The ICP22 Protein of Equine Herpesvirus 1 Cooperates with the IE Protein To Regulate Viral Gene Expression

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The equine herpesvirus 1 (EHV-1) immediate-early (IE) phosphoprotein is essential for the activation of transcription from viral early and late promoters and regulates transcription from its own promoter. The EHV-1 EICP22 protein, a homolog of ICP22 of herpes simplex virus, increases in vitro DNA binding activity of the IE protein for sequences in the IE, early, and late promoters. The EICP22 protein affected the rate as well as the extent of the IE protein binding to promoter DNA sequences. To study the DNA binding activity of the IE protein, Trp493, Gln495, Asn496, and Lys498 of the WLQN region, which is directly involved in DNA binding, were replaced with Ser (IEW493S), Glu (IEQ495E), Ile (IEN496I), and Glu (IEK498E), respectively. Gel shift assays revealed that the glutathione S-transferase (GST)-IEQ495E(407-615) and GST-IEK498E(407-615) proteins failed to bind to the IE promoter, indicating that the Glu and Lys residues are important for the DNA binding activity. In the presence of the GST-EICP22 protein, DNA binding activity of the GST-IEQ495E(407-615) protein was restored, suggesting that the EICP22 protein cooperates with the IE protein to regulate EHV-1 gene expression. Transient-transfection assays also showed that the EICP22 protein allowed the IEQ495E mutant to be functional as a transactivator. These results are unique and may represent an important role for the EICP22 protein in EHV-1 gene regulation.

Equine herpesvirus 1 (EHV-1), an Alphaherpesvirinae member, is a major pathogen of equines, causing respiratory disease, neurological disorders, and spontaneous abortions in pregnant mares (1, 36). The infectious EHV-1 virion is enveloped, possesses an icosahedral capsid, and harbors a linear, double-stranded DNA molecule of approximately 150 kbp (19, 20, 53, 59). The EHV-1 genome, which has been sequenced in its entirety, is a two-isomer structure containing at least 73 unique genes and at least 6 diploid genes (4, 19, 20, 22, 44, 53, 59). During a productive lytic infection, the genes of EHV-1 are coordinately expressed and temporally regulated in immediate-early (IE), early (E), and late (L) fashions (15, 16). The EHV-1 genome, which has been sequenced in its entirety, is a two-isomer structure containing at least 73 unique genes and at least 6 diploid genes (4, 19, 20, 53, 59). During a productive lytic infection, the genes of EHV-1 are coordinately expressed and temporally regulated in immediate-early (IE), early (E), and late (L) fashions (15, 16). The EHV-1 EICP22 protein, of 293 aa (23), exhibits homology to ICP22 of HSV-1 (35), IEP-55 of bovine herpesvirus 1 (46), ORF63 of VZV (9), IR2 of simian varicella virus (14), Rsp40 of PRV (62), and ORF4 of EHV-1 (8). The four proteins share extensive homology with the EHV-1 IE protein (17). On the basis of the predicted amino acid sequences for the VZV, PRV, and HSV-1 homologs of EHV-1 IE, the primary structural classes of these proteins have been conceptually divided into five regions that are colinear among the proteins (7, 17, 34, 55). The four proteins have extensive (approximately 50%) amino acid identity within regions 2 and 4 but have little or no homology within regions 1, 3, and 5, except for a serine-rich tract found in region 1 of all four proteins. Despite the differences among these proteins in regions 1, 3, and 5, they apparently perform similar functions during productive infection. A comparative sequence analysis of the various established classes of DNA binding motifs has identified a sequence (referred to as the WLQN region) that has significant similarity to the homeodomain DNA recognition helix (54). The WLQN region is highly conserved within these alphaherpesvirus ICP4 transactivator homologs. Mutation studies with a conserved sequence within the VZV 140,000-molecular-weight (140 K) DNA binding domain, including residues WLQN, demonstrated that the WLQN homology region is directly involved in the DNA binding interaction (54).

The EHV-1 EICP22 protein, of 293 aa (23), exhibits homology to ICP22 of HSV-1 (35), IEP-55 of bovine herpesvirus 1 (46), ORF63 of VZV (9), IR2 of simian varicella virus (14), Rsp40 of PRV (62), and ORF4 of EHV-1 (8). The EICP22 gene was shown to be expressed as an early 1.4-kb transcript and a late 1.7-kb transcript, and its protein product was identified as a family of proteins ranging in size from 42 to 47 kDa (23). During an EHV-1 lytic infection, the EICP22 protein...
localizes to the nucleus in a diffuse staining pattern and becomes packaged within EHV-1 virions (21).

To elucidate the mechanism of the synergistic effect seen in transient-transfection assays of the IE and EICP22 proteins, DNA binding experiments and transient-transfection assays were performed. In this paper, we report that the EICP22 protein cooperates with the IE protein to regulate EHV-1 gene expression by enhancing the DNA binding capacity of the IE protein.

**MATERIALS AND METHODS**

**Bacteriology** Culture. For the bacterial expression experiments, plasmids were transformed into *Escherichia coli* BL21(DE3)pLysE (52). Suspension cultures of L-M cells were maintained in yeast extract-lactalbumin hydrolysate-peptone suspension medium containing 0.12% methylcellulose, 100 μg of ampicillin per ml, 10 μM of streptomycin per ml, and 5% fetal bovine serum.

**Plasmid constructions.** Plasmids were constructed and maintained in *E. coli* HB101 by standard methods (45). To construct the pGST-EICP22 vector, which encodes the full-length EICP22 (aa 1 to 293), the EcoRI-HindIII fragment of pR4AS containing the EICP22 open reading frame (21) was cloned into EcoRI-HindIII-digested pGEX-KG (18) and was designated pKG-EICP22. The Bal31-EcoRI fragment of pGEX-4T-1 (Pharmacia) was cloned into Bal31-EcoRI-digested pGEX-4T-2 (Pharmacia) to generate the pGST-EICP22 clone. The pGST-EICP22 vector, pGST-EICP22Δ1-239, encoding the carboxy-terminal domain (aa 240 to 293) of the EICP22 protein, was constructed by digesting pKG-EICP22 with EcoRI and XhoI, filling with Klenow fragment, and self-ligating the plasmid. The EcoRI-SalI fragment was ligated into the SalI-XhoI site of pGEX-4T-2 to generate pGST-EICP22 Δ29-293, pGST-EICP22Δ229-293, and pGST-EICP22Δ222-229-293 vectors.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed using the Altered Sites II in vitro mutagenesis system (Promega) according to the manufacturer’s instructions. In this protocol, the DNA fragment of interest was inserted into the multiple cloning site of plasmid pALTER-1 containing a Tetr manufacturer’s site and a mutated Amp’’ site. The plasmids containing the insert (pALT-IE) were subjected to alkaline denaturation. After denaturation, the designed mutagenic oligonucleotides (oligonucleotide I [IEW403S], 5′-AGTTCTGCACCG AGGACATGG-3′; oligonucleotide 2 [IEQ495E], 5′-TAGAGTCTCCGACCG GGA-3′; oligonucleotide 3 [IE963], 5′-AGGCTTAGATGCAGGCCG-3′; and oligonucleotide 4 [IEK498E], 5′-CCCGATGCTAAGTCTGCT-3′), an ampicillin repair oligonucleotide, and a tetacycline koncept oligonucleotide were annealed. Ultimately, the ampicillin gene was repaired, the tetacycline gene was mutated, and the designed oligonucleotide directs mutagenesis of the desired site. Transformants were selected by ampicillin selection. The mutant vectors pALT-EW493S, pALT-IEQ495E, pALT-IEK498E, and pALT-IEK908E were identified by DNA sequencing.

**Gel shift assays.** The DNA fragment of the IE gene promoter from position -144 to +61 (IE2) (28) was end labeled with [α-32P]dATP. DNA binding assays were conducted as previously described (28). The standard DNA binding reaction mixture contained 0.5 to 1 ng of radiolabeled DNA fragments (2 × 10⁴ cpm/ng), 1.5 μg of poly(dI-dC) as a nonspecific competitor, and 1 to 10 μl of bacterial extract in 20 μl of DNA binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM mercaptoethanol, 0.1% [wt/vol] 3-[3-cholamidopropyl]-dimethyl-ammonium-1-propanesulfonate [CHAPS], 100 mM NaCl). After an 80 min incubation at 22°C, 5 μl of loading buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM mercaptoethanol, 0.1% CHAPS, 50% [vol/vol] glycerol, 0.1% bromphenol blue) was added, and the sample was subjected to electrophoresis at 4°C for 3 h. The DnaNase I footprinting assays. DNA footprinting experiments were carried out as previously described (28). One microgram of poly(dI-dC) was added to the 50-μl binding reaction mixture (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM mercaptoethanol, 0.1% CHAPS, 50% [vol/vol] glycerol, 0.1% bromphenol blue) was added, and the sample was subjected to electrophoresis at 4°C for 3 h in an 4% polyacrylamide gel with 0.5% Tris-borate-EDTA running buffer.

DNA transcription and CAT assays. L-M cells seeded at 3 × 10⁴ cells per tissue culture dish (60-mm diameter) in Eagle’s minimum essential medium (with 5% fetal bovine serum) were transfected by the liposome-mediated DNA transfection method 24 h later (25). The pEICP22(E)-CAT plasmid was transfected in 0.45-ml amounts. All effector plasmids were transfected in 0.3-ml amounts. The total amount of DNA per transfection was adjusted to 8 μg by the addition of pUC19. After a further 5 h, the cells were washed and refed with fresh medium; 60 h later, total cell extracts were prepared and chloramphenicol acetyltransferase (CAT) activities were assayed as described previously (48).

**RESULTS**

The EICP22 protein increases the in vitro DNA binding activity of the IE protein. The IE protein mediates DNA binding to the sequence that overlaps the IE transcription initiation site (+1) (28). In transient-transfection assays, the IE gene product trans activates EHV-1 and heterologous viral promoters, from human and other rodent species, is synergistically affected by the EICP22 and EICP27 proteins to trans activate E and L promoters (25, 33, 48, 49, 64). To elucidate the mechanism of the synergistic effect seen with the EICP22 and IE proteins, gel shift assays were performed with the GST-EICP22 (Fig. 1A; see Fig. 3) and GST-IE(407-615) proteins (see Materials and Methods). The GST-EICP22 protein increased in the vitro DNA binding activity of the IE protein (Fig. 1B, lanes 5 to 7). However, GST, bovine serum albumin, and EICP22A (GST-EICP22A) proteins did not increase the DNA binding activity of the IE protein (Fig. 1B, lanes 2 to 4, 11 to 13, and 14 to 16, respectively). To determine whether the concentration of protein nonspecifically affected the DNA binding by the EICP22 protein, gel shift assays were performed with GST protein. As shown in lanes 8 to 10 and 17 to 19 of Fig. 1B, GST in amounts as high as 900 ng could not increase the DNA binding activity of the IE protein. The EICP22 protein by itself did not bind to the IE promoter (Fig. 1B, lanes 20 to 22).

To confirm this synergistic effect, DNAse I footprinting assays were performed (Fig. 1C) with GST-IE(407-615). The amount of protein applied was adjusted by the addition of the

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GST protein. It has been shown by DNase I footprinting analyses that GST-IE(291-634) binds tightly to the IE promoter at position -11 to +14 (IE I) and weakly to the site at position -92 to -77 (IE II), which contains a degenerate version (ATCGA) of the conserved pentanucleotide from position -86 to -82 (28). GST-IE(291-634) generated a clear footprint over the IE I site (Fig. 1C, lanes 5 and 6). GST-EICP22 by itself did not generate a footprint (Fig. 1C, lanes 11 and 12). Since the EICP22 protein increased the DNA binding activity of the IE protein in a DNase I footprinting assay (Fig. 1C, lane 9 with triangles), the stimulatory effect of the EICP22 protein was not an artifact of the gel shift assay. Consequently, the results of the DNase I footprinting analyses were consistent with those of the gel shift assay.

To confirm that the observed complexes were formed by the IE and EICP22 proteins, gel shift assays were performed with monoclonal antibody (MAb) A1.4, which is specific for IE region 1 (aa 323 to 421) (5), and MAb K2, which is specific for EICP22 (6). The GST-IE(291-634) protein bound to the IE promoter as two bands, with the upper band being of relatively weak intensity (Fig. 2, lane 1). Both of these GST-IE(291-634)-DNA complexes were supershifted by the addition of MAb A1.4, and the supershifted complexes (region B) also appeared as two bands (Fig. 2, lane 2). Additionally, a faint band was detected in region C. The GST-IE(291-634)-DNA complexes were not shifted by the addition of MAb K2 (Fig. 2, lane 3). The EICP22 protein increased the DNA binding activity of the IE protein (Fig. 2, lane 4). In the presence of the GST-EICP22 protein, the GST-IE(291-634)–DNA complex was further shifted by the addition of MAb A1.4 (Fig. 2, lane 5), suggesting that the supershifted band (region C) is a DNA-IE protein-EICP22-MAb A1.4 complex or a DNA-multimer IE protein-MAb A1.4 complex. The supershifted band (region C) is probably not a very stable complex, because on occasion it was observed in supershift assays that the DNA-IE protein-MAb A1.4 complex was shifted slightly by the addition of the EICP22 protein (data not shown). In the presence of the GST-EICP22 protein, the GST-IE(291-634)–DNA complex produced a smear of shifted bands after the addition of MAb K2 (Fig. 2, lane 6) (region D). These results suggested that the EICP22 protein may interact weakly with the IE protein to increase the DNA binding activity of the IE protein.

**Region 2 and region 3 domains of the EICP22 protein are important for the synergistic effect.** To define the regions of the IE and EICP22 proteins required for the synergistic effect, various deletion mutants of the EICP22 genes were constructed and expressed in *E. coli* as GST fusion proteins (Fig. 3A and B) (see Materials and Methods). The amino-terminal portion of each fusion protein includes the 26-kDa GST-coding region. These fusion proteins were synthesized after IPTG induction of *E. coli* BL21(DE3)pLysE, purified from bacterial lysates by using glutathione-Sepharose 4B, and electrophoretically analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3B) (see Materials and Methods). Some of

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**FIG. 1.** Synergistic effect of the IE and EICP22 proteins. (A) Schematic diagram of GST-EICP22 fusion proteins. The top diagram depicts the EICP22 open reading frame, showing the four regions of the EICP22 protein. NLS, nuclear localization sequence. (B) Gel shift assays. The radiolabeled probe, IE2 (position −144 to +61), was incubated with various amounts of the protein under the standard conditions described in Materials and Methods. The amount of GST-IE(407-615) used in this experiment (lanes 1 to 19) was 150 ng, no protein added; GST-EICP22, GST-IE(291-634); BSA, bovine serum albumin; GST-EICP22-D, GST-EICP22 (see panel A); triangles, increasing amounts of the protein added, at 1 × (300 ng), 2 × (600 ng), and 3 × (900 ng); rectangles, same amounts of the protein at 1 ×. The position of complexes formed by the IE protein is indicated with an arrow. (C) DNase I footprinting of the IE protein-DNA complexes in the presence or absence of the EICP22 protein. The target DNA was a 220-bp EcoRI-PstI fragment from pUIE12 (28) that contains bp −141 to +61 of the EHV-1 IE gene promoter. The proteins used for the DNA binding reactions are indicated above each lane, and the numbers indicate the relative amounts added in the binding reaction mixture. −−−−, no protein added. The footprinted region is indicated on the left as IE I (position −11 to +14). The arrows indicate increased protection in the presence of the EICP22 protein. Lanes 1 and 13 and lanes 2 and 14 are Maxam-Gilbert G and G+A sequencing reactions for the noncoding strands, respectively.
The IE protein-DNA complex forms rapidly. To test whether the EICP22 protein affects the rate as well as the extent of binding of the IE protein to the IE promoter DNA, complex formation as function of time was monitored by gel shift assays (Fig. 4). GST-IE(291-634) was incubated with the IE promoter probe either in the absence (Fig. 4, lanes 1 to 6) or in the presence (Fig. 4, lanes 7 to 12) of the GST-EICP22 protein. Six identical samples were incubated at 22°C for 0.4, 1, 3, 7, 12, or 25 min and then immediately loaded onto a running polyacrylamide gel. In the absence of the EICP22 protein, the amount of complex formed increased slowly over the time period of 3 to 7 min (Fig. 4, lanes 1 to 4). In contrast, in the presence of the EICP22 protein, nearly all of the probe was bound in the retarded complex after only 0.4 min of incubation (Fig. 4, lane 7). Consequently, formation of a stable IE protein-DNA complex under conditions of electrophoresis occurred rapidly in the presence of the EICP22 protein as compared with the slower formation of DNA-protein complexes observed in the absence of the EICP22 protein.

The EICP22 protein increases the in vitro DNA binding activity of the IE protein for sequences in the E and L promoters. As mentioned above, in transient-transfection assays, the EICP22 protein acts synergistically with the IE protein to trans activate E and L viral promoters. The GST-EICP22 protein increased the in vitro DNA binding activity of the GST-IE(291-634) protein for sequences in the IE promoter (Fig. 1 to 3). To define further this synergistic effect, gel shift assays were performed with EHV-1 E and L promoters. Representative E promoters included those of the EICP22 gene (23), the EICP27 gene (63), the thymidine kinase gene (33, 43), the ICP0 gene (3, 53), and the IR2 gene (19). Sequences of the IR5 gene, a homolog of the HSV-1 US10 gene (24), were employed as the representative L promoter. Previously, it has been shown that the IE protein binds weakly to the E and L promoters and that the IE protein-DNA complex was further shifted by the addition of IE region 2-specific MAb B3.3 (28). When the E and L promoters were examined, three times the amount of GST-IE(291-634) protein was added compared to the amount used for the IE promoter. As shown before, GST-IE(291-634) bound less strongly to the E promoters (Fig. 5A, lanes 1, 4, 7, 10, and 13). Interestingly, in the presence of GST-EICP22, nearly all of the probe was bound in the retarded complex (Fig. 5A, lanes 3, 6, 9, 12, and 15). The GST-IE(291-634) protein bound weakly to the L (IR5) promoter (Fig. 5A, lane 16). However, in the presence of GST-EICP22, nearly all of the probe was bound in the retarded complex (Fig. 5A, lane 18), demonstrating that the EICP22 protein also increased the in vitro DNA binding activity of the IE protein for sequences in both the E and L promoters.

Previously, one-base-substitution mutants of the conserved pentanucleotide 5′-ATCGT-3′ were synthesized and used for gel shift assays to determine which nucleotides are important for the DNA binding of the IE protein (28). Mutation of C or G in the conserved pentanucleotide 5′-ATCGT-3′ critically affected the sequence-specific binding by the IE protein, and mutation of A or T in the conserved pentanucleotide moderately affected the sequence-specific DNA binding by the IE protein (28). Examination of the promoter sequences of EHV-1 E and L genes revealed that each harbors a degenerate version of the consensus binding sequence 5′-ATCGT-3′ (28). To confirm these results, additional gel shift assays were performed with the mutant oligonucleotides (Fig. 5B). These mutant oligonucleotides represent a degenerate version of the consensus binding sequence 5′-ATCGT-3′. In gel shift assays, GST-IE(291-634) bound tightly to the wild-type IE1 oligonucleotide (Fig. 5B, lane 1) but bound weakly to the MIE1.
MIE2, and MIE5 oligonucleotides (Fig. 5B, lanes 4, 7, and 16, respectively). However, no complex was observed with the MIE3 and MIE4 oligonucleotides (Fig. 5B, lanes 10 and 13, respectively). GST-IE(291-634) yielded a pattern with multiple bands. These results were consistent with previous results (28). The EICP22 protein increased the in vitro DNA binding activity of the IE protein for sequences in the IE1, MIE1, MIE2, and MIE5 oligonucleotides (Fig. 5B, lanes 3, 6, 9, and 18, respectively). These results indicated that the EICP22 protein increased the in vitro DNA binding activity of the IE protein for sequences containing a degenerate version of the consensus binding sequence 5'-ATCGT-3'.

The EICP22 protein restores DNA binding activity of the IE mutant. The WLQN region, which is directly involved in the DNA binding interaction of VZV ORF62, is well conserved in the EHV-1 IE region 2 (54). To identify the IE sequences involved in DNA binding, IE residues Trp<sup>493</sup>, Gln<sup>495</sup>, Asn<sup>496</sup>, and Lys<sup>498</sup> of the WLQN homology region were replaced with Ser, Gln, Ile, and Gln, respectively, by in vitro mutagenesis (Fig. 6A) and were used for DNA binding experiments and transient-transfection assays. To study the DNA binding activity of the WLQN region, IE region 2 domains containing these mutagenized WLQN regions were expressed in E. coli as GST fusion proteins [GST-IE(407-615), GST-IEW493S(407-615), GST-IEQ495E(407-615), GST-IEW493S(250-615), and GST-IEW493S(407-615)] (data not shown). Since GST-IEW493S(407-615) was unstable during purification (data not shown), another mutant containing a larger portion, IEW493S(250-615), was used for the gel shift assay. The gel shift assay was performed as described in Materials and Methods. The radiolabeled probe (IE2) was incubated with 100 ng of the GST-IE(407-615) protein, 300 ng of the GST-EICP22 deletion mutants, and 4× GST (1.2 μg) under the standard conditions described in Materials and Methods. The activities of the proteins are shown at the bottom. The position of complexes formed by the IE protein is indicated with an arrow.

FIG. 3. Gel shift assay with the GST-EICP22 deletion mutants. (A) Schematic diagram of recombinant GST-EICP22 deletion mutants. The numbers above the boxes indicate the positions of specific residues with respect to the amino terminus of the native viral protein. The top diagram represents the 293-aa EICP22 protein of EHV-1, which has been divided into four regions. The boundaries of these four regions are indicated by the number of their amino acid residues. NLS, nuclear localization signal. (B) SDS-polyacrylamide gel analysis of the purified GST-EICP22 deletion mutants. The proteins were synthesized after IPTG induction of E. coli BL21(DE3)pLysE and purified from bacterial lysates by using glutathione-Sepharose 4B (see Materials and Methods). The purified samples were electrophoresed through an SDS–10% PAGE gel and stained with Coomassie brilliant blue. Lanes 2 through 11 contain the GST-EICP22 protein and the deletion mutants. The numbers on the right represent molecular mass standards (Bethesda Research Laboratories) in kilodaltons. (C) Gel shift assay. The gel shift assay was performed as described in Materials and Methods. The radiolabeled probe (IE2) was incubated with 100 ng of the GST-IE(407-615) protein, 300 ng of the GST-EICP22 deletion mutants, and 4× GST (1.2 μg) under the standard conditions described in Materials and Methods. The activities of the proteins are shown at the bottom. The position of complexes formed by the IE protein is indicated with an arrow.
of the IE protein was expressed (data not shown). The GST-IEW493S(250-597) protein was more stable than GST-IEW493S(407-615) but still yielded the proteolytic cleavage products (data not shown). Gel shift assays were performed either with or without GST-EICP22 protein. The wild-type (Fig. 6B, lane 3), the IEW493S mutant (Fig. 6B, lane 5), and the IEN496I mutant (Fig. 6B, lane 9) proteins bound to the IE promoter, and as expected, the GST-EICP22 protein increased the DNA binding activity of these proteins (Fig. 6B, lanes 4, 6, and 10, respectively). In contrast, both the IEQ495E mutant and the IEK498E mutant proteins failed to bind to the IE promoter, indicating that the Gln and Lys residues of the WLQN region are important for the DNA binding activity of the IE protein. Interestingly, the GST-EICP22 protein restored the DNA binding activity of the IEQ495E mutant (Fig. 6B, lane 8), but the EICP22 protein did not restore the gel shift pattern to that of the wild-type IE protein, indicating that the EICP22 protein cooperates with IE protein to increase or stabilize the IE DNA binding activity.

To confirm these results, transient-transfection assays were performed with intact IE expression constructs with the same mutations in the DNA binding domain (Fig. 6C). Since the IE protein down-regulates its own promoter (48), the EICP22 early [EICP22(E)] promoter, which is up-regulated by the IE protein, was used for transient-transfection assays. The EICP22 protein alone did not trans activate the EICP22(E) promoter (Fig. 6C, bar 2), whereas the IE protein alone trans activated (7.1-fold) the EICP22(E) promoter (Fig. 6C, bar 3). Cotransfection of pCD4 (EICP22 expression vector) and pSVIE (IE expression vector) resulted in an 18-fold increase in CAT activity (Fig. 6C, bar 4). This result is consistent with our previous results (25). Transfection of pSVIEW493S(1-1487) or pSVIE496I(1-1487) alone resulted in 5.2- and 2.7-fold increases, respectively, in CAT activity (Fig. 6C, bars 5 and 7). In cotransfections of pSVIEW493S(1-1487) or pSVIE496I(1-1487) with the pCD4 construct, CAT activities were increased by 9.8- and 8.4-fold, respectively (Fig. 6C, bars 6 and 8). As expected, pSVIEQ495E(1-1487) and pSVIEK498E(1-1487), when transfected alone, yielded only low levels of CAT activity (Fig. 6C, bars 7 and 11). These results indicated that the Gln and Lys residues of the WLQN region are important for the IE protein to function as a trans activator, an activity that maps within aa 3 to 89 (50). Interestingly, when pSVIEQ495E(1-1487) was cotransfected with pCD4, CAT activity was partially restored. Namely, the EICP22 protein allowed the IEQ495E mutant to be functional as a trans activator (Fig. 6C, bars 7 and 8). Thus, the results from the CAT assays were consistent with the results from the gel shift assays (Fig. 6B). These results suggest that the EICP22 protein may cooperate with the IE protein to regulate EHV-1 gene expression.

![Fig. 4](http://jvi.asm.org/)

**FIG. 4.** The GST-EICP22 protein increases the rate and the extent of IE protein-DNA complex formation. The GST-IE(291-634) protein (100 ng) was incubated with the IE2 probe in either the absence (lanes 1 to 6) or presence (lanes 7 to 12) of 2 μl (300 ng) of the GST-EICP22 protein. Reaction mixtures were incubated at 22°C for 0.4, 1, 3, 7, 12, or 25 min, as indicated above each lane, and then loaded onto a running gel. The region of the shifted bands is indicated with a bracket.

![Fig. 5](http://jvi.asm.org/)

**FIG. 5.** The EICP22 protein increases the in vitro DNA binding activity of the IE protein for sequences containing a degenerate version of the consensus binding sequence 5′-ATCGT-3′. (A) Gel shift assays with EHV-1 E and L promoters. The radiolabeled probe (IE2) was incubated with 300 ng of the GST-IE(291-634) protein either with or without the GST-EICP22 protein, under the standard conditions described in Materials and Methods. The positions of complexes formed by the IE protein are indicated with arrows. TK, thymidine kinase. (B) Gel shift assays with wild-type and mutant oligonucleotides. The mutant oligonucleotides (28) contain a one-base substitution of the conserved pentanucleotide 5′-ATCGT-3′, as follows: IE1, ATCGT; MIE1, T*TCGT; MIE2, AG*CGT; MIE3, AT*GT; MIE4, ATCT*T; and MIE5, ATCGC* (an asterisk indicates a mutated base). The GST-IE(291-634) protein (100 ng) was incubated in either the absence or presence of 2 μl (300 ng) of the GST-EICP22 protein.
DISCUSSION

The EICP22 protein increased the in vitro DNA binding activity of the IE protein for sequences in the IE, E, and L promoters. Examination of the promoter sequences of EHV-1 E and L genes revealed that each harbors a degenerate version of the consensus binding sequence 5′-ATCGT-3′ which does not overlap the transcription initiation site (28). Therefore, the IE protein alone binds weakly to the E and L promoters (28). In contrast, these results suggest that the EICP22 protein increases the expression of the E and L proteins by increasing the DNA binding activity of the IE protein. These findings are consistent with those of transient-transfection assays which showed that the IE gene product trans represses its own expression and acts synergistically with the EICP22 and EICP27 proteins to trans activate E and L promoters (25, 33, 48, 49, 64). Taken together, these results suggest that the EICP22 protein plays a role as a regulatory factor that contributes to the switch from IE to E and L gene expression. Preliminary experiments performed with the two-hybrid system in Saccharomyces cerevisiae have shown that the IE protein may interact with the EICP22 protein (11).

In vitro mutagenesis analysis demonstrated that the Gln and Lys residues of the WLQN region of the IE protein are important for the DNA binding activity of the IE protein. This result differs slightly from those of Tyler et al. (54), who showed that only substitution of Lys^548 of the VZV 140K protein drastically reduced the DNA binding activity of the 140K DNA binding domain. Interestingly, the GST-EICP22 protein partially restored the binding activity of the IEQ495E mutant. Transient-transfection assays also showed that the EICP22 protein allowed the IEQ495E mutant to be functional as a trans activator, suggesting that the EICP22 protein cooperates with the IE protein to regulate EHV-1 gene expression. This finding is unique and may represent an important mechanism for EHV-1 gene regulation.

It is clear that HSV-1 ICP4 is a necessary factor for transcriptional induction of E and L genes (10, 57). ICP4 has nonspecific DNA binding activity (13) but exhibits a marked preference for particular DNA sequences (12). A central question concerning transcriptional regulation in HSV-1-infected cells is how the viral E and L promoters are activated in the apparent absence of specific binding sites for ICP4. In the case of HSV-1 ICP22, also an IE gene, little is known about its functions in lytic infection. The ICP22 gene is nonessential for virus replication in tissue culture (38, 39, 47), and an α22^- (ICP22 knockout) virus is capable of replication in human (Hep-2) and primate (Vero) cells at levels equivalent to those for the wild-type virus (47). However, in cell lines (BHk and RAT-1) of rodent origin, the plating efficiency and yield of the α22^- virus were reduced, and prolonged expression of the E proteins and a decrease in expression of the L proteins were observed. Studies by Purves et al. (41) revealed a decrease in
mRNAs of the IE ICP0 gene and the L Us11 gene in BHK cells infected with the a22- mutant, suggesting that ICP22 might function at the transcriptional level or might function posttranslationally by stabilizing viral mRNAs. Recently, it has been suggested that ICP22 may alter transcriptional specificity in HSV-1-infected cells by modifying the function of RNA polymerase II via a posttranslational modification (42).

There are a number of ways that the EICP22 protein could increase the DNA binding activity of the IE protein: (i) the EICP22 protein promotes the dimerization (or oligomerization) of the IE protein, (ii) the EICP22 protein stabilizes the IE protein-DNA complex by interacting with the IE protein, or (iii) the EICP22 protein binds to DNA and induces a bend in DNA, which directs the IE protein to bind to the DNA. Since the EICP22 protein per se does not bind to the IE and other EHV-1 gene promoters, the third possibility is unlikely. With regard to the mechanism by which viral proteins enhance DNA binding activity, it is interesting that the X gene product encoded by the hepatitis B virus (32, 60) and the Tax protein of human T-cell leukemia virus type 1 (37, 56) stimulate the DNA binding of CREB/ATF-2 protein. The pX protein interacts with the basic region-leucine zipper (bZIP) domain of CREB (60) and increases the affinity of the CRE protein for the CRE site by an order of magnitude, although pX does not alter the rate of CREB dimerization. The Tax protein stimulates the DNA binding of bZIP proteins by enhancing dimerization (37, 56).

It is possible that the EICP22 protein interacts weakly with the IE protein and thereby promotes the dimerization (or oligomerization) of the IE protein. Indeed, it has been shown that oligomeric protein-DNA complexes can result from specific aggregation of the EHV-1 IE protein with itself (28), and a similar finding was reported for the HSV-1 ICP4 protein (61). In this study, multiple protein-DNA bands were also observed in assays with GST-IE(291-634) (Fig. 2 and 3) and GST-IEW493S(250-597) (Fig. 6B, lane 6), and these complexes increased and further shifted in the presence of the EICP22 protein. Interestingly, two of the multiple bands migrated faster than the complex in Fig. 6B, lane 5. There are two possible explanations for these bands. First, dimerized forms of proteolytic cleavage products of the GST-IEW493S(250-597) bands may have migrated faster than the complex. Since the GST-IEW493S(250-597) protein yielded the proteolytic cleavage products, it was possible that the EICP22 protein promoted the dimerization (or oligomerization) of the proteolytic cleavage products of GST-IEW493S(250-597). Second, a distortion in DNA structure may result due to the binding of the EICP22 protein, and this distortion may result in a more rapid migration of the bands. For example, in yeast, the mobility of the holo-TFID-TFIIA complex was the same as that of the TBP-TFIIB complex (30). This was surprising because holo-TFIID is ~750 kDa, whereas TBP is 38 kDa. The solved TBP-TATA box DNA complex structure showed that the DNA is sharply bent and kinked by TBP (29). To address the mechanism of the EICP22 protein function, additional DNA binding assays, glutaraldehyde cross-linking experiments, and methylation interference analyses are in progress. It is hoped that these studies and the use of our panels of GST fusion proteins that harbor specific domains of both the IE and EICP22 proteins will allow us to define more precisely the possible interactions of these herpesvirus proteins and the residues of the EICP22 protein that are essential for its role as an auxiliary regulatory protein.

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