CAT) coding sequence. To generate p62CAT(−578/−36), p62CAT(−578) was digested with *Bsp*1286I and treated with T4 DNA

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TABLE 1. Oligonucleotides used[a]

Oligonucleotide	*cis*-acting elements	Sequence	Mutations
62TAAT1	octa+/TAATGARAT	−259 −247 ctagACAT**ATGTAAATGAAAT**TCACATG TGTATACATTTACTTTAAGTGTACgatc −398 −390	CAT**ATGT** C G **A** C **GAAAT**TC GTATACA G C T G CTTTAAG
62TAAT2	octa−/TAATGARAT	ctagCGACGTGCATTTGATTAACTAGAT GCTGCACG**TAAACTAAT**TGATCTAgatc −100 −92	TGCATTTG C G C AACT ACG**TAAAC** G C G **T**TGA
62TAAT3	octa−/TAATGARAT	ctagATCGTCTTATCATTAAGAATATTTA TAGCAG**AATAGTAAT**TCTTATAAATgatc −345 −338	CGTCTTATC T A G AA GCAG**AATAG** A T C **T**T
62Oct	Octamer	ctagTAAGATGTTTTGCATGTGAGACA ATTCTAC**AAAACGTA**CACTCTGTgatc −206 −201	TGT G G TGCAC GTG AC**A** C C **ACGT** G CAC
62GA	GA-rich (or CGGAAR)	ctagTAAGGTATG**CGGAAA**TAGACACCGG ATTCCATACGCCTTTATCTGTGGCCctag −436 −429	TAT A **CG** T **AAA**TAGA ATA T GC A TTTATCT
62CRE1	CRE	ctagATTGCGCGAACGTAA**TGACGACG**GAGAG TAACGCGCTTGCATTACTGCTGCCTCTCgatc −76 −69	ACGT C T A **GACGACG** G TGCA G A T CTGCTGCC
62CRE2	CRE	ctagATTTACACGG**TGACGACA**CGGGGAG TAAATGTGCCACTGCTGTGCCCCTCctag −117−113	CGG**TG** T G C **ACA**CGG GCCAC A C G TGTGCC
62CCAAT	CCAAT box	ctagAGTCTCGT**CCAAT**CACTACATCGTC TCAGAGCAGGTTAGTGATGTAGCAGctag	CGT G A T **AT**CAC GCA C T A TAGTC

[a] Capital and lowercase letters indicate VZV and linker sequences, respectively. Boldface letters represent putative *cis*-acting elements. Numbers above VZV sequences indicate positions relative to the transcriptional start site of the VZV ORF62 gene. Circled letters indicate mutated nucleotides.

polymerase to blunt the ends. The 0.77-kb fragment was isolated and ligated into the blunted *Sal*I site of pE1bCAT (23). To construct p62CAT(−578/−86), p62CAT(−578) was digested with *Pvu*II and *Ssp*I, and the 0.59-kb fragment was ligated into the *Sal*I site of pE1bCAT. To construct p62CAT(−578/−124), p62CAT was digested with *Pvu*II and *Xho*I, and the 0.45-kb fragment was ligated to pE1bCAT after digestion with *Pst*I (subsequently blunted with T4 DNA polymerase) and *Sal*I. Plasmids p62CAT(−578/−36), p62CAT(−578/−86), and p62CAT(−578/−124) contain the VZV ORF62 promoter sequence (nucleotides −578 to −36, −578 to −87, and −578 to −124, respectively, relative to the ORF10 transcriptional start site) just upstream of the adenovirus E1b TATA box and the CAT gene.

To construct p62CAT(−578)TAAT1-, p62CAT(−578)TAAT2-, p62CAT(−578)TAAT3-, p62CAT(−578)Oct-, p62CAT(−578)GA-, p62CAT(−578)CRE1-, p62CAT(−578)CRE2-, and p62CAT(−578)CCAAT-, site-directed mutagenesis was performed with plasmid p62CAT(−578), using the overlap extension method with PCR (11), as shown in Table 1. Plasmid pPOH2 (the HSV-1 IE ICP4 promoter sequence followed by the CAT gene) was provided by G. Hayward (39). Plasmid pCGoct-2, which expresses human Oct2A, was a gift of L. M. Staut (45).

Transfections and CAT assays. Transient expression and CAT assays were performed in MeWo cells as described previously (34). MeWo cells were used for transactivation experiments because these cells support productive infection with VZV.

Antibodies. Rabbit anti-ORF10 serum 166 was raised against the synthetic oligopeptide corresponding to amino acids (aa) 1 to 13 of ORF10 protein as described previously (17). Rabbit anti-GST (glutathione *S*-transferase)−10 serum was raised against GST-ORF10 (aa 41 to 410) fusion protein (see below). Rabbit anti-VP16 serum C8.5 was kindly provided by S. J. Triezenberg (48). Monoclonal anti-Oct1 antibody YL15 (20) and rabbit anti-recombinant HCF (rHCF) serum (49) were gifts from W. Herr.

Nuclear extracts and mobility shift assays. Nuclear extracts for mobility shift assays were prepared as described previously (21). Nuclear extracts were prepared 48 h after cells were transfected with pCMV (the plasmid lacking any insert), pCMV10, or pCMV16. Probes for mobility shift assays were prepared by annealing complementary oligonucleotides, end labeling with [^{32}P]dCTP and DNA polymerase I (Klenow fragment), and purifying the probe by elution from 10% polyacrylamide gels.

Protein-DNA binding reactions were performed in 20-μl reaction mixtures containing 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 4 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 12% glycerol, 0.25 mg of bovine serum albumin per ml, 2 μg of poly(dI)-poly(dC), 0.05% Nonidet P-40, and 8 μg of nuclear extracts. When indicated, a competitor double-stranded oligonucleotide or antibody to ORF10 protein, VP16, Oct1, or HCF was added to the reaction mixtures. Reaction mixtures were incubated for 15 min at 22°C, radiolabeled probe (2×10^4 cpm; 0.5 ng) was added, and the reaction mixtures were incubated for an additional 30 min. The reactions were run on 4% 37.5:1 acrylamide/bisacrylamide, 0.25× Tris-borate-EDTA gels at 15 V/cm for 2 h at 4°C.

Purification of GST-VZV ORF10 and GST-VP16 fusion proteins. Plasmid pGEX-10(41-410) encodes a GST-VZV ORF10 (aa 41 to 410) fusion protein under the control of *Escherichia coli tac* promoter (31). The *Eco*RI fragment (encoding ORF10 aa 5 to 79) of GAL4-ORF10(5-79) (31) or the *Eco*RI fragment (encoding VP16 amino acids 1 to 490) of pCMV16 (36) was inserted into the *Eco*RI site of plasmid pGEX-3X (after the *Bam*HI site of pGEX-3X had been blunted). The resultant plasmids pGEX-10(5-79) and pGEX-16 encode a GST-VZV ORF10 (aa 5 to 79) fusion protein and a GST-VP16 (aa 1 to 490) fusion protein, respectively, under the control of the *tac* promoter.

E. coli DH5 cells containing pGEX-3X (which contains the coding sequence of GST driven by the *E. coli tac* promoter [Pharmacia]), pGEX-10(41-410), pGEX-10(5-79), and pGEX-16 were grown in LB broth containing 100 μg of ampicillin per ml, induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), incubated for 4 h, and lysed by mild sonication. The lysates were clarified by centrifugation, incubated with glutathione-Sepharose 4B (Pharmacia), and washed, and fusion proteins were eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris (pH 8.0).

Analysis of protein interactions by using GST fusion proteins. Approximately 2 μg of GST fusion protein bound to glutathione-Sepharose was incubated with 10 μg of MeWo cell nuclear extracts in NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.8% Nonidet P-40) for 1 h at 4°C. The Sepharose was washed three times and boiled in sodium dodecyl sulfate (SDS) sample buffer, and the supernatant was fractionated on an SDS–6% polyacrylamide gel. The proteins were transferred to a nitrocellulose filter, reacted with rabbit anti-rHCF antibody, and visualized with goat anti-rabbit antibody conjugated to alkaline phosphatase.

RESULTS

Identification of *cis*-acting elements required for transactivation of the VZV ORF62 promoter by ORF10 protein. The nucleotide sequence of the VZV ORF62 gene promoter contains one TAATGARAT-like element with an overlapping octamer motif (octa+/TAATGARAT) (positions −259 to −247) and two TAATGARAT-like elements without an overlapping octamer motif (octa−/TAATGARAT) (positions −398 to −390 and −100 to −92). We designated these motifs 62TAAT1, 62TAAT2, and 62TAAT3, respectively (Table 1). The former element is located on the plus strand, while the latter two

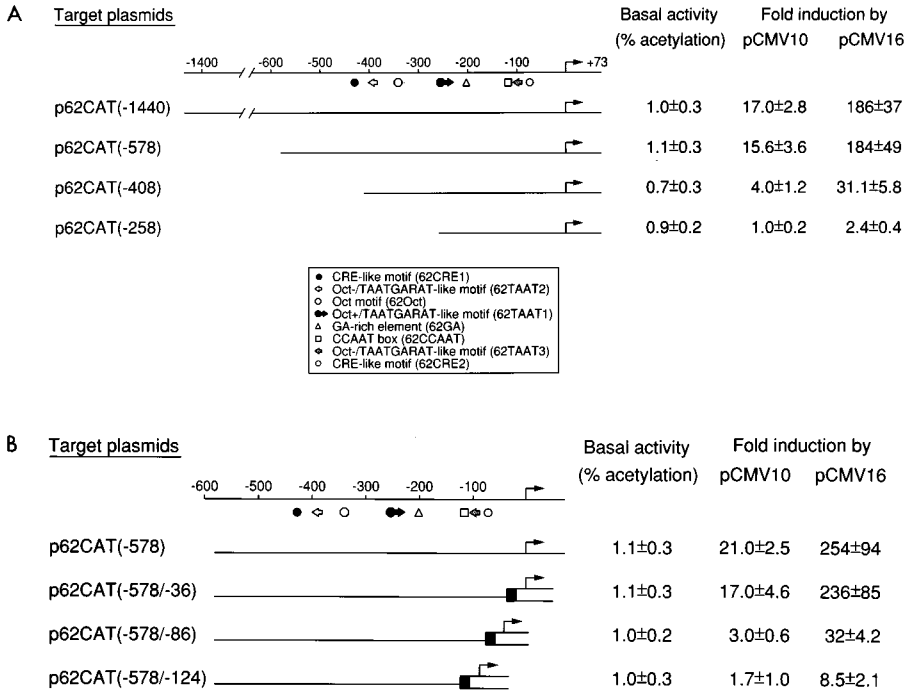


FIG. 1. (A) Effects of VZV ORF62 promoter sequence 5′ deletions on ORF10 protein and VP16 responsiveness. MeWo cells were cotransfected with 250 ng of the indicated target plasmids along with 100 ng of pCMV10 or pCMV16. Fold induction of CAT is the CAT activity relative to the basal activity (shown as percent acetylation) that was obtained for each target plasmid plus pCMV (vector control). Dilutions of cell lysates were made when necessary to accurately calculate fold inductions. (B) Effects of VZV ORF62 promoter sequence 3′ deletions on ORF10 protein and VP16 responsiveness. MeWo cells were cotransfected with 1 μg of the indicated target plasmids along with 100 ng of pCMV10 or pCMV16. Means and standard deviations were calculated from at least three independent transfections. Right- and left-pointing arrows indicate the presence of TAATGARAT-like motifs on the plus and minus strands, respectively. Solid lines indicate the VZV ORF62 promoter sequences, and shaded rectangles indicate the adenovirus E1b TATA box.

elements are located on the minus strand of the virus DNA relative to the ORF62 gene. The ORF62 regulatory region also contains an octamer-like element without TAATGARAT (positions −345 to −338; designated 62Oct). There are a GA-rich (or CGGAAR [4, 46, 48]) element (positions −206 to −201; designated 62GA), two CRE (for a review see reference 22)-like motifs (positions −436 to −429 and −76 to −69; designated 62CRE1 and 62CRE2, respectively), and a CCAAT box (positions −117 to −113; designated 62CCAAT).

To identify DNA sequences required for transactivation by ORF10 protein, a series of 5′ or 3′ truncation mutants of p62CAT was constructed and tested with cotransfected pCMV10 and pCMV16 (which express VZV ORF10 protein and HSV-1 VP16, respectively, under the control of HCMV MIEP). ORF10 protein and VP16 transactivated p62CAT (−1440) up to 17- and 186-fold, respectively (Fig. 1A). Deletion of nucleotides −1440 to −579 had little effect on the responsiveness of the VZV ORF62 promoter to ORF10 protein or VP16. Further deletion to nucleotide −408 resulted in a similar proportional loss of activity of the ORF62 promoter to ORF10 protein or VP16. Additional deletion to nucleotide −258 further reduced activity to the background level. To map the 3′ boundary of the ORF62 promoter, we constructed 3′ truncation mutants of p62CAT(−578) and inserted them upstream of the adenovirus E1b TATA sequence and the CAT gene (Fig. 1B). ORF10 protein and VP16 transactivated p62CAT(−578/−36) up to 17- and 236-fold, respectively, similar to the levels seen with p62CAT(−578). Deletion of nucleotides −87 to −36 resulted in loss of most of the activity of the ORF62 promoter, and further deletion to nucleotide −124 nearly abolished activity. The similar patterns of responsiveness of the ORF62 promoter truncation mutants to both ORF10 protein and VP16 suggest that these two proteins may transactivate by similar mechanisms, although VP16 was approximately 12-fold more potent as a transactivator than ORF10 protein.

To determine the contribution of each *cis*-acting element to transactivation by ORF10 protein, the elements were mutated individually in the context of p62CAT(−578) (Fig. 2). Mutation of 62TAAT2 markedly impaired responsiveness to ORF10 protein, while mutation of 62TAAT1 and 62TAAT3 reduced

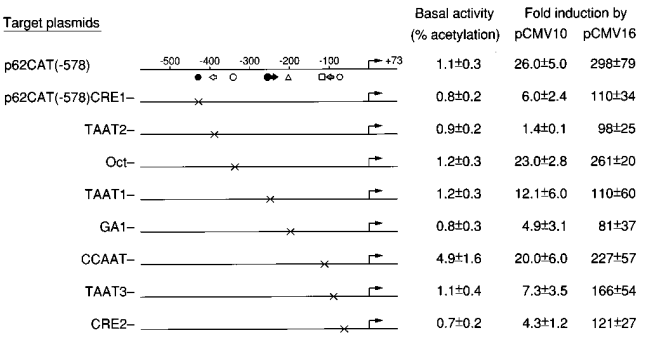


FIG. 2. Effects of site-directed mutagenesis of the VZV ORF62 promoter on ORF10 protein and VP16 responsiveness. MeWo cells were cotransfected with 250 ng of the indicated target plasmids along with 100 ng of pCMV10 or pCMV16. Basal activity of each target plasmid is shown as percent acetylation. Means and standard deviations were calculated from at least three independent transfections. ×'s indicate the mutated sequences summarized in Table 1. Symbols are defined in the legend to Fig. 1.

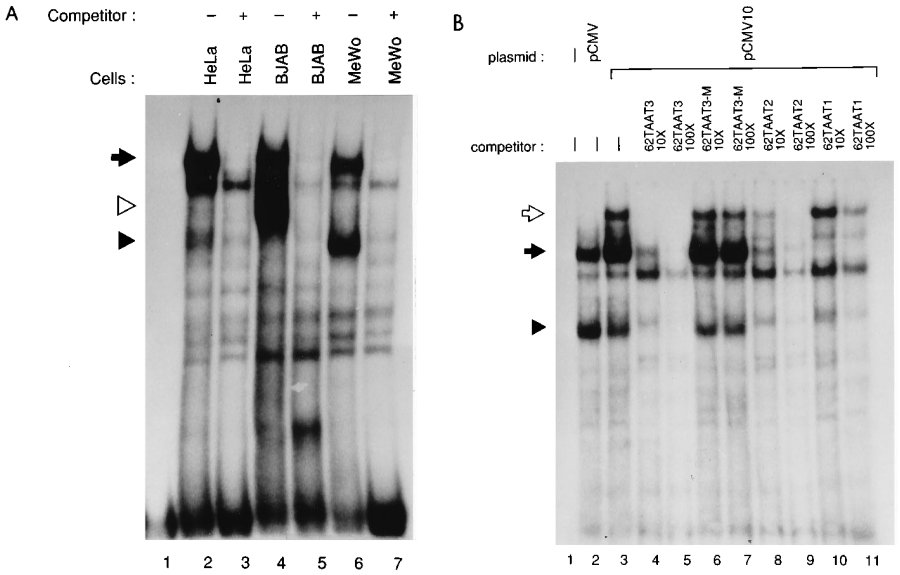


FIG. 3. (A) Complexes formed with the octa−/TAATGARAT-like (62TAAT3) sequence and nuclear extracts from HeLa (lanes 2 and 3), BJAB (lanes 4 and 5), and MeWo (lanes 6 and 7) cell lines. Lane 1 contains free probe only. In lanes 3, 5, and 7, a 100-fold molar excess of unlabeled 62TAAT3 oligonucleotide was added as a competitor. The arrow, open triangle, and solid triangle indicate Oct1, Oct2, and N-Oct3, respectively. The band between Oct1 and Oct2 is nonspecific and is present in each of the cell extracts. (B) Formation of an ORF10-induced complex on the octa−/TAATGARAT-like (62TAAT3) sequence. A radiolabeled oligonucleotide containing the octa−/TAATGARAT-like (62TAAT3) sequence was incubated with nuclear extracts from MeWo cells transfected with pCMV (lane 2) or pCMV10 (lanes 3 to 11). Lane 1 contains free probe only. When indicated, a 10- or 100-fold molar excess of unlabeled 62TAAT3 (lanes 4 and 5), 62TAAT3-M (lanes 6 and 7), 62TAAT2 (lanes 8 and 9), or 62TAAT1 (lanes 10 and 11) oligonucleotide was added as a competitor. The open arrow, solid arrow, and triangle indicate 10IC, Oct1, and N-Oct3, respectively.

activity to 47 and 28% of the wild-type level, respectively. Mutation of the CRE or GA-rich elements reduced activation by ORF10 protein to approximately 20%, while mutation of the Oct element or the CCAAT box retained 80 to 90% of wild-type activity. Mutation of the 62TAAT, CRE, or GA-rich elements also reduced activation by VP16. The patterns of responsiveness of the mutations were similar for ORF10 protein and VP16 except that ORF10 protein was much more

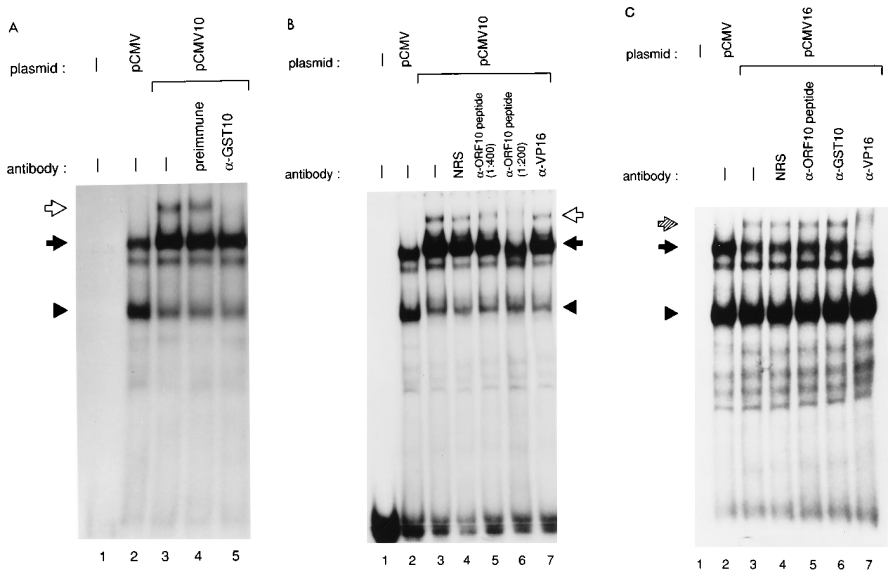


FIG. 4. 10IC contains ORF10 protein. (A) A radiolabeled oligonucleotide containing the octa−/TAATGARAT-like (62TAAT3) sequence was incubated with nuclear extracts from MeWo cells transfected with pCMV (lane 2) or pCMV10 (lanes 3 to 5). Lane 1 contains free probe only. When indicated, preimmune serum (lane 4) or antiserum against GST-ORF10 fusion protein (1:100) (lane 5) was added to the reaction mixtures. The open arrow indicates 10IC, the solid arrow indicates Oct1, and the triangle indicates N-Oct3. (B) A radiolabeled oligonucleotide containing the 62TAAT3 element was incubated with nuclear extracts from MeWo cells transfected with pCMV (lane 2) or pCMV10 (lanes 3 to 7). Lane 1 indicates free probe only. When indicated, normal rabbit serum (NRS; lane 4), anti-ORF10 peptide antibody (lanes 5 [1:400] and 6 [1:200]), or anti-VP16 antibody (1:200) (lane 7) was added to the reaction mixtures. Arrows and triangle are as described for panel A. (C) Formation of 16IC is disrupted by an anti-VP16 antibody but not by anti-ORF10 antibodies. A radiolabeled oligonucleotide containing the 62TAAT3 element was incubated with nuclear extracts from MeWo cells transfected with pCMV (lane 2) or pCMV16 (lanes 3 to 7). When indicated, control rabbit serum (lane 4), anti-ORF10 peptide antibody 166 (1:200) (lane 5), anti-GST-ORF10 antibody (1:100) (lane 6), or anti-VP16 antibody (1:200) (lane 7) was added to the reaction mixtures. Solid and striped arrows indicate Oct1 and 16IC, respectively; the solid triangle indicates N-Oct3.

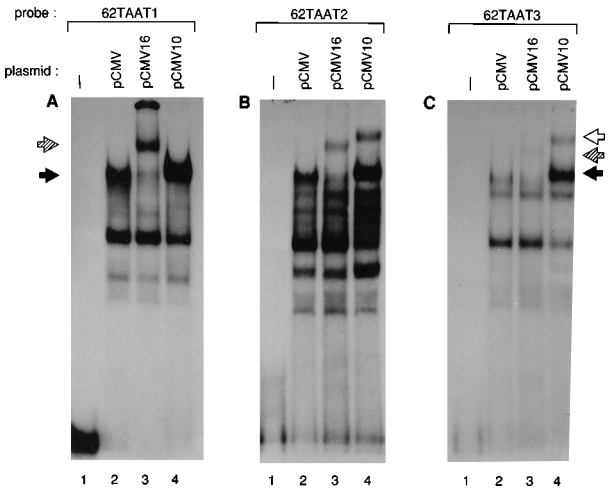


FIG. 5. Different affinities of ORF10 protein- and VP16-induced complexes to the three TAATGARAT-like elements within the VZV ORF62 promoter sequence. A radiolabeled oligonucleotide containing the octa+/TAATGARAT-like (62TAAT1; A), octa−/TAATGARAT-like (62TAAT2; B), or octa−/TAAT GARAT-like (62TAAT3; C) element was incubated with nuclear extracts from MeWo cells transfected with pCMV (lane 2), pCMV16 (lane 3), or pCMV10 (lane 4). Lane 1 indicates free probe alone. Solid, striped, and open arrows indicate Oct1, 16IC, and 10IC, respectively. The uppermost band in lane 3 of panel A is due to probe complexed to protein that has not migrated out of the well.

sensitive than VP16 to mutation of 62TAAT2, 62TAAT3, 62CRE1, and 62CRE2.

Two TAATGARAT-like elements that lack an overlapping octamer-binding motif form a complex with VZV ORF10 protein and cellular factors. The results from the transient expression assays indicated that the individual TAATGARAT-like elements contribute to transactivation of the VZV ORF62 promoter by ORF10 protein. To determine if ORF10 protein, like its HSV homolog VP16, can form a complex with cellular proteins on a TAATGARAT-like sequence, we performed mobility shift assays using labeled double-stranded oligonucleotides containing the 62TAAT1, 62TAAT2, or 62TAAT3 sequence.

Nuclear extracts from HeLa (human cervical carcinoma), BJAB (human B-lymphocyte), or MeWo (human malignant melanoma) cells contain octamer-binding proteins which form a complex with the 62TAAT3 sequence from the VZV ORF62 promoter (Fig. 3A). HeLa cells contain Oct1 (arrow in Fig. 3A), while BJAB cells contain Oct1 and lymphocyte-specific Oct2 (open triangle) (6), and MeWo cells contain Oct1 (as shown below) and neuronal cell-specific N-Oct3 (solid triangle) (as shown in reference 43). Nuclear extracts from MeWo cells transfected with pCMV (vector control) formed two predominant complexes with the 62TAAT3 (TAATGATAA) sequence from the VZV ORF62 promoter containing Oct1 and N-Oct3 (Fig. 3B).

Extracts from MeWo cells transfected with pCMV10 formed an additional, more slowly migrating complex (open arrow in Fig. 3B; designated ORF10-induced complex [10IC]). Addition of excess unlabeled oligonucleotides containing 62TAAT3 or 62TAAT2 eliminated binding of Oct1 and N-Oct3 and formation of 10IC, while an oligonucleotide containing mutated 62TAAT3 (62TAAT3M) did not. Competitor oligonucleotide 62TAAT1 eliminated binding of Oct1 and N-Oct3 but not formation of 10IC. Similar results were obtained for MeWo cells transfected with pCMV10 with the 62TAAT2 (TAAT CAAAT) probe (30).

Addition of rabbit serum raised against either GST-ORF10 or a synthetic ORF10 peptide to nuclear extracts of MeWo cells transfected with ORF10 and incubated with 62TAAT3 disrupted 10IC (Fig. 4A, lane 5; Fig. 4B, lane 6) but did not disrupt the Oct1 or N-Oct3 complex. Addition of preimmune serum, control rabbit serum, or anti-VP16 antibody did not affect 10IC (Fig. 4A, lane 4; Fig. 4B, lanes 4 and 7). Extracts from MeWo cells transfected with pCMV16 also formed a slowly migrating complex (Fig. 5; designated VP16-induced complex [16IC]) which was disrupted by the addition of an anti-VP16 antibody but not an anti-ORF10 antibody (Fig. 4C).

Expression of ORF10 protein enhanced the amount and slowed the mobility of the Oct1 complex and reduced N-Oct3 binding (Fig. 4A, lane 3; Fig. 4B, lane 3; Fig. 5, lane 4), while expression of VP16 reduced Oct1 binding and had little effect on N-Oct3 binding (Fig. 4C, lane 3). These results suggest that ORF10 protein interacts with the TAATGARAT elements somewhat differently than VP16.

Extracts from MeWo cells transfected with pCMV10 and incubated with 62TAAT1 did not show formation of 10IC (Fig. 5A), while 10IC was detected with 62TAAT2 and 62TAAT3 (Fig. 5B and C). Extracts from cells transfected with pCMV16 and incubated with 62TAAT1 did show a slowly migrating complex (16IC) (Fig. 5A); however, extracts from cells transfected with pCMV16 and incubated with 62TAAT2 or 62TAAT3 formed a less prominent 16IC (Fig. 5B and C).

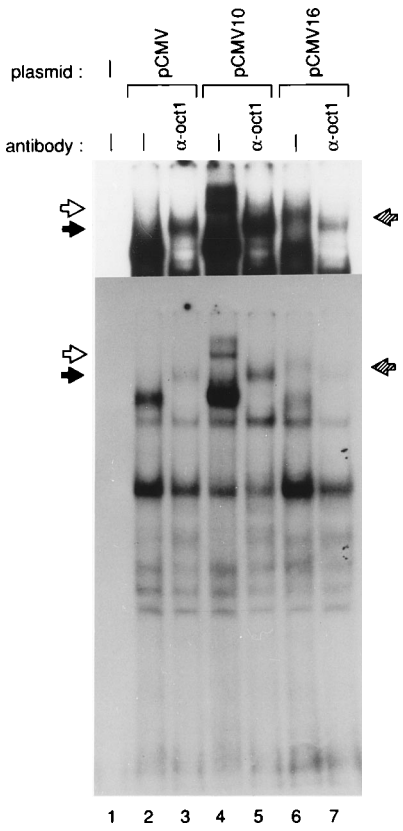
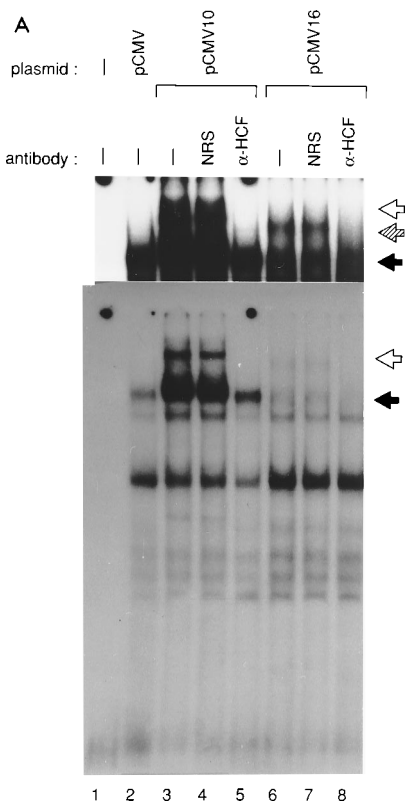


FIG. 6. Involvement of Oct1 in 10IC. A radiolabeled oligonucleotide containing the octa−/TAATGARAT-like (62TAAT3) sequence was incubated with nuclear extracts from MeWo cells transfected with pCMV (lanes 2 and 3), pCMV10 (lanes 4 and 5), or pCMV16 (lanes 6 and 7). A monoclonal anti-Oct1 antibody was added to the reaction mixtures in lanes 3, 5, and 7. Open and striped arrows indicate the positions of 10IC and 16IC, respectively; the solid arrow indicates the shift of Oct1 complex in the presence of the anti-Oct1 antibody. The upper panel is a darker exposure of the lower panel.



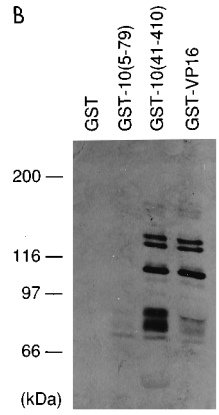


FIG. 7. Involvement of HCF in 10IC. (A) Disruption of 10IC by an anti-HCF antibody. A radiolabeled oligonucleotide containing the octa−/TAATGARAT-like (62TAAT3) sequence was incubated with nuclear extracts from MeWo cells transfected with pCMV (lane 2), pCMV10 (lanes 3 to 5), or pCMV16 (lanes 6 to 8). Lane 1 indicates free probe only. When indicated, normal rabbit serum (NRS; lanes 4 and 7) or antiserum against HCF (lanes 5 and 8) was added to the reaction mixtures. Solid, open, and striped arrows indicate Oct1, 10IC, and 16IC, respectively. (B) Direct interaction of GST-ORF10 fusion protein with HCF. HCF was sequestered from MeWo cell nuclear extracts with immobilized GST-ORF10 (aa 41 to 410) or GST-VP16 (aa 1 to 490) fusion protein. Trace amounts of low-molecular-weight forms of HCF were sequestered with GST-ORF10 (aa 5 to 79) fusion protein but not with GST alone. Numbers at the left indicate molecular masses.

These data indicate that ORF10 protein can form a complex with a cellular factor(s) and either of the two octa−/TAAT GARAT-like elements (62TAAT2 and 62TAAT3), while VP16 forms a complex that is most prominent with the octa+/ TAATGARAT-like element (62TAAT1).

Oct1 and HCF are components of the complex with VZV ORF10 protein and the octa−/TAATGARAT-like element (62TAAT3). To determine whether Oct1 contributes to formation of 10lC, a monoclonal anti-Oct1 antibody was added to nuclear extracts of cells transfected with pCMV, pCMV10, or pCMV16 and incubated with the 62TAAT3 probe. Addition of the monoclonal antibody to Oct1 disrupted formation of both 10IC and 16IC and supershifted the Oct1 complex (Fig. 6). Thus, Oct1 is a component of 10IC as well as 16IC.

To determine whether HCF contributes to 10IC, an anti-rHCF antibody was added to nuclear extracts of cells transfected with pCMV10 or pCMV16 and incubated with the 62TAAT3 probe. Antibody to rHCF disrupted formation of both 10IC and 16IC (Fig. 7A, lanes 5 and 8, respectively). Direct interaction of ORF10 protein with HCF was also demonstrated by the ability of a GST-ORF10 fusion protein to sequester HCF from a cell extract. Two GST-ORF10 fusion proteins, GST-ORF10 (aa 5 to 79) and GST-ORF10 (aa 41 to 410), and a GST-VP16 fusion protein were expressed in *E. coli* and purified on glutathione-Sepharose. Nuclear extracts from MeWo cells were incubated with immobilized GST, GST-ORF10, or GST-VP16. After extensive washing, the Sepharose was boiled and the supernatants were fractionated on an SDS–6% polyacrylamide gel and analyzed by Western blotting (immunoblotting) with the anti-rHCF antibody. Multiple forms of HCF (49) were recognized in samples containing the GST-ORF10 (aa 41 to 410) or GST-VP16 (aa 1 to 490) fusion protein. Trace amounts of HCF were detected with the GST-ORF10 (aa 5 to 79) fusion protein, and no HCF was detected with GST alone (Fig. 7B). Therefore, VZV ORF10 protein can directly interact with HCF, and aa 1 to 40 of the ORF10 protein are dispensable for the interaction.

Oct2 can inhibit ORF10 protein-mediated transactivation of the VZV ORF62 promoter. To establish further that Oct1 plays an important role in transactivation of the VZV ORF62 promoter by ORF10 protein, we expressed Oct2A, a closely related octamer-binding protein, in MeWo cells. Oct2A can bind to the TAATGARAT elements on HSV IE promoters (16) and the TAATGARAT-like elements on the VZV ORF62 promoter (Fig. 3A and 8C) as well as consensus octamer sequences; however, Oct2 cannot form a complex with VP16 because of the difference of the homeodomains of these two proteins (44). Previous studies showed that Oct2 transrepresses the HSV IE promoters (24) and inhibits VP16-mediated transactivation of these promoters (25).

In the absence of ORF10 protein or VP16, Oct2A transactivated p62CAT (the VZV IE ORF62 promoter followed by the CAT gene) up to ninefold and transrepressed pPOH2 (the HSV-1 IE ICP4 promoter followed by the CAT gene) up to 78% (Fig. 8A). When cotransfected with pCMV10 (VZV ORF10 driven by the HCMV MIEP) or pCMV16 (VP16 driven by the HCMV MIEP), Oct2A inhibited ORF10 protein- or VP16-mediated transactivation of the VZV ORF62 promoter in a dose-dependent manner in transient expression assays (Fig. 8B) and disrupted formation of ORF10 protein-induced complex and reduced binding of Oct1 and N-Oct3 in mobility shift assays (Fig. 8C). These results indicate that Oct2A has two different effects on the VZV ORF62 promoter; Oct2A alone transactivates the promoter, and Oct2A inhibits ORF10 protein-mediated transactivation.

DISCUSSION

We have shown that VZV ORF10 protein, the homolog of HSV-1 VP16, forms a complex with either of two octa−/TA

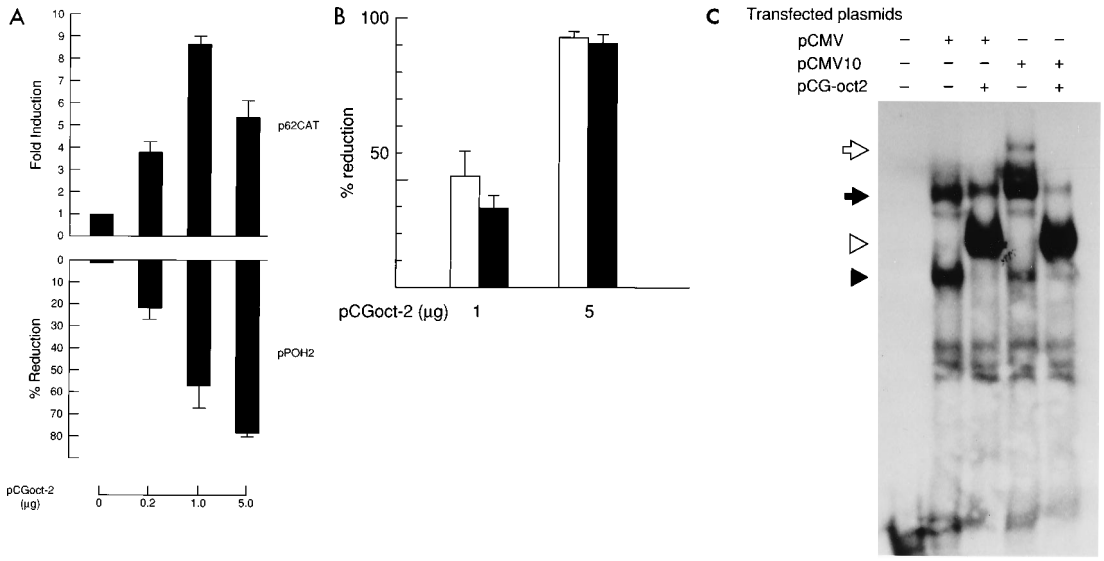


FIG. 8. (A) Oct2 alone transactivates the VZV ORF62 promoter and transrepresses the HSV-1 ICP4 promoter. MeWo cells were cotransfected with 250 ng of p62CAT(−1440) or pPOH2 (the HSV-1 ICP4 promoter followed by the CAT gene) along with various amounts of pCGoct-2 (human Oct2A driven by the HCMV MIEP). Basal activities (expressed as percent chloramphenicol acetylation) with 250 ng of p62CAT(−1440) and pPOH2 were 0.9% ± 0.3% and 10.8% ± 2.8%, respectively. Fold induction and percent reduction are the CAT activities relative to those obtained for p62CAT(−1440) plus pCMV and pPOH2 plus pCMV, respectively. pCMV was added to transfections so that the total amount of plasmid DNA containing the HCMV promoter was 5 μg. The experiments were performed three times, and the means and the standard deviations are shown. (B) Oct2 inhibits ORF10 protein- or VP16-mediated transactivation of the VZV ORF62 promoter. MeWo cells were cotransfected with 250 ng of p62CAT(−1440) plus pCMV, pCMV10, or pCMV16, along with either 1 or 5 μg of pCGoct-2. pCMV was added to transfections so that the total amount of plasmid containing the HCMV promoter was 5 μg. The basal activity (expressed as percent chloramphenicol acetylation) of p62CAT(−1440) (1.0% ± 0.3%) was enhanced up to 21 ± 5- and 248 ± 88-fold with pCMV10 and pCMV16, respectively, in these experiments. Percent reduction of CAT (shown) is the CAT activity obtained for p62CAT and pCGoct-2 plus pCMV10 (open bars) or pCMV16 (closed bars), compared with the CAT activity for p62CAT plus pCMV10 or pCMV16 without pCGoct-2. The experiments were performed three times, and the means and standard deviations are shown. (C) Oct2 inhibits formation of Oct1 complex and 10IEC. Nuclear extracts were prepared from MeWo cells transfected with pCMV (lane 2), pCMV and pCGoct-2 (lane 3), pCMV10 (lane 4), or pCGoct-2 plus pCMV10 (lane 5) and were incubated with the probe containing the octa−/TAATGARAT-like (62TAAT3) sequence. Open arrow, solid arrow, open triangle, and solid triangle indicate the positions of 10IC, Oct1, Oct2, and N-Oct3, respectively.

ATGARAT-like elements in the VZV ORF62 promoter. Addition of an anti-ORF10, Oct1, or HCF antibody disrupted the complex, indicating that these three proteins are components of the complex. Furthermore, a direct interaction between ORF10 protein and HCF was demonstrated by use of a GST-ORF10 fusion protein to sequester HCF from a complex nuclear extract.

The conservation of TAATGARAT-like elements in both VZV and HSV-1 IE promoters suggest that ORF10 protein and VP16 regulate gene expression by similar mechanisms. Analysis of a series of 5′ and 3′ truncation mutants of the VZV ORF62 promoter indicated that they had similar patterns of responsiveness to VZV ORF10 protein and HSV-1 VP16. Similarly, mutagenesis of individual *cis*-acting elements of the ORF62 promoter generally showed similar patterns of responsiveness to ORF10 protein and VP16, with three exceptions. While mutation of octa−/TAATGARAT-like elements (62TAAT2 and 62TAAT3) and a CRE-like motif (62CRE2) reduced responsiveness to VP16 by 67, 44, and 59%, respectively, mutation of these elements had a more profound effect on responsiveness to ORF10 protein (95, 72, and 83% reduction, respectively). Mutation of the 62TAAT2 TAATGARAT-like element abolished the ability of the VZV ORF62 promoter to be transactivated by ORF10 protein above basal levels, while the 62TAAT3 and 62CRE2 mutants of the ORF62 promoter still retained modest (six- to sevenfold) responsiveness to transactivation by ORF10 protein. These experiments suggested that the octa−/TAATGARAT-like elements may be important for interactions with VZV ORF10 protein.

ORF10 protein formed a complex with either of two octa−/TAATGARAT-like elements (62TAAT2 or 62TAAT3), but not an octa+/TAATGARAT-like element (62TAAT1), in the ORF62 promoter. In contrast, VP16 formed a high-affinity complex with the octa+/TAATGARAT-like element but a lower-affinity complex with either of the octa−/TAATGARAT-like elements in the ORF62 promoter. Our results are consistent with those of a prior study (27) that also did not detect an ORF10 protein complex with the octa+/TAATGARAT-like element in the ORF62 promoter. While the octa−/TAATGARAT-like elements are present on the minus strand, relative to the ORF62 gene, the orientation of a corresponding element in ICP4 (the homolog of VZV ORF62) promoter was not essential for transactivating function (4).

The different affinities of ORF10 protein and VP16 for octa+/TAATGARAT- and octa−/TAATGARAT-like elements may be due to differences in amino acid sequences between these two proteins. The two elements that we identified (62TAAT2 and 62TAAT3) do not strictly match the consensus TAATGARAT element that has been identified as interacting with the HSV-1 VP16 multiprotein complex. The ORF62 sequences differ at position 5 (TAATCAAAT for 62TAAT2) or positions 7 and 9 (TAATGATAA for 62TAAT3) of the HSV-1 IE TAATGARAT sequence. They also lack the overlapping octamer motif, which is often found in HSV-1 IE TAATGARAT elements. Recently the sequence TAATGAGCT from the bovine herpesvirus type 1 (BHV-1) IE-1 promoter has been shown to interact with BHV-1 α-gene transinducing factor (29). This element also differs from the classic TAATGARAT at position 8 and lacks the overlapping

octamer motif. Interestingly, BHV-1 α-gene transinducing factor and HSV-1 VP16 have different affinities for TAAT GAGCT (BHV-1 IE-1 promoter) and octa+/TAATGARAT (HSV-1 IE promoter) (28), as seen with VZV ORF10 protein and HSV-1 VP16.

Other *cis*-acting elements in the ORF62 promoter also contributed to transactivation by ORF10 protein. Most of the ORF10 protein-induced transactivation of the ORF62 promoter was lost after mutagenesis of the GA-rich (or CG GAAR) element. This element is also present on the HSV-1 IE promoters, binds a cellular protein, GA-binding protein (46), and has been shown to be involved in transactivation of IE promoters by VP16 (48, 50). The HSV-1 ICP4 promoter contains three imperfect repeats of the hexanucleotide sequence CGGAAR (R = purine), while the VZV ORF62 promoter contains a single CGGAAR element. In contrast, the GA-rich element is absent from the VZV ORF61 and ORF4 promoters, which also lack TAATGARAT-like elements. While these motifs may not interact directly with VP16 (or ORF10 protein), they may enhance the transactivating activity of the protein (2). Mutagenesis of either of two CRE-like motifs in the ORF62 promoter also resulted in marked loss of responsiveness to ORF10 protein. Like the GA-rich element, the CRE-like motifs probably do not interact directly with ORF10 protein but enhance its activity.

In summary, we have shown that (i) ORF10 protein forms a complex with Oct1, HCF, and TAATGARAT-like elements, (ii) TAATGARAT-like elements contribute to ORF10 protein-mediated transactivation of the ORF62 promoter, and (iii) additional *cis*-acting elements are also necessary for full ORF10 protein-mediated transactivation. The complexity of the ORF62 promoter and the numerous cellular factors that can interact with this promoter may be important for ORF10 protein activity in different cell types. These interactions may be part of a system that regulates the ability of VZV to replicate or remain latent in different cell types in vivo.

ACKNOWLEDGMENTS

We thank L. P. Perera for helpful suggestions and providing plasmids p62CAT(−1440), p62CAT(−578), and p62CAT(−258); G. Hayward for plasmid pPOH2; L. M. Staut for plasmid pCGoct-2; P. R. Kinchington for the anti-ORF10 peptide antibody; S. J. Triezenberg for the anti-VP16 antibody; and W. Herr for the anti-Oct1 and anti-HCF antibodies. We thank S. E. Straus for reviewing the manuscript.

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