# Anatomy of the Varicella-Zoster Virus Open-Reading Frame 4 Promoter

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**The regulation of varicella-zoster virus (VZV) gene expression is largely controlled at the transcriptional level by a few key viral proteins cooperating with one another and with cellular transcription factors. However, the mechanisms involved are largely unclear. To identify the sequences important for the transcriptional regulation of open-reading frame (ORF) 4, itself encoding a transcriptional regulator, a mutation analysis of the promoter was done. These studies identified an element between −69 and −59 (relative to the transcriptional start site), which was critical to the activity of the promoter upon stimulation by the VZV transactivator IE62 and by VZV infection. DNA-protein interaction studies revealed that VZV induced the binding of a specific protein complex to this element, which contained the ubiquitous transcription factor USF. ORF 4 is the second VZV gene (in addition to VZV ORF 29) in which USF binding plays a critical role in gene expression.**

The expression of viral genes in all herpesviruses is regulated in a temporal fashion known as the lytic cascade, in which sequential waves of viral genes are expressed under tight transcriptional control [1–3]. Depending upon the timing and requirements for transcription, each herpesvirus gene can be placed into one of three classes: immediate early (IE), early, or late. The IE genes are transcribed without need for new viral protein synthesis and possess promoters that are stimulated by preexisting cellular transcription factors operating alone or in conjunction with virion-associated transactivating proteins [3–6]. Transcription of the early or $\beta$ genes requires one or more of the IE proteins, and late or $\gamma$ gene transcription additionally requires ongoing viral DNA replication before maximal rates of transcription are achieved.

While evidence clearly demonstrates a varicella-zoster virus (VZV) lytic cascade [7], the assignment of VZV genes to specific transcriptional classes has been difficult because of the poor tissue culture growth of VZV and the very low cell-free titers that can be obtained [8]. Single-step, high multiplicity infections cannot be done. It has been assumed that corresponding genes in closely related and better-studied herpesviruses are likely regulated in a fashion similar to that in VZV, and thus they serve as models for comparison. VZV has most often been compared with herpes simplex virus type 1 (HSV-1), which is closely related to VZV both at the level of amino acid homology and the level of genomic organization [8–11].

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HSV-1 expresses five IE genes, of which four have counterparts in VZV [8–10]. All HSV-1 IE gene promoters contain a common *cis* element, known as the TAATGARAT motif, through which transcriptional activation is predominantly mediated upon HSV-1 infection. This is the target for an activating protein complex composed of a virion transactivating protein (VP16) and several cellular factors, including the ubiquitous cellular factor OCT1 [3–6] (reviewed in [12]). Extension to VZV predicts that the VZV homologues of the HSV IE genes (namely, open-reading frames [ORFs] 4, 61, 62, and 63) would also be regulated as IE genes. However, evidence suggests a more complicated picture. Analysis of VZV protein sequences shows that VZV is more closely related to two animal herpesviruses, pseudorabiesvirus and equine herpesvirus type 1, both of which express only one IE gene, the homologue of VZV IE62 [13, 14]. Although homologues of other HSV-1 IE genes are present in these viruses, their transcription is not expressed under IE conditions but rather as early genes. Therefore, there is some ambiguity over the regulatory class of the four ''putative'' VZV IE genes. Adaption of the typical cycloheximide-reversal experiment to VZV, in which only IE genes are allowed to express proteins, has suggested that ORF 62 [15], ORF 63 [16], and possibly ORF 4 [17] are made in some cell-free VZV-infected cells under these conditions.

The promoter structure of these four VZV genes provides some insight into the means by which they are regulated. That of VZV ORF 62 appears to be like a typical alphaherpesvirus IE promoter: It is complex, contains TAATGARAT motifs, and can be transactivated by the virion-associated activation protein from ORF 10, which is the VZV homologue of HSV-1 VP16 [18–20]. In contrast, promoters of VZV ORFs 4, 63, and 61 do not contain any TAATGARAT elements and are not activated by either VZV ORF 10 or HSV-1 VP16 virion proteins [18, 20]. Therefore, either VZV ORFs 4, 61, and 63 are regulated as early genes or they are IE genes and regulated through promoter elements substantially different from their HSV-1 homologues.

To resolve this issue further, we examined in detail the structure of the promoter driving expression of ORF 4.

## Materials and Methods

*Plasmids and DNA construction.* The plasmid pG310 (gift of E. Mocarski, Stanford University, Stanford, CA) is an expression vector, which contains the complete human cytomegalovirus IE promoter and the SV40 late polyadenylation signal. Plasmid pKCMV62 contains the entire coding region of ORF 62 in the vector pG310 so as to be under the control of these elements (sequences present in the construct were from 120,666 to 124,818 with respect to the published VZV DNA sequence [9]). The plasmid pCMVgal, expressing the gene for $\beta$-galactosidase under the control of the CMV IE promoter, was purchased from Life Technologies (Gaithersburg, MD). DNA fragments analyzed for promoter activity were generated by polymerase chain reaction (PCR) amplification methods, using synthetic oligonucleotides containing additional 5′ sequences that added either a *Pst*I or *Hind*III site to the distal end and a *Pst*I site to the proximal end of the amplified DNA fragment to facilitate cloning. The PCR-amplified products were digested with restriction enzymes and cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in the *Hind*III/*Pst*I sites of the reporter vector pCAT-basic (Promega, Madison WI).

Plasmid DNA from correctly oriented positive clones was isolated by use of DNA-binding columns (Qiagen, Santa Clarita, CA), and the insert was completely sequenced. For site-specific mutagenesis, a Mutagene kit (Bio-Rad, Hercules, CA) was used to replace specific 6- to 8-bp segments within the promoter with sequences including a *Bgl*II restriction site (AGATCT) into the construct p-110CAT. All mutagenized DNA inserts were completely sequenced. Figure 1B shows the sequences generated in the −110 promoter background.

*Cells, virus, and transfections.* The Scott strain of VZV (isolate 71004) was used throughout these studies [21] and was grown on the human melanoma cell line MeWo (gift of C. Grose, University of Iowa, Iowa City, IA) at 35°C as previously described [18, 21]. For transfections, MeWo cells were seeded at $5 \times 10^5$ cells/35-mm dish the day before transfection.

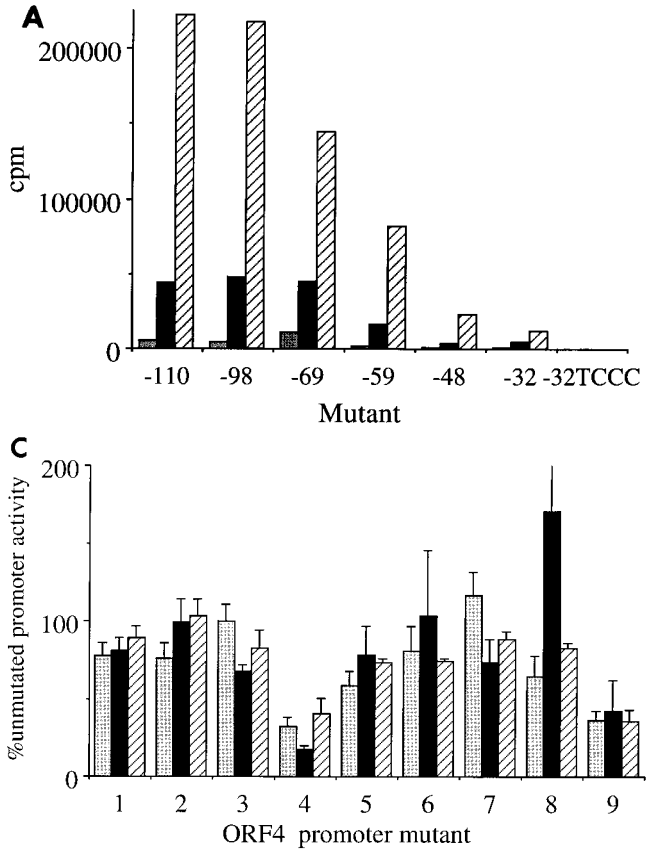
Transfections were initially done using a modification of the HEPES-buffered calcium phosphate coprecipitation procedure as described elsewhere [22] with 0.1–0.3 $\mu$g of effector plasmid to 1.5 $\mu$g of reporter construct plasmid and 24 $\mu$g of sonicated salmon sperm carrier DNA in a total volume of 1 mL. Precipitated DNA (0.5 mL) was added dropwise to cells in 5 mL of medium and incubated at 35°C for 16 h, at which time the medium was replaced with 3 mL of fresh medium per well. In later experiments, we used DNA-Lipofectamine complexes for transfections because of increased transfection efficiency, but ratios of effector and reporter plasmids similar to those just described were used. Cells were exposed to Lipofectamine-DNA complexes for 16 h in serum-free medium. In experiments using VZV infection to stimulate the ORF 4 promoter, cells were overlaid with VZV-infected MeWo cells at $\sim 5 \times 10^4$ VZV-infected cells at the time of medium addition following exposure to DNA complexes. When performed, control, mock-infected transfections were overlaid with $5 \times 10^4$ uninfected cells. In all transfections, cells were harvested 48 h after transfection (24–30 h after infection).

*Reporter assays.* Activity of $\beta$-galactosidase in extracts was measured by a colorimetric assay, using *o*-nitrophenyl-$\beta$-D-galactopyranoside as the substrate and spectrophotometric estimation at 420 nm. CAT activity was assayed by a phase partition assay, using [$^{14}$C]acetyl-CoA as the substrate [23]. A reaction mixture consisting of 50 $\mu$L of lysate, 22.5 $\mu$L 0.1 *M* Tris HCl (pH 8), 50 $\mu$L 2.5 *M* chloramphenicol, and 2.5 $\mu$L (0.05 $\mu$Ci) of [$^{14}$C]acetyl-CoA was incubated at 37°C for 1 h. Acetylated chloramphenicol was phase-extracted into 500 $\mu$L of ethyl acetate, and $^{14}$C counts per minute were determined in a liquid scintillation counter. To control for transfection variability, protein loss, and other variables, all CAT activities from parallel transfections were normalized to $\beta$-galactosidase activity. Reported levels of activity are represented as means (±SD) of results from three separate but identical transfections.

*Electrophoretic mobility shift assays (EMSAs).* Double-stranded oligonucleotide probes spanning regions of the wild-type ORF 4 promoter or containing mutations within it were commercially purchased (BioSynthesis, Lewisville, TX). Double-stranded oligonucleotides representing the consensus binding sites for the transcription factors AP1, SP1, NF$\kappa$B, Oct1, CREB, and TFIID were purchased from Promega, and those for the USF consensus binding sequence and the mutated sequence were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotide probes were end-labeled to high specific activity using [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase.

VZV-infected nuclear extracts were prepared from confluent 175-cm$^2$ flats of attached VZV-infected MeWo cells or from uninfected MeWo cells. Nuclear extracts were prepared using a modification of an established protocol [24]. In brief, washed and harvested cells were suspended in a 5× pellet volume of hypotonic buffer (10 m*M* HEPES, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.2 m*M* phenylmethanesulfonyl [PMSF], 0.5 m*M* dithiothreitol [DTT]), and nuclei were released by 10–20 strokes of a type B pestle in a glass Dounce homogenizer. Pelleted nuclei were resuspended at 100 $\mu$L/175-cm$^2$ flat in low-salt buffer (20 m*M* HEPES, 25% glycerol, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.02 *M* KCl, 0.2 m*M* PMSF, 0.5 m*M* DTT), and an equal volume of high-salt buffer (20 m*M* HEPES, 25% glycerol, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 1.2 *M* KCl, 0.2 m*M* PMSF, and 0.5 m*M* DTT) was added dropwise. Nuclei were then extracted for 30 min at 4°C and pelleted by centrifugation at 25,000 *g* at 4°C for 30 min. The supernatant containing the nuclear extract was dialyzed against a buffer containing 20 m*M* HEPES, 20% glycerol, 100 m*M* KCl, 0.2 m*M* EDTA, 0.2 m*M* PMSF, and 0.5 m*M* DTT for 3–5 h at 4°C. The extracts were snap-frozen and stored in liquid nitrogen.

EMSA assays were done according to the method of Chodosh [25]. Nuclear extract (2–5 $\mu$g) was incubated in gel shift binding buffer (4% glycerol, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* DTT, 50 m*M* NaCl, 10 m*M* Tris-HCl [pH 7.5], 50 $\mu$g/mL poly[dI-dC]) for 10 min. One microliter (50,000–200,000 cpm) of the end-labeled oligonucleotide probe was added and incubated for 20 min at room temperature. In competition assays, a 10- to 200-fold excess of unlabeled oligonucleotide competitor was added and incubated for 10 min prior to the addition of the labeled probe. For supershifts, 1–2 $\mu$g of antibodies was added following incubation with the labeled probes and incubated for an additional 30



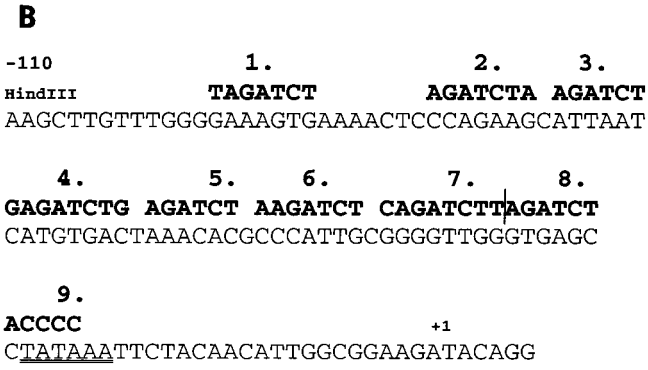




**Figure 1.** **A**, Representative experiment showing relative levels of CAT activity expressed from open-reading frame (ORF) 4 promoter and deletions therein. Counts are expressed as cpm per reaction of [$^{14}$C] acetylated chloramphenicol and are normalized to levels of galactosidase activity as described in Materials and Methods. *x* axis represents base pairs from which upstream sequences (given with respect to transcriptional initiation site of ORF 4) were deleted. Construct −32TCCC has all sequences upstream of −32 deleted and has TATA box mutated to TCCC. **B**, Sequence of −110 to +6 ORF 4 promoter. Bold indicates altered sequences introduced by site-specific mutagenesis over respective bases to create *Bgl*II site in 9 respective constructs. Promoter sequences in p110CAT construct included sequences from −110 to +32; *Hind*III site located at −110 is natural within sequence. **C**, CAT activities of wild-type and mutated promoters outlined in **B**, expressed as proportion of activity of unmutated −110 to +32 construct. Each is result of 3 independent transfections normalized to levels of $\beta$-galactosidase activity expressed from cotransfected plasmid containing *Lac*Z under cytomegalovirus immediate-early promoter. Bars: gray, unstimulated; hatched, stimulated by IE62; black, activation following superinfection with VZV-infected cells. Stimulation of CAT activity from full-length unmutated p110CAT construct by VZV infection was 19-fold over base level and 84-fold over base level when coexpressed with pKCMV62.

min at room temperature. All reaction products were placed in loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 6 m*M* EDTA, 1× TBE, 3% ficoll) and resolved on nondenaturing 4% polyacrylamide gels by electrophoresis in 0.5× TBE (0.045 *M* Tris-borate, 0.001 *M* EDTA, pH 8.0). The gels were dried and visualized by autoradiography. Antibodies to USF and Max transcription factors were obtained from Santa Cruz Biotechnology.

## Results

*Sequence elements required for ORF 4 promoter function.* The approach used to identify the key ORF 4 promoter *cis* elements is based on previous mapping studies of the ORF 4 transcriptional initiation site [18] and is similar to the approach used to dissect the ORF 28 and 29 promoters [26]. Regions of the ORF 4 promoter containing all sequences upstream of the start codon of the ORF were derived by PCR and tested for activity to drive transcription of CAT. A series of ORF 4 promoters was generated that contained deletion of the promoter sequences from the distal end.

Figure 1A shows the levels of CAT expression from cells 48 h after transfection from constructs under three conditions: alone; following cotransfection with plasmid pKCMV62, expressing the activator IE62 [20, 27]; and following superinfection with VZV-infected cells. Deletion of sequences from −201 to −110 had little effect on the activation of the promoter by either VZV infection or by IE62 (data not shown). Figure 1A shows a representative experiment of constructs deleted within position −110 of the ORF 4 promoter. VZV activated the −110 promoter and larger promoters to a level ~17-fold over base levels, and IE62 caused an activation of 95- to 105-fold over base levels. CAT levels following activation by IE62 were consistently higher than that generated by VZV infection, most likely because the levels of IE62 expressed from the CMV IE promoter were probably much higher than that generated by VZV infection. Deletion of sequences between −98 and −69 resulted in a reduced level of activation of the promoter by IE62

but did not significantly affect activation by VZV infection. However, there was a substantial decrease in CAT activity expressed from constructs with deletions −69 or closer to the transcription start site, with activity falling as sequences internal to −59 were deleted. This suggests that elements within position −69 were important for maximal gene transactivation of the ORF 4 promoter. Of interest, deletion of sequences up to the minimal TATA box did not eliminate activation of the promoter, and only when the TATA box was mutated was promoter activity abolished, suggesting that there may be mechanisms of nonsequence-specific, low-level transactivation occurring.

To identify additional elements that might be important for promoter activity, we altered the −110 construct by site-specific mutagenesis to insert base substitutions into regions of the ORF 4 promoter sequence, creating a *Bgl*II site. Figure 1B shows the original ORF 4 promoter sequence within −110 to +6 and the mutations introduced. Each mutation was assessed in the context of the full −110 to +32 promoter. Figure 1C shows the combined results of three experiments for all nine constructs under unstimulated conditions, cotransfection with pKCMV62, or superinfection with VZV. All levels are equalized to constitutively expressed $\beta$-galactosidase and are shown as a percentage of the full-length construct. In these assays, VZV infection stimulated the wild-type −110 promoter a mean of 19-fold over the base level, and coexpression with IE62 resulted in a >84-fold activation over the base level. Cotransfection with the empty CMV IE promoter vector or superinfection with uninfected cells did not result in significant activation of the promoter over the base expression (data not shown).

The transactivated activity of the promoter fell to 26%–37% when the sequences between −69 and −59 were altered (p4CAT) for all conditions. Mutation of the sequences flanking this region (p3CAT and p5CAT) also had significant, but less, effect on the activity of the promoter. As expected, mutation of the putative TATA box (p9CAT) resulted in a substantial loss of CAT activity, although surprisingly, it did not completely eliminate activity. However, in mutant 9, there remains an AATT sequence, which might still be sufficient to allow some initiation of transcription. These results were consistent with the deletion studies and pointed to an element between approximately −69 and −59 that was critical for function of the ORF 4 promoter. Additional but less important factors might be affected by the scanning mutations introduced into the promoter, as indicated by mutants p3CAT and p4CAT. Furthermore, the mutation of sequences immediately upstream of the TATA box in mutant 8 resulted in a slightly more efficient activation of the promoter by VZV infection. The underlying cause of this is not known, although it is feasible that a repressor activity is removed by mutations introduced in mutant 8, thus allowing better VZV activation. However, the most critical region affecting transcription was located between −69 and −59, and it was therefore examined for binding of transcriptional regulatory proteins.

*Proteins binding to the ORF 4 promoter sequences.* A radioactive double-stranded DNA probe designated $W_0$ was used in EMSA assays, representing sequences from −82 to −49, to identify the proteins that bound to the ORF 4 promoter within the critical region identified by mutational analysis. In addition, a similar probe of the same length, which contained the sequence changes between −69 and −59, was tested ($M_0$). When assayed with nuclear extracts obtained from VZV-infected cells that had been harvested at progressive days after infection, a change was identified in the proteins binding to the wild-type ORF 4 promoter probe (figure 2A). At least six gel-retardation complexes were identified. Complexes D and E were prominent in uninfected cells and formed with both the wild-type ($W_0$) and mutant ($M_0$) probes, but both became much less prominent as VZV infection progressed (specific activity of probe $M_0$ is 2- to 3-fold higher than probe $W_0$ in figure 2A). A weak binding activity of a wide mobility range was seen with uninfected cell extracts on overexposure and was designated complex C (figure 2B, lane 2). Three VZV-induced complexes were designated A, A′, and B (figure 2A, lanes 4–6). Mutations of the critical region identified in the functional promoter assays, as presented with probe $M_0$, resulted in considerable reduction in binding of the complexes A and A′ (figure 2A, lanes 8–10), suggesting that the proteins forming complex A or A′ (or both) were likely those required for activation of the ORF 4 promoter.

Computer sequence analysis predicted a consensus binding site for the bHLH family of transcription factors at −69 to −63 (5′-CATGTG), which differed by only 1 base from the reported binding site for the cellular transcription factor USF (5′-CACGTG) [25, 26]. In addition, two potential binding sites for the transcription factor AP1 were predicted at −74 to −68 and −65 to −59. To identify which proteins played a role in the formation of complexes A and A′, double-stranded oligonucleotides that contained consensus sequences for transcription factors were used in competition experiments. The weak complex C identified in uninfected cells (figure 2B, lane 2) was specifically competed by AP1 consensus oligonucleotides (figure 2B, lane 1) but not by SP1, Oct1, or NF$\kappa$-B sequences (data not shown). However, the AP-1 oligonucleotides were found not to compete for the complexes A, A′, and B in VZV-infected cells, suggesting that AP-1 was not involved. However, consensus oligonucleotides for USF competitively inhibited the formation of VZV-specific complex A (figure 2B, lane 5). A 2-bp mutation within the consensus USF site in the USF competing oligonucleotide resulted in the inability of the competitor to compete with complex A (figure 2B, lane 6). Competitor probes for transcription factors AP-1, Oct-1, and Nf$\kappa$-B also failed to compete with any VZV-specific induced complexes (data not shown), suggesting that results indicating binding of USF were highly specific.

To confirm these results, we used antibodies to USF in supershift experiments. Because other bHLH cellular transcription factors, notably Myc and Max, also can bind to the USF site [28] and could also possibly participate in protein binding

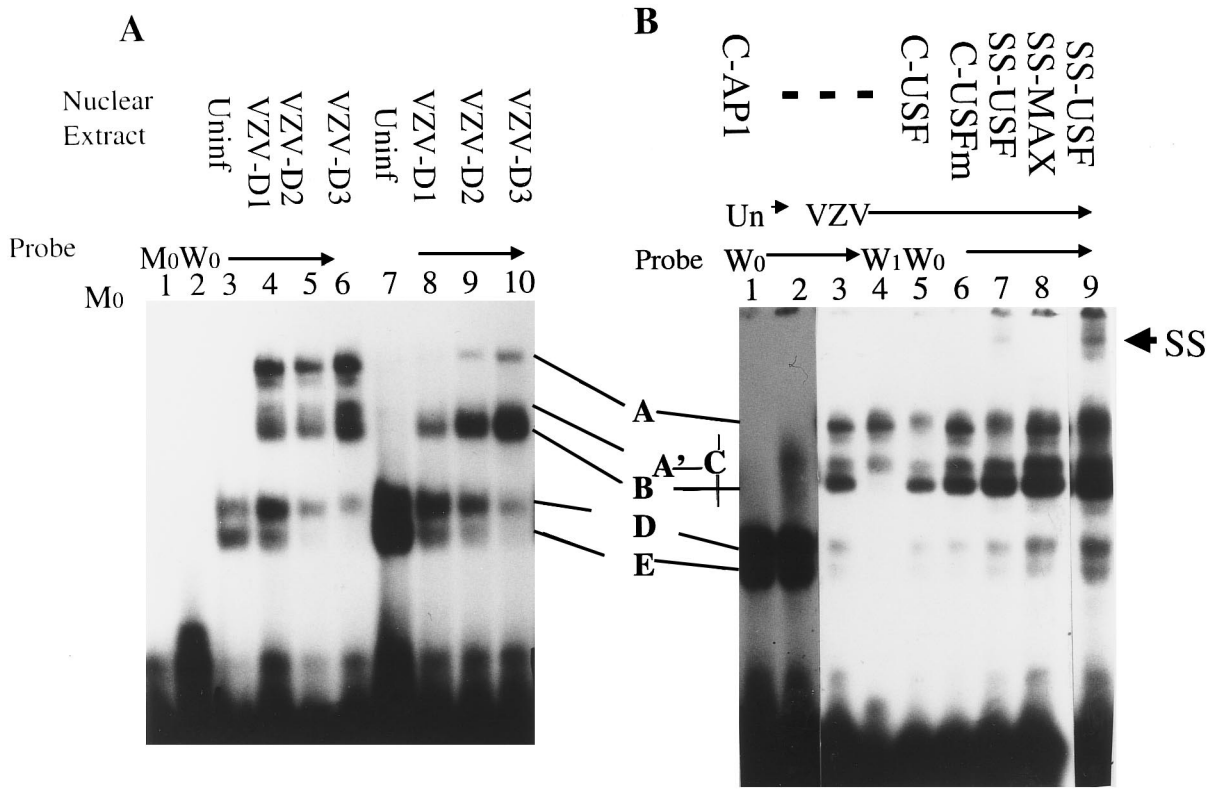



**Figure 2.** Electrophoretic gel mobility shift assays to examine open-reading frame (ORF) 4 promoter. **A**, Double-stranded radiolabeled wild-type probe ($W_0$) or probe containing mutations as discussed in text ($M_0$) were reacted with nuclear extracts from uninfected cells (Unif; lanes 3 and 7) or VZV-infected cells at days 1, 2, or 3 after infection (VZV-D1, -D2, -D3). Complexes were resolved by acrylamide gel electrophoresis under native conditions. Lanes 1 and 2 contain probes only with no nuclear extracts. **B**, Analysis of complexes formed by uninfected cell extracts (Un) or day 2 VZV–infected cell extracts (VZV) with wild-type probe $W_0$ or probe $W_1$ deleted for 8 bp (representing deletion of sequences from −32 to −40 on ORF 4 promoter). Complexes were either untreated (horizontal bar), reacted in competition assay with 50-fold cold consensus double-stranded oligonucleotides as described in text (C-AP1, C-USF, C-USFm), or subsequently reacted with antibodies to USF (SS-USF) or to MAX transcription factor (SS-MAX). Arrow indicates supershifted complex in lane SS-USF. Lane 9 is identical to lane 7 but is exposed to show supershifted complex more clearly.

to the ORF 4 promoter, we also tested the ability of anti-Max antibodies to supershift the fragment. When tested, the USF-specific antibodies resulted in a very slow mobility complex (SS in figure 2B, lane 9), and part of the complex that formed at the position characteristic for complex A was reduced (figure 2B, lane 7). Max antibodies failed to alter the pattern of gel-retarded complexes or yield a supershift (figure 2B, lane 8). Together with the lack of binding of complex A to the mutant probes, these results strongly suggest that complex A contains USF as a component and that its binding is required for the efficient transcriptional stimulation of ORF 4.

Complex B, the faster migrating form of the VZV-specific complexes, was competitively inhibited by a consensus oligonucleotide derived from the VZV IE62 binding site described by Wu and Wilcox [28] (data not shown). Deletion of 8 bp from the right-hand end of the wild-type probe $W_0$ resulted in a gel-retardation profile that lacked complex B but retained the ability to bind complex A (figure 2B, lane 4). In addition, when the VZV IE62 binding site oligonucleotide was used as a probe, a single complex formed that was competitively inhibited by the full-length $W_0$ probe but only weakly inhibited by probe $W_1$, in which the right-hand 8 bp had been deleted (data not shown). This suggests that complex B represents binding outside the critical USF binding region and might represent a high-affinity binding of IE62 to the promoter sequences between the USF binding site and the TAATA box. Mutations created in constructs 4n6CAT are within this region, but they do not strongly affect the activation of the promoter by VZV infection and coexpression with IE62. This suggests that complex B may be supplemental but not essential for the efficient transcriptional activation of the ORF 4 promoter.

## Discussion

In this study, an element in the ORF 4 promoter was identified that was critical for its optimal activation by VZV transactivators. The element was delineated to sequences between −69 and −59 and was shown to bind complexes containing the

cellular transcription factor USF. This is the second VZV gene in which USF has been shown to play an important role.

USF is comprised of two factors, USF-1 [43-kDa) and USF-2 [44-kDa) [29, 30], which are members of the bHLH family of transcription factors and bind to the consensus DNA sequence 5′-CACGTG-3′ [31]. While USF is present in a wide range of mammalian cells [32], our results demonstrated that binding to the ORF 4 promoter occurred only following VZV infection, suggesting that VZV employs a mechanism that recruits a USF-containing complex to the promoter. Since IE62 efficiently stimulates this promoter and since mutations that fail to bind USF are poorly activated by IE62, it seems likely that IE62 may facilitate this binding. The marked increase in activity over base levels with both infection and IE62 coexpression implies that both IE62 and USF are necessary for maximal stimulation of the ORF 4 promoter and that these two proteins act in conjunction. These findings are similar to those of Meier et al. [26] for the presumed early regulated ORF 28 and ORF 29 promoters. Meier et al. demonstrated that cellular USF in conjunction with viral IE62 activates the bidirectional promoter regulating these genes, and they proposed a model in which USF targets the promoter for activation by IE62 and in which both USF and IE62 bind to the promoter and interact to stabilize the transcription complex. The precise mechanisms by which this occurs remain undefined.

USF binding has been described for the promoters of the VZV late protein encoded by ORF 10 and the late HSV-1 and HSV-2 genes encoding Vmw65 [33], and it has also been shown to be important in the regulation of the HSV-1 latency-associated transcript promoter 1 [34, 35]. We are not aware of any reports of USF serving as an activator of alphaherpesvirus IE genes. Taken into account with the lack of TAATGARAT elements within the promoter of ORF 4 [18, 20] and the small minimal region defined in this work for the promoter, it could be argued that the VZV ORF 4 promoter has more similarity to the components of early genes. The ORF 4 homologue in the closely related alphaherpesviruses (pseudorabiesvirus and equine herpesvirus) are regulated as either early or late genes [13, 14]. Alternatively, ORF 4 may be regulated as an IE gene by virion-associated IE62 [14] interacting with USF. Results from our laboratory and those from others [17] have suggested that ORF 4 can be detected in typical cycloheximide-reversal type experiments in which only IE proteins are made. However, in our experience, such expression is restricted to a small fraction (15%–26%) of cell-free VZV-infected cells that are expressing the IE-regulated ORF 62 (unpublished data). The resolution of these issues will require a more detailed analysis of ORF 4 expression in the context of the virion, and although this is a somewhat difficult task, it may reveal novel mechanisms of alphaherpesvirus gene expression.

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