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Characterization of the molecular and biological properties between the equine herpesvirus type 1 immediate-early protein and the general transcription factor human TFIIB

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Abstract

The equine herpesvirus type 1 (EHV-1) immediate- early (IE) protein is a potent transactivator responsible for the activation of both early and late genes during the course of infection and is comprised of discrete functional domains that mediate its many functions. Interaction between transactivators such as the IE protein and various components of the RNA polymerase II transcription initiation machinery has been demonstrated to be critical for transactivation. In the present report, it is addressed the hypothesis that the IE protein interacts with various components of transcription machinery to mediate transactivation of target viral genes. In these studies, it is demonstrated that *in vitro* transcribed and translated IE protein interacts with TFIIB-agarose conjugate but not with TFIID-agarose conjugate. Additional immunoprecipitation studies using nuclear extracts derived from EHV-1 infected RK-13 cells confirmed that the IE protein interacts strongly with TFIIB, but fails to interact with TFIID. IR2, a truncated form of the IE protein lacking the potent transactivation domain and involved in the down-regulation of the IE gene, also interacted with TFIIB but not with TFIID. Studies were also performed to ascertain if particular TBP-associated factors (TAFs) could mediate IE or IR2 binding to TFIID. *In vitro* transcribed and translated TAF250 added to nuclear extracts generated from EHV-1 infected cells also failed to mediate an interaction between the IE protein or the IR2 protein and TFIID. This study demonstrated that the IE protein mediates transactivation of target viral genes by a mechanism that involves TFIIB. This is in contrast to mechani는 that have been proposed for both the herpes simplex virus ICP4 and VP16 protein which have been proposed to transactivate viral genes through interactions involving both TFIIB and TFIID. This study also intimates that IR2 mediate its repressive effects during the course of EHV-1 infection by a mechanism that involves sequestration of various transcription factors.

Key words : Equine herpesvirus type 1, EHV-1, IE protein, TFIIB

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Introduction

Equine herpesvirus type 1 (EHV-1) is a member of the subfamily *Alphaherpesvirinae*, that encodes 77 genes which are temporally in an immediate-early (IE), early and late fashion[1-3]. EHV-1 encodes a single IE protein that is expressed in the absence of prior viral protein synthesis due to the expression of potent virion transactivating protein referred to as E-TIF[4]. The sole IE protein of EHV-1 is a phosphorylated, 200 kDa regulatory protein responsible for the transactivation of both early and late genes during the course of a productive infection[5,6]. The IE protein down-regulates gene expression directed by its own promoter, activates gene expression directed by EHV-1 early promoters, and cooperates in a synergistic fashion with viral accessory regulatory proteins to induce gene expression directed by early and late viral promoters[6-15].

Genetic analyses of *Alphaherpesvirinae* ICP4 homologs revealed that these potent viral transactivators contain conserved regions that harbor discrete functional domains[16]. Sequence alignment of the EHV-1 IE protein and its ICP4 homologs have defined five colinear regions. Region 1 contains a potent transcriptional activation domain (TAD) with its first 89 amino acids (aa) and a serine rich tract (SRT; aa 181-220) that may contain a site for phosphorylation[13,17]. Regions 2 and 3 harbor a DNA-binding domain, while the nuclear localization signal (NLS) lines with region 3[18,19]. Regions 4 and 5 contain a putative transcriptional enhancement domain[19,20]. Studies have shown that the minimal TAD, the SRT, the DNA-binding domain, and the NLS of the IE protein are required for EHV-1 growth in cell[21]. A novel feature of the IE open reading frame is that an early gene, IR2, maps within the IE gene and encodes a 5′ truncated version of the IE protein lacking amino acids 1 to 322[22]. Although the IR2 protein lacks the transactivation domain and is not able to transactivate any viral gene tested to date, it harbors the DNA-binding domain and is capable of down-regulating the EHV-1 IE promoter[18,22].

The mechanism by which the IE protein is able to transactivate target viral genes has not yet been fully elucidated. However, it has been shown that transcription of viral genes in a productive infection is mediated by the interaction of virus-encoded regulatory proteins and various components of the RNA polymerase II cellular transcription machinery[23-33]. The large T antigen of simian virus 40, the E1A protein of adeno-virus, and the E2 protein of papilloma-virus are other noteworthy examples of viral transcriptional activators that control both activation and repression of viral genes through interactions with cellular transcription factors[24,27,29,30,33]. Many viral transcriptional activators have been shown to interact with a number of general transcription factors (GTFs) such as the TATA-binding protein (TBP), TBP-associated factors (TAFs), TFIIB and TFIID[26,32,34,35]. TFIID is a complex of proteins that includes TAFs and the TBP, which recognizes and binds to TATA boxes within the promoter[25,26,34,36,37]. TFIIB is a single protein that binds to both TBP and TFIID[31,35].

Transcriptional activators typically bind

to the specific sequences within the target promoter as well as recruit and enhance the assembly of the GTFs in a defined and highly ordered fashion[26,34,35,38,39]. Briefly, the assembly of the transcription initiation complex begins with the binding of TFIID to the promoter[34,39]. Subsequent to TFIID binding, TFIIA, TFIIB, TFIIF and RNA polymerase II sequentially bind followed by the binding of TFIIE, TFIIJ and TFIIH[39-42].

Studies examining the transcriptional activities of the herpes simplex virus type 1 (HSV-1) ICP4 protein revealed that ICP4 forms a tripartite complex with both TFIID and TFIIB[31]. Further, the interaction between ICP4 and TFIID is mediated through an interaction between the ICP4 protein and the TBP-associated factor TAF250. This interaction is mediated by the C-terminal region of the ICP4 protein which has been shown to be critical for transactivation function[25,28]. Interestingly, the relative affinity of the ICP4 molecule for binding sites within target viral promoters directly influenced the recruitment of GTFs and resulted in the activation or repression of the target promoter[28,31]. It has been hypothesized that the EHV-1 IE protein interacts with TFIIB and TFIID and transactivates viral promoters in a manner similar to that observed for the HSV-1 ICP4 protein. To ascertain if both the IE and IR2 proteins are able to interact with TFIIB and TFIID, immunoprecipitation analyses were further performed to extend the previous studies[23,44]. Interestingly, in experiment performed in which the IE and IR2 proteins were immunoprecipitated from either *in vitro* transcription and translation reactions or nuclear extracts derived from EHV-1 infected RK-13 cells, it was found that the IE protein strongly interacted with TFIIB but failed to interact with TFIID. The addition of TAF250 in immunoprecipitations using *in vitro* transcribed and translated IE protein and TFIID-agarose conjugate beads did not result in an interaction between the TFIID and IE proteins. Further, the IR2 protein, which lacks a transactivation domain, interacted with TFIIB but failed to interact with TFIID. These results suggest that the IE protein transactivates target viral promoters by a mechanism that differs from that elucidated for the HSV-1 ICP4 protein.

Materials and Methods

Cell culture and virus titration

Rabbit kidney cells (RK-13) were maintained in complete Eagle's minimum essential medium (EMEM) supplemented with penicillin (100 μg/mℓ), streptomycin (100 μg/mℓ), nonessential amino acids, and 5% fetal bovine serum (FBS)[21].

Preparation of nuclear and infected-cell extracts

Nuclear extracts of infected cells were prepared as described previously, with some modifications[45]. RK-13 cells were infected with wild-type EHV-1 Kentucky A (KyA) strain at a multiplicity of infection (MOI) of 15 to 20 PFU per cell. At 6 hours post infection, cells were scraped into phosphate-buffered saline (PBS) containing 0.1 mM each of TLCK

and TPCK, pelleted, and resuspended in 4 volumes of buffer A (10 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP40, 0.5 mM DTT, 0.1 mM TLCK, and 0.1 mM TPCK). After incubation for 10 min on ice, the nuclei were pelleted at 14,000 rpm for 5 min in a micro-centrifuge. The supernatant was discarded, and proteins were eluted from the nuclei by incubation for 30 min on ice in 2 volumes of buffer B (10 mM HEPES at pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.1 mM TLCK, and 0.1 mM TPCK). The nuclear debris were pelleted by centrifugation at 14,000 rpm for 15 min in a microcentrifuge, and the supernatants containing the nuclear IE proteins were stored at -70℃.

Plasmids

All recombinant DNA methods were performed according to standard protocols [46]. Plasmid pG3IE was constructed by cloning an *NheI/DraI* fragment containing the entire 4,773 bp IE open reading frame in to the *XbaI/SmaI* sites of plasmid pGEM-3Z (Promega) according to the previous study[44]. Plasmid pGEM44, which express IE (323-1487; IR2 protein), has been described previously[22]. Plasmid pG3hIIB, which expresses the entire TFIIB gene in *in vitro* transcription and translation reactions, was newly constructed as earlier studies described elsewhere[23,44]. Two expression plasmids (pN254[47] and pM270) carrying the entire human TFIIB gene were kindly provided by Dr. D. Reinberg (Massachusetts Institute of Technology, Cambridge) and Dr. M. Hampsey (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey). Plasmid pTβHAX-h encoding the full-length, human TAF250 gene under the control of the T7 promoter was a kind gift from Dr. Robert Tjian (University of California, Berkeley).

In vitro transcription and translation

In vitro transcription and translation reactions to express the IE, IR2, and TAF250 proteins were performed by using the TNT-coupled transcription and translation rabbit reticulocyte lysate system as per the manufacture's instructions (Promega). Briefly, 1 ㎍ of each plasmid was incubated with 25 ㎕ of rabbit reticulocyte lysate, 1 ㎕ of polymerase, and [^{35}S]methionine (40 Ci/㎖; specific activity, 1,175 Ci/mmol), and reaction mixtures were incubated for 2 hours at 30℃. Radioactive products were analyzed by SDS-PAGE followed by autoradiography, and either stored at -70℃ or used immediately for *in vitro* protein-binding assays.

Immunoprecipitations

Approximately, 25 ㎕ of the transcription and translation lysates were subjected to immunoprecipitation with rocking at 4℃ overnight, using TFIIB-agarose conjugate (Santa Cruz Biotechnology) or TFIID-agarose conjugate (Santa Cruz Biotechnology. Briefly, immunoprecipitations were performed using either 20 ㎕ of nuclear extract or 25 ㎕ each of *in vitro* transcribed and translated IE, IR2 or TAF250 proteins. To these

reactions, 10 μℓ of either TFIIB-agarose conjugate or 10 μℓ of TFIID-agarose conjugate was added together eith 75 μℓ of bovine serum albumin (BSA; 10㎎/㎖). Reaction volume were adjusted to 500 μℓ of NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl at pH 8.0, 0.5% NP40), and placed on a shaking platform at 4℃ overnight. After approximately 24 hours, beads were then centrifuged and washed five times with 500 μℓ of NETN buffer. The bound proteins were eluted by boiling for 5 min in 20 μℓ of 2× SDS sample buffer (120 mM Tris-HCl at pH 6.8, 4% SDS, 20% glycerol, 0.001% bromphenol blue, 2% 2-mercaptoethanol). The proteins were separated by SDS-PAGE through a 4% stacking gel and a 10% resolving gel. The gels were subjected to Western blot analyses (below) or were dried, and the bands were allowed to expose Kodak XAR-5 X-Omat AR film.

SDS-PAGE and Western blot analysis

Protein samples were boiled for 5 min with an equal volume of 2× Laemmli sample buffer. Proteins were separated by SDS-PAGE through a 4% stacking gel and a 10% resolving gel, and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) at 100V for 1 hour at 4℃. After transfer, the membrane was blocked for 1 hour at RT in TBST buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat powdered milk. The membrane was then incubated with anti-IE peptide-specific antiserum at a dilution of 1 : 1,000 in TBST for 30 min at RT. After three 10 min washes with TBST, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma), diluted in TBST at a dilution of 1 : 5,000, for 30 min at RT, and followed with three TBST washes to remove unbound anti-body. Immunocomplexes were visualized by incubation in AP buffer (100 mM Tris-HCl at pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing the AP substrates BCIP/NBT (0.165 ㎎/㎖ of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt [BCIP] and 0.3 ㎎/㎖ of nitroblue tetrazolium chloride [NBP]; Gibco BRL). The anti-IE peptide-specific antiserum was raised against a peptide derived from amino acids 925 to 943 of the IE protein and has been demonstrated in previous studies to be highly reactive to the IE protein[13,21].

Results

Immunoprecipitation of *in vitro* transcribed and translated IE and IR2 proteins

The IE protein is a potent trans-activator of EHV-1 genes. As discussed above, the IE protein harbors a potent TAD within its first 89 amino acids, which was hypothesized to be the domain likely to interact with components of the transcription machinery. Interestingly, the IR2 protein, a 5′ truncated form of the IE protein (aa 322-1,487), lacks the potent TAD but does contain the DNA-binding, nuclear localization, and carboxyl terminus that harbors a putative transactivation enhancement domain. Because the IR2 protein does not contain the TAD, it was

hypothesized that IR2 would fail to interact with GTFs. The experiments that follow were performed to address if either the IE and/or IR2 proteins interact with the transcription factor TFIIB and TFIID which are a critical components required for the initiation of the transcriptional initiation complex.

Fig 1. Immunoprecipitations using *in vitro* transcribed and translated IE and IR2 proteins, and TFIIB-agarose conjugate. *In vitro* transcription and translation reactions to express the IE and IR2 proteins followed by immunoprecipitations using TFIIB-agarose conjugate were performed as described in Materials and Methods. *In vitro* transcribed and translated IE (lane 1), IR2 (lane 2), and luciferase (lane 3) proteins were shown in the respective lanes. The IR2 protein (lane 4), an equimolar (1:1 ratio) mix of the IE and IR2 proteins (lane 5), or the IE protein alone (lane 6) were immunoprecipitated using TFIIB-agarose conjugate. Both the IE and IR2 proteins were shown to interact with TFIIB *in vitro*.

Initial experiments were performed to determine if the IE and IR2 proteins interact with TFIIB. Both the IE and IR2 proteins were *in vitro* transcribed and translated and assayed in immuno-precipitations experiments (Fig 1) using agarose beads conjugated to TFIIB, as described in the Materials and Methods. Transcription and translation events were not evident in either of the reactions (Fig 1, lanes 1 and 2). Transcription and translation reactions of the luciferase protein were also performed (Fig 1, lane 3), and the luciferase protein wea added to each immunoprecipitation as a non-specific protein control. Reactions with the IE protein and TFIIB-agarose conjugated beads showed that the IE protein was able to interact with the TFIIB protein (Fig 1, lane 6).

Interestingly, when the immuno-precipitation reactions were performed with the IR2 protein, a 130 kDa band was visible on SDS-PAGE gels indicating that the IR2 protein also interacted with the TFIIB protein (Fig 1, lane 4). When the IE and IR2 proteins were mixed in a 1 : 1 ratio and subjected to immuno-precipitation, both proteins were evident in roughly equimolar quantities, suggesting that TFIIB does not have a significantly greater affinity for either the IE or IR2 protein (Fig 1, lane 5). These experiments suggest that while the TAD of the IE protein may interact with various transcr iption factors, other domain(s) of the IE and IR2 proteins are capable of mediating the interaction with TFIIB.

Similar experiments were performed to address putative interactions between IE or IR2 and the transcription factor TFIID (Fig 2). Immunoprecipitations were performed using *in vitro* transcribed and translated IE and IR2 proteins and TFIID-agarose conjugated beads. Again, luciferase was used as a non-specific protein control. Immunoprecipitations using TFIID-agarose beads and the luciferase protein alone indicated that the TFIID-

agarose beads have no affinity for the luciferase protein (Fig 2, lane 2). As a positive control, the IE and IR2 proteins were immunoprecipitated with TFIIB-agarose conjugated beads (Fig 2, lane 3). When immunoprecipitations were perfor med with the TFIID-agarose beads and either the IE or IR2 proteins, no reactive protein bands were evident (Fig 2, lanes 4 and 5), suggesting that the IE and IR2 proteins fail to interact with transcription factor TFIID. These experiments suggest that while the ICP4 protein, the HSV-1 homolog, is able to interact with both the TFIIB and TFIID proteins, the EHV-1 IE protein interacts only with transcription factor TFIIB[25,28,31]. These results indicated that the IE protein may mediate transactivation in a manner that differs from that described for the HSV-1 ICP4 protein.

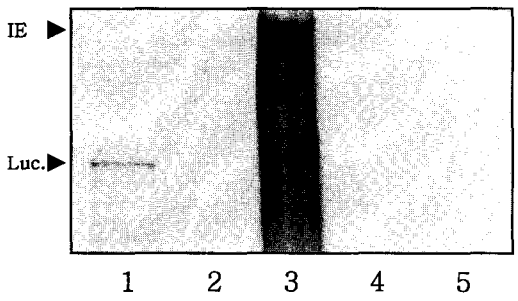

Fig 2. Immunoprecipitations using *in vitro* transcribed and translated IE and IR2 proteins and TFIID-agarose conjugate. The IE and luciferase proteins were transcribed and trans lated as described in Materials and Methods. Transcribed and translated luciferase protein control (lane 1). The luciferase (lane 2), IE (lane 4) and IR2 (lane 5) proteins were immunoprecipitated with TFIID-agarose conjugate. No interaction was observed between the luciferase, IE, or IR2 proteins and TFIID. As a control, the IE protein was immunop recipitated with TFIIB-agarose conju gate (lane 3).

Immunoprecipitation of the IE and IR2 proteins from nuclear extracts

The experiments described above show that *in vitro* transcribed and translated IE and IR2 proteins interact directly with the transcription factor TFIIB. It was next interested in examining the interaction between the IE and IR2 proteins and both TFIIB and TFIID within the context of a viral infection. RK-13 cells were either moch-infected or infected with EHV-1 at an MOI of 10. Nuclear extracts derived from mock-infected and EHV-1 infected RK-13 cells were generated as described in the Materials and Methods, and immunoprecipitations were performed using either TFIIB- or TFIID-agarose conjugated beads (Fig 3). Subsequent to immunoprecipitation, Western blot analyses were performed using an anti-IE peptide specific antibody that reacts with both the IE and IR2 proteins[21]. Both a 200 kDa and 130 kDa immunoreactive bands representing the IE and IR2 proteins, respectively, were detected in immunopre cipitation reactions using nuclear extracts generated from EHV-1 infected RK-13 cells and TFIIB-agarose conjugated beads (Fig 3, lane 4). However, no immunorea ctive bands corresponding to either the IE or IR2 proteins were detected in immuno-precipitation reactions using nuclear extracts from EHV-1 infected cells and TFIID-agarose conjugated beads (Fig 3, lane 6). As expected, no immunoreactive bands were detected in nuclear extracts derived from mock-infected RK-13 cells

(Fig 3, lanes 1, 3, and 5). Similar to the findings of the *in vitro* transcription and translation studies, these data show that the IE and IR2 proteins strongly interact with TFIIB, but fail to associate with transcription factor TFIID.

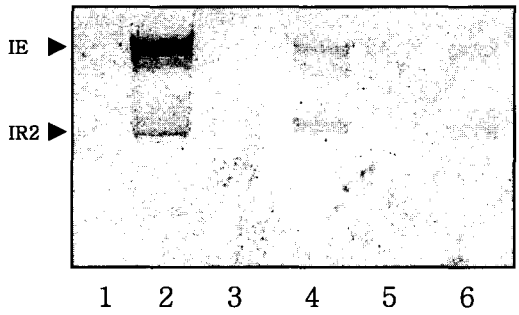

Fig 3. Immunoprecipitation of the IE and IR2 proteins from nuclear extracts derived from EHV-1 infected RK-13 cells using TFIIB- and TFIID-agarose conjugates. Nuclear extracts derived from either mock-infected or EHV-1 infected RK-13 cells were generated and incubated with TFIIB- or TFIID-agarose conjugates as described in the Materials and Methods. Proteins were resolved by SDS-PAGE, immunoblotted and visualized using an anti-IE peptide antibody. Staining for the IE protein in nuclear extracts derived from mock-infected (lane 1) or EHV-1 infected (lane 2) RK-13 cells. Immuno-precipitations using nuclear extracts derived from mock-infected RK-13 cells and TFIIB- (lane 3) or TFIID-agarose conjugates (lane 5) were performed as described in the Materials and Methods. Immunoprecipitations using nuclear extracts generated from EHV-1 infected RK-13 cells and TFIIB- (lane 4) or TFIID-agarose conjugates (lane 6). Both the IE and IR2 proteins interacted with TFIIB but failed to interact with TFIID.

Immunoprecipitation of the IE protein and TAF250

Earlier studies showed that the HSV-1 ICP4 protein interacts with TFIID via its carboxyl terminus and further analyses demonstrated that the interaction between TFIID and ICP4 is mediated by the TAF250[25,28,31]. The initial investigations indicated that the EHV-1 IE protein fails to interact with commercial TFIID-agarose conjugates. The commercially prepared TFIID-agarose conjugate is a preparation that is predominantly TBP and does not contain any of the TAFs, such as TAF250, that are normally associated with TBP and that constitute what is referred to as TFIID. It was interested in determining if TAF250 could mediate an interaction between the IE protein and TBP, therby permitting the IE protein to associate with TFIID in the transcription initiation complex. TAF250 was *in vitro* transcribed and translated, and incubated with nuclear extracts derived from either mock-infected of EHV-1-infected RK-13 cells. Immuno-precipitations reactions were then performed using the either TFIIB-agarose or TFIID-agarose conjug ates followed by Western blot analyses using the anti-IE peptide antiserum (Fig 4). While the IE protein interacted with TFIIB in either the presence or absence of TAF250 (Fig 4, lanes 5 and 7), the IE protein failed to interact with the TFIID conjugates in the presence or absence of TAF250 (Fig 4, lanes 6 and 8). The IE protein was not detected in any of the immunoprecipi-tations using nuclear extracts derived from mock-infected RK-13 cells, demonstrating that the interaction between the IE protein and TFIIB does not arise from a non-specific binding event (Fig 4, lanes 1 and 3). These

results suggest that the IE protein interacts with TFIID and that the putative interaction between the two proteins is not mediated by TFA250.

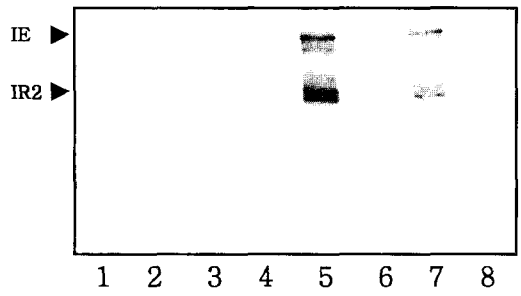

Fig 4. Immunoprecipitations mediated by TAF250. TAF250 was transcribed and translated and added to nuclear extracts generated from either mock-infected or EHV-1-infected RK-13 cells. Immunoprecipitations were performed as described in the Materials and Methods using TFIIB- or TFIID-agarose conjugates. Proteins were resolved by SDS-PAGE, immunoblotted and visualized using an anti-IE peptide antibody. No immunoreactive bands from immunoprecipitations were observed when nuclear extracts derived from mock-infected RK-13 cells were incubated with TFIIB-agarose conjugate (lane 1), TFIID-agarose conjugate (lane 2), TAF250 plus TFIIB-agarose conjugate (lane 3), or TAF250 plus TFIID-agarose conjugate (lane 4). The IE and IR2 proteins were immunoprecipitated both in the absence (lane 5) and presence (lane 7) of TAF250 using nuclear extracts derived from EHV-1 infected RK-13 cells. However, both the IE and IR2 proteins failed to interact with TFIID in the absence (lane 6) and presence (lane 8) of TAF250 indicating that TAF250 does not mediate IE binding to TFIID.

Discussion

The EHV-1 IE protein is a multifunctional protein that regulates both early and late viral gene expression. The many functional domains within the IE protein may serve as sites for putative interactions with both viral and cellular proteins. Specifically, the IE protein possesses a potent transactivation domain that is hypothesized to interact with various components of the transcription machinery to initiate transactivation of target genes. Studies examining the transcriptional functions of the HSV-1 ICP4 and VP16 proteins have revealed that these transactivators are able to mediate transcription through interactions with both the TFIIB and TFIID as well as through a number of TAFs such as TAF250 and $hTAF_{II}31$[26,31,32,34,35,37-39]. In this report, it is described experiments that tested the hypothesis that the EHV-1 IE protein contains an acidic TAD similar to that contained within the ICP4 and VP16 proteins which has been shown to interact with TFIIB and TFIID. Interestingly, the present studies revealed that while the IE protein interacted strongly with TFIIB, it fails to interact with TFIID. Further, the IR2 regulatory protein, a truncated form of the IE protein lacking aa 1-322 that harbors the TAD and SRT domains, was able to interact with TFIIB but not with TFIID. These findings suggest that the EHV-1 IE protein transactivates viral genes by a mechanism that differs that of the ICP4 and VP16 proteins of HSV-1.

EHV-1 mutants that express mutant forms of the IE protein are being generated[48].

Initial characterization of these mutant viruses has revealed that the TAD, DNA-binding domain, SRT, and NLS are essential for IE function and viral growth in cell culture. Further, it has been shown that while the TAD is required for IE transactivation activity, the carboxyl terminus of the IE protein is also required for full IE transactivation function[20,48]. IE mutant viruses KyAn1029 and KyAn1411 that harbor nonsense codons at aa 1029 and 1411, respectively, display a signif icant growth defect in cell culture. Additionally, transient transfection assays that employ constructs encoding forms of the IE protein containing nonsense codons at aa 1029 and 1411 also show a significant defect in transactivation function[13,19,20,48]. Because the carboxyl terminus contributes significantly to the transactivation function of the IE protein, it is possible that this region may recruit members of the transcription initiation machinery and/or stabilize the transcri ptional initiation complex at target viral promoters. This possibility is supported by findings described here that the IR2 protein is able to interact with TFIIB. The IR2 protein lacks the TAD domain but does retain both the DNA-binding domain and the carboxyl terminus[22]. The IR2 protein is responsible for down-regulating the expression from the IE promoter at both early and late times during infection[22], and it is therefore possible that sequestration of transcription factors may be a component of the mechanism by which IR2 mediates its repressive effect.

Studies described here demonstrate that neither the IE nor IR2 proteins are unable to interact with transcription factor TFIID. Additional experiments using TAF250 indicated that TAF250 is unable to mediate an interaction between the IE protein and TFIID. These findings are in contrast to those described for the HSV-1 ICP4 protein which interacts with TFIID by a mechanism involving TAF250. Interestingly, the TFIID used in our experiments was commercially purchased and consisted predominantly of TBP. TFIID is normally comprised of TBP and a number of associated TAFs. Experi ments are planned to ascertain if other TAFs normally associated with TBP are able to mediate an interaction between the IE protein and TBP. Studies examining the mechanism by which the potent transactivator VP16 mediates transactiv ation have revealed that different activators are able to interact with specific TAFs in the TFIID complex to initiate transcription[49]. Thus, while initial studies indicate that the EHV-1 IE protein appears to mediate transcription through a mechanisml involving TFIIB, it is possible that TAFs other than TAF250 in the TFIID complex contribute to an interact ion between the EHV-1 IE protein and TFIID.

In addition to the above results, we recently showed that EICP0 protein, which is another potent transactivator of EHV-1 promoters[5,7], directly interacts with TFIIB, TBP, and the IE protein, suggesting that the IE protein may antagonize the transactivation ability of the EICP0 protein by their interaction and/or by competition for TFIIB and TBP[50]. There is consider able information but no vital *in vivo* data regarding the antagonism. However, possible explanation of the antagonistic relationship is that the IE and

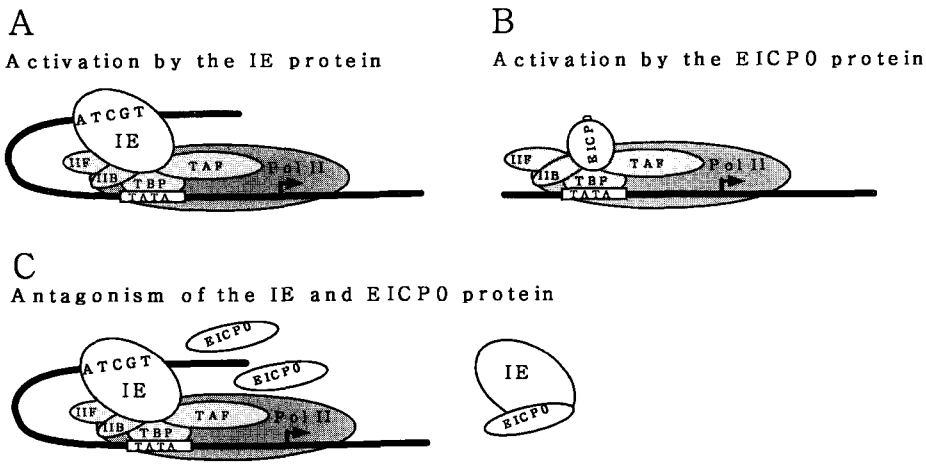

Fig 5. Models of how the IE and EICP0 proteins may activate transcription. Panel A, The IE protein binds to the IE protein-binding consensus sequence of an EHV-1 promoter and then interacts with the basal transcription factors TFIIB and TBP to transactivate EHV-1 promoters. Panel B, The EICP0 protein directly interacts with TFIIB and TBP to transactivate EHV-1 promoters. Panel C, The mechanism of the antagonism observed between the IE and EICP0 proteins. Two possibilities were proposed that the IE and EICP0 proteins firstly antagonize by an interaction between the two proteins, and the IE and EICP0 proteins secondly antagonize by competing for binding to TFIIB and TBP. The arrows indicate the transcription initiation sites.

EICP0 proteins compete for binding to cellular transcription factors. The two-hybrid system in *Saccharomyces cervisiae* has shown that a physical interaction occurred between the EICP0 protein and TFIIB[50]. Further, it was previously showed that the IE protein interacts with the domain within TFIIB at aa 125 to 174, as well as with TBP[23]. Interestingly, the EICP0 protein interacts with the same domain within TFIIB at aa 125 to 174 and is able to compete with the IE protein for interaction with TFIIB in competition assays. Further, the EICP0 protein also specifically and directly interacts with human TBP[50]. These results suggest that the IE protein may antagonize the transactivation ability of the EICP0 protein by competing for binding to these transcription factors. The E2 protein of bovine papillomavirus type 1 binds directly to TFIID and TFIIB and competes with VP16 for factors important for transactivation in vivo[24,30,33]. Taken together with all of our recent studies[23,44,50] including the present results, it could propose a model to explain the mechanism of the antagonism between the IE and EICP0 proteins (Fig 5). Firstly, the IE protein binds to the IE binding consensus sequence of EHV-1 promoters and then interacts with the basal transcription factors TFIIB and TBP to transactivate EHV-1 promoters (Fig 5, A).

Secondly, the EICP0 protein also interacts with TFIIB and TBP by direct protein-protein interaction to trans-activate EHV-1 promoters (Fig 5, B). From these results, two possibilities are suggested that the antagonism is firstly attributed to interactions between the IE and EICP0 proteins, and the antagonistic relationship between the IE and EICP0 proteins is secondly mediated by competition for TFIIB and TBP (Fig 5, C).

Interactions between the IE protein and various components of the cellular transcriptional machinery mediate the formation of a complex that ultimately permits RNA polymerase II transcription. The mechanism by which the IE and IR2 proteins are able to perform their respective functions have not yet been fully elucidated, but it is clear that interactions between these proteins and both viral and cellular factors are critical components for EHV-1 replicate in cell culture. Studies to identify additional interactions between the IE and IR2 proteins and cellular transcription factors hopefully will lead to a better understanding of the mechanism by which the IE protein governs viral gene expression.

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