Published in final edited form as:

Virus Res. 2012 April ; 165(1): 52–60. doi:10.1016/j.virusres.2012.01.005.

Characterization of *cis*-acting elements required for autorepression of the equine herpesvirus 1 IE gene

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Abstract

The immediate-early protein (IEP), the major regulatory protein encoded by the IE gene of equine herpesvirus 1 (EHV-1), plays a crucial role as both transcription activator and repressor during a productive lytic infection. To investigate the mechanism by which the EHV-1 IEP inhibits its own promoter, IE promoter-luciferase reporter plasmids containing wild-type and mutant IEP-binding site (IEBS) were constructed and used for luciferase reporter assays. The IEP inhibited transcription from its own promoter in the presence of a consensus IEBS (5'-ATCGT-3') located near the transcription initiation site but did not inhibit when the consensus sequence was deleted. To determine whether the distance between the TATA box and the IEBS affects transcriptional repression, the IEBS was displaced from the original site by the insertion of synthetic DNA sequences. Luciferase reporter assays revealed that the IEP is able to repress its own promoter when the IEBS is located within 26-bp from the TATA box. We also found that the proper orientation and position of the IEBS were required for the repression by the IEP. Interestingly, the level of repression was significantly reduced when a consensus TATA sequence was deleted from the promoter region, indicating that the IEP efficiently inhibits its own promoter in a TATA box-dependent manner. Taken together, these results suggest that the EHV-1 IEP delicately modulates autoregulation of its gene through the consensus IEBS that is near the transcription initiation site and the TATA box.

Keywords

Equine herpesvirus 1; Immediate-early protein; IEP-binding site; IE gene autorepression; TATA box-dependent manner

1. Introduction

The equine herpesvirus 1 (EHV-1), a member of *Alphaherpesvirinae*, is a major pathogen causing respiratory disease, neurological disorders, and abortion in horses (Allen and Bryans, 1986; O'Callaghan and Osterrieder, 2008). The 78 EHV-1 genes are temporally and coordinately regulated in an immediate-early (IE), early (E), and late (L) fashion during a

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lytic infection by six regulatory proteins: the sole immediate-early protein (IEP), four early proteins (IR2P, EICP0P, UL5P, and IR4P), and the late tegument protein ETIF (Albrecht et al., 2004; Bowles et al., 1997; Caughman et al., 1985; Elliott and O'Hare, 1995; Elliott, 1994; Gray et al., 1987; Holden et al., 1995; Kim et al., 1997, 2006; Lewis et al., 1993; Smith et al., 1992; Zhao et al., 1995). In addition, it has recently been reported that the IR3 gene encodes a transcript that is antisense to the IE mRNA (Holden et al., 1992) and functions as a negative regulatory molecule in IE gene expression (Ahn et al., 2007, 2010).

The IEP is a regulatory phosphoprotein of 1,487-amino acid (aa) that functions as both transcriptional activator and repressor during a productive EHV-1 infection. The IEP not only *trans*-activates E and some L viral promoters, but also down-regulates its own promoter and the late *glycoprotein K* (*gK*) promoter (Kim et al., 1999; Purewal et al., 1992; Smith et al., 1992). Moreover, the IEP cooperates synergistically with two early auxiliary regulatory proteins IR4P and UL5P to *trans*-activate early and γ 1 late promoters and interacts physically with the IR4P to enhance IEP DNA-binding activity (Derbigny et al., 2002; Holden et al., 1995; Kim et al., 1997; Smith et al., 1992; Zhao et al., 1995). Interestingly, the IEP acts antagonistically with an early regulatory EICP0 to selectively repress the expression of all classes of viral promoters including γ 2 late promoters (Bowles et al., 2000; Kim et al., 1999, 2003). Our previous results showed that the IEP directly interacts with the general transcription factors (GTFs) TFIIB and TATA box-binding protein (TBP), and these protein-protein interactions are required for full *trans*-activation of the viral promoters by the IEP (Albrecht et al., 2003; Jang et al., 2001; Kim et al., 2003, 2006). Furthermore, DNase I footprinting assay showed that the IEP strongly binds to a consensus pentanucleotide sequence (5'-ATCGT-3') at -11 to +14 nucleotide (nt) relative to the transcription initiation site and weakly binds to a degenerate version (5'-ATCGA-3', -92 to -82 nt) of this conserved sequence (Kim et al., 1995). Also, the C or G nucleotide in the consensus 5'-ATCGT-3' is important for the DNA-binding capability of IEP, and residues 422 to 597 of the IEP are sufficient for its sequence-specific DNA-binding activity (Kim et al., 1995, 1999).

RNA polymerase II-mediated transcription machinery requires the GTFs such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and is initiated through the assembly of the preinitiation complex (PIC) (Hampsey, 1998; Orphanides et al., 1996). The efficiency of the formation of the PIC is a critical step in determining the rate of transcription and is a common site of both positive and negative transcriptional regulation (Arnosti et al., 1993; Hampsey, 1998). Some general transcriptional repressors have been recently shown to regulate the expression of their target genes through core promoter elements and factors that affect TBP function. These repressors include human Dr1-DRAP1/NC2 complex (Inostroza et al., 1992), mouse Dfa (Brower et al., 2010), and YY1 protein (Chang et al., 2011). In addition, several studies reported that some immediate-early proteins of alphaherpesviruses autorepress the transcription of their own promoters via the binding of the corresponding proteins to conserved *cis*-acting elements. In the case of herpes simplex virus 1 (HSV-1), ICP4 has been shown to repress its own promoter by binding to the ICP4-binding site that overlaps the transcription initiation site and by the interaction between TBP, TFIIB, and ICP4 (Gu et al., 1995; Kuddus et al., 1995; Smith et al., 1993). Moreover, IE180 of pseudorabies virus (PRV) and IE62 of varicella-zoster virus (VZV) also down-regulate their own promoters through binding to specific sites located near the transcription initiation site (Disney et al., 1990; Ihara et al., 1983; White et al., 2010). However, the exact mechanisms of negative regulation of EHV-1 IEP including the autorepression of its own promoter remain unclear.

In this study, we generated EHV-1 IE promoter-luciferase reporter plasmids containing wild-type or mutant IEP-binding site (IEBS; 5'-ATCGT-3') and performed transient-

transfection and luciferase reporter assays to elucidate the mechanism by which the EHV-1 IEP represses transcription from its own promoter. Our results revealed that the IEP inhibits its own promoter through a consensus IEBS that is proximal to the transcription initiation site, and the short distance between the TATA box and the IEBS plays an important role in the repression by the IEP. In addition, deletion of a consensus TATA sequence from the IE promoter leads to a significant reduction in the transcriptional repression, suggesting that the EHV-1 IEP efficiently modulates autoregulation of its gene through two *cis*-acting elements: a consensus IEBS and the TATA box.

2. Materials and methods

2.1. Cell culture

Rabbit kidney RK13 cells were used for transient transfection. Cells were cultured in Eagle's minimal essential medium supplemented with 100 units/ml of penicillin, 100 µg/ml of streptomycin, nonessential amino acids, and 5% fetal bovine serum at 37°C in 5% CO₂ incubator.

2.2. Plasmid constructs

To generate luciferase reporter plasmids containing wild-type and synthetic EHV-1 IE promoter region from -802 to +73 relative to the transcription initiation site, PCR products were amplified by using proper primers, Accuprime *pf*x polymerase, and template plasmid containing the IE promoter region. PCR product harboring the IE promoter region [IE(-802/-60)] was amplified by using primers (5'-ttt ggt acc taa cac acg agg gcg ccc tcg tgc gga-3'/5'-ttt ctc gag tgg gag tgg cca gcc cac act atc gat-3'), digested with KpnI and XhoI, and cloned into the KpnI and XhoI sites of pGL3-basic (Promega, Madison, WI) [designated pIE(-802/-60)-Luc]. PCR product containing IE promoter region [IE(-66/+73)] was amplified using primers (5'-ttt ctc gag gga agg caa aac tcc ctc gta gta gta-3'/5'-ttt aag ctt tcc aag atg cga tcg ata gtc ctc gaa-3'), and digested with XhoI and HindIII. The digested PCR product was subcloned into pIE(-802/-60)-Luc digested with XhoI and HindIII to generate pIEwtIEBS-Luc. To construct mutant reporter plasmids deleted of the IEBS (5'-ATCGT-3') within the IE promoter region, DNA fragment (5'-ggt ctc gag gga agg caa aac tcc ctc gta gta gta taa agc acc tgt tgc tta ccc att caa gca tcg cgg act aga gag cct ttc agc tca ctg gac cag cca gcc ttc gag gac tat cga tcg cat ctt gga aag ctt ggg-3') digested with XhoI and HindIII was cloned into the XhoI and HindIII sites of pIEwtIEBS-Luc (designated pIEΔIEBS-Luc). Synthetic IE promoters with different distances between the TATA box and the IEBS were constructed by digestion of pIEwtIEBS-Luc with XhoI and HindIII followed by insertion of synthetic DNA fragments of various lengths between the TATA and the IEBS. The synthetic DNA fragments IE-i8 (5'-gtc tcg agg gaa ggc aaa act ccc tcg tag tag tat aaa gca cct cca gtc cgg ttg ctt acc cat cgt agc atc gcg gac tag aga gcc ttt cag ctc act gga cca gcc agc ctt cga gga cta tcg atc gca tct tgg aaa gct tgg g-3'), IE-i16 (5'-gtc tcg agg gaa ggc aaa act ccc tcg tag tag tat aaa gca cct cca gtc cga ggt cag agt tgc tta ccc atc gta gca tcg cgg act aga gag cct ttc agc tca ctg gac cag cca gcc ttc gag gac tat cga tcg cat ctt gga aag ctt ggg-3'), IE-i24 (5'-gtc tcg agg gaa ggc aaa act ccc tcg tag tag tat aaa gca cct cca gtc cga ggt cag att ctt gga gtt gct tac cca tcg tag cat cgc gga cta gag agc ctt tca gct cac tgg acc agc cag cct tcg agg act atc gat cgc atc ttg gaa agc ttg gg-3'), and IE-i32 (5'-gtc tcg agg gaa ggc aaa act ccc tcg tag tag tat aaa gca cct cca gtc cga ggt cag att ctt gga cat acc ttg ttg ctt acc cat cgt agc atc gcg gac tag aga gcc ttt cag ctc act gga cca gcc agc ctt cga gga cta tcg atc gca tct tgg aaa gct tgg g-3') were digested with XhoI and HindIII, and subcloned into pIEwtIEBS-Luc to generate pi8-IEBS-Luc, pi16-IEBS-Luc, pi24-IEBS-Luc, and pi32-IEBS-Luc, respectively. The underlined sequences are the inserted DNA sequence.

To generate a mutant promoter with the IEBS oriented in the opposite direction relative to the TATA box, the PCR product was amplified from pIEWtIEBS-Luc using primers (5'-ttt ggt acc taa cac acg agg gcg ccc tgc tgc gga cgt gta tga agg cgc atg taa aac cgt gtt ttg aaa cag cgc cac cgc ctg gct cct tg-3'/5'-ttt aag ctt tcc aag atg cga tgc ata gtc ctc gaa ggc tgg ctg gtc cag tga gct gaa agg ctc tct agt ccg cga tgc tta gca ggg taa gc-3'), digested with KpnI and HindIII, and cloned into pIEWtIEBS-Luc (designated pIEorIEBS-Luc). Furthermore, two synthetic DNA fragments IE-rvIEBS (5'-ggt ctc gag gga agg caa aac tcc ctc gta gta gta taa agc acc tgt tgc tta ccc cga tgc tac gat cgg act aga gag cct ttc agc tca ctg gac cag cca gcc ttc gag gac tat cga tgc cat ctt gga aag ctt ggg-3') and IE-upIEBS (5'-ggt ctc gag gga agg atc gta gca tgc caa aac tcc ctc gta gta gta taa agc acc tgt tgc tta ccc cgg act aga gag cct ttc agc tca ctg gac cag cca gcc ttc gag gac tat cga tgc cat ctt gga aag ctt ggg-3') were subcloned into pIEWtIEBS-Luc digested with XhoI and HindIII to construct pIErvIEBS-Luc and pIEupIEBS-Luc which displaced the IEBS to the complementary strand of the original site and to upstream promoter region (31 to 27 nt upstream relative to the TATA box), respectively. To generate mutant IEBS promoters pIEBSΔ/wt2-Luc and pIEBSΔ1/Δ2-Luc which are deleted upstream IEBS (5'-ATCGA-3'; positions -86 to -82 nt), PCR products were amplified by using pIEWtIEBS-Luc as a template and primers (5'-ttt ggt acc taa cac acg agg gcg ccc tgc tgc gga cgt gta tg-3'/5'-ttt ctc gag tgg gag tgg cca gcc cac act att aat tgt gat tg-3'), digested with KpnI and XhoI, and cloned into pIEWtIEBS-Luc and pIEΔIEBS-Luc, respectively. The PCR products used for cloning of pIEBSΔ1/wt2-Luc was digested with KpnI and XhoI and cloned into pi32IEBS-Luc to construct pi32IEBS-Luc lacking the upstream IEBS (designated pi32IEBSΔ1/wt2-Luc). To mutate both the upstream and downstream IEBS or downstream IEBS in pi32IEBS-Luc, PCR product was amplified by using pi32IEBSΔ1/wt2-Luc and pi32IEBS-Luc as a template and primers (5'-ttt ggt acc taa cac acg agg gcg ccc tgc tgc gga cgt gta tga agg cgc atg taa aac cgt gtt ttg aaa cag cgc cac cgc ctg gct cct tg-3'/5'-ttt aag ctt tcc aag atg cga tgc ata gtc ctc gaa ggc tgg ctg gtc cag tga gct gaa agg ctc tct agt ccg cga tgc ttg aat ggg taa gc-3') and digested with KpnI and HindIII. Each PCR product was cloned into pIEWtIEBS-Luc digested with KpnI and HindIII, respectively (designated pi32IEBSΔ1/Δ2-Luc and pi32IEBSwt1/Δ2-Luc). PCR product was amplified from wild-type IE promoter region using primers (5'-ttt ctc gag gga agg caa aac tcc ctc gta gta ggc acc tgt tgc tta ccc atc-3'/5'-tct tcc atg gtg gct tta cca aca gta ccg gaa tgc caa gct tac tta gat cgc-3') to generate mutant IE promoter lacking a consensus TATA box. The amplified PCR product was digested with XhoI and NcoI, and cloned into pIEWtIEBS-Luc (designated pIEΔTATA-Luc).

To construct IE promoter mutant containing two consensus TATA sequences which are located at -24 to -29 nt and -85 to -80 nt relative to the transcription initiation site (pIE-2TATA-Luc), and a mutant promoter displaced a consensus TATA at -85 to -80 nt (pIE-upTATA-Luc), PCR product was amplified from wild-type IE promoter region (primers 5'-ttt ggt acc taa cac acg agg gcg ccc tgc tgc gga cgt gta tg-3'/5'-ttt ctc gag tgg gag tgg cca gcc cac act tta tat tgt gat tg-3'). PCR products digested with KpnI and XhoI were cloned into pIEWtIEBS-Luc and pIEΔTATA-Luc, respectively. In addition, PCR product was amplified from plasmid pIEΔIEBS-Luc (primers 5'-ttt ctc gag gga agg caa aac tcc ctc gta gta ggc acc tgt tgc tta ccc att-3'/5'-tct tcc atg gtg gct tta cca aca gta ccg gaa tgc caa gct tac tta gat cgc-3'), digested with XhoI and NcoI, and cloned into pIEWtIEBS-Luc to construct pIEΔTATA/IEBS-Luc lacking both a consensus TATA and the IEBS. To further generate four synthetic IE promoter-reporter plasmids containing the various distances between the TATA box and the IEBS, the following primers were used for PCR-amplification (for pTA/IEBSΔ4bp-Luc, 5'-ttt ctc gag gga agg caa aac tcc ctc gta gta taa agt gtt gct tac cca-3'/5'-tct tcc atg gtg gct tta cca aca gta ccg gaa tgc caa gct tac tta gat cgc-3'; for pTA/IEBSΔ9bp-Luc, 5'-ttt ctc gag gga agg caa aac tcc ctc gta gta gta taa agc tta ccc atc gta-3'/5'-tct tcc atg gtg gct tta cca aca gta ccg gaa tgc caa gct tac tta gat cgc-3'; for pTA/IEBSΔ13bp-Luc, 5'-ttt ctc gag gga agg caa aac tcc ctc gta gta gta taa agc cca tgc tag

cat-3'/5'-tct tcc atg gtg gct tta cca aca gta ccg gaa tgc caa gct tac tta gat cgc-3'; for pTA/IEBSΔ16bp-Luc, 5'-ttt ctc gag gga agg caa aac tcc ctc gta gta gta taa aga tcg tag cat cgc-3'/5'-tct tcc atg gtg gct tta cca aca gta ccg gaa tgc caa gct tac tta gat cgc-3'). The PCR products were digested and cloned into the XhoI and NcoI sites of pIEWtIEBS-Luc. Plasmids pSVIE, pSVIR2, and pSVSPORT1 (Gibco, BRL, Grand Island, NY) have been described previously (Kim et al., 2006). Plasmid pGST-IE(1-1487) expressing glutathione *S*-transferase (GST)-full-length IE fusion protein have been described previously (Kim et al., 1995). All the plasmid constructs were sequenced to confirm the identity of the constructs.

2.3. Luciferase reporter assay

The luciferase reporter assay was performed as previously described (Kim et al., 2006). RK13 cells were grown to 70% confluence in 24-well plates. Cells were co-transfected with 0.15 pmol of reporter plasmid and 0.3 pmol of effector plasmids using lipofectin reagents (Invitrogen, Carlsbad, CA). Six microliters of lipofectin were mixed with 300 μl of Opti-MEM medium (Gibco, BRL) and incubated at room temperature for 45 min. The reporter and effector plasmids were mixed with 300 μl of Opti-MEM medium, and the total amount of DNA was adjusted to the same amount with pSVSPORT1. The mixtures were combined and incubated at room temperature for 15 min, and one-third of the total mixture was transferred into each of three wells. Following incubation for 5 h, lipofectin-DNA mixture was removed and medium was replaced with normal growth medium. Luciferase assay was performed on cell lysate at 40 h after transfection with luciferase assay kit (Promega) and POLARstar OPTIMA plate reader (BMG LABTECH Inc., Durham, NC) according to the manufacturer's instructions.

2.4. Purification of the GST-IE(1-1487) fusion protein

Purification of the GST fusion protein expressing EHV-1 IEP (aa 1-1487) was conducted as described previously (Albrecht et al., 2003). The GST-IE(1-1487) plasmid was transformed into *Escherichia coli* [BL21(DE3)pLysE] cells (Novagen, Madison, WI). The transformed bacteria were incubated for 2 h at 37°C in 2× YT medium [16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per liter, 2% (wt/vol) glucose] containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol. GST-fusion protein synthesis was induced by addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG), and cells were incubated for another 3 h after induction. The GST-IE(1-1487) fusion protein was purified with the GST Bind Kit (Novagen). Briefly, bacteria were lysed in the BugBuster protein extraction reagent. The insoluble debris was removed by centrifugation, and the soluble GST fusion proteins were purified using the GST-bind resin. The eluted protein was then filtered using Amicon ultra centrifugation filter devices (Millipore, Billerica, MA), desalted, and concentrated. The purified protein was separated by SDS-PAGE and visualized by staining the gel with Coomassie blue.

2.5. Gel shift assay

The DNA-binding assay was performed as previously described (Kim et al., 1995). Two double-stranded and annealed DNA oligonucleotides were used in gel shift assay. The wild-type IEBS probe consisted of a 38-bp sequence containing a consensus IEBS (underlined) in the EHV-1 IE promoter (positions -16 to +22 nt relative to the transcription initiation site, 5'-ttg ctt acc c atcgt agc atc gcg gac tag aga gcc tt-3'/5'-tta agg ctc tct agt ccg cga tgc tac gat ggg taa gc-3'). A mutant IEBS probe lacking a consensus IEBS sequence was annealed by using oligonucleotides (5'-ttg ctt acc cat tca agc atc gcg gac tag aga gcc tt-3'/5'-aag gct ctc tag tcc gcg atg ctt gaa tgg gta agc aa-3'). Each oligonucleotide annealed was end-labeled with [γ -³²P]ATP (New England Nuclear Corporation, Boston, MA) and T4 polynucleotide kinase (Promega) according to the manufacturer's directions. The DNA-binding reactions were carried out in a total volume of 20 μl containing approximately 1 ng of radiolabeled

double-stranded DNA fragments (2×10^5 cpm/ng), 30 ng of poly(dI-dC) as a nonspecific competitor, binding buffer (20 mM HEPES-KOH [pH 7.9], 0.5 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, 0.025% NP-40, 25 mM KCl, 2 mM MgCl₂), and 100 ng of indicated protein. The DNA binding reaction mixtures were incubated at room temperature for 20 min, and 5 μ l of loading buffer (200 mM HEPES-KOH [pH 7.9], 50% [vol/vol] glycerol, 0.02% bromophenol blue) was added. The reaction samples were separated by electrophoresis on a 3% polyacrylamide gel with 0.5 $\times$ TBE running buffer for 3 h at 200 V. Following drying, the gels were exposed to a phosphor screen and scanned on the molecular imager FX system (Bio-Rad Laboratories).

3. Results

3.1. The EHV-1 IEP inhibits its own promoter through an IEP-binding site (IEBS) located near the transcription initiation site

To investigate the effect of the presence of a consensus IEBS (5'-ATCGT-3') on transcriptional repression of the IE promoter by the IEP, we constructed luciferase reporter plasmids harboring the IE promoter sequence from -802 to +73 relative to the transcription initiation site or a mutant promoter sequence deleted of the IEBS. The IEBS which is located at 18-bp downstream relative to the consensus TATA box (TATAAA) lies upstream by 2-bp of the transcription initiation site (Fig. 1A). We determined if the IEP specifically binds to the consensus IEBS sequence by gel shift assays with GST-IE(1-1487) fusion protein and probes wtIEBS or Δ IEBS containing the IE promoter (-16 to +22 nt) as described in Materials and methods. The conserved pentanucleotide 5'-ATCGT-3' was substituted with 5'-ATTCA-3' in the Δ IEBS mutant probe. Gel shift assay showed that GST-IE(1-1487) strongly bound to the IE promoter containing a consensus IEBS (Fig. 1B, lane 3) but bound only poorly to the promoter lacking the IEBS (Fig. 1B, lane 6), indicating that the DNA-binding ability of EHV-1 IEP depends on the consensus IEBS.

To examine the effect of the IEBS on transcription from wild-type and mutant IEBS promoters, RK13 cells were co-transfected with reporter plasmids (pIEwtIEBS-Luc or pIE Δ IEBS-Luc) and effectors (pSVSPORT1, pSVIE, or pSVIR2), and luciferase reporter assays were performed. There was no significant difference in the basal transcription level between pIEwtIEBS-Luc and pIE Δ IEBS-Luc deleted of the IEBS (Fig. 1C, bars 4 and 7). Consistent with our previous results (Kim et al., 2006; Smith et al., 1992), luciferase activity of the wild-type IE promoter was decreased by ~80% as compared to the reporter alone in the presence of the IEP (Fig. 1C, bar 5). Interestingly, the IEP *trans*-activated the mutant promoter by 3.5-fold as compared to the reporter only when the consensus IEBS was deleted (Fig. 1C, bar 8). These results revealed that the IEP inhibits its own promoter through the consensus IEBS that is located near the transcription initiation site. However, the IR2 protein, a truncated form of the IEP, repressed both wild-type and mutant IEBS promoter by 95% and 94% as compared to each reporter only, respectively (Fig. 1C, bars 6 and 9). This result suggested that the IR2P strongly inhibits the IE promoter regardless of the presence of the IEBS.

3.2. The short distance between the TATA box and the IEBS is important for transcriptional repression by the IEP

To determine the effect of the distance between the TATA box and the IEBS on the transcriptional repression of the IE promoter by the IEP, the 18-bp downstream IEBS relative to the TATA box was displaced by the insertion of 8-, 16-, 24-, or 32-bp of synthetic DNA sequences between the TATA box and the IEBS. Therefore, the consensus IEBS is located at 18-bp downstream of the TATA box in pIEwtIEBS-Luc, but is located at 26-, 34-, 42-, and 50-bp downstream from the TATA box in pi8-IEBS-Luc, pi16-IEBS-Luc, pi24-

IEBS-Luc, and pi32-IEBS-Luc, respectively (Fig. 2A). The inserted DNA sequences do not have any significant match to the consensus sequences for binding of the IEP and general transcription factors (GTFs). Reporter plasmids pIEWtIEBS-Luc, pi8-IEBS-Luc, pi16-IEBS-Luc, pi24-IEBS-Luc, and pi32-IEBS-Luc were co-transfected into RK13 cells in the presence or absence of the effector pSVIE, and cell lysates were employed in luciferase assay at 40 h after transfection. IEP expression decreased luciferase activities of pIEWtIEBS-Luc and pi8-IEBS-Luc by 80% and 70%, respectively (Fig. 2B, bars 4 and 6). However, luciferase activities of pi16-IEBS-Luc, pi24-IEBS-Luc, and pi32-IEBS-Luc were increased by 1.3-, 1.4-, and 4.6-fold as compared to that of each reporter only, respectively (Fig. 2B, bars 8, 10, and 12). Therefore, when the distance between the TATA box and a consensus IEBS is greater than 34-bp, the IEP was unable to repress transcription from its own promoter. In contrast to the IEP, the addition of the IR2P decreased more than 90% of the luciferase activities of wild-type and all mutant promoters as compared to each reporter only (Fig. 2C), suggesting that the IR2P strongly inhibits the IE promoters independent of the distance between the TATA box and the IEBS. These results demonstrated that the short distance between the TATA box and the IEBS is important for repression of the IE promoter by the IEP, and implied the possibility that the ability of IEP to inhibit the transcription may be closely related to the protein-protein interactions between the IEP and GTFs associated with the TATA box as well as the presence of the IEBS.

3.3. The proper orientation and position of the IEBS are required for efficient repression by the IEP

To examine whether the orientation of the IEBS affects transcriptional repression by the IEP, we generated a mutant promoter harboring the IEBS oriented in the direction opposite to the wild-type IE promoter (Fig. 3A). Luciferase activity of pIEorIEBS-Luc increased by 5.4-fold compared to that of the reporter alone in the presence of the IEP (Fig. 3B, bar 8), similar to the level of luciferase activity of pIEΔIEBS (Fig. 3B, bar 6). These results revealed that the consensus IEBS (ATCGT) must be oriented in the 5' to 3' direction for the IEP to repress its own promoter. To determine if the position of the consensus IEBS affects transcriptional repression, we generated two reporter plasmids pIErvIEBS-Luc and pIEupIEBS-Luc as shown in Fig. 3C. pIErvIEBS-Luc contains the IEBS (5'-ATCGT-3') on the complementary strand of wild-type IE promoter sequences, and pIEupIEBS-Luc positioned the IEBS at 31- to 27-bp upstream from the TATA box while the consensus sequence is located at 18-bp downstream from the TATA box in wild-type IE promoter. Luciferase activity of pIErvIEBS-Luc was not efficiently decreased in the presence of the IEP (Fig. 3D, bar 6). This result demonstrated that the proper position of IEBS is required for efficient repression by the IEP. Interestingly, luciferase activity of pIEupIEBS-Luc was reduced by 52% compared to that of the reporter alone in the presence of the IEP (Fig. 3D, bar 8). This finding suggested that the IEP was able to repress transcription from the mutant promoter even though IEBS is displaced at 27-bp upstream of the TATA box.

3.4. The upstream IEBS (positions -86 to -82 nt) is not necessary for the transcriptional repression by the IEP

It has been previously shown by DNase I footprinting that the EHV-1 IEP binds to two IEBS sequences located at -11 to +14 nt and -92 to -82 nt relative to the transcription initiation site in the IE promoter region (Kim et al., 1995). We also found that the downstream IEBS (5'-ATCGT-3') located at -6 to -2 nt is critical for the inhibition of transcription from the IE gene by the IEP. To determine whether the upstream IEBS (5'-ATCGA-3') located at -86 to -82 nt is required for the down-regulation by the IEP, the mutant promoters deleted of the upstream and/or downstream IEBS were generated and used for luciferase reporter assay (Fig. 4A). Moreover, we deleted the upstream IEBS from pi32IEBS-Luc to examine the contribution of the upstream IEBS on repression by the IEP

when the downstream IEBS was displaced at 50-bp from the TATA box. Luciferase activities of pIEBSwt1/wt2-Luc and pIEBSΔ1/wt2-Luc which deleted the upstream IEBS but retained the downstream IEBS were decreased by 67% and 54%, respectively, as compared to that of each reporter only in the presence of the IEP (Fig. 4B, bars 2 and 6). However, luciferase activities of pIEBSwt1/Δ2-Luc and pIEBSΔ1/Δ2-Luc, in which the downstream IEBS was deleted, were 5.3- and 7.4-fold increased compared to that of the reporter only in the presence of the IEP, respectively (Fig. 4B, bars 4 and 8). These results showed that the ability of IEP to repress its own promoter is retained without the upstream IEBS, although the inhibition level was increased by 13% in the presence of two IEBS sequences compared to a mutant promoter containing the downstream IEBS alone. Mutant promoters pi32IEBSwt1/wt2-Luc, pi32IEBSwt1/Δ2-Luc, pi32IEBSΔ1/wt2-Luc, and pi32IEBSΔ1/Δ2-Luc were strongly *trans*-activated by the IEP regardless of the presence or absence of the upstream IEBS (Fig. 4B, bars 10, 12, 14, and 16). These findings suggested that the upstream IEBS (positions -86 to -82 nt) is not essential for repression by the IEP.

3.5. The IEP efficiently inhibits its own promoter in a TATA box-dependent manner

To ascertain if other *cis*-acting elements modulate the transcriptional repression of the IE gene by the IEP, we investigated if a consensus TATA is necessary for full repression by the IEP. We constructed mutant promoters deleted of a consensus TATA box and/or the downstream IEBS (Fig. 5A). In luciferase reporter assay, the activity of pIEWtIEBS-Luc that harbors a consensus TATA box (TATAAA) was reduced by 63% compared to the reporter only in the presence of the IEP (Fig. 5B, bar 4), but the activity of pIEΔTATA-Luc deleted of a consensus TATA box was decreased by 31% compared to the reporter only (Fig. 5B, bar 6). When both the TATA box and downstream IEBS were deleted from the IE promoter, luciferase activity was increased 1.8-fold with the IEP compared to that of the reporter alone (Fig. 5B, bar 8). However, the activity of pIEΔIEBS-Luc that lacked the downstream IEBS but retained a consensus TATA was increased by 8.4-fold compared to the reporter only in the presence of IEP (Fig. 5B, bar 14). These results indicated that the ability of IEP-mediated *trans*-activation of mutant IE promoters relies on a functional TATA box. The IEP *trans*-activated pIE-2TATA-Luc and pIE-upTATA-Luc by 2.7- and 4.6-fold compared to each reporter only, respectively (Fig. 5B, bars 10 and 12). These results suggested that a consensus IEBS and functional TATA box are required for efficient repression by the IEP and that the short distance between these two elements is a crucial factor in determining the efficacy of transcriptional repression.

To further investigate the effect of distance between the TATA box and the IEBS on full *trans*-repression of the IE promoter, promoters that varied the distance between the two *cis*-acting elements were generated and employed in luciferase reporter assays (Fig. 5C). A consensus IEBS is 18-bp downstream of the TATA box in wild-type IE promoter but is 14-, 9-, 5-, and 2-bp downstream from the TATA box in pTA/IEBSΔ4bp-Luc, pTA/IEBSΔ9bp-Luc, pTA/IEBSΔ13bp-Luc, and pTA/IEBSΔ16bp-Luc, respectively (Fig. 5C). Transcriptional inhibition levels of pTA/IEBSΔ4bp-Luc, pTA/IEBSΔ9bp-Luc, pTA/IEBSΔ13bp-Luc, and pTA/IEBSΔ16bp-Luc by the IEP were more dramatically increased as compared to that of wild-type (Fig. 5D). These results revealed that the distance between the TATA box and the IEBS required for efficient *trans*-repression by the IEP is less than 14-bp.

4. Discussion

The efficient regulation of the essential IE gene plays a critical role in initiation of the viral gene regulatory cascade since the EHV-1 genes are coordinately expressed and regulated in three kinetic classes, immediate-early, early, and late, and this virus has a sole IE gene. Although studies on the mechanism by which IEP *trans*-activates the expression of EHV-1

viral genes have been reported (Albrecht et al., 2003; Buczynski et al., 1999; Derbigny et al., 2002; Jang et al., 2001), the mechanism and function of negative regulation by the IEP in viral gene expression are poorly understood. Here, we present results that the EHV-1 IEP represses transcription from its own promoter through the consensus IEBS (5'-ATCGT-3') which is located at -6 to -2 nt relative to the transcription initiation site. HSV-1 ICP4, PRV IE180, and VZV IE62, IEP homologs of other alphaherpesviruses, also *trans*-activate early gene promoters and exhibit autorepression (Disney et al., 1990; Douville et al., 1995; Gomez-Sebastian and Tabares, 2004; Gu et al., 1995; Ihara et al., 1983; Kuddus et al., 1995; Leopardi et al., 1995; Lium et al., 1996; Smith et al., 1992; White et al., 2010). Each of these immediate-early genes contains a consensus binding sequence (5'-ATCGT-3') that interacts with its gene product. ICP4 represses the transcription from its own promoter through the bipartite consensus ICP4-binding sequence (ATCGTCNNNNYCGRC) that overlaps the transcription start site (Gu et al., 1995; Smith et al., 1993). Like VZV IE62, the DNA-binding ability of EHV-1 IEP depends on the conserved pentanucleotide sequence (ATCGT), whereas mutations in both the 5' and 3' sequences of the bipartite consensus ATCGTCNNNNYCGRC affected ICP4 binding (Betz and Wydoski, 1993; Tyler and Everett, 1993, 1994).

Consistent with the results from HSV-1 ICP4, the distance between the TATA box and the IEBS is important for efficient repression by the IEP of EHV-1. The IEP was able to repress transcription from its own promoter when the IEBS is located within 26-bp from the TATA box, but not when the distance between the two elements is greater than 34-bp. The ICP4 did not inhibit transcription from its own promoter when the distance between TATA box and the ICP4-binding site is greater than 29-bp (Kuddus et al., 1995). Previous studies revealed that ICP4 cooperatively forms tripartite complexes with TFIIB and the TATA box-binding protein (TBP) on DNA containing an ICP4-binding site *in vitro*, and the formation of this complex inhibits activated transcription (Gu et al., 1993, 1995; Kuddus et al., 1995; Smith et al., 1993). Recently, it has also been reported that ICP4 interacts with components of TFIID and Mediator as well as TBP, TFIIB, the TBP-associated factor 1 (TAF1), and RNA polymerase II in the context of viral infection (Lester and DeLuca, 2011). According to our previous publications, the IEP directly interacts with TFIIB and TBP (Albrecht et al., 2003; Jang et al., 2001; Kim et al., 2003). The importance of the short distance between the two elements in the IEP-mediated repression implies that protein-protein interactions between the IEP and GTFs associated with the TATA box including TFIID are required for efficient autorepression by the IEP.

Mutational analysis of orientation of the IEBS revealed that a consensus IEBS must be oriented 5' to 3' direction relative to the TATA box for efficient repression. In contrast to wild-type IEBS promoter, the consensus IEBS (5'-ATCGT-3') was mutated to 5'-TGCTA-3' in pIEorIEBS-Luc. We also confirmed by gel shift assay that this change leads to loss of DNA-binding ability of the IEP in a sequence-specific manner. Interestingly, the IEP was able to repress transcription from pIEupIEBS-Luc which moved the consensus binding site to 27-bp upstream from the TATA box, indicating that the repressive role of the IEBS positioned within 27-bp upstream as well as 26-bp downstream of the TATA box. In addition, results obtained with the pIEΔTATA-Luc construct demonstrated that a functional TATA box is required for efficient repression by the IEP. Direct interaction of consensus TATA box and TBP, a subunit of TFIID, is essential for RNA polymerase II-mediated transcription in TATA-containing promoters, whereas transcription from TATA-less promoters is mediated by RNA polymerase II with Initiator and its binding proteins such as TFIID and TFII-I (Emami et al., 1997; Hampsey, 1998; Smale, 1997). EHV-1 IEP directly binds to the consensus IEBS located between the TATA box and the transcription initiation site and forms complexes with GTFs including TBP and TFIIB. These DNA-protein and

protein-protein interactions may prevent the assembly of the transcription preinitiation complex (PIC) and/or block to the recruitment of RNA polymerase II to the promoter.

We recently observed that EHV-1 early regulatory proteins IR2P, UL3P, and UL4P act as the negative regulatory protein for EHV-1 gene expression (Ahn et al., 2011; Charvat et al., 2011; Kim et al., 2006, 2011). Unlike the IEP, these negative regulatory proteins have a broad inhibitory function that may prevent transcription of a variety of EHV-1 promoters by interfering with the interaction between the GTFs. In this study, the IR2P strongly inhibited the transcription from the IE promoter regardless of the presence of the IEBS and the distance between the TATA box and the IEBS. Our findings indicated that the mechanism of negative regulation of IR2P is quite distinct from that of IEP-mediated repression.

Overall, the mechanism of autorepression by EHV-1 IEP is similar to that of repression with IEP homologs of other alphaherpesviruses. Therefore, the autorepression of immediate-early genes with the IEP homologs requires several common features (i) a sequence-specific DNA-binding ability of IEP, (ii) the consensus binding site located at or near the transcription initiation site, (iii) the short distance between the TATA box and the consensus binding site, and (iv) the proper positioning and orientation of consensus binding site relative to the TATA box in the corresponding gene. In contrast to HSV-1 ICP4, the IEP of EHV-1 is capable of repression of basal as well as activator-mediated transcription and represses its own promoter through a consensus binding site located either upstream or downstream of the TATA box. Thus we suggest that autorepression of immediate-early genes via their gene products-binding site is a common mechanism for autoregulation of the essential IE gene of alphaherpesviruses.

Acknowledgments

We thank Mrs. Suzanne Zavec for excellent technical assistance. This research was supported by Agriculture and Food Research Initiative Competitive Grant 2008-35204-04438 from the USDA National Institute of Food and Agriculture, and by NIH center grant P20-RR018724 from the National Center for Research Resources.

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Research Highlights

We investigated the mechanism by which the EHV-1 IEP inhibits its own promoter. > The IEP represses transcription from its gene through the consensus IEP-binding site (IEBS). > The short distance between the TATA box and the IEBS is important for IEP-mediated repression. > The IEP efficiently modulates autorepression of its gene in a TATA box-dependent manner.

Fig. 1. The consensus IEBS is important for the transcriptional repression of its own promoter by the EHV-1 IEP but not for the repression by the IR2P

(A) Schematic diagram of luciferase reporter plasmids pIEWtIEBS-Luc and pIEΔIEBS-Luc. pIEWtIEBS-Luc possesses two IEBS sequences located at -86 to -82 nt (5'-ATCGA-3') and -6 to -2 nt (5'-ATCGT-3'), but pIEΔIEBS-Luc lacks the downstream IEBS (positions -6 to -2 nt). (B) Gel shift assay using GST-IE(1-1487) fusion protein and wild-type (lanes 1 to 3) and mutant IEBS probes (lanes 4 to 6). The wtIEBS probe containing the EHV-1 IE promoter region (positions -16 to +22) and the ΔIEBS probe deleted of the consensus IEBS were radiolabeled with [γ - 32 P]ATP, and were incubated with 100 ng of purified GST (lanes 2 and 5) or GST-IE(1-1487) protein (lanes 3 and 6). (C) Effect of the IEBS on repression by the IEP or the IR2P. RK13 cells were co-transfected with 0.15 pmol of reporter plasmids (pGL3basic-Luc, pIEWtIEBS-Luc, and pIEΔIEBS-Luc) and 0.3 pmol of effector plasmids (pSVSPORT1, pSVIE, or pSVIR2) as described in Materials and methods. Luciferase activity was measured at 40 h post transfection and expressed as relative luciferase units (RLU). Each experiment was carried out in triplicate. Error bars represent standard deviation of three independent experiments.

Fig. 2. The short distance between the TATA box and the IEBS is critical for IEP-mediated autorepression

(A) Schematic diagram of luciferase reporter plasmids containing wild-type and mutant IEBS. (B and C) Effect of the distance between the TATA box and the IEBS on the inhibition of transcription by the IEP or the IR2P. RK13 cells were co-transfected with 0.15 pmol of reporter plasmids (pGL3basic-Luc, pIEWtIEBS-Luc, pi8-IEBS-Luc, pi16-IEBS-Luc, pi24-IEBS-Luc, and pi32-IEBS-Luc) and 0.3 pmol of effector plasmids [pSVSPORT1, pSVIE (B), and pSVIR2 (C)]. Error bars represent the mean $\pm$ SD of three independent experiments.

Fig. 3. Effect of the orientation and the position of the IEBS on repression by the IEP

(A) Schematic diagram of mutant IEBS-luciferase reporter plasmids. In contrast to pIEWtIEBS-Luc, the consensus IEBS is in an opposite orientation (5'-TGCTA-3') relative to the TATA box in pIEorIEBS-Luc. The orientation of the IEBS is shown by the arrows. The sequences of wild-type and mutant IEBS in the promoters are shown in the right panels. (B) Effect of the orientation of IEBS on the repression by the IEP. Luciferase reporter assay was performed by using 0.15 pmol of reporter plasmids (pGL3basic-Luc, pIEWtIEBS-Luc, pIEΔIEBS-Luc, and pIEorIEBS-Luc) and 0.3 pmol of effector plasmids (pSVSPORT1 and pSVIE). (C) Schematic diagram of mutant promoters altered in the position of the IEBS. The pIErvIEBS-Luc contains a consensus IEBS located on the complementary strand of its original site, and the downstream IEBS was displaced to upstream of the TATA box in pIEupIEBS-Luc. The position and orientation of the IEBS are shown by the arrows, and the sequences of the IEBS are shown in the right panels. (D) Effect of the position of the IEBS on the transcriptional repression by the IEP. Reporter plasmids (pGL3basic-Luc, pIEWtIEBS-Luc, pIErvIEBS-Luc, and pIEupIEBS-Luc) and effector plasmids (pSVSPORT1 and pSVIE) were used for transfection and luciferase reporter assay as described in Materials and methods.

Fig. 4. The upstream IEBs (positions -86 to -82 nt) is not necessary for the down-regulation by the IEP

(A) Schematic of wild-type and mutant IEBs promoters. The mutant promoters were deleted of the upstream and/or downstream IEBs (see Materials and methods). The IEBs1 is an upstream IEBs (5'-ATCGA-3') located at -86 to -82 nt relative to transcription start site, and the IEBs2 is the downstream IEBs (5'-ATCGT-3') located at -6 to -2 nt. In pi32IEBSwt1/wt2-Luc, IEBs2 is located at 50-bp downstream relative to the consensus TATA. (B) Luciferase reporter assay. RK13 cells were co-transfected with 0.15 pmol of reporter plasmids (pIEBSwt1/wt2-Luc, pIEBSwt1/Δ2-Luc, pIEBSΔ1/wt2-Luc, pIEBSΔ1/Δ2-Luc, pi32IEBSwt1/wt2-Luc, pi32IEBSwt1/Δ2-Luc, pi32IEBSΔ1/wt2-Luc, and pi32IEBSΔ1/Δ2-Luc) and 0.3 pmol of effector plasmids (pSVSPORT1 and pSVIE).

Fig. 5. The consensus TATA box is required for efficient repression by the IEP

(A) Schematic diagram of mutant IE promoters deleted of the TATA box and/or the IEBS. The wild-type IE promoter contains a consensus TATA box (positions -29 to -24 nt) and two IEBS sequences (positions -86 to -82 nt and -6 to -2 nt). However, pIEΔTATA-Luc and pIEΔTATA/IEBS-Luc contain the TATA-less promoter, and pIE-2TATA-Luc contains two consensus TATA boxes which are located at -85 to -80 nt and -29 to -24 nt from the transcription start site. In the pIE-upTATA-Luc plasmid, the consensus TATA box was displaced from its original site to -85 to -80 nt. (B) Luciferase reporter assay using reporter plasmids (pGL3basic-Luc, pIEwtIEBS-Luc, pIEΔTATA-Luc, pIEΔTATA/IEBS-Luc, pIE-2TATA-Luc, pIE-upTATA-Luc, and pIEΔIEBS-Luc) and effector plasmids (pSVSPORT1 and pSVIE). *P<0.001 versus the corresponding control value. (C) DNA sequences and distance between the TATA box and the IEBS in wild-type and mutant promoters. The deleted sequences between the TATA box and the IEBS are indicated by carets. (D) Luciferase reporter assay was performed by using reporter plasmids pGL3basic-Luc, pIEwtIEBS-Luc, pTA/IEBSΔ4bp-Luc, pTA/IEBSΔ9bp-Luc, pTA/IEBSΔ13bp-Luc, and pTA/IEBSΔ16bp-Luc and effector plasmids (pSVSPORT1 and pSVIE).