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The Transcriptional Activation Domain of Varicella-Zoster Virus Open Reading Frame 62 Protein Is Not Conserved with Its Herpes Simplex Virus Homolog

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Varicella-zoster virus (VZV) open reading frame 62 (ORF62) encodes an immediate-early protein that transactivates expression of VZV, herpes simplex virus (HSV), and cellular genes in transient expression assays. VZV ORF62 is homologous to HSV ICP4 and pseudorabies virus immediate-early (IE180) proteins. All three viral proteins have conserved DNA binding domains that recognize similar sites in their corresponding promoters. Here, we show that the transcriptional activation domain of ORF62 is located near the amino terminus of the protein and is not conserved with the activation domain of ICP4. A 161-amino-acid activation domain of ORF62 activates transcription to a level comparable to that of the potent HSV VP16 activation domain; much of the activity is contained in the first 90 amino acids of ORF62. Deletion of the activation domain from full-length ORF62 markedly reduced transactivating activity. These experiments indicate that while VZV ORF62 and HSV ICP4 have conserved amino acid sequences, including their DNA binding domains, the transcriptional activation domains are poorly conserved.

Varicella-zoster virus (VZV), herpes simplex virus (HSV), and pseudorabies virus (PRV) are members of the alphaherpesvirus subfamily. The genomes of these viruses show similar organization, and many of their gene products have conserved amino acid sequences and similar locations within the genome (7). Four HSV immediate-early genes, ICP0, ICP4, ICP22, and ICP27, are homologous with VZV genes ORF61, ORF62, ORF63, and ORF4, respectively (6). Similarly, PRV encodes an immediate-early protein, IE180, that is homologous to VZV ORF62 and HSV ICP4 (2) and an early protein, EP0, that is homologous to VZV ORF61 and HSV ICP0 (3).

VZV ORF62 transactivates expression of VZV putative immediate-early, early, and late gene promoters (16, 27), HSV immediate-early and early gene promoters (12), and other viral (e.g., human immunodeficiency virus long terminal repeat) and cellular (e.g., c-*myc*) gene promoters (12, 17). VZV ORF62 represses its own promoter in simian kidney cells (11, 13) and activates its own promoter in human T and rat neuronal cell lines (28). While the transcriptional activation domain of VZV ORF62 is unknown, an internal domain (termed region 2) is required for repression of the ORF62 promoter (11). VZV ORF62 is a 175-kDa phosphorylated protein (14) and is a structural protein of the viral tegument (19). VZV ORF62 is a target for both cytotoxic and helper T lymphocytes (1, 23).

As a homolog of HSV ICP4, VZV ORF62 can complement an HSV mutant that is deleted for ICP4 (10, 13). VZV ORF62 is also homologous to the PRV immediate-early (IE180) protein (2). All three proteins transactivate expression of early genes of their corresponding viruses, and all down-regulate expression from their own promoters. These three viral proteins have extensive homology with each other in conserved regions 2 and 4 (2, 25). All three proteins have conserved DNA-binding domains that recognize similar sites in their corresponding promoters (32); however, the transcriptional activation domains of HSV ICP4 and PRV IE180 are not conserved (24). Here, we show that the transcriptional activation domain of ORF62 resides within a 90-amino-acid sequence located near the amino terminus of the protein. This sequence is not conserved with the activation domain of HSV ICP4.

MATERIALS AND METHODS

Cell lines. MeWo cells, a human melanoma cell line that supports the replication of VZV, were kindly provided by Charles Grose (15). MeWo cells were grown in Eagle's minimal essential medium with 10% fetal bovine serum. COS cells, a simian virus 40-transformed simian cell line, were grown in Dulbecco's modified Eagle medium supplemented with sodium pyruvate, nonessential amino acids, and HEPES (4-[2-hydroxyethyl]-1-piperzine-ethanesulfonic acid). Chinese hamster ovary (CHO) cells were grown in minimal essential (alpha-modified) medium containing ribonucleosides and deoxyribonucleosides.

Activator plasmids. Plasmid GAL4 contains the DNA binding domain (amino acids 1 to 147 [18]) of the yeast GAL4 protein preceded by the simian virus 40 early promoter and followed by a polylinker sequence (29). Plasmid GAL4E was constructed by cutting GAL4 with *Bam*HI and inserting a 13-bp double-stranded oligonucleotide (GATCGATATCG GG). The resulting plasmid contains a new *Eco*RV site.

A series of activator plasmids containing the DNA binding domain of GAL4 or GAL4E fused to portions of the VZV ORF62 gene was constructed (Fig. 1A; amino acids designated in parentheses). Plasmids GAL62(1-299), GAL62(1-569), GAL62(40-90), GAL62(40-161), GAL62(306-406), GAL62(407-569), GAL62(417-861), and GAL62(734-1310) were constructed by ligating the *Bst*XI-*Sty*I, *Bst*XI-*Kpn*I, *Bst*E2-*Sna*BI, *Bst*E2-*Stu*I, *Sma*I-*Hin*cII, *Sal*I-*Kpn*I, *Cla*I-*Xho*I, and *Bam*HI-*Tth*111I fragments, respectively, from an ORF62 plasmid to GAL4 or GAL4E to maintain an open

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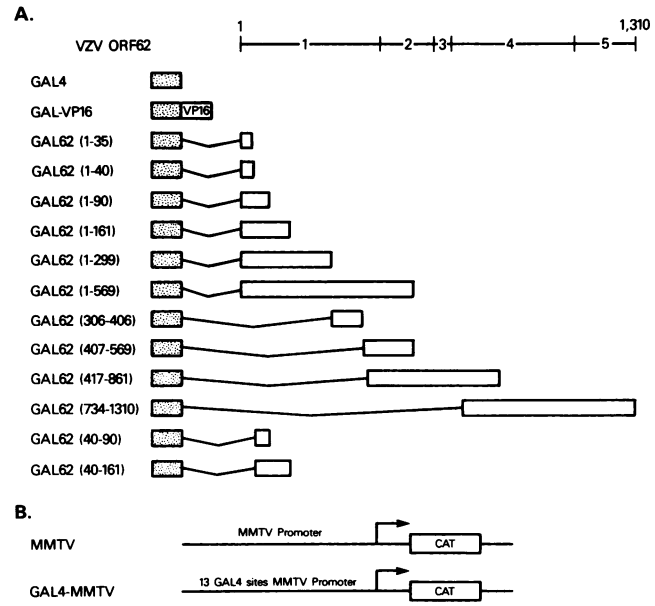

FIG. 1. GAL4 fusions with VZV ORF62. (A) Activator plasmids contain the GAL4 DNA binding domain or gene fusions between this domain and the HSV VP16 activating domain or portions of the VZV ORF62 gene under the control of the SV40 early promoter. The VZV ORF62 protein (top line) contains domains that are conserved (regions 2 and 4) with HSV ICP4 (25) and PRV IE180 (2). (B) Target plasmids contain the murine mammary tumor virus (MMTV) promoter followed by the CAT gene with or without 13 upstream GAL4 binding sites.

reading frame. Plasmids GAL62(1-35), GAL62(1-40), GAL62(1-90), and GAL62(1-161) were constructed by deleting the *Apa*I-*Xba*I, *Bst*EII-*Xba*I, *Sna*BI-*Xba*I, and *Stu*I-*Xba*I fragments, respectively, from GAL(1-299). For all constructs 5′ overhangs were filled in with the large (Klenow) fragment of DNA polymerase I and 3′ overhangs were removed with T4 DNA polymerase. The nucleotide sequence of the GAL4-ORF62 junction was determined to verify that the open reading frame was maintained. GAL-VP16, which contains the 79-amino-acid activation domain of HSV VP16 fused to GAL4 (29), was a positive control for activation.

Plasmid pCMV62 contains the ORF62 gene, inserted into plasmid pG310, under the control of the immediate-early human cytomegalovirus promoter (27). Plasmid pCMV62d 8-86 was constructed by cutting plasmid pGi26 (13) with *Eco*47III and *Sna*BI and inserting a double-stranded oligonucleotide, GTAGTCGTCTT, to maintain the open reading frame. The resulting plasmid was cut with *Sca*I and *Bgl*II and inserted into the *Eco*RI site of plasmid pG310 (27) and contains the ORF62 gene with a deletion of the nucleotides encoding amino acids 8 to 86, under the control of the human cytomegalovirus promoter. Plasmid pCMV62d39-90 was constructed by cutting pGI26 with *Sna*BI and *Sfi*I, and inserting a double-stranded oligonucleotide, CCGGGCCCT GTGTTCGGCGGCCGCGG, to result in an in-frame deletion of the nucleotides encoding amino acids 39 to 90. The resulting plasmid was cut with *Sca*I and *Bgl*II and inserted into plasmid pG310 to yield pCMV62d39-90. The nucleotide sequence of the junctions of the deletions was determined to verify that the open reading frame was maintained.

Reporter plasmids. Reporter plasmids GAL4-MMTV and MMTV contained the murine mammary tumor virus promoter with or without 13 upstream GAL4 DNA binding sites, respectively, followed by the chloramphenicol acetyltransferase (CAT) gene (Fig. 1B). Plasmid P4CAT (27) contains the VZV ORF4 gene promoter fused to the CAT gene.

Transfections and CAT assays. MeWo cells were transfected with GAL4 fusion plasmids by the lipofection procedure (Lipofection Reagent; Life Technologies). About 1.5×10^6 cells were plated 1 day before transfection onto 60-mm dishes. Activator and target plasmids (10 μg each) were added to the lipofection reagent and incubated with the cells in serum-free medium. After 6 h, an equal volume of medium containing 20% fetal bovine serum was added. About 48 h after transfection, cell extracts were prepared for CAT assays as described previously (4). Cell extracts were normalized for protein content before CAT assays were performed. CAT activity was defined as the ratio of counts in the acetylated [^{14}C]chloramphenicol spots to the sum of the acetylated and unacetylated spots. All CAT assays were performed at least twice from two or more independent transfections.

CHO cells were transfected with 2 μg each of activator and target plasmids as described previously (4), and MeWo cells were transfected with pCMV62 or pCMV62 deletion mutants by the lipofection procedure.

Immunoprecipitations. COS cells were transfected with activator plasmids and labeled with [^{35}S]methionine, and cell lysates were incubated with anti-GAL4(1-147) antibody and protein A-Sepharose as described previously (4). Immune complexes were washed and fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Immunofluorescence assays. MeWo cells grown on microscope slides were transfected with pCMV, pCMV62, or deletion mutants of pCMV62. After 72 h the cells were fixed with acetone, air dried, incubated with monoclonal antibody to ORF62 (2XF1 [14]), washed, and incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody. Slides were washed with phosphate-buffered saline, and fluorescence microscopy was performed.

Computer analysis. Predicted amino acid sequences of VZV ORF62, HSV ICP0, and PRV IE180 were simultaneously compared by using the Pileup and Pretty Programs from the Sequence Analysis Software Package (9) (Genetics Computer Group, Inc., Madison, Wis.).

RESULTS

VZV ORF62 contains a transcriptional activation domain. Activator plasmids containing GAL4-ORF62 fusions and a reporter plasmid with the CAT gene and 13 GAL4-binding sites upstream of a TATA box were cotransfected into MeWo cells. An activator plasmid with the GAL4 DNA binding domain alone was a negative control for activation, while a reporter plasmid with the CAT gene with no GAL4 binding sites was a control for specificity of activation. A plasmid with the 79-amino-acid activation domain of VP16 fused to GAL4 served as a positive control for activation.

Analysis of the GAL4-ORF62 constructs indicated that VZV ORF62 contains a strong transcriptional activating domain (Fig. 2). The amino third of the VZV ORF62 molecule (amino acids 1 to 569) weakly activated transcription, while constructs from the middle or carboxy portions of VZV ORF62 failed to activate transcription. Successive carboxy-terminal deletions from the amino terminus showed that maximal activity was in amino acids 1 to 161. This

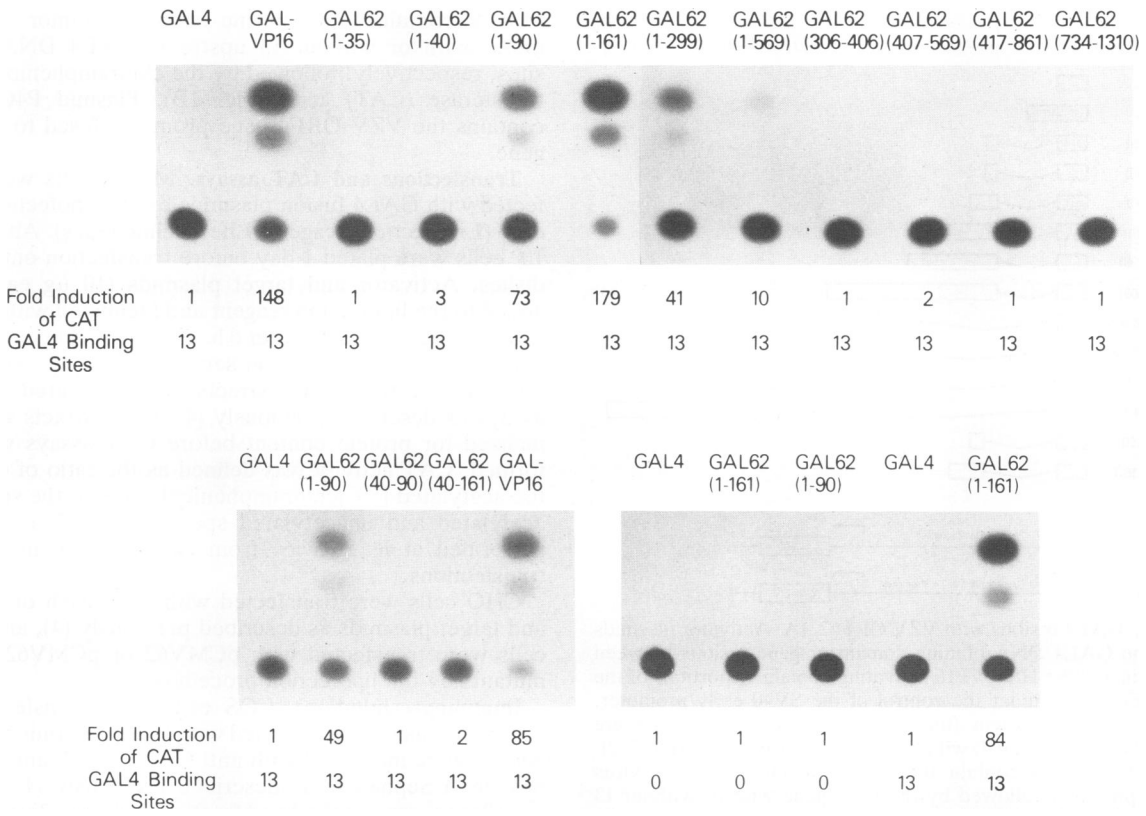

FIG. 2. VZV ORF62 activates transcription in MeWo cells. Activator and target plasmids were cotransfected into MeWo cells, and cell lysates were assayed for CAT activity. Activator plasmids are shown at the top of each lane, and the number of GAL4-binding sites are shown below each lane. Fold induction of CAT (below each lane) is the CAT activity relative to that obtained with the activator containing GAL4 alone. The variation for fold induction of CAT for two separate transfection experiments was <30%.

domain was able to activate transcription 179-fold. The level of transcriptional activation seen with GAL62(1-161) was comparable to that of the potent activator HSV VP16.

Transcriptional activity was reduced with further carboxy-terminal deletions proximal to amino acid 161, although much of the activity was present in the first 90 amino acids of the protein. Attempts to further delineate the transcriptional activating activity of VZV ORF62 were unsuccessful. While amino acids 1 to 35 or 1 to 40 had little or no activity, amino acids 40 to 90 or 40 to 161 also had no activity. Thus, this analysis indicates that amino acids 1 to 90 are required for much of the transcriptional activating activity by VZV ORF62.

The specificity of activation was shown by the need for GAL4 DNA binding sites on the reporter plasmid. Cotransfection of two of the strongest activators [GAL62(1-90) and GAL62(1-161)] with a reporter plasmid lacking GAL4 sites failed to activate transcription (Fig. 2).

Expression of GAL4-VZV ORF62 proteins in transfected cells. Since the various levels of activation seen with the different GAL4-ORF62 constructs could be due to different levels of expression or stability in the transfected cells, it was important to verify that each of the constructs could express similar amounts of protein. To accomplish this, COS cells were transfected with activator plasmids and fusion proteins were isolated by immunoprecipitation from the cells with anti-GAL4(1-147) antibody (4). Immunoprecipitations indicated that nearly all of the activator constructs were expressed at comparable levels in transfected cells (Fig. 3);

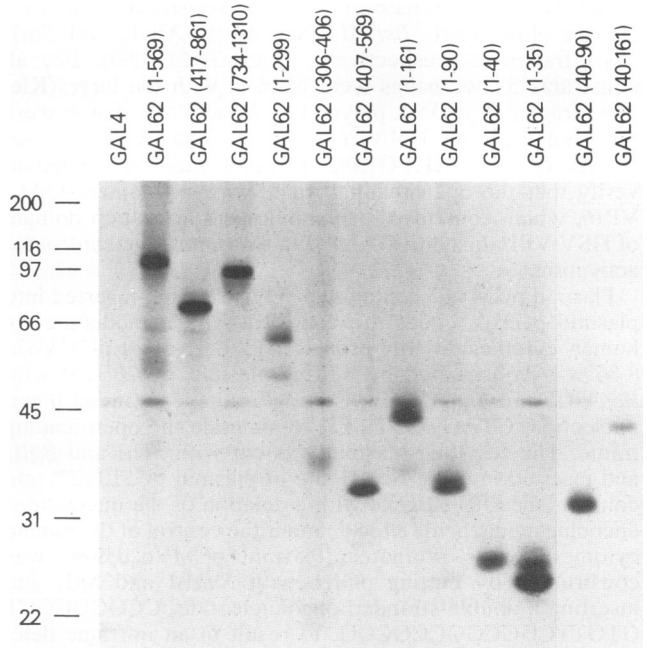

FIG. 3. Expression of GAL4-VZV ORF62 proteins in transfected cells. COS cells were transfected with activator plasmids, radiolabeled with [^{35}S]methionine, and immunoprecipitated with anti-GAL4 antibody. Immune complexes were fractionated on an SDS–10% polyacrylamide gel. Activator plasmids are shown at the top of each lane, and protein sizes in kilodaltons are indicated at left.

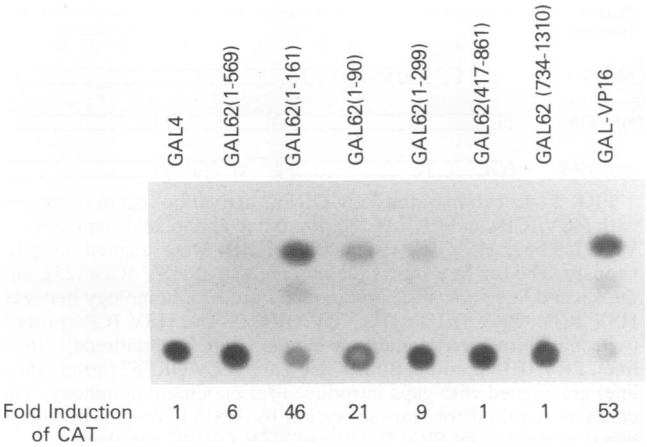

FIG. 4. VZV ORF62 activates transcription in CHO cells. Activator and target plasmids (2 μg each) were cotransfected into CHO cells with DEAE-dextran, and lysates were assayed for CAT activity. Fold induction of CAT, relative to GAL4 alone, is shown below each lane.

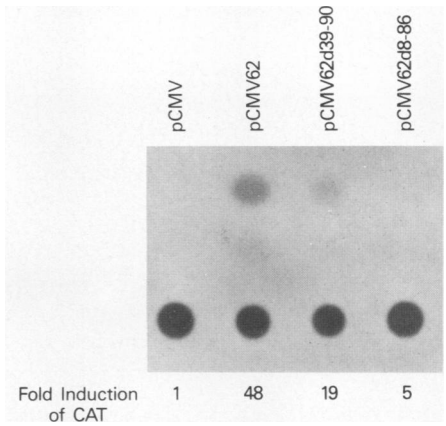

FIG. 5. VZV ORF62 amino acids 8 to 86 are important for transactivation. Activator plasmids (5 μg) expressing ORF62 (pCMV62), ORF62 deletions (pCMV62d8-86, pCMV62d39-90), or vector control plasmid pCMV (pG310 [28]) were cotransfected with 2 μg of target plasmid containing the VZV ORF4 promoter followed by CAT (p4CAT). Fold induction of CAT, relative to p4CAT alone, is shown below the autoradiogram. The variation for fold induction of CAT with repeated experiments was <35%.

GAL62(40-161) expressed a slightly lower level of activator protein on repeated experiments.

VZV ORF62 is a transcriptional activator in CHO cells. GAL4 constructs containing VZV ORF62 also activated transcription when transfected into CHO cells (Fig. 4). The most active constructs in MeWo cells [GAL62(1-161), GAL62(1-90)] were also the most active constructs in CHO cells. These data indicate that VZV ORF62 does not require additional factors present in primate cells in order to activate transcription.

VZV ORF62 amino acids 8 to 86 are important for transactivation. To verify that the transcriptional activation domain is required for activation when full-length VZV ORF62 interacts with a VZV promoter, we assayed plasmids expressing ORF62 or ORF62 deletion mutants for their ability to transactivate a VZV putative immediate-early gene promoter. pCMV62 contains the VZV ORF62 gene driven by the HCMV promoter (27). pCMV62d8-86 and pCMV62d39-90 contain the ORF62 gene with in-frame deletions of nucleotides encoding amino acids 8 to 86 or 39 to 90, respectively, under the control of the HCMV promoter. Cotransfection of pCMV62, which expresses full-length VZV ORF62 protein, with a reporter plasmid containing the VZV ORF4 promoter fused to the CAT gene (P4CAT) in MeWo cells activated the ORF4 promoter 48-fold. In contrast, cotransfection of plasmids pCMV62d8-86 or pCMV62d39-90 with p4CAT resulted in 5- or 19-fold activation of the VZV ORF4 promoter (Fig. 5). Thus, a 79-amino-acid sequence in ORF62 (amino acids 8 to 86) is essential for full transactivation by VZV ORF62.

To verify that each of the deletion mutants expressed VZV ORF62 protein, MeWo cells were transfected with pG310 (pCMV vector), pCMV62, pCMV62d8-86, and pCMV62d39-90 and stained with monoclonal antibody to VZV ORF62 (14). Characteristic granular nuclear and nuclear membrane staining was present in the cells transfected with pCMV62 (Fig. 6A) or the pCMV62 deletion mutants (Fig. 6B and C); in contrast, no staining was present in cells transfected with the pCMV vector. Thus, the reduced ability of the ORF62 deletion mutants to transactivate a VZV promoter is not due to reduced expression or stability of the ORF62 protein.

The activation domain of VZV ORF62 is not highly conserved with HSV ICP4. VZV ORF62 is homologous to HSV ICP4 (25) and PRV IE180 (2). The transcriptional activation domain of PRV IE180 has been mapped to the first 34 amino acids of the protein (24), while deletion of the first 89 amino acids of ICP4 does not reduce the transactivating activity of ICP4 (8). Further experiments indicate that transactivation by HSV ICP4 requires three domains defined by amino acids 142 to 210, 263 to 338, and 449 to 487 (30). An additional domain, defined by amino acids 840 to 1,100, also contributes to transactivation, but to a lesser extent than the domains in the amino half of the molecule (26).

Alignment of the VZV ORF62, HSV ICP4, and PRV IE180 sequences indicated that the activation domain of VZV ORF62 is not highly conserved with the transactivation domains of the other two proteins (Fig. 7). While the activation domains of VZV ORF62 and PRV IE180 are both at the amino termini of their corresponding proteins, amino acids 1 to 40 of VZV ORF62 (which align with the activation domain [amino acids 1 to 33] of PRV IE180) cannot activate transcription alone. VZV ORF62, HSV ICP4, and PRV IE180 share two highly conserved domains, region 2, which includes the DNA binding domain, and region 4 (Fig. 7) (25); however, the transcriptional activation domain of VZV ORF62 is divergent from the other two proteins. The activation domain of ORF62 (amino acids 1 to 90) contains 18 negatively charged amino acids, a portion of which (amino acids 19 to 38) has a putative alpha-helical structure. Acidic domains with predicted alpha-helical structures in other viral proteins have been shown to activate transcription, including the activation domains of HSV VP16 (29) and Epstein-Barr virus EBNA-2 (4).

DISCUSSION

We have shown that VZV ORF62 contains a strong transcriptional activation domain. The loss of transactivation after deletion of most of the activation domain from the full-length protein indicates that the domain is required for activation. In addition, the necessity of GAL4 DNA binding sites on the target DNA for interaction with the GAL4-

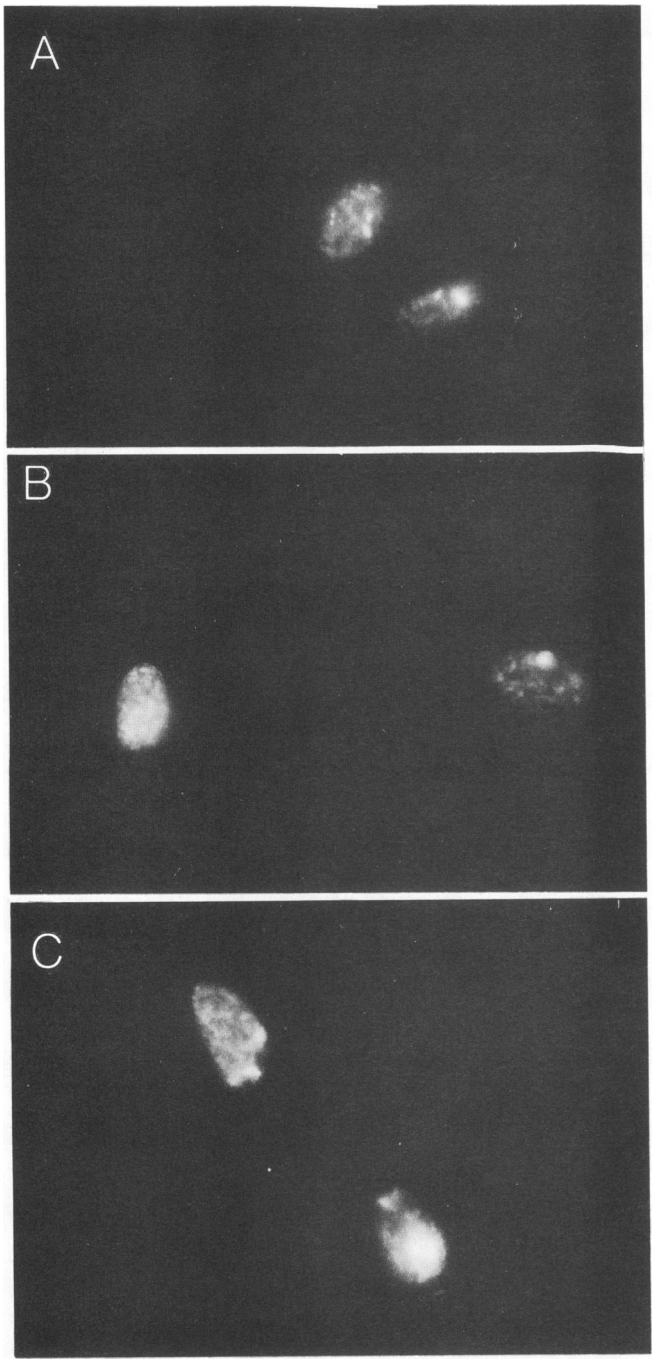

FIG. 6. Expression of VZV ORF62 deletion mutants in MeWo cells. Plasmids (10 μg) expressing ORF62 (pCMV62 [A]) or ORF62 deletion mutants (pCMV62d8-86 [B] and pCMV62d39-90 [C]) were transfected into MeWo cells and stained with monoclonal antibody to VZV ORF62. Granular nuclear and nuclear membrane staining, characteristic of ORF62, is present.

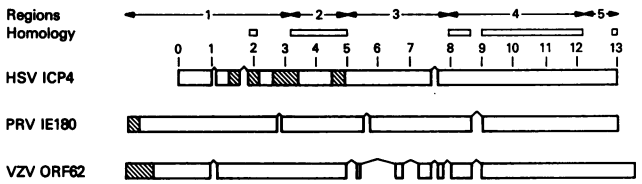

FIG. 7. Location of the VZV ORF62 activation region compared with HSV ICP4 and PRV IE180. Predicted amino acid sequences of VZV ORF62, HSV ICP4, and PRV IE180 were aligned simultaneously. The top line shows the five regions of HSV ICP4 (25), and the second line shows the predominant areas of homology between HSV ICP4, PRV IE180, and VZV ORF62. The HSV ICP4 protein (with numbers corresponding to amino acids [in hundreds]) (third line), PRV IE180 protein (fourth line), and VZV ORF62 protein (fifth line) are aligned with gaps introduced for maximum homology. The domains required for transactivation by HSV ICP4 and transcriptional activation by PRV IE180 and VZV ORF62 are shaded.

ORF62 protein indicates that the activation domain must be directed to a transcriptional initiation site. Previous experiments have shown that VZV ORF62 possesses a DNA binding domain that is specific for a sequence in the ORF62 promoter (32). Our findings indicate that VZV ORF62 is similar to other transcriptional activators containing both a modular activation domain and a region that can target the protein to a promoter (21, 24).

Amino acids 1 to 161 compose the activation domain of VZV ORF62. Most of the activity is in the first 90 amino acids of the molecule. Neither the amino half (amino acids 1 to 40) nor the carboxy half (amino acids 40 to 90) of the domain exhibit full activity alone. Thus, either most of the entire 90-amino-acid portion is critical for transcriptional activation or the middle portion of this region contains a sequence necessary for activation. The 90-amino-acid region contains 18 negatively charged amino acids. A portion of the domain (amino acids 19 to 38) has a putative alpha-helical structure with four negatively charged amino acids. While many transcriptional activators contain negatively charged, putative alpha-helical regions, recent studies indicate that an alpha-helical structure might not be necessary for activation (5).

Transcriptional activators are thought to function by interacting with cellular proteins or basic transcriptional factors at the transcriptional complex. Recent studies indicate that other viral activators (e.g., HSV VP16 [22, 31], adenovirus E1a [20]) can bind TFIID or TFIIB, and in doing so may mediate part of their roles in activation. At present it is unknown whether VZV ORF62 interacts with one or more of these factors.

VZV ORF62 shares amino acid homology with HSV ICP4, and ORF62 can functionally replace HSV ICP4. While both proteins have extensive homology in their DNA binding domains (region 2) and an additional domain (region 4), the transcriptional activation domains are not conserved. It would seem likely that these two proteins could have evolved from a single ancestral protein. Yet during evolution the DNA binding domains have been conserved while the transactivating domains have diverged. The results of this process have been sequence divergence as well as some functional differences in activation by these proteins. Specifically, VZV ORF62 (or infection with VZV) transactivates the VZV and HSV thymidine kinase promoters, while HSV ICP4 (or infection with HSV-1) transactivates the HSV, but not the VZV, thymidine kinase promoter (17). Thus, despite the modular feature of their activating domains and conservation of their DNA binding domains, the immediate-early genes of these two alphaherpesviruses may have evolved different mechanisms to activate specific viral promoters.

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