The equine herpesvirus 1 EICP27 protein enhances gene expression via an interaction with TATA box-binding protein

Randy A. Albrecht, a Seong K. Kim, a Yunfei Zhang, a Yuhe Zhao, 1 and Dennis J. O’Callaghan a,*

Center for Molecular and Tumor Virology and Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, Shreveport, LA 71130-3932, USA

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Abstract

The mechanism(s) by which the early EICP27 gene product cooperates with other equine herpesvirus 1 (EHV-1) regulatory proteins to achieve maximal promoter activity remains unknown. Transient transfection assays revealed that deletion of residues 93–140 of the 470-aa EICP27 protein substantially diminished its activation of the immediate-early (IE) promoter, whereas deletion of residues 140–470 that contain a zinc-finger motif abolished this activity. Fluorescence microscopy of cells expressing the full-length EICP27 protein or portions of this protein revealed that an arginine-rich sequence spanning residues 178–185 mediates nuclear entry. Experiments employing the mammalian Gal4 two-plasmid system revealed that the EICP27 protein does not possess an independent trans-activation domain (TAD). Protein–protein interaction assays using purified proteins revealed that residues 124–220 of the EICP27 protein mediate its direct interaction with TATA box-binding protein (TBP). Partial deletion of this TBP-binding domain attenuated the ability of the EICP27 protein to stimulate the IE and early EICP0 promoters by 68% and 71%, respectively, indicating the importance of this protein–protein interaction.

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Introduction

Equine herpesvirus 1 (EHV-1) is a member of the Alpha-herpesvirinae subfamily that also includes herpes simplex virus (HSV) types 1 and 2 and varicella zoster virus (VZV). Our laboratory employs EHV-1 as a model to investigate aspects of Alphaherpesvirinae gene regulation, pathogenesis, and persistent infection mediated by defective interfering (DI) particles (O’Callaghan and Osterrieder, 1999). During lytic infection, the 78 EHV-1 genes are temporally transcribed as immediate-early (IE), early, leaky late (γ1), and true late (γ2) messenger RNA (Breeden et al., 1992; Caughman et al., 1985; Cohen et al., 1975; Gray et al., 1987a, 1987b; Telford et al., 1992) in a manner that is analogous to the gene regulation programs of other Alphaherpesvirinae members (Clements et al., 1977; Honess and Roizman, 1974; Roizman et al., 1975; Weinheimer and McKnight, 1987; Weir, 2001). Six virally encoded regulatory proteins (the IE protein; the early EICP0, EICP22, EICP27, and IR2 proteins; and the late E-TIF) orchestrate the temporal transcription of the EHV-1 genes. The sole IR1 gene encodes the IE regulatory protein that trans-activates early and some late promoters (Smith et al., 1992, 1995), and whose functions are essential for lytic replication (Garko-Buczynski et al., 1998). The early EICP0 regulatory protein promiscuously trans-activates early and late EHV-1 promoters (Bowles et al., 1997, 2000), but is not essential for viral replication (Yao et al., 2003). The early EICP22 and EICP27 regulatory proteins synergize the trans-activation functions of the IE and EICP0 proteins (Bowles et al., 2000; Holden et al., 1995; Kim et al., 1997; Smith et al., 1993b; Zhao et al., 1995). The early IR2 protein is hypothesized to negatively regulate EHV-1 gene expression by interfering with the trans-activation function of the IE protein (Harty and O’Callaghan, 1991; Smith et al., 1994). The late E-TIF regulatory protein is a tegument protein...
that specifically trans-activates the IE promoter (Kim and O’Callaghan, 2001; Lewis et al., 1993, 1997).

The early \textit{EICP27 (U\textsubscript{i,5})} gene encodes a 470-amino-acid (aa) phosphoprotein with an apparent molecular weight of 51.4 kDa (Zhao et al., 1992). The EICP27 protein has limited homology with the ICP27 protein of HSV-1 (32%) and the IE4 protein of VZV (13%) (Piette et al., 1995; Zhao et al., 1992). Furthermore, the 3’ end of the \textit{EICP27} gene encodes a conserved zinc-finger motif that was demonstrated to be functional in its HSV-1 ICP27 homologue (Vaughan et al., 1992). Transient transfection assays revealed that the EICP27 protein: (i) enhanced gene expression from the IE promoter (Zhao et al., 1995), (ii) could not alleviate the negative autoregulation of the IE promoter by the IE protein (Zhao et al., 1995), (iii) cooperated with the IE protein in trans-activation of representative early and late viral promoters (Smith et al., 1992, 1993b), and (iv) functioned in a synergistic manner with the EICP0 protein to stimulate expression of a representative early promoter (Bowles et al., 2000).

Interestingly, EICP27 residues 402–470 that encompass the conserved zinc-finger motif are present within the EICP22/EICP27 hybrid protein encoded by the genome of EHV-1 DI particles (Chen et al., 1996; O’Callaghan and Osterrieder, 1999). EHV-1 infections enriched for DI particles expressing this \textit{EICP22/EICP27} hybrid gene were shown to result in persistent infection (Dauenhauer et al., 1982; Gray et al., 1989; Robinson et al., 1980). The \textit{EICP22/EICP27} hybrid gene originated from a homologous recombination event that involved an identical 8-bp sequence present within the \textit{EICP22} and \textit{EICP27} genes (Chen et al., 1996; Yalamanchili et al., 1990). Thus, the hybrid gene also encodes EICP22 residues 1–196, which harbors

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Fig. 1. Expression of the native \textit{EICP27} gene and \textit{EICP27} truncation and deletion mutants in transiently transfected L-M cells. (Panel A) Schematic representation of the EICP27 protein indicating the amino acid positions of the structural motifs. The amino acid sequence deleted in each EICP27 mutant polypeptide is indicated at the left of the figure. (Panel B) Western blot analyses of full-length EICP27 and EICP27 mutant polypeptides synthesized in transiently transfected L-M cells. The EICP27 mutants EICP27\Delta291–343, EICP27\Delta343–395, EICP27\Delta380–402, EICP27\Delta216–446, EICP27(1–281), and EICP27(1–422) yielded less intense bands, suggesting that this region may harbor the majority of the immunodominant epitopes present within the full-length EICP27 protein. The sizes of the molecular weight markers (kDa) are indicated at the left of the panel.
domains for both EICP22 protein self-dimerization and IE protein-binding (Chen et al., 1996; Derbigny et al., 2000). The hybrid protein was demonstrated to repress both activation of the IE promoter and trans-activation of early promoters by the IE protein; however, the EICP22/EICP27 protein stimulated the ability of the IE protein to trans-activate late promoters (Chen et al., 1999). How the EICP27 zinc-finger contributes to the functions of the EICP27 protein during lytic infection or to those of the hybrid protein during persistent infection remains unknown.

The mechanism(s) by which the EICP27 protein increases the expression of viral genes is obscure; therefore, EICP27 deletion mutants were tested in transient transfection assays to delineate the functional regions of this protein. Because the EICP27 protein cooperates with the IE and EICP0 proteins to activate EHV-1 promoters, it was of interest to determine if this cooperation was mediated, at least in part, by an interaction with one or more cellular transcription factors. In this manuscript, we document the direct interaction of the EICP27 protein with TATA box-binding protein (TBP).

Results

**EICP27 residues 279–470 are essential to enhance IE promoter activity**

Prior data from transient transfection assays indicated that the EICP27 protein independently enhances the activity of the IE promoter (Smith et al., 1993b; Zhao et al., 1995). To map the domain(s) within the EICP27 protein necessary for this function, a panel of EICP27 deletion and truncation mutants was constructed (Fig. 1A). To facilitate the detection of the EICP27 deletion mutants, a polyclonal antibody that recognizes the full-length EICP27 protein (aa 1–470) was generated. Western blot assays showed that this antibody detects the EICP27 protein produced during EHV-1 infections (Fig. 2A) and by in vitro transcription/translation reactions (Fig. 2B). Extracts of L-M cells that were transfected with constructs encoding the EICP27 deletion mutants were examined by Western blot analysis with the anti-EICP27 protein polyclonal antibody to verify the expression of the encoded EICP27 sequence (Fig. 1B). These analyses revealed that the EICP27 sequence within each construct expressed a protein of the expected size, and that the protein was readily detected by Western blotting.

The ability of each EICP27 mutant to enhance expression of the IE promoter-chloramphenicol Acetyl-transferase (CAT) reporter chimera, pIE-CAT [pIE (−802/+73)], was compared to that of the native EICP27 in transient transfection assays. As consistent with previous findings (Smith et al., 1992), the full-length EICP27 protein enhanced expression from the IE promoter by 12-fold (Fig. 3, panel A, Bar 2) and by 9-fold (Fig. 3, panel B, Bar 2). The deletion mutants EICP27D41–89 and EICP27D89–124 (Fig. 3, panel A, Bars 3 and 5), respectively enhanced reporter expression from the IE promoter with efficiencies that were comparable to that of the native EICP27 protein. An EICP27 deletion mutant that lacked residues 8–57 activated the IE promoter to a level comparable to that of the full-length EICP27 protein (data not shown). The ability of the EICP27D93–140 polypeptide to activate the IE promoter was reduced by 68% (Fig. 3, panel A, Bar 4). Conversely, the EICP27 derivatives EICP27D93–220, EICP27D140–220, EICP27D41–279, EICP27D291–343, EICP27D343–395, EICP27D380–402, EICP27D216–446, EICP27D1–281, and EICP27D1–422 were completely devoid of this functional activity (Fig. 3, panel B, Bars 3–11, respectively). These results argue that the sequence spanning aa 1–140 is not essential for this
The ineffectiveness of some of the EICP27 mutants to activate the IE promoter could be explained by the loss of a nuclear localization signal. The intracellular distribution of the EICP27 protein derivatives within RK-13 cells transfected with each of the EICP27 expression plasmids was assessed by indirect immunofluorescence (IF) microscopy. As shown in Fig. 4A, the anti-EICP27 protein antiserum showed a punctate staining pattern of the EICP27 protein within the nuclei of EHV-1-infected cells. The NLS was subsequently mapped by examining the intracellular distribution of each EICP27 polypeptide in cells transfected with the corresponding EICP27 expression plasmids. The data demonstrated that the full-length EICP27 protein as well as the EICP27 polypeptide that contained residues 1–281 localized within the nucleus. The EICP27 polypeptides that lacked residues 41–279 or 140–220 accumulated within the cytoplasm of transfected cells, although the EICP27Δ41–279 (25.5 kDa) and EICP27Δ140–220 (42.9 kDa) failed to enter the nucleus, suggesting that residues 140–220 harbor an NLS.

To elucidate further the NLS, the intracellular distribution of several EICP27 linker insertion mutants was examined by IF microscopy (Fig. 4B). The linker insertion mutants harbor a BglII linker encoding the four aa sequence Gly-Cys-Thr-Thr. The presence of the BglII linker should generate conformational changes in the EICP27 protein within the region that spans the insertion site for the linker sequence. The results of these assays demonstrated that the EICP27Δ140, EICP27Δ151, and EICP27Δ220 polypeptides localized within nuclear punctuate foci similar to that observed for the full-size EICP27 protein. However, the EICP27Δ176 polypeptide failed to localize within the nucleus, suggesting that the BglII linker insertion at codon 176 disrupted its NLS function.

To delineate the minimal NLS, sequences encoding EICP27 residues 1–470, residues 140–220, or portions of this region were cloned in-frame with a sequence encoding the green fluorescent protein (GFP). The data obtained from fluorescence microscopy of the expression of these GFP-EICP27 fusion plasmids in transfected RK-13 cells are shown in Fig. 4C. The micrographs revealed punctuate accumulations of the GFP-EICP27(1–470) within the nucleus as observed by IF microscopy of the nuclear localization of the EICP27 protein in cells either infected with EHV-1 or transfected with the pSVEICP27 expression plasmid (Fig. 4A). In addition, EICP27 residues 140–220 and residues 176–216 also mediated the nuclear accumulation of the GFP fusion protein; however, the nuclear distribution of these GFP-EICP27 fusion proteins was more diffuse than the punctuate nuclear pattern observed for the GFP-EICP27(1–470) fusion protein. Sequence analysis of residues 176–216 revealed an arginine-rich stretch of basic residues within codons 176–188 (underlined). The aa composition of this sequence closely resembles the requirements of polybasic nuclear localization signals (such as arginine-rich sequences) (Christophe et al., 2000), suggesting that this sequence mediates the nuclear entry of the EICP27 protein. Indeed, the GFP-EICP27(178–185)
fusion protein with the arginine-rich sequence RVHRNRRR efficiently localized within the nucleus, but did not show a punctuate staining pattern similar to that observed with the GFP-EICP27(1–470) fusion protein. The diffuse nuclear distribution of some GFP fusion proteins was not surprising as these proteins express only 2–17% of the 470 residues of the full-length EICP27 protein. Overall, these studies indicate that the arginine-rich sequence spanning EICP27 residues 178–185 functions as an NLS.

The EICP27 protein does not contain an independent trans-activation domain

The ability of the EICP27 protein to enhance IE promoter activity may be ascribed to the function of an independent trans-activation domain (TAD) within the region spanning aa 279–470. To test whether the EICP27 protein contains a TAD, the mammalian Gal4 two-plasmid system was employed as used previously to map the TAD of other herpesvirus regulatory proteins (Perera et al., 1994; Smith et al., 1994; Xiao et al., 1997). The native EICP27 gene as well as portions of this gene were cloned in-frame with the sequence encoding the Gal4 DNA-binding domain (Fig. 5A). Analyses of the expression of these constructs in transfected RK-13 cells by Western blot and IF microscopy revealed that the Gal4-EICP27 fusion proteins were synthesized as fusion proteins with the green fluorescent protein (GFP). RK-13 cells were transfected with 7.5 pmol of either the empty pEGFP-C3 cloning plasmid, or one of the EICP27 expression plasmids pGFP-EICP27(1–470), pGFP-EICP27(140–220), pGFP-EICP27(176–216), and pGFP-EICP27(178–185). At 24 h post-transfection, fluorescence microscopy assessed the ability of the encoded EICP27 sequence to localize the GFP moiety to the nucleus. DAPI (blue fluorescence) was included in the mounting solution to visualize the nuclei (as shown in the bottom row of panel C).
These results indicate that the EICP27 protein does not harbor a trans-activation domain, and thus suggest that the inability of some of the EICP27 deletion mutants to increase expression from the IE promoter is not attributed to the deletion of a TAD.

The EICP27 protein directly interacts with TATA box-binding protein

The IE protein trans-activates EHV-1 early and late promoters via a mechanism that involves its interaction with TBP and TFIIIB (Albrecht et al., 2003; Jang et al., 2001). The cooperation between the EICP27 and the IE proteins may be due to the ability of the EICP27 protein to stabilize the interactions of the IE protein with cellular TBP and TFIIIB (Smith et al., 1993b; Zhao et al., 1995). One prediction of this model would be that the EICP27 protein directly associates with one or both of these transcription factors. The demonstration that the EICP27 protein interacts with one or both of these transcription factors, possibly within the RNA polymerase II preinitiation complex, would elucidate one mechanism by which this viral protein stimulates gene expression. To test this possibility, GST-pulldown assays were performed to determine whether the GST-EICP27(1–470) protein precipitated 35S-labeled TBP, but not 35S-labeled TFIIIB (Fig. 6A). To ascertain whether the EICP27 protein directly interacts with TBP, GST-pulldown assays that employed purified, full-length GST-EICP27 protein and purified TBP were performed. Purification of GST-EICP27 protein and TBP is described in Materials and methods. The presence of purified TBP within the precipitated GST-EICP27 fusion protein complexes was determined by Western blot analysis with an anti-TBP monoclonal antibody. The results showed that GST-EICP27(1–470) directly precipitated a considerable
amount of the purified TBP (Fig. 6B), whereas GST alone precipitated only a trivial amount of the purified TBP.

Co-immunoprecipitation assays were conducted as an alternative approach to validate the results of the GST-pull-down experiments that demonstrated a direct interaction between the EICP27 protein and TBP (Fig. 6C). In these assays, 35S-labeled TBP was examined for its co-immunoprecipitation with 35S-labeled luciferase (negative control), 35S-labeled TFIIB (positive control), and the 35S-labeled EICP27(1–470) protein. The negative control reaction indicated that the anti-TBP polyclonal antibody failed to co-immunoprecipitate 35S-TBP with 35S-luciferase (negative control, lane 6), 35S-labeled TFIIB (positive control, lane 7), or 35S-labeled EICP27(1–470) (lane 8). Some of the reactions (lanes 9 and 10) included pre-immune sera that served as a control for the specificity of the anti-TBP polyclonal antibody. The input lanes [luciferase, lane 2; TBP, lane 3; TFIIB, lane 4; and EICP27(1–470), lane 5] contain 50% of the amount of 35S-labeled protein included in the co-immunoprecipitation reactions and served to indicate the efficiency with which TBP co-immunoprecipitated with the test proteins. The sizes of the molecular weight markers (kDa) are indicated at the left of each panel (lane 1).

**EICP27 residues 124–220 harbor a TBP-binding domain**

The domain(s) within the EICP27 protein that mediates its interaction with TBP was mapped by a series of GST-pulldown assays. In these assays, GST-TBP precipitated a minimal amount of the 35S-luciferase protein, which was employed as a negative control (Fig. 7). GST-TBP was next tested for its ability to precipitate 35S-labeled full-length EICP27 protein and EICP27 deletion and truncations.
tion polypeptides. The data revealed that GST-TBP precipitated radiolabeled full-size 35S-EICP27(1–470) as well as 35S-EICP27(1–422), 35S-EICP27(1–281), 35S-EICP27Δ8–59, 35S-EICP27Δ41–89, and 35S-EICP27Δ89–124 polypeptides with comparable efficiencies (Fig. 7). However, GST-TBP was incapable of precipitating the deletion mutants 35S-EICP27Δ93–140 and 35S-EICP27Δ140–220. Overall, these findings indicate that EICP27 residues 124–220 mediate its direct interaction with TBP.

The TBP-binding domain is important for the EICP27 protein to activate the EICP0 promoter

Transient transfection assays demonstrated that deletion of EICP27 residues 93–140 attenuated the ability of the EICP27 protein to activate the IE promoter by 68% (Fig. 3). An additional experiment was conducted to determine whether deletion of residues 93–140 also attenuated the ability of the EICP27 protein to activate EHV-1 promoters in addition to the IE promoter. These assays examined whether the EICP27Δ93–140 mutant was impaired in activating expression of the EICP0 promoter-CAT reporter chimera, which harbors the EICP0 promoter sequence −335 to −21, relative to the translation initiation site. As shown in Fig. 8, the IE protein that served as the positive control stimulated a 45-fold increase in reporter expression from the EICP0 promoter (Fig. 8, Bar 2). The full-length EICP27 protein also enhanced
expression from the EICP0 promoter by 32-fold (Fig. 8, Bar 3). However, removal of residues 93–140 from the EICP27 protein attenuated the ability of this EICP27 mutant to stimulate this EHV-1 promoter by approximately 71% (Fig. 8, Bar 4). Collectively, the results of the transient transfection assays that employed the IE and early EICP0 promoters suggest that residues 93–140 that contribute to the TBP-binding domain of the EICP27 protein are important for this protein to stimulate representative EHV-1 promoters.

**Discussion**

Prior investigation of the EHV-1 EICP27 protein revealed that it independently increased expression from the IE promoter, but failed to activate early or late viral promoters (Smith et al., 1993b). These experiments also showed that the EICP27 protein cooperated with either the IE or EICP0 protein to trans-activate representative early and late viral promoters (Bowles et al., 2000; Smith et al., 1993b; Zhao et al., 1995). These results suggest little about the exact function of the EICP27 protein during infection, except that it serves as an early auxiliary regulatory protein. To elucidate the functional domains of the EICP27 protein, EICP27 deletion and truncation mutants were examined in transient transfection assays for their ability to activate the IE promoter. The data illustrated that removal of aa 140–220 or 279–470 rendered the EICP27 protein inactive. A possible explanation for the loss of function of some EICP27 deletion mutants is the absence of an NLS, which was mapped in this study to lie within aa 178–185. These residues may provide another function other than serving as a nuclear localization signal. Studies on the nuclear entry of proteins revealed that 50 kDa is the upper size limit that allows the unrestricted diffusion of proteins across the nuclear membrane (reviewed in Macara, 2001). Because the full-length EICP27 protein (51 kDa) and the GFP-EICP27 fusion proteins (approximately 30 kDa each) accumulated within nuclei, residues 178–185 may function as a nuclear retention signal. Commonly, an NLS is composed of either a short stretch of polybasic residues or two clusters of basic residues separated by a minimal cluster of uncharged residues (Christophe et al., 2000). In the case of the EICP27 protein, the arginine/glycine-rich residues within aa 176–188 were shown to be necessary to mediate nuclear entry. In the case of the HSV-1 ICP27 protein, the first arginine-rich domain (RGG box, aa 139–153) did not function as an independent NLS, but contributed to the nucleolar localization of this immediate-early protein (Hibbard and Sandri-Goldin, 1995; Mears et al., 1995).

The EICP27 homologues of the alphaherpesviruses appear to vary considerably in their functions in virus gene regulation. For example, the EICP27 gene of EHV-1 is an early gene (Zhao et al., 1992), whereas its homologues in HSV-1 and VZV are immediate-early genes (Defechereux et al., 1997; Honess and Roizman, 1974). Our observation that the early EICP27 protein does not contain an independent trans-activation domain differs from the findings for the immediate-early IE4 protein of VZV (Perera et al., 1994). Although Perera et al. demonstrated the IE4 gene product activated reporter expression in the Gal4 two-plasmid system, the authors did not map the minimal domain that mediates this activation function. Additional investigations revealed that the IE4 protein increased transcription from heterologous HIV-1 and SV40 promoters, and that this function of the VZV protein required cis-acting binding sites for cellular factors, such as NF-κB and Sp1 (de Maisieres et al., 1998; Perera et al., 1994). In the case of HSV-1, the ICP27 protein governs gene expression by several mechanisms that include: (i) an interaction with RNA polymerase II (Zhou and Knipe, 2002); (ii) an RNA-binding activity (Brown et al., 1995; Ingram et al., 1996; Mears and Rice, 1996; Sandri-Goldin, 1998); (iii) the preferential export of viral transcripts into the cytoplasm (i.e. the leaky-late U1,24 transcript) (Pearson et al., 2004; Phelan and Clements, 1997; Phelan et al., 1996; Sandri-Goldin, 1998; Soliman et al., 1997); (iv) the regulation of pre-mRNA processing (Bryant et al., 2000, 2001; Hann et al., 1998; Hardy and Sandri-Goldin, 1994; McGregor et al., 1996; McLauchlan et al., 1992); and (v) the redistribution of cellular factors that mediate RNA-splicing and mRNA export (Phelan et al., 1993; Sandri-Goldin, 1995). The consequences of these functions of the ICP27 protein are that late gC and U1,47 genes and early genes encoding factors involved in HSV-1 DNA replication are efficiently expressed (Jean et al., 2001; McCarthy et al., 1989; Rice and Knipe, 1990; Rice et al., 1989; Sacks et al., 1985; Uprichard and Knipe, 1996). Association of the ICP27 protein with RNA polymerase II combined with its necessity for the efficient transcription of the late gC and U1,47 genes suggests that this protein directly stimulates transcription from HSV-1 promoters. However, a recent study revealed that the HSV-1 ICP27 protein increases gC gene expression posttranscriptionally in a manner that is dependent on sequences within the gC open reading frame, but is not dependent on the regulatory elements of the gC gene (i.e. the promoter and polyadenylation site) (Perkins et al., 2003). To date, studies in this laboratory indicate that the EHV-1 EICP27 protein does not function as a major RNA-binding protein and appears not to shuttle viral transcripts from the nucleus. These differences between the EICP27 protein and HSV-1 ICP27 protein are not surprising because the proteins exhibit only 32% identity at the amino acid level.

Recent studies revealed that the EHV-1 IE and early EICP0 regulatory proteins have an antagonistic relationship, possibly due to their competition for binding to cellular transcription factors, such as TBP and TFIIIB (Albrecht et al., 2003; Jang et al., 2001; Kim et al., 2003). As noted above, the EICP27 protein functions in a cooperative manner with either the IE or the EICP0 protein (Smith et al., 1993b; Zhao et al., 1995), and this cooperation may involve the EICP27 protein stabilizing the interaction between these viral proteins and a subset of cellular transcription factors. Evidence
supporting this possibility was obtained from protein–protein interaction studies that revealed that EICP27 residues 124–220 interact directly with purified TBP. The VZV IE4 protein also associates with TBP, suggesting that the interaction of these herpesviral proteins with cellular transcription factors contributes to their regulatory roles. In this regard, TBP is a component of TFIID and recognizes the cognate TATA box element, and initiates the assembly of the RNA polymerase II preinitiation complex (Hampsey, 1998). The recruitment of TFIID to promoters is an absolute requirement for transcription, in that artificial tethering of TBP to promoters is sufficient to initiate transcription (Gonzalez-Couto et al., 1997; Huh et al., 1999; Xiao et al., 1995). Numerous viral regulatory proteins target TBP as a means to regulate transcription of viral promoters (Abmayr et al., 1988; Carrozza and DeLuca, 1996; Caswell et al., 1993; Damania et al., 1998; Grondin and DeLuca, 2000; Hall et al., 1999; Ingles et al., 1991; Lieberman and Berk, 1991; Lieberman et al., 1997; Qadri et al., 1995; Smith et al., 1993a). The binding of viral trans-activators to TBP results in the: (i) preferential assembly of the preinitiation complex on viral promoters (Lieberman et al., 1997), (ii) increased stability of the TFIIID-TFIIA complex on viral promoters (Abmayr et al., 1988; Damania et al., 1998; Grondin and DeLuca, 2000; Smith et al., 1993a), and (iii) increased reinitiation of transcription by stabilizing the transcription intermediate composed of TFIID, TFIIB, TFIH, TFIIE, and Mediator (Yudkovsky et al., 2000).

Although the EICP27 protein does not possess an independent TAD, it appears to stimulate promoter activity by binding to TBP and perhaps stabilizing the association of TBP with the IE protein and other general transcription factors. The EICP27 protein failed to bind directly to TFIIB; however, one consequence of its interaction with TBP is that this viral protein may associate indirectly with other cellular and viral transcription factors, such as TFIIB and the EHV-1 IE protein. Whether an interaction between the EICP27 and IE proteins influences the DNA-binding activity of the IE protein or the interactions of the IE protein with general transcription factors will be assessed in future studies. In this regard, our recent studies indicate that a physical interaction does occur between the IE and EICP27 proteins (R.A. Albrecht and D.J. O’Callaghan, unpublished observations). As a result of its interaction with TBP, the EICP27 protein might also stabilize the TFIID-TFIIA transcription reinitiation intermediate or promote transcription elongation by RNA polymerase II. These and other questions about the role of the EICP27 protein in EHV-1 gene programming are being addressed at the present time.

Materials and methods

Virus and cell culture

EHV-1 (KyA strain) was passaged in murine fibroblast L-M cells as described previously (Perdue et al., 1974). L-M cells were expanded as suspension cultures in YELP medium [Eagle’s minimum essential medium (EMEM) supplemented with yeast extract, lactalbumin hydrolysate, and peptone] with 100 units of penicillin/ml, 100 μg of streptomycin/ml, nonessential amino acids, and 5% fetal bovine serum (FBS). Rabbit kidney cells (RK-13 cells; ATCC CCL 37) and equine transitional cell carcinoma (ETCC) cells (Kim et al., 2003) were grown as monolayer cultures in EMEM supplemented with 100 units of penicillin/ml, 100 μg of streptomycin/ml, nonessential amino acids, and 5% FBS.

Plasmid construction

Cloning of the expression plasmids was performed according to standard procedures (Sambrook et al., 1989). The mammalian expression plasmid pSVEICP27 (formerly pSVUL3) that encodes EICP27 aa 1–470 was described previously (Smith et al., 1993b; Zhao et al., 1995). pGex-EICP27 (1–470), which expresses the EICP27 gene as a GST fusion protein, was created by digesting pSVEICP27 with EcoRI and HindIII. The resulting enzyme fragment was then cloned into the EcoRI and HindIII sites of pGex-Kg (Guan and Dixon, 1991).

EICP27 truncation and deletion mutants

pSVEICP27Δ41–89 is a product of the EICP27 linker insertion mutants pSVEICP27in41 and pSVEICP27in89. pSVEICP27in41 was cloned by inserting the 12-bp BglII linker 5′-d(pGGAAGATCTTCC)-3′ (New England Biolabs, Beverly, MA) into the HincII site of pSVEICP27 (codon 41). pSVEICP27in89 was cloned by inserting a BglII linker into the HaelII site of pSVEICP27 (codon 89). pSVEICP27Δ41–89 was generated by ligating the EcoRI–BglII fragment of pSVEICP27in41 with the EcoRI–BglII digestion product of pSVEICP27in89. pSVEICP27Δ41–279 is a derivative of pSVEICP27in41 and pSVEICP27in279. pSVEICP27in279 was cloned by inserting a BglII linker at the HincII site of pSVEICP27 (codon 279). pSVEICP27Δ41–279 was subsequently generated by ligating the EcoRI–BglII fragment of pSVEICP27in41 with the EcoRI–BglII fragment of pSVEICP27in279. pSVEICP27Δ89–124 was cloned by self-ligation of the NaeI restriction enzyme digestion product of pSVEICP27. pSVEICP27Δ93–140 is a derivative of the linker insertion mutants pSVEICP27in93 and pSVEICP27in140. pSVEICP27in93 was cloned by inserting a BglII linker into the HaelII site of pSVEICP27 (codon 93). pSVEICP27in140 was cloned by inserting a BglII linker into the Rsal site of pSVEICP27 (codon 140). pSVEICP27Δ93–140 was subsequently cloned by ligating the EcoRI–BglII fragment of pSVEICP27in93 to the EcoRI–BglII fragment of pSVEICP27in140. pSVEICP27Δ93–220 was derived from the plasmids pSVEICP27in93 and pSVEICP27in220. pSVEICP27in220 was created by inserting a BglII linker into the AluI site of pSVEICP27 (codon 220). pSVEICP27Δ93–220 was subsequently cloned by ligating the EcoRI–BglII fragment of pSVEICP27in93 to the EcoRI–BglII fragment of...
pSVEICP27in220. pSVEICP27Δ140–220 was derived from the linker insertion mutants pSVEICP27in140 and pSVEICP27in220. pSVEICP27in140 was cloned by inserting a BglII linker into the Rsal site of pSVEICP27 (codon 140). pSVEICP27Δ140–220 was derived by ligating an EcoRI– BglII fragment of pSVEICP27in140 with EcoRI–BglII digested pSVEICP27in220. pSVEICP27Δ291–446 was cloned by digesting pSVEICP27 with PstI at codons 216 and 446, and the resulting fragment was self-ligated. pSVEICP27Δ291–343 is a derivative of the EICP27 linker insertion mutants pSVEICP27in291 and pSVEICP27in343. pSVEICP27in291 was generated by inserting a BglII linker into the HaelIII site of pSVEICP27 (codon 291). pSVEICP27in343 was produced by inserting a BglII linker into the HaelIII site of pSVEICP27 (codon 343). pSVEICP27Δ291–343 was cloned by ligating the EcoRI–BglII fragment of pSVEICP27in291 to the EcoRI–BglII digested pSVEICP27in343. pSVEICP27Δ343–395 was derived from the plasmids pSVEICP27in343 and pSVEICP27in395. pSVEICP27in395 was generated by inserting a BglII linker into the Alul site of pSVEICP27 (codon 395). pSVEICP27Δ343–395 was subsequently cloned by ligating the EcoRI–BglII fragment of pSVEICP27in343 with the EcoRI–BglII fragment of pSVEICP27in395. pSVEICP27Δ380–402 was cloned by a multistep process. First, the EHV-1 subgenomic plasmid pHa was digested with AcyI and self-ligated to create pdHA. Second, pdHA and pSVUL3EH (Zhao et al., 1995) were digested with HindIII. Finally, the HindIII fragment of pdHA was ligated to the HindIII site of pSVUL3EH. pSVEICP27(1–281) was cloned by partially digesting pSVEICP27 with Rsal and inserting a 14-bp Xbal oligonucleotide linker 5'(dTATATAGTACTAG)3' (New England Biolabs), which contains an amber codon in all three reading frames, at codon 282. pSVEICP27(1–422) was generated by partially digesting pSVEICP27 with Rsal, and inserting a 14-bp Xbal oligonucleotide linker at codon 423.

**GFP-EICP27 fusion vectors**

pGFP-EICP27(1–470) was created by cloning the EcoRI–BamHI fragment of pSVEICP27 into the same sites of pEGFPC3 (Clontech, Palo Alto, CA). pGFP-EICP27(140–216) was generated by first PCR amplifying the EICP27 sequence that encoded aa 140–216 and subcloning the Rsal and Xbal restriction enzyme sites (sites are underlined in the sequence) to facilitate cloning of EICP27 codons 178–185 into the Xhol and BamHI sites of pEGFPC3.

**EICP27 in vitro transcription/translation expression plasmids**

pGemEICP27(1–470) was constructed by digesting pSVEICP27 with EcoRI and SphI, and ligating the resulting fragment with pGem7z (Promega, Madison, WI) that was digested with EcoRI and SphI. pGemEICP27(1–422) was generated by inserting the EcoRI–SphI fragment of pSVEICP27(1–422) into the same sites of pGem7z. pGemEICP27(1–281) was created by subcloning the EcoRI–SphI fragment of pSVEICP27(1–281) into the same sites of pGem7z. The plasmids pGemEICP27Δ41–89, pGemEICP27Δ93–140, pGemEICP27Δ89–124, and pGemEICP27Δ140–220 were created by subcloning the EcoRI–SphI fragment from the EICP27 expression plasmids pSVEICP27Δ41–89, pSVEICP27Δ93–140, pSVEICP27Δ89–124, and pSVEICP27Δ140–220, respectively, into the EcoRI and SphI sites of pGem3z. pGemEICP27Δ8–59 was created by a two-step process. First, pSVEICP27Δ8–59 was generated by double digestion of pSVEICP27 with PvuII and Eco47III, and self-ligating the resulting restriction enzyme fragment. pGemEICP27Δ8–59 was then created by subcloning the EcoRI–SphI fragment of pSVEICP27Δ8–59 into the same sites of pGem3z.

**TATA box-binding protein and TFIIIB plasmids**

pGST-hTBP(1–339) was generated by subcloning the BamHI–EcoRI fragment containing the TBP open reading frame from pG3htBP(1–339) into the same restriction enzyme sites of pGEX-Kg. pG3htBP(1–339) was created by first PCR amplifying the TBP gene from pQE30-6His-hTBP by a two-step process.
(1–339), that was kindly provided by Dr. A. J. Berk (University of California at Los Angeles, CA). The forward primer was TBPfor and the reverse primer was TBPrev. The restriction enzyme sites for BamHI and EcoRI within the forward and reverse primers, respectively, are underlined. The PCR-amplified TBP open reading frame was digested with BamHI and EcoRI and was cloned into the same sites in pGem-3z. The construction of pG3hIIB that expresses full-length TFIIB (1–316) was described previously (Jang et al., 2001).

Primer sequences

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>NLS1:</td>
<td>5'-GTCAAGCGGTTAGCTGGACCAAGCGGTCGAGCCTGACCATCGGCC-3'</td>
</tr>
<tr>
<td>NLS2:</td>
<td>5' -AAGACGAGAAACGCCAAAAGCGTGGGCA-GAGTTCACCGCAACCAGCGCAAGGGG-3'</td>
</tr>
<tr>
<td>NLS3:</td>
<td>5' -CGCCTACAGACGTCCGGCAGATCTCGAGTGCGTACCTCAAAATG-3'</td>
</tr>
<tr>
<td>TBPre:</td>
<td>5'-CGCGGATCCATGGATCAGAACAAAGACGAGAACGCAAGAAGC-3'</td>
</tr>
<tr>
<td>TBPfor:</td>
<td>5'-CGCGGATCCATGGATCAGAACAAAGACGAGAACGCAAGAAGC-3'</td>
</tr>
<tr>
<td>TBPrev:</td>
<td>5'-CCCGATTCTTACACGCTTCTCAGCTCC-3'</td>
</tr>
</tbody>
</table>

Expression and purification of GST fusion proteins

The induction of GST fusion protein synthesis has been described elsewhere (Jang et al., 2001; Kim et al., 1995). The pGex plasmids were transformed into the Escherichia coli BL21(DE3)LYS strain (Novagen, Madison, WI) or the trxB(DE3)LYS strain (Novagen) by heat shock. Following the induction of protein synthesis, the bacteria were pelleted and lysed in 10 ml of Bughoster Reagent (Novagen) supplemented with benzonuclease (Novagen) and the protease inhibitors PMSF, TPCK, TLCK, and aproin (Sigma, St. Louis, MO). The purification and assessment of the concentration of the GST-fusion proteins were performed as described elsewhere (Jang et al., 2001). To prepare TBP devoid of the GST fusion tag, GST-TBP was treated with thrombin (Sigma) to release the GST moiety. TBP was then separated from the GST moiety by centrifugation in a 30-kDa size exclusion Centricon spin column (Millipore Corporation, Bedford, MA).

Generation of anti-EICP27 protein polyclonal antiserum

Two New Zealand White rabbits (approximately 3 kg per rabbit) were each immunized intramuscularly in a hind leg with 500 µg of purified GST-EICP27(1–470) diluted in sterile phosphate-buffered saline (PBS) and emulsified in Freund’s complete adjuvant. The immunized rabbits were administered a booster injection of 500 µg of recombinant protein emulsified in incomplete Freund’s adjuvant every fourth week for a total of four booster injections per rabbit. Pre-immune serum and the anti-GST-EICP27(1–470) antiserum were separated from the plasma by storing the blood for 4 h at room temperature, and then incubating at 4 °C overnight. After removing the clot, the antiserum was cleared of any remaining blood cells by centrifugation for 10 min at 2700 rpm in an Allegra 6R centrifuge (Beckman Coulter, Fullerton, CA).

Plasmid transfection and chloramphenicol Acetyl-transferase (CAT) assays

The EHV-1 IE promoter-CAT reporter chimera, pIE-CAT [pIE (−802/+73)], was described elsewhere (Smith et al., 1992). The cloning of the EICP0 promoter-CAT reporter chimera, pEICP0-CAT [pEICP0 (−335/−21)] was previously detailed (Buczynski et al., 1999). L-M or RK-13 cells were seeded as monolayers of 5 × 10^5 cells per 60-mm-diameter dish. Transfection of cell monolayers with the reporter and effector plasmids was performed as described previously (Holden et al., 1995; Jang et al., 2001). In the transient transfection experiments that employed the pIE-CAT reporter, cells were transfected with 0.5 pmol of the IE-CAT [pIE(−802/+73)] reporter plasmid alone or were cotransfected with the IE-CAT reporter plasmid and 0.3 pmol of the indicated EICP27 expression plasmid. For the Gal4 two-plasmid assay, cells were transfected with 1.4 pmol of the pG5EC reporter plasmid alone or were cotransfected with the pG5EC reporter plasmid and 0.3 pmol of the indicated Gal4 expression plasmid. In the transient transfection assays that employed the pEICP0-CAT reporter, cells were transfected with 1.4 pmol of the pEICP0-CAT reporter plasmid alone or were cotransfected with 1.4 pmol of the pEICP0-CAT and 0.3 pmol of one of the effector constructs indicated in the figure legend. At 48 h post-transfection, the cell monolayers were harvested, and protein quantitation and CAT assays were performed as described elsewhere (Holden et al., 1995; Smith et al., 1992). For each CAT assay, every sample was assessed in triplicate, and each assay was independently repeated at least three times. The statistical significance of the data was determined with the Student’s t test.

Western blot analyses

Western blot analyses was performed as described elsewhere (Jang et al., 2001), except for the following modifications. After washing the membranes 3 × with TBST buffer, the membranes were incubated with either pre-immune serum, the anti-GST-EICP27 polyclonal antibody (1–5000 dilution in TBST), or with the anti-TBP monoclonal antibody [1–500 dilution in TBST, BD Biosciences Pharmingen (Transduction Laboratories), San Diego, CA] for 1 h at RT.

Fluorescence microscopy

RK-13 cells cultured in two-well Falcon chamber slides (BD Biosciences) were transfected with 7.5 pmol of the indicated EICP27 expression plasmids as described above.
At 24 h post-transfection, the cells were fixed with 1 ml of fixation buffer (3% paraformaldehyde and 2% sucrose in PBS) for 15 min at room temperature. Next, the cells were incubated in permeabilization buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100) for 10 min at room temperature and then washed 3× with PBS. To examine the expression of the EICP27 gene and EICP27 deletion mutants, the wells were incubated with the anti-GST-EICP27 polyclonal antiserum diluted 1:1500 in PBS for 1 h at room temperature. After washing the wells 3× with PBS, FITC-conjugated goat anti-rabbit IgG antiserum diluted 1:1000 was added, and the slides were incubated for 1 h at room temperature. IF images were captured with a Sensys digital camera that was mounted to an Olympus BX50 fluorescent microscope, and the images were analyzed with the Metamorph analysis software. To examine the expression of the GFP fusion proteins harboring portions of the EICP27 protein, cells were transfected as described above with the indicated GFP expression construct. At 24 h post-transfection, the cells were fixed with 1 ml of fixation buffer, and coverslips were mounted with the SlowFade antifade solution containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), as per the manufacturer’s directions (Molecular Probes, Eugene, OR). Fluorescence microscopy was performed as described above.

**In vitro transcription/translation reactions**

The T7 promoter controlled expression of the pGem-EICP27 derivatives, although the Sp6 promoter regulated expression from pG3hTBP(1–339) and pG3hIIB. The proteins were synthesized with the TnT-coupled rabbit reticulocyte lysate in vitro transcription/translation (IVTT) kit (Promega) as per the manufacturer’s directions. To generate ³⁵S-labeled proteins, [³⁵S]methionine (40 μCi/ml; specific activity, 1175 Ci/mmol; New England Nuclear Corporation, Boston, MA) was included in the IVTT reactions. The synthesis of ³⁵S-labeled proteins was assayed by SDS-PAGE analysis and autoradiography, and the proteins were either employed in GST-pulldown assays or stored at −20°C.

**GST-pulldown assays**

To determine whether the EICP27 protein directly interacts with TBP, GST-pulldown assays were performed as detailed previously (Jang et al., 2001), with some modifications of the procedure. One microgram of either GST or GST-EICP27(1–470) was mixed with 1 μg of GST-free TBP in 600 μl of NETN buffer [100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl (pH8.0), and 0.5% NP-40]. The samples were incubated for 90 min at room temperature, then supplemented with 40 μl of a 50% mix of GST-Bind resin (Novagen) and incubated an additional 90 min at room temperature. The samples were then centrifuged and washed 5× with 600 μl of NETN buffer. Precipitated TBP was processed for Western blot analysis as described above. To examine the interactions of ³⁵S-labeled proteins with GST fusion proteins in vitro, GST-pulldown assays were performed. Briefly, 2 μg of either GST alone or a GST fusion protein was mixed with ³⁵S-labeled proteins in 600 μl of NETN buffer. The GST-pulldown reactions were processed as described for the direct interaction assay. The ³⁵S-labeled proteins that were precipitated by the GST proteins were visualized by SDS-PAGE followed by autoradiography using phosphorstorage screens. The ³⁵S-labeled proteins were visualized by analyzing the screens in the Storm PhosphorImager (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ).

**Co-immunoprecipitation studies**

³⁵S-radiolabeled luciferase, ³⁵S-TBP, ³⁵S-TFIIB, and the ³⁵S-EICP27(1–470) protein were produced by in vitro transcription and translation reactions as described above. The binding reactions included 10 μl of ³⁵S-labeled TBP incubated with 10 μl of one of the ³⁵S-labeled proteins [luciferase, TFIIB, or EICP27(1–470)] diluted in 600 μl of immunoprecipitation buffer [10 mM Tris–HCl (pH 8.0), 140 mM NaCl, 0.025% NaN₃, 0.1% Triton X-100, and 5 mM MgCl₂]. Additionally, the binding reactions contained either 5 μl of the anti-TBP polyclonal antibody (SI-1, Santa Cruz) or rabbit pre-immune sera. Following an overnight incubation of the binding reactions at 4°C, 40 μl of a 50% slurry of Protein A agarose beads (Sigma) were added to each reaction. Following a 1.5-h incubation at room temperature, co-immunoprecipitated proteins bound to the Protein A agarose beads were washed five times with 600 μl of immunoprecipitation buffer. Following the last wash, the Protein A agarose beads containing the immunoprecipitated proteins were processed for SDS-PAGE, and the co-immunoprecipitated ³⁵S-labeled proteins were visualized as described above.

**Acknowledgments**

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