The EICP27 protein of equine herpesvirus 1 is recruited to viral promoters by its interaction with the immediate-early protein

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Abstract

The equine herpesvirus 1 (EHV-1) EICP27 protein cooperates with either the immediate-early (IE) or the EICP0 protein to synergistically *trans*-activate viral promoters. GST-pulldown and co-immunoprecipitation assays revealed that the EICP27 protein's cooperation with the IE or the EICP0 protein involves its physical interaction with these viral proteins. In the case of the IE–EICP27 protein interaction, IE residues 424 to 826 and EICP27 residues 41 to 206 harbor the interactive domains. Electrophoretic mobility shift assays (EMSA) suggested that the EICP27 protein is not a sequence-specific DNA-binding protein as it fails to directly bind to the IE promoter, the early EICP27, EICP0, and TK promoters, or the late gD and IR5 promoters. However, EMSA studies also showed that the interaction of the IE and EICP27 proteins results in the recruitment of the EICP27 protein to representative early promoters. These results support our hypothesis that the EICP27 protein participates in the *trans*-activation of EHV-1 promoters, and suggest its presence within RNA polymerase II preinitiation complexes that assemble at viral promoters.
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Introduction

The sole *immediate-early* (*IE*) gene of equine herpesvirus 1 (EHV-1) encodes a spliced viral transcript that is translated to a 1,487-aa (200 kDa) phosphoprotein (Grundy et al., 1989; Harty et al., 1989). The IE protein performs two well-described regulatory activities: (i) repression of its own promoter and the late glycoprotein K promoter (Kim et al., 1999; Smith et al., 1992), and (ii) *trans*-activation of EHV-1 early and late promoters and some heterologous promoters (Buczynski et al., 1999; Smith et al., 1992, 1993). Recently, the IE protein was demonstrated to *trans*-activate the EICP0 promoter that is repressed by an as-yet unidentified cellular DNA-binding protein that binds to a *cis*-negative regulatory element (NRE; Kim et al., 2004). These findings suggest that the IE protein can mediate the derepression of viral promoters. Infections with the mutant virus KyAΔIE that lacks the *IE* (*IR1*) gene demonstrated that *IE* gene expression is essential for virus replication (Garko-Buczynski et al., 1998). The block in virus replication presumably occurs because RNA polymerase II preinitiation complexes ineffectively assemble at the core elements of EHV-1 early promoters in the absence of the IE protein. Thus, the IE protein *trans*-activates viral promoters by facilitating the assembly of preinitiation complexes at viral promoters. Indeed, our previous investigations revealed that the cellular transcription factors TATA box-binding protein (S. K. Kim and D. J. O'Callaghan, unpublished observations) and TFIIB (Albrecht et al., 2003; Jang et al., 2001) are interactive partners of the IE protein.

The IE protein possesses several domains that are essential to its *trans*-activation function: (i) an acidic

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trans-activation domain (TAD; aa 3 to 89; Buczynski et al., 1999; Harty and O'Callaghan, 1991; Smith et al., 1994), the interactive partners of which have not been elucidated, (ii) a serine-rich tract that binds to the cellular EAP protein (Kim et al., 2001), (iii) a nuclear localization signal (aa 963 to 970) that is comprised of nonpolar and basic amino acids (Smith et al., 1995), and (iv) a WLQN region (residues 422 to 597) that mediates the IE protein's binding to the consensus DNA sequence 5′-ATCGT-3′ (Kim et al., 1995). The location of this pentanucleotide sequence relative to the transcription initiation site dictates whether the IE protein *trans*-activates or *trans*-represses promoters (Kim et al., 1995, 1999). For example, the IE protein autorepresses its own promoter and represses the late gK promoter because a consensus sequence of this *cis* element is adjacent to the transcription initiation sites of these promoters (Grundy et al., 1989; Kim et al., 1999).

The *EICP27* (U_L5) gene is transcribed early in infection to a 1.6-kb transcript that is translated to a 470-aa nuclear phosphoprotein that harbors an arginine-rich nuclear localization signal (aa 176 to 185; Albrecht et al., 2004; Zhao et al., 1992). Sequence alignment of the EICP27 protein (51.4 kDa) indicated that this protein shares limited homology with its *Alphaherpesvirinae* counterparts in herpes simplex virus type 1 (32% with the HSV-1 ICP27 protein) and varicella-zoster virus (18% with the VZV IE4 protein) (Piette et al., 1995; Zhao et al., 1992). Our previous studies demonstrated that this protein: (i) independently stimulates reporter gene expression from the IE and early EICP0 promoters (Albrecht et al., 2004; Zhao et al., 1995); (ii) cooperates in a synergistic manner with the IE protein to achieve maximal activation of EHV-1 early and some late promoters (Smith et al., 1992, 1993); and (iii) potentiates the activation of EHV-1 promoters by the EICP0 protein (Bowles et al., 2000). The direct interaction of EICP27 residues 124 to 220 with TBP is one mechanism by which the EICP27 protein: (i) contributes to the *trans*-activation of EHV-1 promoters by the IE and EICP0 proteins, and (ii) independently stimulates the IE and early EICP0 promoters (Albrecht et al., 2004).

In this manuscript, we present evidence that the contributions of the EICP27 protein to viral gene expression do not involve its direct binding to promoter sequences. The cooperation between the EICP27 and IE proteins involves their physical interactions, and the sequences within these two regulatory proteins that mediate this interaction were mapped. The interaction of the EICP27 and IE proteins results in the recruitment of the EICP27 protein to viral promoters, where it may influence the regulatory properties of the IE protein. The data are consistent with our hypothesis that the EICP27 protein is incorporated into preinitiation complexes, where it may stimulate transcription initiation and/or elongation by cellular RNA polymerase II.

Results

The EICP27 protein is not a sequence-specific DNA-binding protein

Our recent studies demonstrated that the EICP27 protein activates the IE promoter [−802/+73, relative to the transcription initiation site] and the EICP0 promoter [−277/−50, relative of the ATG start codon] that controlled expression of the chloramphenicol acetyl-transferase (CAT) reporter gene (Albrecht et al., 2004). It was of interest to ascertain whether the activation of these viral promoters by the EICP27 protein involved its direct binding to a specific sequence present within these viral promoters. Our interest in determining whether the EICP27 protein binds directly to viral promoters was based on several observations: (i) the HSV-1 ICP27 and EHV-1 EICP27 proteins possess a conserved zinc-finger domain within their C-terminal region (Fig. 1A), (ii) the positions of cysteine and histidine residues within these alphaherpesvirus zinc-finger domains align with those present in the sequence for zinc-finger modules (Rhodes and Klug, 1993), and (iii) zinc-finger domains can mediate a DNA-binding activity (Kohn et al., 1997; Rhodes and Klug, 1993). Electrophoretic mobility shift assays (EMSA) examined whether the EICP27 protein directly binds to the IE promoter, the early TK, EICP0, and EICP27 promoters, and the late gD and IR5 promoters. Similar results were obtained for each of the indicated promoters, and the data presented in Figs. 1B and C depict our findings with the IE and EICP0 promoters, respectively, and are representative of the results obtained with the other indicated early and late promoters. The IE (407–757) polypeptide served as a positive control as it binds to all classes of viral promoters (Kim et al., 1995, 2004). In agreement with our previously published results (Kim et al., 1995), the EMSA results demonstrated that the IE polypeptide bound the IE promoter sequence [−151/+65] (Fig. 1B; lane 8) that contains the pentanucleotide 5′-ATCGT-3′ element (Grundy et al., 1989). Similarly, the IE polypeptide bound the EICP0 promoter sequence [−277/−50, relative of the ATG start codon] (Fig. 1C, lane 8) that contains a permutation of the pentanucleotide *cis*-element (Bowles et al., 1997). GST alone failed to bind to either of the viral promoter sequences (Figs. 1B and C, lane 2). Unlabeled IE and EICP0 promoter sequences (Figs. 1B and C; lane 10, respectively) but not unlabeled SV-Sport-1 sequence (Figs. 1B and C; lane 9) competed for binding of the IE polypeptide to the probe sequence, which indicated the specificity of this interaction. Additionally, the anti-IE protein monoclonal antibody A1.4 (Figs. 1B and C; lane 12), but not the control monoclonal antibody (Figs. 1B and C; lane 11), generated a super-shifted complex, confirming the presence of the IE protein at these promoter sequences. However, purified GST–EICP27 protein failed to exhibit sequence-specific binding to the IE promoter sequence (Figs. 1B and C; lanes 3 to 7) as well as to any of the other
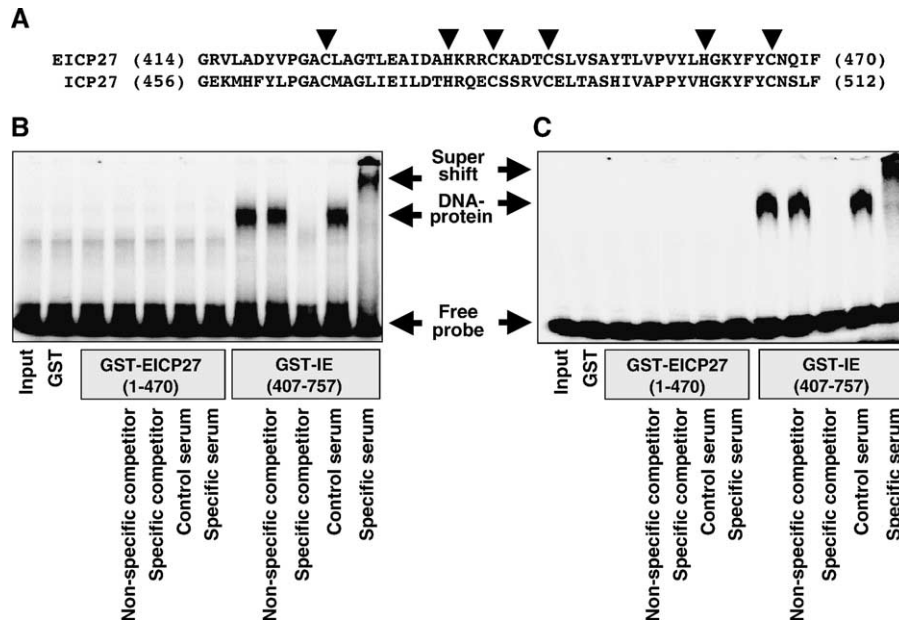
Fig. 1. The EICP27 protein is not a sequence-specific DNA-binding protein. (Panel A) Alignment of the zinc-finger domains contained within the EHV-1 EICP27 sequence (aa 414 to 470) and within the HSV-1 ICP27 sequence (456 to 512). The conserved cysteine and histidine residues are denoted by the black arrows. (Panels B and C) Electrophoretic mobility shift assays examined whether the purified GST-IE (407–757) protein or the purified GST–EICP27 protein directly bound the ^{32}P-labeled IE promoter [−175/+57] (Panel B) or the ^{32}P-labeled EICP0 promoter [−277/−50, relative of the ATG start codon] (Panel C). Each reaction contained ~12 ng of either the ^{32}P-labeled IE promoter (Panel B) or the ^{32}P-labeled EICP0 promoter (Panel C). The lane marked Input (lane 1) lacked any GST protein and served to indicate the position of the ^{32}P-labeled IE promoter and the ^{32}P-labeled EICP0 promoter (Panels B and C, respectively). The DNA-binding specificity for each GST fusion protein was examined by including ~300 ng of either unlabeled IE promoter or unlabeled EICP0 promoter as specific competitors (Panels B and C, respectively, lanes 5 and 10) or ~300 ng of an unlabeled sequence derived from the plasmid pSV-Sport1 as a non-specific competitor (Panels B and C, respectively, lanes 4 and 9). The identity of the IE polypeptide within the DNA–protein complexes was confirmed by including either the anti-IE protein A1.4 monoclonal antibody (Panels B and C, lane 12, super-shift indicated by the solid arrow) or the negative control anti-Gal4 DNA-binding domain-specific monoclonal antibody (Panels B and C, lane 11). The inclusion of either the anti-GST–EICP27 polyclonal antibody (Panels B and C, lane 7) or preimmune serum (Panels B and C, lane 6) served to confirm the absence of the EICP27 protein as a component of any DNA–protein complexes.

four representative early and late EHV-1 promoter sequences (data not shown).

The EICP27 protein stably associates with the IE protein in vitro

Transient transfection assays revealed that the EICP27 protein cooperates with the IE and EICP0 proteins to achieve maximal *trans*-activation of EHV-1 early and late promoters (Bowles et al., 2000; Smith et al., 1992; Zhao et al., 1995). The cooperation between the EICP27 protein and the IE and EICP0 proteins may involve an interaction of the EICP27 protein with one or both of the EHV-1 regulatory proteins. GST-pulldown assays initially tested whether the GST–EICP27 protein precipitated ^{35}S-labeled IE protein, ^{35}S-labeled E-TIF, and/or ^{35}S-labeled EICP0 protein. The data presented in Fig. 2A are representative of three independent experiments and show that the GST–EICP27 protein efficiently precipitated the IE and EICP0 proteins from in vitro transcription/translation reactions, but did not efficiently precipitate E-TIF above background levels. GST by itself as a negative control failed to precipitate any of the ^{35}S-labeled proteins. Since the EICP0 regulatory protein lacks DNA-binding activity (S. K. Kim and D. J. O'Callaghan, unpublished observations, Everett et al., 1993), studies of the

EICP27–EICP0 protein interaction would not elucidate whether the EICP27 protein is recruited to viral promoters. Therefore, this investigation focused on the EICP27–IE protein interaction in order to ascertain whether this interaction resulted in the recruitment of the EICP27 protein to IE protein-containing preinitiation complexes at viral promoters. GST-pulldown assays examined the stability of this EICP27–IE protein interaction in the presence of increasing concentrations of salt. The data revealed that the GST–EICP27 protein efficiently precipitated ^{35}S-labeled IE protein in the presence of salt concentrations ranging from 100 mM to 800 mM (Fig. 2B; lanes 4 to 8). At the 400 mM salt concentration, the capability of the GST–EICP27 protein to bind the IE protein was reduced to approximately half of that observed at the 100 mM salt concentration (Fig. 2B; lane 6 versus lane 4). The 1000 mM salt concentration nearly abolished the precipitation of the ^{35}S-labeled IE protein by the GST–EICP27 protein (Fig. 2B; lane 9). GST alone precipitated only a minimal amount of the labeled IE protein (Fig. 2B; lane 3). These results suggest that the EICP27 protein stably interacts with the IE protein at salt concentrations that exceed physiological conditions by as much as 3-fold. High salt concentrations reportedly weaken electrostatic interactions between proteins, while hydrophobic associations are enhanced (Tanford, 1961). The decreased
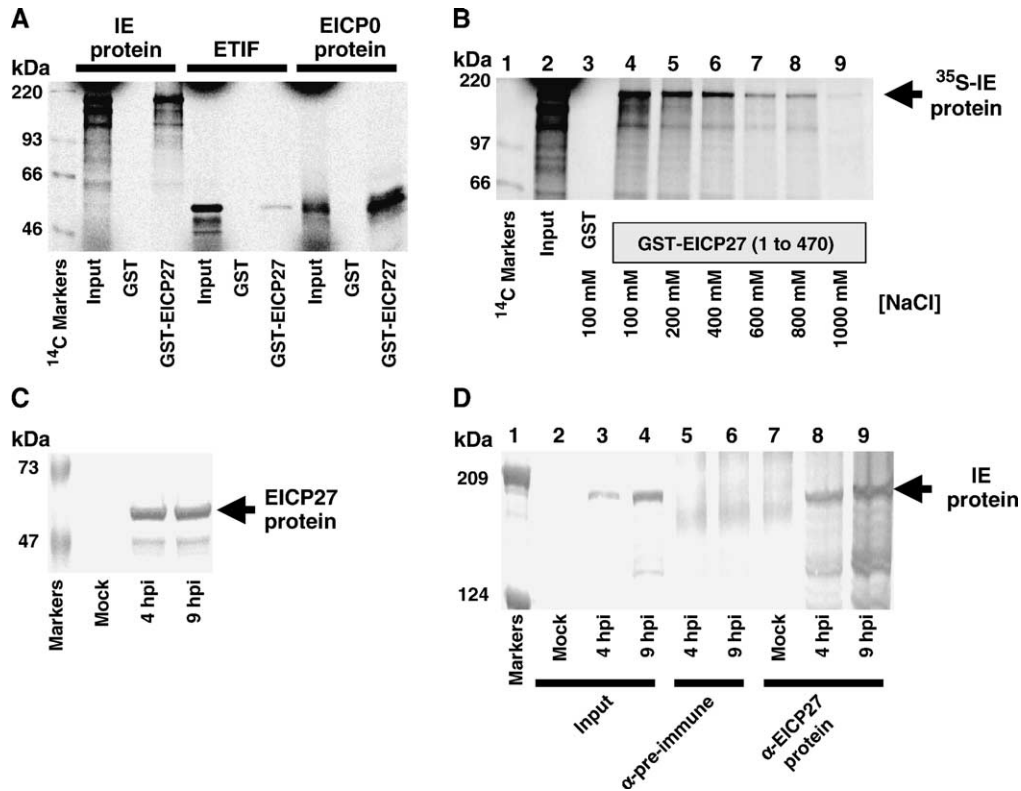
Fig. 2. The EICP27 protein interacts with the IE protein. (Panel A) The GST–EICP27 protein specifically interacts with the IE and EICP0 proteins. GST-pulldown assays assessed whether the GST–EICP27 protein precipitated ^{35}S-labeled IE, ^{35}S-labeled E-TIF, and/or ^{35}S-labeled EICP0 proteins from IVTT reactions. (Panel B) GST–EICP27 precipitates the IE protein at high salt concentrations. GST-pulldown assays examined whether the GST–EICP27 protein precipitated ^{35}S-labeled IE protein from IVTT reactions in the presence of increasing molar concentrations (100 mM to 1000 mM) of salt. For Panels A and B: (i) the input lanes show the amount of ^{35}S-labeled IE protein in each reaction, in order to estimate the relative binding efficiency of the GST–EICP27 protein, and (ii) the sizes of the ^{14}C molecular weight markers (kDa) are indicated at the left of the panel. (Panel C) The EICP27 protein is stably synthesized in EHV-1-infected RK-13 cells. Western blot analysis with the anti-EICP27 antibody confirmed the presence of the EICP27 protein in RK-13 cell extracts prepared at 4 and 9 h post-infection, but not in extracts prepared from mock-infected cells. (Panel D) Co-immunoprecipitation assays demonstrated that the anti-EICP27 antibody co-precipitates the IE protein with the EICP27 protein from infected cell extracts. EICP27–IE protein complexes were co-immunoprecipitated by the anti-EICP27 protein antibody from cell extracts that were prepared from either mock-infected RK-13 cells or infected cells at 4 or 9 h post-infection. The presence of the IE protein within the immunoprecipitates was assayed by Western blot analyses with the anti-IE protein antibody.

stability of this interaction at the 400 mM salt concentration suggests that a strong electrostatic interaction mediates the EICP27–IE protein interaction.

The interplay between the EICP27 and IE proteins was confirmed by co-immunoprecipitation assays that examined whether the anti-EICP27 protein antibody co-immunoprecipitated the native IE protein with the native EICP27 protein from EHV-1-infected cell extracts (Fig. 2D). Whole-cell extracts were prepared from mock-infected RK-13 cells or from cells infected for 4 h (early) or 9 h (late) with EHV-1. Western blot analysis confirmed the presence of the native 51.4 kDa EICP27 protein in the virus-infected cell extracts (Fig. 2C; lanes 3 and 4), and its absence in mock-infected cell extracts (Fig. 2C; lane 2). Western blot analyses that employed the anti-IE peptide antibody to detect the IE protein in the co-immunoprecipitates revealed that the anti-EICP27 protein antibody co-immunoprecipitated the native IE protein with the EICP27 protein from virus-infected cell extracts (Fig. 2D; lanes 8 and 9). The anti-IE peptide antibody did not react with any cellular proteins that were co-immunoprecipitated from

the mock-infected cell extracts by the anti-EICP27 protein antibody (Fig. 2D; lane 7). The negative controls in which virus-infected cell extracts were incubated with pre-immune serum failed to precipitate the IE protein (Fig. 2D; lanes 5 and 6). The input lanes contained 10% of the cell extracts that were included in each respective sample, and served to indicate the amount of the IE protein that was precipitated from the virus-infected extracts (Fig. 2D; lanes 3 and 4).

The EICP27 protein co-localizes with the IE protein during EHV-1 infection

To support the data obtained from the GST-pulldown and co-immunoprecipitation experiments, laser scanning confocal microscopy was conducted to examine the co-localization of these EHV-1 regulatory proteins in EHV-1-infected cells (Fig. 3). EHV-1-infected RK-13 cells were incubated with the anti-EICP27 protein polyclonal antibody and the anti-IE protein monoclonal antibody A1.4. At early (4 h) and late (9 h) times of infection, the EICP27 protein (green fluorescence)
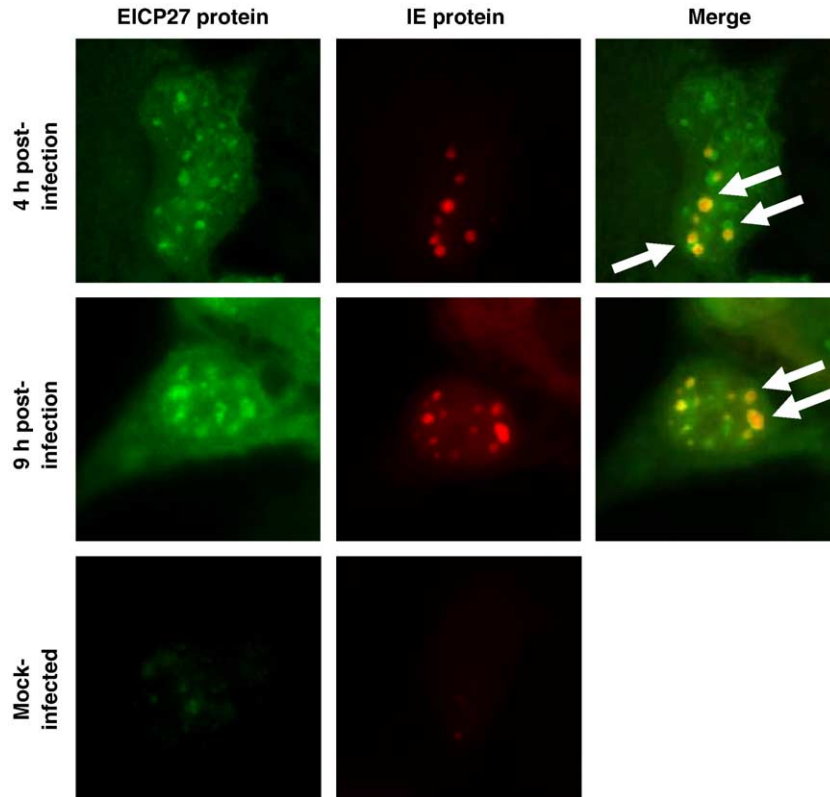
Fig. 3. Photomicrographs depicting the colocalization of the EICP27 protein with the IE protein. RK-13 cells were either mock-infected or infected with EHV-1 at an MOI of 5 pfu per cell. Infected cells were methanol-fixed at either early (4 h) or late (9 h) times post infection. Colocalization of the EICP27 protein foci (green) with the IE (1–1487) protein foci (red) is indicated by the yellow foci in the photomicrographs labeled Merge (arrows).

exhibited a diffuse nuclear staining pattern with some of the protein accumulating within foci. The IE protein (red fluorescence) predominantly localized within discrete nuclear foci at both time points. Merging of the immunofluorescence images revealed that some of the EICP27 protein foci overlapped the IE protein foci within the infected nucleus at early and late times of infection (yellow foci indicated by the arrows). Based on the protein–protein interaction data, the colocalization of the EICP27 and IE proteins in the nuclei of EHV-1-infected cells is consistent with the data from the protein–protein interaction assays (Figs. 2A and D) which showed that these EHV-1 regulatory proteins interact.

IE residues 424 to 826 harbor the EICP27 protein-binding domain

To delineate the region within the IE protein that mediates its interaction with the EICP27 protein, GST-pulldown assays tested the ability of the GST–EICP27 protein to precipitate ^{35}S-labeled truncation and deletion mutant polypeptides of the full-length IE protein (Fig. 4A). The GST–EICP27 protein precipitated the C-terminal truncation mutants IE (1–1254), IE (1–904), and IE (1–620), but not the mutants IE (1–424) and IE (1–289) (Fig. 4B). In addition, the GST–EICP27 protein interacted with the N-terminal truncation mutants IEΔ2–289, IEΔ2–421, IEΔ2–642, and IEΔ2–719, but did not

interact with IEΔ2–826 or IEΔ2–905 (Fig. 4B). These data indicated that the EICP27 protein-binding domain maps within IE residues 424 to 826. We next sought to map the minimal EICP27 protein-binding domain by additional GST-pulldown assays that tested the ability of the GST–EICP27 protein to precipitate IE derivatives that harbored specific deletions within the region spanning residues 424 to 826 (Fig. 5A). These results demonstrated that the GST–EICP27 protein efficiently precipitated the full-length ^{35}S-IE protein and the ^{35}S-IE deletion mutants IEΔ407–757, IEΔ621–757, and IEΔ644–824 (Fig. 5B). The negative control GST alone did not precipitate any of these proteins. Overall, these findings indicate that IE residues 424 to 826 harbor multiple domains that mediate the IE protein's interaction with the EICP27 protein. IE residues 424 to 620 harbor one EICP27 protein-binding domain, while residues 719 to 826 harbor another domain. The reduced efficiency by which the GST–EICP27 protein precipitated the IE (1–620) C-terminal truncation mutant is consistent with this IE mutant harboring only one of the two EICP27 protein-interactive domains.

EICP27 residues 41 to 206 harbor the IE protein-binding domain

The region within the EICP27 protein that mediates its interaction with the IE protein was mapped by GST-
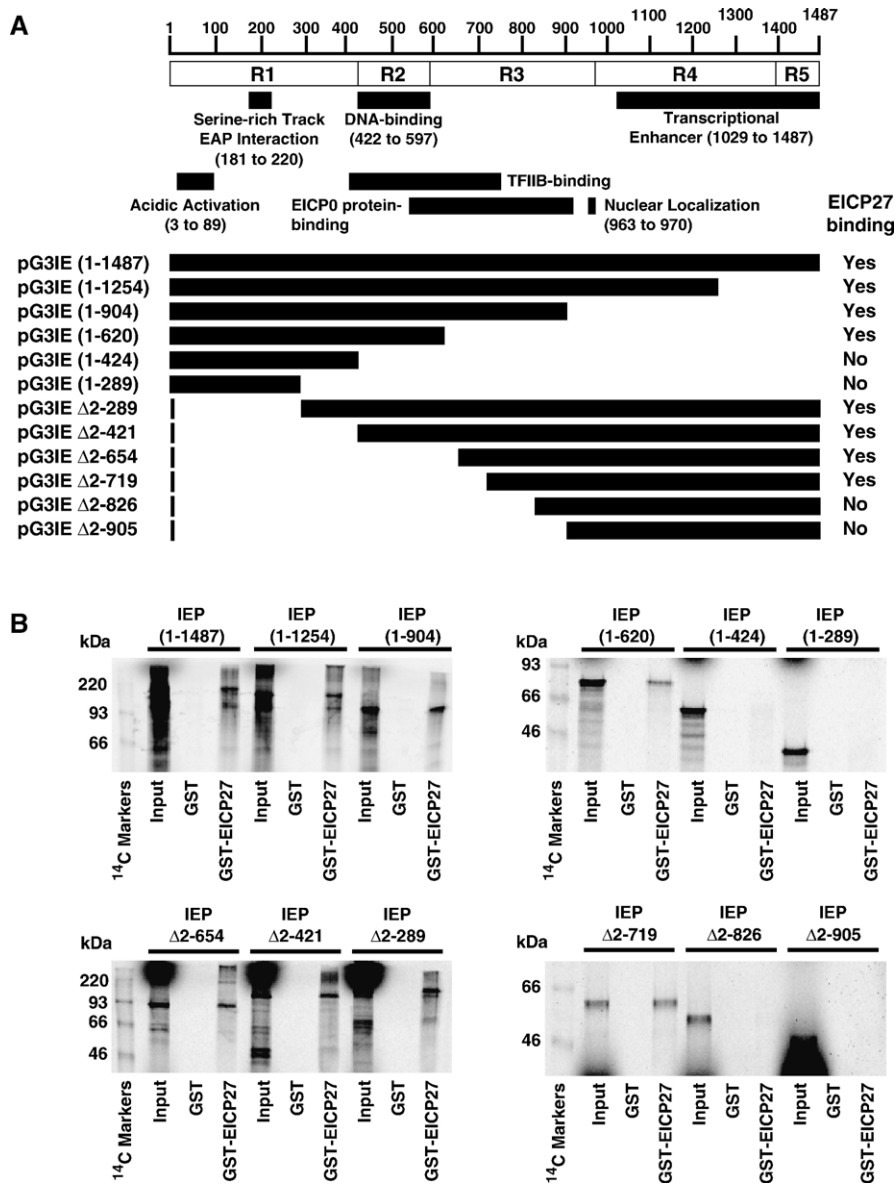
Fig. 4. Mapping the EICP27 protein-binding domain within the IE protein. (Panel A) Schematic representation of the functional domains present within the full-length and mutant IE polypeptides. The colinear regions R1 to R5 that are conserved among the alphaherpesvirus IE homologues, and the amino acid positions of the functional domains that contribute to the regulatory functions of the IE protein are illustrated. The amino acid sequence deleted in each truncation and deletion mutant IE polypeptide is indicated at the left of the figure. The ability of the GST–EICP27 protein to precipitate each radiolabeled IE polypeptide is indicated at the right of each polypeptide. (Panel B) IE residues 424 to 826 harbor an EICP27 protein-binding domain. GST-pulldown assays assessed the ability of the GST–EICP27 protein to precipitate the indicated ^{35}S-labeled IE polypeptide from IVTT reactions. The input lanes indicate the amount of each ^{35}S-labeled IE polypeptide included in each reaction, in order to estimate the relative binding efficiency of the GST–EICP27 protein. The sizes of the ^{14}C-labeled molecular weight markers (kDa) are indicated at the left of each panel.

pulldown assays that examined the ability of the GST-IE protein to precipitate ^{35}S-radiolabeled truncation and deletion mutants of the full-length EICP27 protein (Fig. 6A). The GST-IE protein precipitated the radiolabeled EICP27 (1–470), EICP27 (1–281), EICP27Δ93–140, EICP27Δ41–89, EICP27Δ8–59, EICP27Δ140–220, and EICP27Δ216–446 polypeptides (Fig. 6B). However, the GST–IE fusion protein failed to precipitate the radiolabeled EICP27 (226–470) and EICP27Δ41–279 polypeptides (Fig. 6B). These observations suggest that EICP27 residues 41 to 279 harbor one or more domains that mediate its interaction with the IE

protein. Deletions within the EICP27 protein sequence could result in EICP27 mutants that assume an incorrect conformation and thus fail to bind to the IE protein as would the native EICP27 protein. Therefore, additional assays were performed that examined the ability of GST-fusion proteins containing portions of EICP27 residues 41 to 282 (Fig. 7A) to precipitate radiolabeled IE protein and thus elucidate the minimal IE protein-binding domain of the EICP27 protein. These data would elucidate whether the inability of the EICP27Δ41–279 polypeptide to precipitate the IE protein is attributed to the removal of an IE protein-
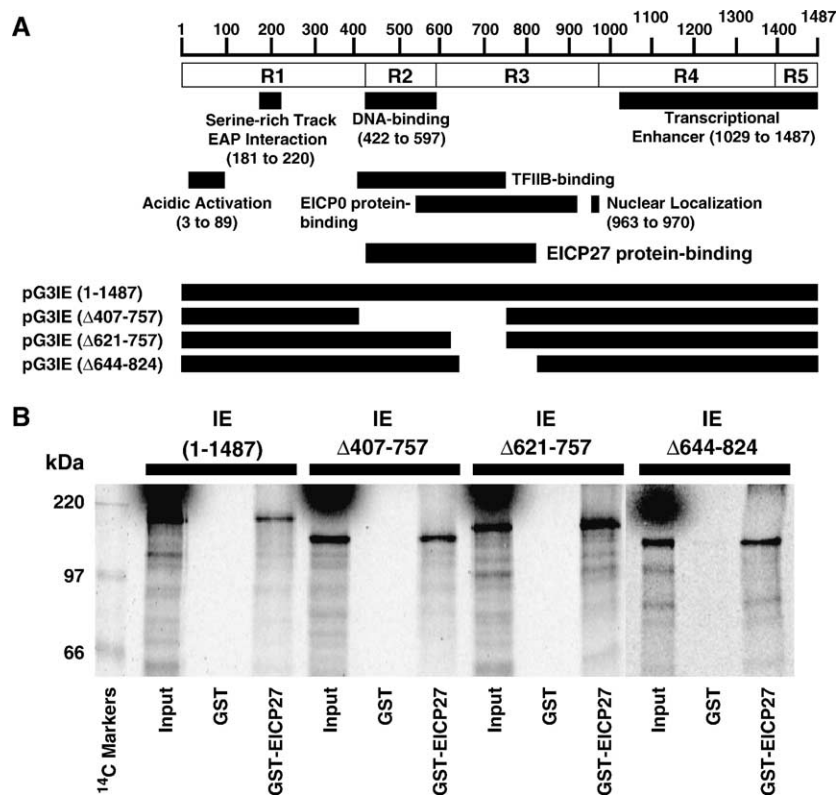
Fig. 5. (A) IE residues 407 to 824 mediate an interaction with the EICP27 protein. (B) GST-pulldown assays examined the ability of the GST–EICP27 protein to precipitate the indicated ^{35}S-labeled IE polypeptide from IVTT reactions. The input lanes indicate the amount of each ^{35}S-labeled IE polypeptide included in each reaction, in order to estimate the relative binding efficiency of the GST–EICP27 protein. The sizes of the ^{14}C-labeled molecular weight markers (kDa) are indicated at the left of each panel.

binding domain or because the polypeptide assumed an incorrect conformation. These data revealed that the ^{35}S-labeled IE protein was efficiently precipitated by the full-length GST–EICP27 fusion protein and by GST fusion proteins harboring EICP27 residues 41 to 282, 41 to 153, and 89 to 206 (Fig. 7B, upper autoradiograph). These results suggest that EICP27 residues 41 to 206 harbor one or more domains that mediate an interaction with the IE protein.

Previous studies on the regulatory functions of the EICP27 protein revealed that amino acids 124 to 216 mediate its interaction with TATA box-binding protein (TBP) (Albrecht et al., 2004). Consistent with our previous findings, GST fusion proteins harboring the entire EICP27 protein as well as residues 41 to 282, 41 to 153, and 89 to 206 precipitated ^{35}S-labeled TBP (Fig. 7B, lower autoradiogram). However, GST alone, the negative control, failed to precipitate any of the radiolabeled IE protein and precipitated background amounts of radiolabeled TBP. The data suggest that the same region of the EICP27 protein interacts with the IE protein and TBP. Therefore, GST-pulldown assays examined whether EICP27 residues 89 to 206 formed a tripartite complex with the IE protein and TBP. In these experiments, the GST–EICP27 (89–206) polypeptide was examined for its ability to precipitate radiolabeled TBP in the presence of increasing amounts of either ^{35}S-labeled luciferase or ^{35}S-labeled IE protein (Fig. 7C). In all reactions

including those in which the amount of ^{35}S-labeled IE protein varied by as much as 20-fold in the presence of a constant amount of ^{35}S-labeled TBP, the GST–EICP27 polypeptide was able to precipitate similar amounts of ^{35}S-labeled TBP. The results revealed that the GST–EICP27 (89–206) polypeptide simultaneously precipitated ^{35}S-TBP and the ^{35}S-IE protein (Fig. 7C; lanes 8 to 10). The GST–EICP27 polypeptide precipitated only background amounts of ^{35}S-luciferase (Fig. 7C; lanes 5 and 6). Thus, EICP27 residues 89 to 206 simultaneously interact with the IE protein and TBP to form a tripartite complex. In addition, in the presence of increasing amounts of ^{35}S-labeled IE protein, the GST–EICP27 protein precipitated a constant amount of IE protein. These data suggest that all of the available GST–EICP27 interacted with a limiting amount of ^{35}S-labeled IE protein.

The IE protein recruits the EICP27 protein to viral promoters

Although the EICP27 protein does not directly bind to viral promoters, its interaction with the IE protein may allow its recruitment to EHV-1 regulatory sequences. To address this possibility, EMSAs examined the capacity of the purified IE (323–1487) polypeptide to recruit the GST–EICP27 protein to the EHV-1 early EICP27 promoter (Fig.
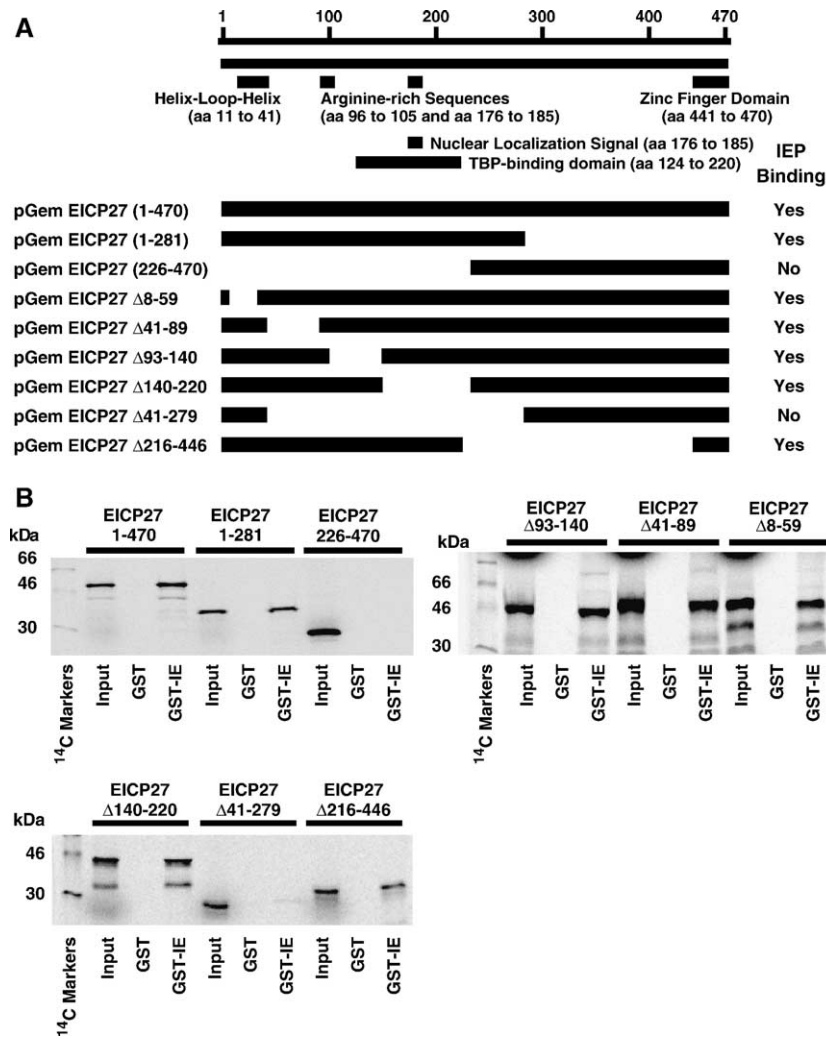
Fig. 6. Mapping the IE protein-binding domain within the EICP27 protein. (Panel A) Schematic representation of the functional domains present within the full-length and mutant EICP27 polypeptides. The amino acid positions of the TBP-binding domain, nuclear localization signal, and zinc finger domain that contribute to the regulatory functions of the EICP27 protein are illustrated. The amino acid sequence present in each EICP27 truncation mutant and deletion mutant is indicated at the left of the figure. The ability of the GST-IE protein to precipitate each radiolabeled EICP27 polypeptide is indicated at the right of each polypeptide. (Panel B) EICP27 residues 41 to 216 harbor an IE protein-binding domain. GST-pulldown assays assessed the ability of the GST-IE protein to precipitate the indicated ^{35}S-labeled EICP27 polypeptide from IVTT reactions. The input lanes indicate the amount of each ^{35}S-labeled EICP27 polypeptide included in each reaction, in order to estimate the relative binding efficiency of the GST-IE protein. The sizes of the ^{14}C-labeled molecular weight markers (kDa) are indicated at the left of each panel.

8). Similar experiments were performed that employed the EHV-1 early EICP0 and late IR5 promoters, and the results presented for the EICP27 promoter are representative of findings for all promoters. The results indicated that the IE polypeptide recognized the ^{32}P-labeled EICP27 promoter (−241/+8) (Fig. 8; lane 4), whereas the EICP27 protein failed to do so (Fig. 8; lane 3). In the presence of increasing amounts of the EICP27 protein, the IE protein complex at the EICP27 promoter migrated more slowly than that observed for the complex containing the IE polypeptide alone (Fig. 8; lanes 5 to 7). These data suggest that the increasing amounts of the GST–EICP27 protein included in some of the reactions resulted in increased ratios of the EICP27 protein relative to the limiting amount of IE protein, hence the generation of DNA–protein complexes

of greater molecular size. The inclusion of the anti-IE (925–943) peptide antibody (Jang et al., 2001) generated a super-shifted complex, confirming the presence of the IE protein at this promoter (Fig. 8; lanes 8 and 9). Similarly, inclusion of the anti-EICP27 protein antibody resulted in a super-shifted complex, demonstrating the presence of the EICP27 protein within this complex (Fig. 8; lanes 10 to 13). The negative control GST alone did not bind to the promoter sequence (Fig. 8; lane 2), and as expected preimmune serum did not generate a super-shifted complex (Fig. 8; lane 14). Overall, these results demonstrated that the EICP27 protein does not physically interact with EHV-1 promoters, and that the EICP27 protein associates with viral promoter complexes via its interaction with the IE protein.
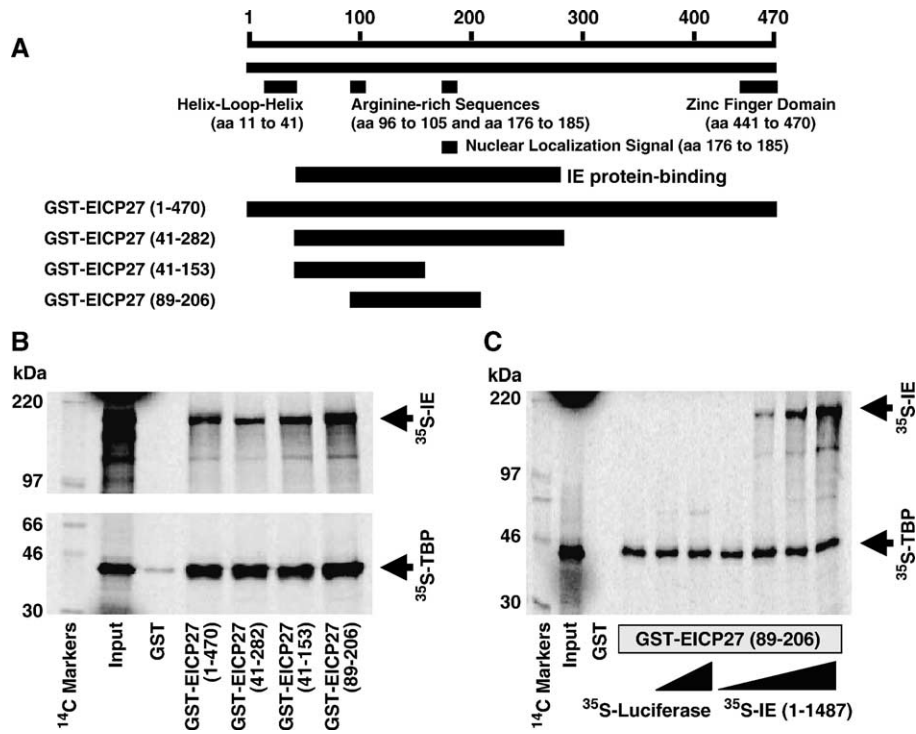
Fig. 7. The EICP27 protein forms a trimeric complex with the IE protein and TBP. (Panel A) Schematic representation of the functional domains present within the full-length and EICP27 polypeptides that were synthesized as GST fusion proteins. The amino acid sequence present in each EICP27 polypeptide is indicated at the left of the figure. (Panel B) GST-pulldown assays tested the ability of each GST–EICP27 fusion protein to precipitate either full-length ^{35}S-labeled IE protein (upper autoradiogram) or full-length ^{35}S-labeled TBP (lower autoradiogram) from IVTT reactions. The input lanes indicate the amount of either ^{35}S-labeled IE protein or ^{35}S-labeled TBP included in each reaction, in order to estimate the relative binding efficiency of the indicated GST–EICP27 protein. The sizes of the ^{14}C-labeled molecular weight markers (kDa) are indicated at the left of each panel. (Panel C) EICP27 residues 89 to 206 simultaneously co-precipitated the IE protein and TBP. GST-pulldown assays examined the ability of the GST–EICP27 (89–206) polypeptide to co-precipitate ^{35}S-labeled TBP and ^{35}S-labeled IE protein. The amount of ^{35}S-labeled TBP included in each reaction remained constant, whereas some of the samples included increasing amounts (as much as 20-fold) of ^{35}S-labeled IE protein or ^{35}S-labeled luciferase as indicated. The input lane indicates the amount of ^{35}S-labeled TBP included in each reaction, which served to monitor the relative binding efficiency of the GST–EICP27 (89–206) polypeptide. The sizes of the ^{14}C-labeled molecular weight markers (kDa) are indicated at the left of each panel.
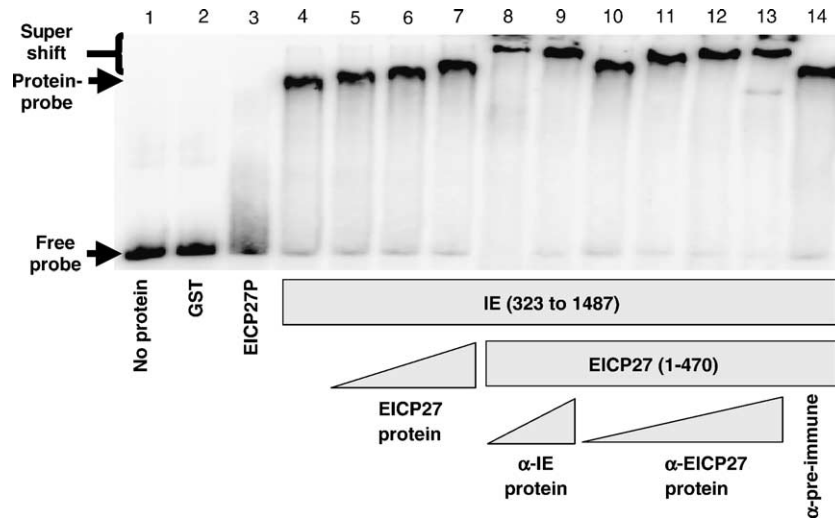

Fig. 8. The IE (323–1487) polypeptide physically recruits the EICP27 protein to an early viral promoter. Electrophoretic mobility shift assays that included the low ionic strength 0.5× tris-boric-EDTA (TBE) buffer that favors the formation of protein–protein interactions at viral promoters tested whether the IE (323–1487) polypeptide recruited the EICP27 protein to the ^{32}P-labeled EICP27 promoter [−241/+8]. The ^{32}P-labeled EICP27 promoter was incubated with the GST-IE (323–1487) polypeptide alone (lane 4) or with increasing amounts of the GST–EICP27 protein (lanes 5 to 7). The presence of the IE and EICP27 proteins within the protein–DNA complexes was demonstrated by including either the anti-IE (925–943) polyclonal antibody (lanes 8 and 9), the anti-GST–EICP27 polyclonal antibody (lanes 10 to 13), or preimmune serum in some reactions (lane 14). The lane marked No Protein (lane 1) lacked GST protein and served to indicate the position of the ^{32}P-labeled EICP27 promoter.

Discussion

Previous work on EHV-1 gene regulation demonstrated that the EICP27 protein cooperates with either the IE protein or EICP0 protein, resulting in the synergistic *trans*-activation of EHV-1 promoters (Bowles et al., 2000; Smith et al., 1992, 1993; Zhao et al., 1995). However, these experiments did not address the mechanism(s) by which the EICP27 protein contributes to the *trans*-activation functions of the IE and EICP0 proteins. Although the EICP27 protein lacks a *trans*-activation domain, residues 124 to 220 of the EICP27 protein mediate an interaction with the cellular transcription factor TBP (Albrecht et al., 2004).

In this manuscript, in vitro protein binding studies revealed that the IE and EICP0 proteins, but not ETIF, are interactive partners of the GST–EICP27 protein. The EICP27–EICP0 protein interaction is consistent with our prior observations that these two regulatory proteins cooperate to synergistically *trans*-activate EHV-1 early and late viral promoters (Bowles et al., 2000). The current EMSA studies on the EICP27 protein and previous studies of the EICP0 protein revealed that these EHV-1 proteins are incapable of directly binding to viral promoter sequences in a sequence-specific manner (S. K. Kim and D. J. O'Callaghan, unpublished observations, Everett et al., 1993). Considering these observations, it remains to be ascertained how the EICP27 and EICP0 proteins specifically stimulate EHV-1 promoters and whether the physical interaction between these proteins influences their activation of viral promoters.

The IE–EICP27 protein interaction was examined more extensively because this association may elucidate one mechanism by which the EICP27 protein is specifically incorporated into preinitiation complexes assembling at viral promoters. The binding of these EHV-1 proteins in the presence of high salt concentrations (400 mM NaCl) indicated the stability of this association. The IE protein-binding domain mapped within a region of the EICP27 protein (aa 41 to 206) that also contains a NLS and harbors a TBP-binding domain (Albrecht et al., 2004). The EICP27 protein-binding domain was mapped to a region of the IE protein (aa 424 to 826) that harbors its binding domains for DNA, TFIIB, and the EICP0 protein (Jang et al., 2001; Kim et al., 2003, 1995). An antagonistic relationship exists between the IE and EICP0 proteins that may be mediated by the physical association of these two EHV-1 regulatory proteins during infection (Kim et al., 2003). Since the EICP27 and EICP0 proteins may compete for binding to the same region of the IE protein, the EICP27–IE protein interaction may perturb the ability of the IE protein to antagonize the regulatory functions of the EICP0 protein. One consequence of the interaction between EICP27 and IE proteins is that the EICP27 protein associates with viral promoter complexes. In addition, the EICP27 protein forms a tripartite complex with the IE protein and TBP, a finding that is consistent

with our hypothesis that this early regulatory protein is incorporated into IE protein-governed preinitiation complexes. Spengler et al. (2000) also demonstrated a physical interaction between the varicella-zoster virus IE4 and IE62 proteins. However, the authors did not map the IE62 protein-binding domain within the IE4 protein and did not examine whether the interaction of the IE4 protein with the IE62 protein resulted in the recruitment of the IE4 protein to viral promoter complexes.

Our speculation that the EICP27 protein regulates transcription is consistent with several findings of the HSV-1 immediate-early ICP27 protein. Panagiotidis et al. (1997) demonstrated by gel shift assays that HSV-1 ICP27 associated with protein complexes at viral promoters, possibly due to its interaction with the HSV-1 ICP4 protein. Jenkins and Spencer (2001) demonstrated the presence of ICP27 within RNA polymerase II holoenzyme complexes that were devoid of TFIIE. Co-immunoprecipitation studies showed an association of this HSV-1 protein with RNA polymerase II, suggesting one mechanism by which ICP27 incorporates within preinitiation complexes (Zhou and Knipe, 2002). Interactions of ICP27 with RNA polymerase II factors may explain how this HSV-1 regulatory protein increases transcription of late genes (Jean et al., 2001) and expression of viral genes encoding proteins necessary for replication of the viral genome (McCarthy et al., 1989; Rice and Knipe, 1990; Rice et al., 1989; Sacks et al., 1985; Uprichard and Knipe, 1996). Lastly, HSV-1 ICP27 is present within ND10 structures that serve as sites for viral genomic replication and transcription (Everett et al., 2004; Tang et al., 2003; Zhong and Hayward, 1997). Together, these observations suggest that this protein is incorporated within an RNA polymerase II preinitiation complex; however, few studies have elucidated exactly how these protein interactions involving HSV-1 ICP27 contribute to transcription of viral genes.

The functions of HSV-1 ICP27 in post-transcriptional processes have been investigated, and the findings from several laboratories were discussed in a recent review (Sandri-Goldin, 2004). These studies revealed that ICP27: (i) binds viral transcripts; (ii) regulates the processing of the 3′ ends of pre-mRNA; (iii) shuttles between the nucleus and cytoplasm, (iv) facilitates the nuclear export of viral intron-less transcripts; (v) inhibits splicing of intron-containing pre-mRNA; and (vi) influences the intranuclear distribution of small nuclear ribonucleoproteins. Whether the EHV-1 EICP27 protein, which is an early gene product that exhibits limited homology with its HSV-1 counterpart, mediates any of these post-transcriptional functions remains to be determined.

The prior observation that the EICP27 protein interacts with TBP (Albrecht et al., 2004) combined with the findings presented here that this early viral protein is physically recruited to viral promoters by its interaction with the IE protein are consistent with our hypothesis that the EICP27 protein actively participates within RNA

polymerase II preinitiation complexes. Whether the association of the EICP27 and IE proteins influences the interactions of the IE protein with additional viral and/or cellular transcription factors will be addressed in future studies. Other questions to be addressed are whether the EICP27 protein contributes to the assembly and stability of preinitiation complexes at viral promoters, and whether the EICP27 protein stimulates transcription elongation by RNA polymerase II.

Materials and methods

Virus and cell culture

The propagation of EHV-1 (KyA strain) in murine fibroblast L-M cells and the plaque assays to titer EHV-1 were described previously (Perdue et al., 1974). The propagation of L-M cell as suspension cultures in YELP medium and rabbit kidney cells (RK-13 cells; ATCC CCL 37) in EMEM was described elsewhere (Albrecht et al., 2004).

Cloning of expression plasmids

The procedures for cloning the expression plasmids employed in this investigation were outlined elsewhere (Sambrook et al., 1989). DNA sequencing to confirm that the indicated coding sequences were cloned in-frame into the expression plasmids employed the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB Corporation, Cleveland, OH) per the manufacturer's directions. Some plasmids were sequenced by the DNA Laboratory at Arizona State University, Tempe, AZ.

Bacterial expression plasmids

pGexEICP27 (1–470) that harbors the *EICP27* open reading frame (ORF) cloned in-frame with the sequence encoding GST was described previously (Albrecht et al., 2004). pGexEICP27 (41–282) was created by digesting pSVEICP27in41 (Albrecht et al., 2004) with *Bgl*II and *Hind*III, and ligating the released fragment into pGex-Kg (Guan and Dixon, 1991) that was digested with *Bam*HI and *Hind*III. pGexEICP27 (41–153) was created by digesting the pSVEICP27in41 with *Bgl*II and *Sac*I, and ligating the released fragment with pGexKg that was digested with *Bam*HI and *Sac*I. pGexEICP27 (89–206) was created by digesting pSVEICP27in89 (Albrecht et al., 2004) with *Bgl*II and *Not*I, and cloning the released fragment into pGex4T-2 (Amersham Biosciences, Piscataway, NJ) that was digested with *Bam*HI and *Not*I. Plasmids pGexIE (1–1487), pGexIE (407–757) and pGexIE (323–1487) in which the indicated coding sequence of the *IE* gene is cloned in-frame with the sequence encoding GST were detailed earlier (Jang et al., 2001; Kim et al., 1995).

In vitro transcription/translation expression plasmids

pGemEICP0 that expresses the entire *EICP0* gene and pGemTBP that expresses the *TBP* gene were described earlier (Albrecht et al., 2004; Bowles et al., 1997). pGemETIF that expresses the *ETIF* gene was created by subcloning the *Hind*III–*Xba*I fragment of pCETIF (Kim and O'Callaghan, 2001) into the same sites of pGem3z (Promega Corporation, Madison, WI). pG3IE (1–1487), pG3IE (1–1254), pG3IE (1–904), pG3IE (1–620), pG3IE (1–424), pG3IE (1–289), pG3IEΔ2–289, pG3IEΔ2–421, pG3IEΔ2–654, pG3IEΔ2–719, pG3IEΔ2–826, pG3IEΔ2–905, pG3IEΔ407–757, pG3IEΔ621–757, and pG3IEΔ644–824 that express the indicated *IE* coding sequence were described previously (Jang et al., 2001). Plasmids pGemEICP27(1–470), pGemEICP27(1–281), pGemEICP27Δ8–59, pGemEICP27Δ41–89, pGemEICP27Δ93–140, and pGemEICP27Δ140–220 that express the indicated *EICP27* codons were described previously (Albrecht et al., 2004). pGemEICP27Δ41–279 and pGemEICP27Δ216–446 were generated by subcloning the *Eco*RI–*Sph*I fragment of pSVEICP27Δ41–279 and pSVEICP27Δ216–446, respectively (Albrecht et al., 2004), into the same sites of pGem3z. pGemEICP27(226–470) was created by digesting pSVEICP27 (Albrecht et al., 2004) with *Not*I and *Sph*I and cloning the resulting fragment into the same sites of pGem5z (Promega).

Laser scanning confocal microscopy

RK-13 cell monolayers of 2×10^4 cells per well of two-well Lab-Tek chamber slides (Nagle Nunc International, Naperville, IL) were either mock-infected or infected with EHV-1 at an MOI of 5 pfu per cell. At early (4 h) and late (9 h) times post-infection, the cells were fixed onto the glass slides with 100% ice-cold methanol for 10 min. The slides were washed 3 times with phosphate buffered saline (PBS) for 10 min per wash and then incubated with the primary antibodies anti-GST–EICP27 polyclonal antibody (Albrecht et al., 2004; antibody diluted 1:1000 in PBS) and anti-IE monoclonal A1.4 antibody (Caughman et al., 1995; antibody diluted 1:200) for 1 h. Following 3 washes with PBS, the slides were incubated with the secondary antibody FITC-conjugated goat anti-rabbit IgG antibody or TRITC-conjugated goat anti-mouse IgG antibody for 1 h. After 3 washes with PBS, the slides were mounted with a solution of 50% glycerol and 0.1% *p*-phenylenediamine in PBS. Images were obtained with a laser scanning confocal microscope system (MRC600; Bio-Rad Laboratories, Hercules, CA) attached to a Nikon Diaphot microscope as described previously (O'Callaghan et al., 1994).

Expression and purification of GST fusion proteins

The expression and purification of GST-fusion proteins have been described elsewhere (Albrecht et al., 2003; Kim et

al., 1995). The bacterial strains *Escherichia coli* BL21(DE3) pLysE (Novagen, Madison, WI) or *trxB*(DE3)pLysE (Novagen) were employed for induction of GST-fusion protein synthesis. Antibiotic selection involved 100 μg ampicillin per ml and either 50 μg kanamycin per ml for the *trxB*(DE3)LysE strain or 25 μg chloramphenicol per ml for the BL21(DE3)pLysE strain. The methods for desalting and for ascertaining the concentration of the purified GST-fusion proteins were described elsewhere (Jang et al., 2001). The purified GST–IE protein and the GST–EICP27 protein were centrifuged through 100 kDa and 50 kDa Centricon size exclusion spin columns (Millipore, Bedford, MA), respectively, to separate the majority of the proteolytic/degradation products from the full-length GST fusion proteins.

In vitro transcription/translation reactions

The Sp6 promoter controlled expression of the ETIF, EICP0, IE, and TBP pGem expression plasmids, while the T7 promoter controlled expression of the EICP27 pGem expression plasmids. In vitro transcription/translation (IVTT) reactions were performed with the TnT quick-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's directions. ^{35}S-labeled proteins were synthesized by including [^{35}S]methionine (40 μCi/ml; specific activity, 1175 Ci/mmol; New England Nuclear Corporation, Boston, MA) in the IVTT reactions. Synthesis of the ^{35}S-labeled proteins was checked by SDS-PAGE analysis and autoradiography.

GST-pulldown assays

GST-pulldown assays that employed ^{35}S-labeled polypeptides were performed as detailed previously (Albrecht et al., 2003). Briefly, 2 μg of either GST alone or the indicated GST fusion protein were mixed with ^{35}S-labeled proteins in 600 μl of NETN buffer. The ^{35}S-labeled proteins precipitated by the GST fusion protein were analyzed by SDS-PAGE followed by autoradiography using phosphorstorage screens (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ). The ^{35}S-labeled proteins were visualized by analyzing the screens in the Storm phosphorimager (Molecular Dynamics) or the Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA).

Co-immunoprecipitation assays

To confirm the data obtained from the GST-pulldown assays, co-immunoprecipitation assays were performed as described elsewhere (Albrecht et al., 2004). Briefly, 5×10^6 L-M cells were either mock-infected or infected with EHV-1 at an MOI of 10 plaque forming units per cell. At 4 and 9 h post-infection, whole cell extracts were prepared by precipitating the cells by centrifugation and lysing the cells in lysis buffer (1% Triton X-100, 50 mM Tris–HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, and 0.02% sodium azide). The

protein concentration of each sample was determined with the BCA kit (Pierce Endogen, Rockford, IL). Each co-immunoprecipitation reaction included 200 μg of cell extract incubated with 2 μl of either pre-immune serum or anti-GST–EICP27 protein antibody in 600 μl of immunoprecipitation buffer (Albrecht et al., 2004). After processing the reactions as detailed previously (Albrecht et al., 2004), the pelleted protein A agarose beads containing the co-immunoprecipitated protein complexes were resuspended in 2× Laemmli buffer (Sigma, St. Louis, MO) and subjected to Western blot analysis with the anti-IE peptide antibody (Albrecht et al., 2003).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed essentially as described (Kim et al., 1995). The ^{32}P-labeled probe harboring the IE promoter sequence [−175/+57, relative to the transcription initiation site] was generated by PCR amplification with the forward primer IEgsaF 5′-AAAGCGCATGCAAATGCAAAGCGCGGGACC-3′ and reverse primer IEgsaR 5′-ATAGTCCTCGAAGGCTG-GCTGG-3′ and plasmid pIE [−802/+73]-CAT. The ^{32}P-labeled probe harboring the EICP0 promoter sequence [−277/−50, relative of the ATG start codon] was generated by PCR amplification. The PCR reactions consisted of plasmid pEICP0 [−339/−21]-CAT (Kim et al., 2004), the forward primer EICP0FP 5′-AACCACCGTGTGCT-CACCCG-3′, and the reverse primer EICP0RP 5′-TTAAAACCAGGCTTGGTGCC-3′. The ^{32}P-labeled probe harboring the EICP27 promoter sequence [−241/+8] was generated by PCR amplification. The PCR reactions consisted of plasmid p*Xba*I-G (Zhao et al., 1992), the forward primer RAE27FP 5′-AACCCCCCGACTCCCG-3′, and reverse primer 5′-GCCAAGAGTGCGGACC-3′. The DNA fragments were end-labeled with [γ-^{32}P]dATP (New England Biolabs, Beverly, MA) and T4 polynucleotide kinase (Promega). The DNA-binding reactions contained approximately 20,000 CPM of radiolabeled DNA probe (approximately 12 ng), 1.5 μg of poly[dI-dC] as a nonspecific competitor, 50 or 100 ng of the indicated protein in 20 μl of binding buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 10 mM mercaptoethanol, 0.1% (wt/vol) CHAPS, 100 mM NaCl, 10% glycerol). DNA–protein complexes were allowed to assemble for 30 min at room temperature. The non-specific competitor DNA was isolated by PCR amplification of the pSV-Sport1 sequence spanning bp 1378 to 1618 with the forward primer 5′-CTGTTCCGACCCTGCCGC-3′ and the reverse primer 5′-TGGCTGCTGCCAGTGGCG-3′. To confirm the presence of the IE protein within DNA–protein complexes, either the anti-IE protein monoclonal antibody A1.4 or the anti-IE (925–943) polyclonal antibody was included in some reactions. The presence of the EICP27 protein was demonstrated by including the anti-GST–EICP27 protein polyclonal antibody in some reactions. The control antibodies included the anti-Gal4 DNA-binding

domain-specific monoclonal antibody (RK5C1, Santa Cruz Biotech, Santa Cruz, CA) and rabbit preimmune serum. In the antibody-containing reactions, the indicated antibody was added after the initial 30-min incubation, and the sample was incubated an additional 30 min. Following the incubation period, the DNA–protein complexes were resolved by polyacrylamide gel electrophoresis at 20 mAmps in a non-denaturing gel containing 4% acrylamide/bis-acrylamide, 10% glycerol and either $0.5\times$ TBE buffer ($10\times$ stock solution: 89 mM Tris base, 89 mM boric acid, 20 mM EDTA, and 25 mM $MgCl_2$) or $0.75\times$ TGE buffer ($10\times$ stock solution: 25 mM Tris base, 1.9 M glycine, 10 mM EDTA, and 25 mM $MgCl_2$).

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