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Varicella-Zoster Virus Open Reading Frame 4 Encodes a Transcriptional Activator That Is Functionally Distinct from That of Herpes Simplex Virus Homolog ICP27

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Varicella-zoster virus is the etiological agent of chickenpox and zoster in humans and belongs to the *Alphaherpesvirinae* subfamily within the family *Herpesviridae*. Much of the current understanding of gene regulation in alphaherpesviruses has been derived from studies of the prototype herpes simplex virus (HSV). In HSV, two virus-encoded, *trans*-regulatory proteins, ICP4 and ICP27, are essential for the replicative cycle of the virus. ICP4 is important in modulating HSV genes of all three kinetic classes, whereas the *trans*-regulatory effects of ICP27 are primarily associated with the expression of late genes. Recent evidence indicates that the *trans*-regulatory effects of ICP27 involve posttranscriptional processing of target gene transcripts (R. M. Sandri-Golding and G. E. Mendoza, Genes Dev. 6:848–863, 1992). The ICP27 homolog in varicella-zoster virus is a 452-amino-acid polypeptide encoded by the open reading frame 4 (ORF4) gene. Contrary to what is found with ICP27, we show that the ORF4 polypeptide is a transcriptional activator of diverse target promoters and has a critical requirement for the presence of upstream elements within these promoters to mediate its transcriptional effects. Evidence is also presented to implicate a critical role for the cysteine-rich, C-terminal region of the ORF4 polypeptide in its *trans*-regulatory functions. Specifically, by oligonucleotide-directed site-specific mutagenesis, we demonstrate that of 10 cysteine residues in the ORF4 polypeptide, only C-421 and C-426 are essential for transactivator function and suggest that these cysteine residues may participate in critical protein-protein interactions rather than protein-nucleic acid interactions to mediate ORF4 inducibility.

Varicella-zoster virus (VZV) is a neurotropic alphaherpesvirus and is the etiological agent of chickenpox and zoster in humans. The other members of the *Alphaherpesvirinae* subfamily include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), pseudorabies virus, and equine herpesvirus type 1. The alphaherpesviruses are enveloped particles with relatively large double-stranded DNA genomes. The emerging evidence, by direct analyses and by sequence analogies, indicates that they are closely related genetically and that their genomes are essentially collinear with respect to gene distribution and genetic topology.

Our understanding of gene regulation in alphaherpesviruses is far from being complete, much of the current knowledge about it having been derived from studies of the prototype HSV-1 system. In HSV-1, the expression of viral genes during lytic infection occurs in a tightly regulated cascade pattern (6, 14, 20, 21, 45). Depending on the kinetics of expression and responses to inhibitors of macromolecular synthesis, the viral genes are classified into three broad classes: the immediate-early (α), the early (β), and the late (γ) genes (6, 7, 14, 19–21). The first set of genes to be expressed are the five α genes; α0, α4, α22, α27, and α47. The expression of α genes does not require de novo protein synthesis and is stimulated by a virion-associated factor, VP16 (also known as α-TIF or Vmw65), which itself is encoded by a γ gene (3, 5). With the exception of ICP47, all immediate-early proteins, i.e., ICP0, ICP4, ICP22, and ICP27, have been shown to possess regulatory functions that influence the expression of early and late genes (reviewed in reference 45). However, of these five immediate-early proteins, only ICP4 and ICP27 play an essential role in the infectious cycle of the virus. Temperature-sensitive mutants and mutants with deletions in either α4 or α27 genes are not viable under nonpermissive conditions (10, 11, 32, 45).

ICP27 is a 63-kDa phosphoprotein and localizes to the nucleus of infected cells (1, 27, 60). Studies with ICP27 temperature-sensitive and null mutants have revealed that the protein is involved in the negative regulation of viral immediate-early and early genes and is needed for the expression of late genes (32, 45, 47). Recent evidence indicates that the *trans*-regulatory effects of ICP27, at least in part, involve posttranscriptional processing of target gene transcripts and that the negative effects on gene expression are related to the presence of introns within the target gene whereas positive effects are mediated through 3′-end processing of target gene transcription (50). Although the specific function of ICP27 in the HSV replicative cycle is not clear, it is likely that this function is of central importance since homologs of ICP27 are conserved in other alphaherpesviruses including VZV and equine herpesvirus type 1 (8, 57).

A 452-amino-acid polypeptide encoded by the open reading frame 4 (ORF4) gene is the ICP27 homolog in VZV, which

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TABLE 1. Oligonucleotides used for hybrid promoter construction and in PCR

Oligonucleotide sequence	Purpose
5′ GATCCAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGA 3′	First strand for 2KB+3SpI cartridge
5′ GATCTCGCCACTCCCCAGTCCCGCCCAGGCCACGCCTCCCTGGAAAGTCCCCAGCGGAAAGTCCCTG 3′	Second strand for 2KB+3SpI cartridge
5′ GATCCGGGACTTTCCGCTGGGGACTTTCCA 3′	First strand for 2KB cartridge
5′ GATCTGGAAAGTCCCCAGCGGAAAGTCCCG 3′	Second strand for 2KB cartridge
5′ GATCCGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAA 3′	First strand for 3SpI cartridge
5′ GATCTTCGCCACTCCCCAGTCCCGCCCAGGCCACGCCTCCCG 3′	Second strand for 3SpI cartridge
5′ GATCCGGGATATCTGGCCTGTCTAGATCCGTGCACTGGCGAA 3′	First strand for 3mutSpI cartridge
5′ GATCTTCGCCAGTGCACGGATCTAGACAGGCCAGATATCCCG 3′	Second strand for 3mutSpI cartridge
5′ CGTCTCAGCCAATCCCTGGGTGAGTTTCACCAG 3′	CAT PCR primer 1
5′ GCCCTGCCACTCATCGCAGTACTGTTG 3′	CAT PCR primer 2
5′ CCCATTCATCAGTTCCATAGGTTGGAATCT 3′	CAT PCR primer 3
5′ GCGGCATCAGCACCTTGTCGCCT 3′	CAT PCR probe
5′ ATGGATGATGATATCGCCGCG 3′	5′ β-actin PCR primer
5′ CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC 3′	3′ β-actin PCR primer
5′ CTGCCTGACGGCCAGGTCATCACCATTGGC 3′	β-actin PCR probe

shares an overall amino acid homology of 27% (8). Certain regional identities, especially in the carboxy end of the polypeptides, can be as high as 45%. Although the functional role of the ORF4 gene product in VZV replicative cycle is even less well understood than that of ICP27, we and others have previously demonstrated that it is capable of upregulating the expression of both VZV gene promoters and certain heterologous viral promoters (9, 22, 40). The pattern of the effects of ORF4 protein on VZV gene promoters belonging to each kinetic class is reminiscent of that of the effects of ICP27 on diverse HSV gene promoters and suggests a corresponding regulatory role for the ORF4 protein in the ordered expression of VZV genes in its replicative cycle.

With the goal of defining the structure-function relationships of the ORF4 *trans*-regulatory protein, we sought to identify the critical *cis* elements in the ORF4-responsive promoters and to delineate the critical domains of the ORF4 polypeptide which endow it with transactivator function.

MATERIALS AND METHODS

Nomenclature. For the description of amino acids, the single-letter code was used. Mutations were designated as described by Knowles (28); the number defines the position of a mutated amino acid in relation to the first methionine residue, and the substituted amino acid is given after the number.

Cell lines. The CD4-positive continuous human T-cell line A 3.01 (15) was obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases. The A 3.01 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Plasmid constructions. All recombinant plasmids were generated by standard procedures (49). Plasmids pCMV4, pUIIIRCAT, p−167HIVCAT, p−57HIVCAT, pSV2CAT, pA10CAT, E1bCAT, and 5GE1bCAT have been described previously (18, 29, 30, 40, 46). Briefly, in pCMV4, the coding region of VZV ORF4 gene is placed under the strong constitutive promoter of human cytomegalovirus immediate-early promoter and expresses the ORF4 gene product. Plasmids p-167HIVCAT and p-57HIVCAT are deletion derivatives of the human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, driving the chloramphenicol acetyltransferase (CAT) gene. pA10CAT is a derivative of pSV2CAT (18) in which the enhancer region in the simian virus 40 (SV40) promoter has been deleted. In E1bCAT, the CAT gene is expressed from the minimal E1b promoter of adenovirus. Plasmid 5GE1bCAT is identical to E1bCAT except that five tandem copies of the 17-bp element that binds the yeast transcriptional activator GAL4 are positioned immediately upstream of the promoter.

To create a panel of plasmids with different elements (Table 1) positioned immediately upstream of a basal minimal promoter-driven CAT gene, we used the pIFNTATA plasmid as the "parent" construct. Plasmid pIFNTATA contains the promoter elements of the murine α4 interferon gene extending from −39 to +22 (+1 being the transcription initiation site) with a unique *Bgl*II site immediately upstream of −39: this construct is a derivative of pαCAT (25). In pRB45, a segment of the HIV LTR promoter extending from −104 to −32 has been cloned into the unique *Bgl*II site in pIFNTATA (37). Plasmid p2KB+3SpI is a derivative of pIFNTATA in which a 67-bp oligonucleotide cassette (Table 1) containing two NF-κB and three SpI transcription factor-binding elements (as found in the HIV LTR) cloned into the *Bgl*II site. To create plasmid p2KB, a 30-bp oligonucleotide cassette containing two tandemly arranged NF-κB-binding sites (as shown in Table 1) was cloned into the *Bgl*II site in pIFNTATA. Similarly, a 42-bp oligonucleotide cassette containing three tandem copies of SpI-binding sites was cloned into the *Bgl*II site in pIFNTATA to generate plasmid p3SpI. Plasmid pMutSpI was created by cloning a 42-bp oligonucleotide cassette with mutated SpI sites (Table 1) into the *Bgl*II site of pIFNTATA.

To create a panel of SV40 promoter-driven CAT constructs differing only in their 3′ RNA processing signals, the SV40 promoter, including the 72-bp enhancer element, was isolated from pSV2CAT as a 323-bp fragment after digestion with *Pvu*II and *Hin*dIII. This fragment was then cloned into the pSKII (Stratagene) vector, which had been digested with *Eco*RV and *Hin*dIII. The resultant plasmid was then linearized with *Hin*dIII, and cut ends were blunted before cloning of a 800-bp fragment containing the coding region of the CAT gene derived from pSV2CAT as an *Xho*II-*Hin*dIII fragment (both ends blunted prior to cloning) to obtain a plasmid with an SV40 promoter-driven CAT gene. This plasmid was designated as pSVCATNP. This construct does not contain any eukaryotic RNA-processing signals 3′ to the terminator codon of the CAT gene. To create a plasmid similar to pSVCATNP with the exception of having RNA-processing signals of the SV40 early region 3′ to the CAT reporter gene, pSVCATNP was first linearized with *Kpn*I. After blunting the cut ends and redigesting with *Sal*I, an 850-bp *Xho*II fragment (cut ends blunted)

containing the SV40 early-region splice signals and poly(A) sequence from pSV2CAT was cloned to obtain pSVCATSV40. To obtain a derivative of pSVCATNP with the RNA-processing signals of human beta interferon, a 650-bp fragment containing the 3′ untranslated region with the natural poly(A) signal of human beta interferon was derived from plasmid pIRF (38) as a *Kpn*I-*Xho*I fragment. The ends of this fragment were flushed and cloned into pSVCATNP that had been digested with *Xho*I and *Kpn*I (cut ends blunted) to obtain pSVCATβIFN. To create pSVCATmyc, a 617-bp fragment containing the 3′ untranslated region of c-*myc* with the natural poly(A) site was isolated from the genomic c-*myc* clone pHSR-1 (2) after digestion with *Ssp*I and *Eco*RI. This 617-bp fragment was then cloned into pSKII vector that had been digested with *Hin*cII and *Eco*RI to obtain pJM52 (39). Next, pJM52 was linearized with *Pst*I and a 1.1-kb *Pst*I fragment containing the SV40 promoter-driven CAT gene from pSVCATNP was cloned to obtain pSVCATmyc. In pSVCATmyc, the SV40 promoter-driven CAT reporter gene contains RNA-processing signals of human c-*myc* 3′ to the terminator codon of the CAT gene.

To express segments of the VZV ORF4 gene as chimeric proteins containing the DNA-binding domain of yeast transcriptional activator GAL4 (24, 48), we used the fusion vector pBSGAL4-1 (41). First, pCMV4 was digested with *Bgl*II and *Bam*HI to isolate a 1.7-kb fragment, which was then cloned into the *Bgl*II-linearized pBSGAL4-1. The resultant plasmid with the 1.7-kb fragment inserted in the correct orientation was designated p4GAL121–452: it contains the coding region of ORF4 from codon 121 through 452. To generate p4GAL2–121, a new *Bgl*II site was introduced by changing codon 2 (GCC) to AGA in pCMV4 by oligonucleotide-directed site-specific mutagenesis as described previously (16). After digestion with *Bgl*II, a 365-bp fragment was isolated and cloned into *Bgl*II-linearized pBSGAL4-1 to obtain p4GAL2–121 with the 365-bp fragment in the correct orientation. Plasmid p4GAL2–121 contains the coding region of ORF4 from codons 2 through 282. To generate p4GAL2–282, using three oligonucleotide primers, two novel *Bgl*II sites were created by changing codons 2 and 282 to AGA simultaneously while altering codon 122 to AGG to destroy the original *Bgl*II site in the ORF4 coding region. The resultant plasmid was then digested with *Bgl*II to isolate an 840-bp fragment. This 840-bp fragment was then cloned into *Bgl*II-linearized pBSGAL4-1 vector to obtain p4GAL2–282, which contains the ORF4 coding region from codons 2 through 282 fused in frame with the GAL4 DNA-binding segment. To create a GAL4 chimeric protein with the entire coding region of VZV ORF4, pBSGAL4-2 fusion vector (41) was first digested with *Eco*RI. An 800-bp fragment containing the SV40 promoter-driven GAL4 DNA-binding domain segment was then cloned into pNED4 (40) after being linearized with *Eco*RI. The resultant construct with the SV40 promoter-driven GAL4 DNA-binding segment in frame with the VZV ORF4 was designated p4GAL1–452.

A series of substitution mutants of the ORF4 gene product were created by site-specific mutagenesis with pCMV4 as the parent construct (40). All 10 cysteine codons in the ORF4 coding region were mutated to codons specifying serine residues individually. The resultant mutant constructs were then designated as pCMV4C16S, pCMV4C237S, pCMV4C335S, pCMV4C363S, pCMV4C393S, pCMV4C406S, pCMV4C421S, pCMV4C426S, pCMV4C447S, and pCMV4C452S. In addition, codons 417 and 434, specifying histidines in the wild-type ORF4 polypeptide, were changed to codons specifying prolines individually, and the resultant constructs were then designated pCMV4H417P and pCMV4H434P, respectively. Similarly, to generate a mutant ORF4 gene product devoid of the C-terminal cysteine cluster, codon 393 was changed to an ochre terminator (TAA) codon and the resultant construct was designated pCMV4/393T. All recombinant plasmids generated in this study were sequenced across their manipulated regions to ensure the integrity of the constructions.

cDNA synthesis and PCR analysis. A 3.01 cells were electroporated with pSVCATSV40 plasmid DNA alone or in combination with pCMV4 plasmid DNA as described below. After 18 h, total cellular RNA was extracted with RNAzolB (Tel Test B Inc.) as specified by the manufacturer. Total cellular RNA (2 μg) was then treated with 10 U of DNase I for 15 min at room temperature. Samples were then heated to 95°C for 5 min to inactivate the DNase I before 1 μl of hexamer random primers was added (BRL-GIBCO, Gaithersburg, Md.), and the mixture was incubated at 65°C for 10 min. Samples were rapidly chilled on dry ice, and 9 μl of reverse transcription buffer (50 mM Tris [pH 8.3], 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.75 mM deoxynucleoside triphosphate [dNTP] mix) was added along with 200 U of Moloney murine leukemia virus reverse transcriptase. The reverse transcription reaction was performed at 37°C for 1 h. A 2-μl sample from the reverse transcription reaction mix was used for subsequent PCR amplification. The PCR mixture consisted of 50 mM KCl, 10 mM Tris (pH 8.3), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1.5 U of *Taq* polymerase, and 0.5 μM each primer in a total volume of 30 μl. The amplification profile included a preheating step for 3 min at 94°C followed by 30 cycles, each consisting of a 94°C denaturation segment for 1 min, a 60°C annealing segment for 1 min, and a 72°C extension segment for 3 min; the cycles were followed by a final extension at 72°C for 7 min. Amplified samples were then resolved on a 1.25% agarose gel and blotted on to Nylon-I membrane (Bethesda Research Laboratories) by capillary transfer. Blots were then hybridized with oligonucleotide probes that had been labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase.

DNA transfections. All DNA transfections for transient-expression assays were done by electroporation with a Gene-Pulser electroporator (Bio-Rad). For electroporation of A 3.01 cells, cells in log-phase growth were resuspended at a density of 5×10^7 cells per ml in RPMI 1640 medium with 20% fetal calf serum but without glutamine or antibiotics. Aliquots (350 μl) of the cell suspension were mixed with plasmid DNA in sterile 1.5-ml cryovials (Nunc) and incubated on ice for 15 min. The cells were then transferred into an electroporation cuvette with a 0.4-cm electrode gap and were electroporated with a single pulse with the settings at 0.20 V and 960 μF with a capacitance extender. After pulsing, the cells were immediately transferred into 24-well tissue culture plates that had been chilled on ice and incubated for a further 15 min on ice before addition of the growth medium. The cells were then grown for 48 h before harvesting.

CAT assay. Cells were harvested 48 h after DNA transfection and CAT assays were performed essentially as described by Gorman et al. (18). Briefly, the cells were washed once with phosphate-buffered saline, resuspended in 0.25 M Tris-HCl (pH 7.8), and disrupted by three cycles of freeze-thawing. Protein concentrations in cell lysates were determined by using the Bio-Rad protein assay kit as specified by the manufacturer. The CAT activity was assayed by using the same amount of total protein for all samples in an individual experiment and quantitated by using a Phosphor Image scanner with Image-Quant software (Molecular Dynamics Inc., Sunnyvale, Calif.). All experiments were repeated at least three times with independent DNA transfections.

Immunoblot analysis. The plasmid DNA (25 μg) was trans-

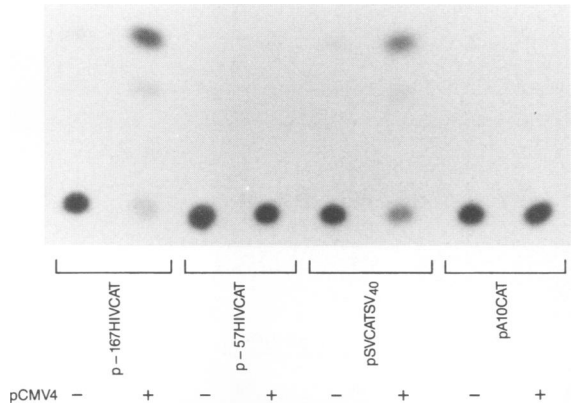

FIG. 1. Importance of enhancer elements for ORF4 inducibility. Equal amounts (5 μg) of effector and target plasmid DNA were electroporated into A 3.01 cells. Cells were harvested 48 h after DNA transfections, and the levels of CAT activity in the cell lysates were determined by using the same amount of total protein for all samples, as indicated in Materials and Methods.

fected into cells by electroporation as described above. After the transfection, cells were plated on 100-mm tissue culture dishes. The cells were harvested 48 h after transfection and used for immunoblot analysis to detect the proteins expressed from the transfected plasmid DNA as described previously (26) with a 1:50 dilution of the polyclonal rabbit antiserum raised against the VZV ORF4 gene product. The bound antibody was then detected with ^{125}I-labeled protein A.

RESULTS

Identification of critical *cis* elements in ORF4-responsive promoters. It has been shown previously that the gene product of VZV ORF4 effectively activates heterologous viral promoters including the HIV LTR promoter and certain VZV gene promoters such as ORF62 and ORF29 (9, 22). To delineate the critical *cis* elements in promoters which render them ORF4 responsive, we chose the HIV LTR promoter as a model system for investigation. The HIV LTR promoter has been extensively characterized and shown to contain numerous *cis* elements that interact with both cellular and viral transcription factors (46). When a panel of diverse plasmid constructs with progressive nested 5′ deletions of the HIV LTR promoter fused to the CAT gene was cotransfected with pCMV4 (ORF4 expression plasmid), there was clear demarcation of the HIV LTR promoter region responsive to ORF4-mediated activation. Plasmid p−167HIVCAT, which contains the sequence elements from +80 to −167 (+1 being the transcription start site) of the HIV LTR promoter, was fully responsive to the ORF4 gene product. In contrast, p−57HIVCAT, bearing only the sequence elements from +80 to −57 of the HIV LTR promoter, was refractory to ORF4-mediated activation (Fig. 1). However, the failure of p-57HIVCAT to respond was not due to a "killed" or nonfunctional promoter, since it retained responsiveness to other viral transactivators such as the VZV immediate-early protein IE62 (data not shown).

The sequence elements between −167 and −57 of the HIV LTR promoter needed for ORF4 inducibility contain the well-defined core enhancer elements of the HIV LTR promoter (46). Two tandemly arranged NF-κB-binding elements and three tandemly arranged SpI-binding elements constitute the HIV LTR core enhancer elements.

To discern whether there are any consensus motifs in different ORF4-responsive promoters, we next examined another well-characterized promoter, i.e., the SV40 promoter, for ORF4 inducibility. As shown in Fig. 1, the SV40 promoter containing the enhancer elements showed strong inducibility in the presence of the ORF4 gene product. However, the ORF4 inducibility was dependent on the presence of the enhancer elements in the SV40 promoter, since pA10CAT, which lacks the enhancer elements, was completely refractory to ORF4-mediated activation.

Evaluation of different transcription factor-binding motifs for ORF4 inducibility. A comparison of enhancer elements in the HIV LTR promoter and the SV40 promoter reveals that they both share the SpI and NF-κB transcription factor-binding motifs (13, 46, 51, 53). To evaluate the contribution of each of these motifs to ORF4 responsiveness, we performed transient-expression studies with a panel of constructs with defined upstream elements. A minimal promoter driving the CAT gene (pIFNTATA) was used as the "parent" plasmid, and synthetic oligonucleotides corresponding to different segments in the HIV LTR enhancer were placed immediately upstream of this minimal promoter. Each of these constructs was then cotransfected with pCMV4.

As shown in Fig. 2, the minimal promoter pIFNTATA was totally refractory to ORF4-mediated activation. However, pRB45, which contains the HIV LTR sequences extending from −104 to −32, was responsive to ORF4-mediated activation. Similarly, p2KB+3SpI, containing two copies of NF-κB sites and three SpI sites, demonstrated significant induction when cotransfected with the pCMV4 plasmid. Next, in an attempt to discern whether the NF-κB-binding motifs or the SpI-binding motifs were responsible for the mediation of ORF inducibility, we tested hybrid minimal promoters containing either two tandemly arranged NF-κB-binding elements (p2KB) or three tandemly arranged SpI-binding elements (p3SpI). Both hybrid minimal promoters were induced by pCMV4, although the NF-κB element-containing construct was more responsive to ORF4-mediated induction (Fig. 2). Insertion of nonfunctional DNA (with mutated SpI sites) did not confer any inducibility to the minimal promoter. The higher basal activity of NF-κB-containing promoters or, alternatively, the preferential association of ORF4 polypeptide with the NF-κB enhancer functions may be responsible for the differences in the magnitude of induction seen with hybrid promoters. Thus, it can be concluded that the presence of upstream elements alone is sufficient to confer ORF4 inducibility to a nonresponsive promoter. The ability of ORF4 polypeptide to enhance the transcriptional activity of target promoters on its own clearly distinguishes itself from the HSV ICP27, which has little or no effect on its own on most target genes in transient-expression assays (52).

Effects of 3′ RNA-processing signals on ORF4-mediated activation. Modulation of gene expression by HSV ICP27 was recently shown to be mediated via a novel, promoter-independent mechanism in transient-expression assays (50). The activation of target promoters correlated with different polyadenylation sites, whereas the repressor activity of ICP27 appeared to be associated with the presence of introns either 5′ or 3′ to the target gene-coding sequences. To determine whether the VZV ORF4 polypeptide possesses the same pattern of promoter-independent gene-modulatory activities, we created a panel of SV40 promoter-driven CAT constructs differing only in the 3′ RNA-processing signals to use as targets in the cotransfection assays. The target plasmid pSVCATNP does not contain any eukaryotic RNA-processing signals 3′ to the CAT gene. Although the inherent basal activity of this

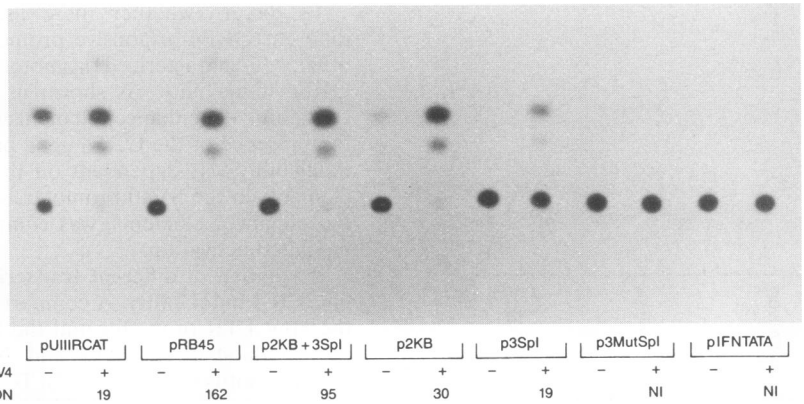

FIG. 2. ORF4 inducibility of minimal promoters with NF-κB- and SpI-binding elements. Equal amounts (5 μg) of effector and target plasmid DNA were electroporated into A 3.01 cells. The CAT activity in cell lysates was measured after 48 h and quantitated with a Phosphor-Imager scanner (Molecular Dynamics) and Image-Quant software. The basal activities of the promoter-CAT constructs in the absence of the effector were 48% (pUIIIRCAT), 4.3% (pRB45), 3.4% (p2KB+3SpI), 6.5% (p2KB), 1% (p3SpI), 0.5% (p3MutSpI), and 0.5% (pIFNTATA). Induction represents the relative increase in the CAT activity in cotransfections with the effector plasmid pCMV4. NI denotes no measurable induction.

plasmid was the lowest, it was still responsive to ORF4-mediated inducibility, as shown in Fig. 3. Interestingly, addition of SV40 early-region RNA-processing signals resulted in the highest basal activity of the target gene, presumably as a result of enhanced stability of this transcript. Addition of either the beta interferon or c-*myc* RNA-processing signals devoid of any intron sequences resulted in intermediate levels of basal activity, a finding consistent with the reported instability of transcripts bearing these RNA-processing signals (4, 58, 59). However, regardless of the 3′ RNA-processing signals, all four targets were induced in cotransfection assays in the presence of ORF4 polypeptide. Thus, from the data shown in Fig. 3, it is apparent that the RNA-processing signals of targets are not the dominant determinants of ORF4-mediated responsiveness of a gene construct.

The data shown in Fig. 1 to 3 collectively suggest that the ORF4-mediated induction of gene expression occurs primarily at the level of transcription. To further substantiate this, we

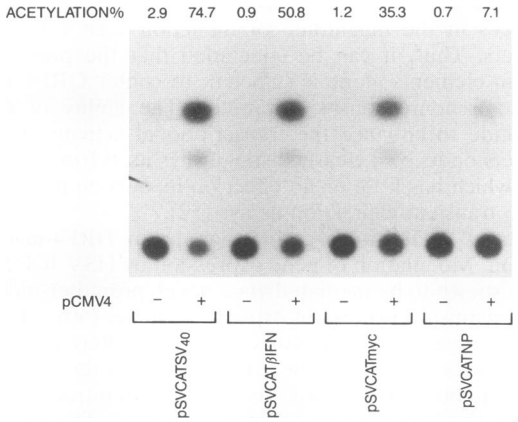

FIG. 3. Effects of 3′ RNA-processing signals in target constructs on ORF4-mediated activation. Equal amounts (15 μg) of target and effector plasmid DNA were used in cotransfections. Increased amounts (15 μg) of target plasmid DNA were needed in transfection assays to obtain reproducible differences in the basal activities of these target constructs with different 3′ RNA-processing signals. Values above the panel are the ratios of acetylated chloramphenicol to its unacetylated form, expressed as percentages.

devised a strategy depicted schematically in Fig. 4A. The nascent primary transcripts (pre-mRNAs) have extremely short intracellular half-lives and are rapidly processed into more stable mature forms. Although an increase in the steady-state levels of a transcript could indicate either an increase in the transcription or an enhanced stabilization of preexisting transcript, the elevated levels of a primary transcript correlate directly with increased transcriptional activity. To detect and quantitate the nascent primary transcript (pre-mRNA) from the target plasmid pSVCATSV40 following transfection in either the presence or absence of pCMV4, we performed PCR amplifications with two combinations of primers in parallel. Primers 1 and 3, when used in the PCR, should detect only the primary transcript with the intact intron sequences and will result in a 487-bp fragment. In contrast, amplification with the CAT coding-region primers 1 and 2 would not differentiate between mature processed transcript and the unprocessed pre-mRNA and should result in an amplified fragment of only 220 bp. As shown in Fig. 4B, we were able to detect the primary CAT transcript with the primer combination 1 and 3, and amplification resulted in a fragment of the expected size (487 bp). The fact that we were able to detect this form of the transcript even when pSVCATSV40 was transfected alone illustrates the extreme sensitivity of the technique used. There was a threefold increase in the primary CAT transcript in cotransfections with pCMV4, although the increase in CAT activity was approximately ninefold in this experiment (data not shown). It should be noted, however, that such a nonlinear relationship between the transcription and CAT activity is not unexpected considering the complexities associated with transcription and translation processes. Nonetheless, the data in Fig. 4 conclusively indicate that the ORF4-mediated induction of target genes occurs transcriptionally.

Localization of the activation domains in the ORF4 gene product. Many *trans*-regulatory proteins that control transcriptional initiation by RNA polymerase II have modular configurations with two domains, one which binds to DNA and the other which activates transcription (reviewed in references 34 and 43). A widely used strategy to localize the activation domains of complex *trans*-regulatory proteins makes use of domain swap assays with the yeast transcriptional activator GAL4 (17, 30, 31, 41). In this system, fusion of the GAL4 DNA-binding domain [amino acids 1 through 147 of the GAL4

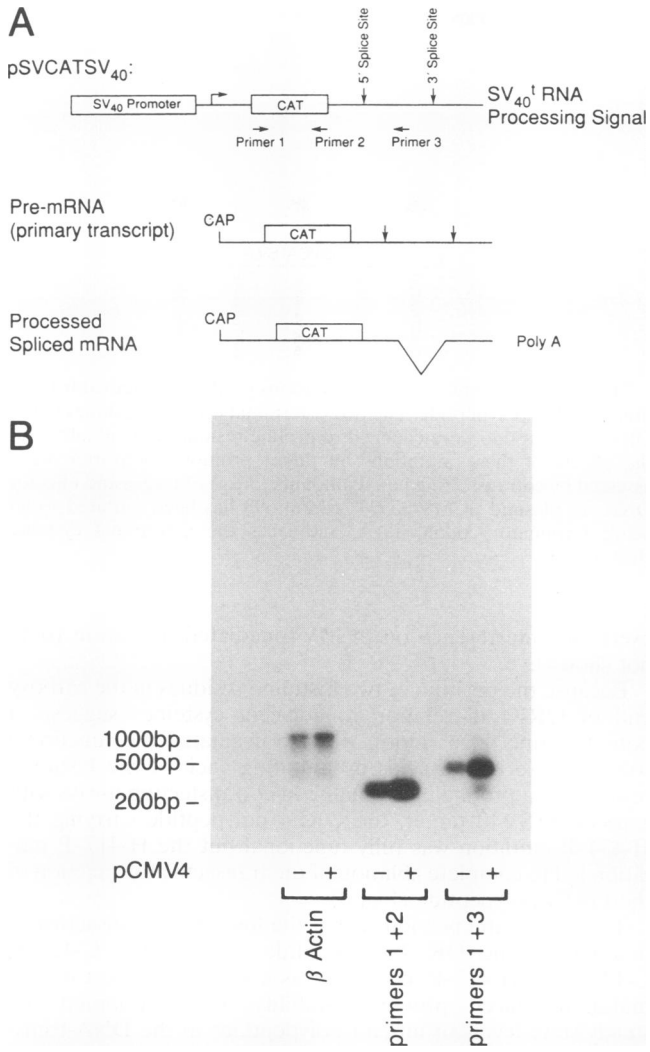

FIG. 4. Detection of nascent primary CAT transcripts in DNA-transfected A 3.01 cells. (A) Schematic drawing of the strategy used to detect primary CAT transcripts following transfection of effector (pCMV4) and target (pSVCATSV40) plasmid DNA. (B) Total cellular RNA was isolated 18 h after transfection of plasmid (5 μg of effector and 5 μg of target) DNA, and cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase as described in Materials and Methods. Following PCR amplification, the amplified DNA was resolved by 1.25% agarose gel electrophoresis, subjected to Southern blotting, and hybridized with ^{32}P-labeled oligonucleotide probes. The sequences of the specific primers used in PCR and hybridization are given in Table 1.

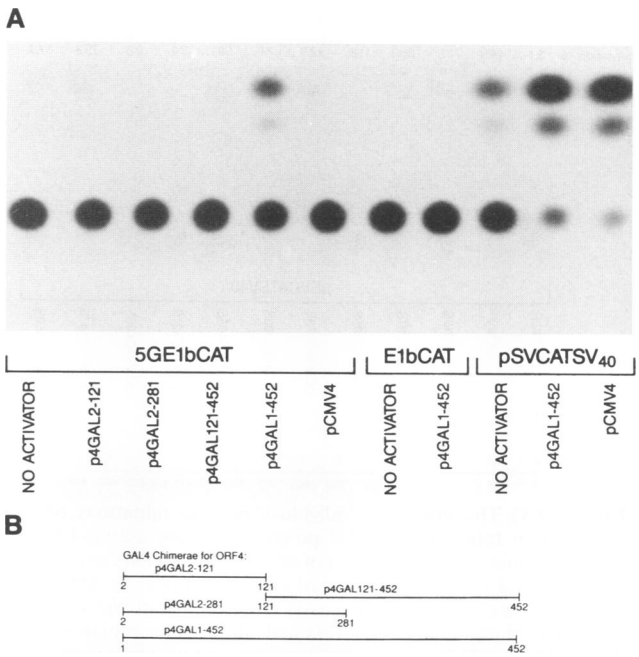

FIG. 5. Use of GAL4-ORF4 chimerae to define the boundaries of ORF4 activation domain. (A) Equal amounts (10 μg) of target and effector plasmid DNA were used for cotransfections. (B) Schematic diagram of different segments of the ORF4 coding region used in the GAL4 fusion constructs.

polypeptide, GAL4(1–147)] with sequences derived from other heterologous transactivator proteins results in chimeric proteins that can specifically activate target promoters containing GAL4-binding DNA elements if the fused heterologous segment contained an activation domain.

As illustrated in Fig. 5B, four overlapping segments representing the entire coding region of VZV ORF4 were used to create GAL4-ORF4 chimeras. When cotransfected with a target promoter bearing five tandem copies of 17-bp GAL4-binding elements (5GE1bCAT), the only chimeric construct that was able to activate the target promoter was p4GAL1–452 (Fig. 5A). In the p4GAL1–452 hybrid plasmid, the entire coding region of VZV ORF4 is present in frame with the GAL4(1–147) segment. That the full-length native ORF4 gene product (from pCMV4) was unable to activate the 5GE1bCAT target plasmid and the p4GAL1–452 construct could not activate the E1bCAT plasmid, lacking any GAL4-binding elements, clearly demonstrate the specificity of the system. To verify that the GAL4 DNA-binding domain in the chimeric polypeptide does not interfere with the natural target promoter recognition by the ORF4 polypeptide, we cotransfected p4GAL1–452 with a natural target promoter, pSVCATSV40 (Fig. 5A). Thus, despite our failure to localize a discrete activation domain to a single region of the polypeptide, the use of the GAL4 chimeric system unambiguously confirms a transcriptional role for the ORF4 polypeptide.

Identification of functionally critical cysteine and histidine residues in the ORF4 polypeptide. The schematic diagram shown in Fig. 6B illustrates the distribution of cysteine residues in the ORF4 polypeptide. There is a conspicuous clustering of cysteine residues in the C-terminal part of the polypeptide. The importance of cysteine residues to the structure and function of proteins is well documented. Cysteines are involved in the formation of critical disulfide linkages essential for proper folding of the polypeptides, oligomerization and subunit assembly of multicomponent proteins, protein-nucleic acid interactions, and metal ion binding. We therefore examined the functional importance of cysteine residues in the ORF4 gene product by mutating in turn each individual cysteine residue to a serine residue. The functional effects of these mutations were then assessed by cotransfecting the mutant plasmids with the pSVCATSV40 target construct. As shown in Fig. 6A, no significant perturbation of transactivator function was seen with C-16–S, C-237–S, C-363–S, C-393–S, C-406–S, C-447–S, and C-452–S mutations. In contrast, the C-421–S and C-426–S mutations completely abrogated the activator function of the ORF4 polypeptide. The C-335–S

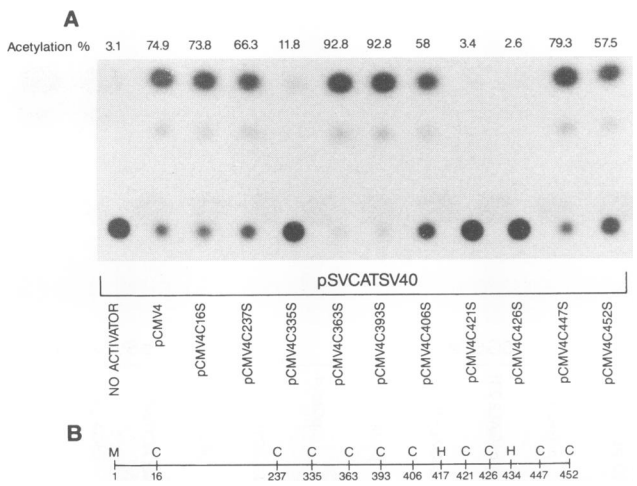

FIG. 6. (A) The effects of individual cysteine mutations on the transactivation function of ORF4 polypeptide were assessed by cotransfecting equal amounts (15 μg) of target plasmid DNA and mutant effector plasmid DNA. The increased amounts of plasmid DNAs (15 μg) were necessary in transfection assays to demonstrate the low levels of transactivating ability still retained in certain mutants (e.g., pCMV4C335S). (B) Distribution of cysteine and histidine residues in the ORF4 polypeptide. Each cysteine residue was individually mutated to a serine residue by site-specific mutagenesis as described in Materials and Methods.

mutation, although it drastically affected the transactivation ability, did not completely abolish this function of the protein. Since mutants of ICP27 with in-frame insertions in the C-terminal cysteine cluster were shown previously to interfere with the gene regulatory activities of the native protein (54), we assessed the effects of coexpression of pCMV4C426S on ORF4-mediated target activation. As shown in Fig. 7, in contrast to what has been reported for ICP27 mutants, pCMV4C426S failed to exert any effects on pCMV4-mediated activation of the target. Similarly, coexpression of pCMV4 C421S or pCMV4/393T in competition experiments failed to

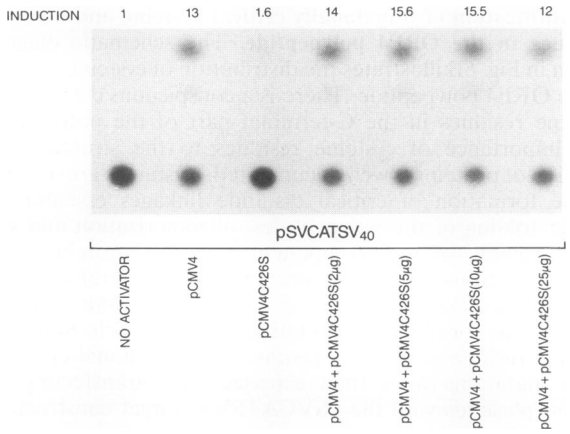

FIG. 7. Effects of transactivation-deficient mutant on native ORF4-mediated activation. Various amounts of pCMV4C426S plasmid DNA were cotransfected with 5 μg of wild-type effector pCMV4. The amount (5 μg) of target plasmid pSVCATSV40 was kept constant in all cotransfections.

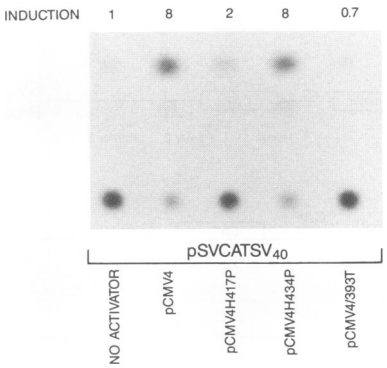

FIG. 8. Effects of C-terminal mutations on the transactivator function of ORF4 polypeptide. The two C-terminal histidine residues in the ORF4 polypeptide were changed to proline residues individually, and the effects of these mutations on target promoter activation were assessed by cotransfecting equal amounts (5 μg) of target and effector DNA. In plasmid pCMV4/393T, codon 393 has been mutated to an ochre terminator codon, TAA, and lacks the C-terminal cysteine cluster.

exert any interference on pCMV4-mediated activation (data not shown).

Because the position of two histidine residues in the carboxy end of ORF4 in relation to adjacent cysteines suggests a potential zinc finger motif, we also evaluated the functional role of these amino acids by mutating each of the histidine residues to a proline individually. In cotransfection assays with a pSVCATSV40 target, the ORF4 polypeptide carrying the H-434–P mutation was fully functional but the H-417–P mutation led to complete ablation of the transactivator function of the ORF4 polypeptide (Fig. 8).

To rule out the possibility that the loss of the transactivator function of the ORF4 polypeptide carrying the C-421–S, C-426–S, or H-417–P mutation was a secondary effect due to mutation-induced protein instability, we determined the steady-state levels of mutant polypeptides in the DNA-transfected cells. As shown in Fig. 9, the steady-state levels of mutant and wild-type proteins were comparable as determined

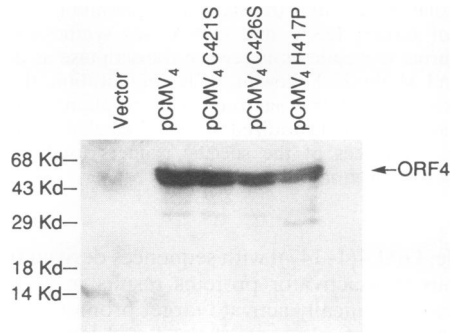

FIG. 9. Expression of wild-type and transactivation-deficient mutants of the ORF4 polypeptide from the transfected plasmid DNA. A 3.01 cells were transfected with 25 μg of plasmid DNA by electroporation. The cells were harvested 48 h after transfection, and the presence of ORF4 polypeptide was detected by immunoblot analysis with equal amounts of crude protein from each sample. The primary antibody used was a rabbit polyclonal antibody raised against the full-length ORF4 polypeptide. The bound antibody was then detected with ^{125}I-labeled protein A. The approximate sizes of marker proteins are shown on the left.

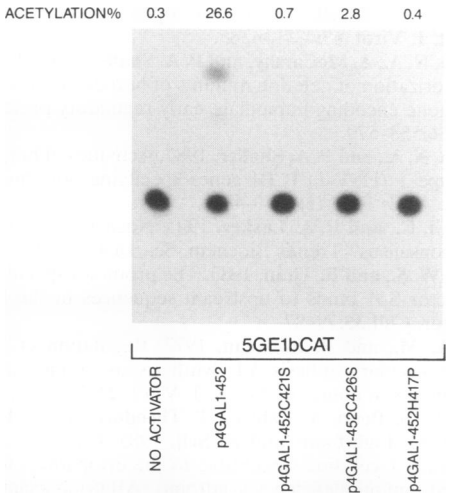

FIG. 10. Transactivation ability of full-length, mutant ORF4 polypeptides fused to the GAL4 DNA-binding domain. To ascertain whether C421S, C426S, or H417P mutations affect the transcriptional activity or the recognition of target elements, we introduced these individual mutations into the chimeric construct p4GAL1-452 by site-specific mutagenesis. The mutant constructs were then cotransfected with the target plasmid 5GE1bCAT as described in Materials and Methods.

by immunoblot analysis with a polyclonal antibody raised against the ORF4 gene product. In addition, the amino acids surrounding the critical cysteines and histidine (ALITHQREC GRVECTLST) do not conform to predicted characteristics of the nuclear localization signals of other nuclear proteins (12), thus making it less likely that the loss of transactivation ability is secondary to defective nuclear localization.

To verify that the C-421, C-426, and H-417 amino acids either are essential for recognition of specific DNA *cis* elements in ORF4-responsive promoters or are important for interactions with the transcriptional complex, we assessed the effects of substitution mutations in these codons in the context of GAL4-ORF4 chimeric proteins with 5GE1bCAT as a target. If these amino acids were essential for the recognition of DNA *cis* elements, the introduction of those mutations should not affect the activation of the 5GE1bCAT target by GAL4-ORF4 chimeric protein, since the tethering of chimeric protein to the target promoter would now occur via the GAL4 DNA-binding domain. Conversely, if the mutations affect the potential interactions of the ORF4 polypeptide with the transcriptional complex, the ability of the GAL4-ORF4 chimeric protein to activate the target promoter would be compromised.

As shown in Fig. 10, all three mutant constructs, p4GAL1-452C421S, p4GAL1-452C426S, and p4GAL1-452H417P failed to activate the 5GE1bCAT target in cotransfection assays. Thus, these findings suggest that the C-421, C-426, and H-417 amino acids in the ORF4 polypeptide may play an essential role in the transcriptional activity of the polypeptide.

DISCUSSION

Currently, five VZV gene products are known to possess *trans*-regulatory activities in transient-transfection assays; these are the gene products of ORF4, ORF10, ORF61, ORF62, and ORF63 (22, 23, 35, 36, 40, 42). Although accumulating evidence suggests that the IE62 protein (the product of ORF62) is a potent promiscuous transactivator, the other VZV *trans*-regulatory proteins have been shown to modulate the expression of only a limited set of VZV promoters (9, 22, 23, 35, 36). Whatever their specificity, however, the molecular mechanism(s) underlying the *trans*-regulatory activities of any of these VZV gene products remains to be elucidated.

In the present study, we have attempted to dissect the *trans*-regulatory activities of the ORF4 gene product by examining both *cis* elements in responsive promoters as well as functional domains of the polypeptide itself, using a variety of genetic approaches.

A striking feature we identified in ORF4-responsive promoters is the presence of upstream elements (13, 46, 51, 53). Accordingly, the removal of selected upstream elements from the responsive promoters renders them refractory to ORF4-mediated inducibility. Furthermore, insertion of these elements upstream of a basal promoter renders it ORF4 responsive (Fig. 2). In our studies, both NF-κB and SpI can effectively confer ORF4 inducibility to a nonresponsive basal promoter. Thus, it may be concluded that upstream elements are required to mediate ORF4 inducibility, but there is no strict specificity as to a particular upstream element.

Two mechanistic models consistent with the upstream element requirement for ORF4 inducibility can be proposed. First, it is possible that the upstream elements merely function to boost the basal transcriptional activity of the promoter and that activation by the ORF4 gene product is not discerned until a certain threshold of ongoing transcription is achieved. A second possibility is that the upstream elements and their associated proteins interact more directly with the ORF4 gene product. These interactions, in turn, facilitate the engagement of the polymerase complex more efficiently, thereby leading to increased transcription. This model mandates the prior interaction of the ORF4 gene product with upstream element function before it could modulate the polymerase complex.

However, our findings with the GAL4 chimeric gene constructs show that if the ORF4 gene product is guided to the vicinity of the polymerase complex by tethering the fusion protein to the promoter-proximal DNA, it is capable of augmenting the transcriptional activity of the promoter over 80-fold. This argues against the first model, which specifies a high level of basal transcription for ORF4 inducibility. In addition, the results of studies with the GAL4 chimeric system raise an intriguing possibility, namely that the upstream elements and their associated proteins may be involved in recruiting the ORF4 polypeptide to the promoter-proximal vicinity in a manner analogous to the GAL4 component in the chimeric system. Such involvement of cellular intermediates in recruiting transcriptional activators is not without precedence; for example, the HSV VP16 transactivator is targeted to responsive TAATGARAT elements by the cellular Oct-1 protein (56). Thus, our data favor the second model, i.e., that it is the nature of the transcriptional complex influenced by the promoter and promoter-upstream elements rather than the number of template-engaged complexes that specifies the ORF4 inducibility.

Although we have clearly established a transcriptional role for the VZV ORF4 gene product in this study, we cannot rule out any additional posttranscriptional role for this protein in gene regulation. However, posttranscriptional effects of the ORF4 polypeptide, if any, do not appear to be specific for a particular type of 3′ RNA-processing signal as has been suggested for HSV ICP27.

The gene product of VZV ORF4, as well as its homologs, the ICP27 and UL3 gene products of HSV-1 and equine herpesvirus type 1, respectively, contains a highly conserved, cysteine-rich region in the C-terminal portion of the polypeptide. Moreover, it has been suggested that the arrangement of

cysteine residues in this region resembles zinc finger motifs seen in DNA-binding proteins (33, 44). In ICP27, certain mutations in this region not only result in ablation of the *trans*-regulatory functions of this protein but also generate mutant proteins that effectively interfere with the regulatory activities of the native protein (54). Similarly, when the carboxy-terminal region of the UL3 gene, the equine herpesvirus type 1 homolog of ICP27, is deleted, the mutant protein is no longer able to induce the equine herpesvirus type 1 late-gene promoters in transient-expression assays (55). Consistent with the above findings, when we mutated cysteine codon 393 (the first of the C-terminal cysteine cluster) in VZV ORF4 to a TAA ochre codon, the mutant protein was completely devoid of any transactivation ability. Taken together, these findings clearly argue for a critical role for the C-terminal portion of these related proteins. However, our mutational analyses of the C-terminal cysteine and histidine residues of the ORF4 polypeptide do not support the presence of a functional zinc finger motif. First, of six cysteine residues in this region, only two, C-421 and C-426, were critical for ORF4-mediated inducibility of target promoters. Second, the relative position of the critical histidine residue (H-417) to the other two essential cysteines (C-421 and C-426) does not conform to any hitherto described zinc finger motif. Finally, if these three amino acids, i.e., C-421, C-426, and H-417, were an integral component of a functionally essential DNA-binding domain, GAL4-ORF4 chimeric proteins with identical mutations in these residues should still be functional, since in the chimeric proteins the tethering to DNA occurs via the GAL4 DNA-binding domain. As shown in Fig. 10, this clearly is not the case. Thus, our findings are most compatible with a model in which protein-protein interactions are essential for ORF4 inducibility and in which amino acids C-421, C-426 and H-417 play a critical role in these interactions.

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