Identification and control of the cis-acting elements of the immediate early gene of equid herpesvirus type 1

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Consensus cis-acting DNA sequences upstream of the immediate early (IE) gene of equid herpesvirus type 1 (EHV-1, strain Ab4) were identified. One copy of the conserved motif TAATGARATTC, which is the binding site for the host cellular factor Oct-1 and herpes simplex virus type 1 (HSV-1) virion protein, VmW65, complex, was identified at positions −630 to −620. Using transient transfections and chloramphenicol acetyltransferase assays the IE promoter of EHV-1 was shown to be trans-activated by VmW65 within the region −685 to +73. Ultraviolet light-inactivated EHV-1 was able to stimulate the expression of the IE gene of EHV-1 as well as HSV-1, indicating that EHV-1 possesses a protein equivalent to VmW65. The ubiquitous equid herpesvirus type 2 (EHV-2), which is not known to be a primary pathogen, was also able to trans-activate the EHV-1 and HSV-1 IE genes. Further work is being performed in order to identify the nature of the EHV-1 and EHV-2 trans-activating proteins.

Introduction

Equine herpesvirus type 1 (EHV-1), an alphaherpesvirus, causes abortions in infected mares (for review see Allen & Bryans, 1986). After primary infection, the virus becomes latent in lymphoid tissues, in particular those draining the respiratory tract (for review see Edington, 1991; Welch et al., 1992). In this state the virus remains quiescent until reactivation initiates viral replication and subsequent host cell lysis. The prevalence of latency and the factors initiating reactivation are important in the epidemiology and control of infection.

Gray et al. (1987) demonstrated that EHV-1 gene expression is co-ordinately regulated with immediate early (IE), early (E) and late (L) phases. This temporal regulation of polypeptide synthesis has been intensively studied with the prototype alphaherpesvirus, herpes simplex virus type 1 (HSV-1) (Hones & Roizman, 1974; Clements et al., 1977). The five IE genes are the first to be transcribed after infection, and their expression does not require de novo protein synthesis, whereas at least three IE proteins are important trans-activators required for maximal E and L gene expression (for review see Everett, 1987). Cis-acting sequences upstream of the HSV-1 IE genes include at least one copy of the consensus element TAATGARATTC (where R represents purine) (Preston et al., 1984). This is the binding site for the viral tegument protein VmW65 (also known as α-TIF and VP16) (Campbell et al., 1984; Preston et al., 1984). VmW65 does not attach directly to DNA (Marsden et al., 1987), but binds one or more host cellular factors (including Oct-1) to form a multiprotein complex IEC which interacts with TAATGARATTC (O'Hare et al., 1988; Preston et al., 1988). After interaction of the multiprotein complex with the cis-acting sequences upstream of the mRNA start site, the strongly acidic C-terminal 80 amino acids of VmW65 bind the transcription complex and enhance transcription (Triezenberg et al., 1988; Sadowski et al., 1988; Cousens et al., 1989). The importance of the function of the C-terminal region is further substantiated by the findings that the VmW65 homologue of varicella zoster virus (VZV), open reading frame (ORF) 10, which lacks this acidic region, is unable to trans-activate IE gene expression (McKee et al., 1990). Interestingly, VZV IE (ORF62), which possesses upstream cis-acting sequences similar to TAATGARATTC, was trans-activated at low levels by HSV-1 VmW65.

Gray et al. (1987) identified a single IE transcript of approximately 6 kb from the EHV-1 inverted repeat regions (map units 0.78 to 0.83 and 0.95 to 1.0). Grundy et al. (1989) sequenced the ORF of the IE gene of EHV-1 which was found to be 4461 bp (1487 codons) encoding a protein of 155K. Furthermore, the deduced amino acid sequence for this protein has a high degree of similarity to the HSV-1 IE-3 and VZV ORF62 proteins, which implies that control of expression of EHV-1 IE may share a similar pathway. However, little is known about the regulation of the EHV-1 IE gene, which could have important implications for the replication of the virus in
vivo (Ace et al., 1989), and in particular the reactivation of latent EHV-1 which is the cause of major loss to the horse-racing industry.

An interesting aspect of latent EHV-1 is its relationship to EHV-2. Previous workers have described EHV-2 as a cytomegalovirus (Colacino et al., 1989) which is present in 90% of the equine population (Kemeny & Pearson, 1970). Although EHV-2 has frequently been recovered from leukocytes (Studdert, 1974) as well as other tissues, including pneumonic lungs, experimental work has never shown it to be a primary pathogen (Studdert, 1974; Blakeslee, 1975; Fu et al., 1986). However, recent work using polymerase chain reaction and co-cultivation studies has shown that latent EHV-1 in outbred animals is always detected in lymphoid tissues in the presence of EHV-2 (for review see Edington, 1991). It is also reported that recovery of EHV-2 precedes EHV-1 when corticosteroids are used in immunosuppression (Edington et al., 1985). In contrast to previous findings (Allen & Turtinen, 1982; Staczeck et al., 1983), recent work in our laboratory has shown that EHV-1 DNA cross-hybridizes significantly with EHV-2 DNA. Approximately 40% of this cross-hybridization is also shared by the IE-3 probe of HSV-1 (A. S. Purewal, unpublished data). This series of observations led us to investigate the influence of EHV-2 on IE gene expression. We have sequenced upstream of the EHV-1 IE mRNA initiation site (Harty et al., 1989) and identified consensus cis-acting elements. In vitro trans-activation studies were used to investigate the control of expression of EHV-1 IE.

Methods

Plasmids. Plasmid pAA104 contains the region from -1806 to +73 of the EHV-1 (strain Ab4; Patel & Edington, 1983) IE gene inserted into plasmid pUC18 (Fig. 1). This 188 kb HindIII fragment was subcloned from an EcoRI library of EHV-1 as described by Maniatis et al. (1982). Plasmid pCAT (which was supplied by Dr R. Everett) is derived from pBLW2 (Gaffney et al., 1985). It carries the chloramphenicol acetyltransferase (CAT) gene and contains restriction sites for HindIII, PstI, Smal and BamHI upstream of the CAT coding region. Plasmid pCAT1.8Hin contains the EHV-1 IE region -1806 to +73 inserted with the correct orientation at the HindIII site of pCAT. This region contains the TATA box, the 5' mRNA start site, as well as a part of the untranslated leader sequence (Fig. 1). Plasmid pCATAKH is pCAT containing the region from -994 to +73, which is a KpnI-HindIII fragment subcloned from plasmid pAA104. The 3' and 5' overhangs of this KpnI-HindIII fragment were filled as described by Perbal (1988) and ligated into pCAT. A correctly orientated recombinant was selected for study. Plasmid pCATRV contains the region from -685 to +73 (Fig. 1; Harty et al., 1989) in Smal-digested pCAT. This region contains a proposed RNA splice donor site at position +113 (identified by Hartly et al., 1989). The correctly orientated recombinant was selected for further work. Plasmid pCATARVH carries region -685 to +73 in Smal-digested pCAT. The 5' HindIII overhang was filled as described above, and the correctly orientated recombinant vector was identified and used in trans-activation studies. Plasmid pCATATAAT contains region -359 to +73 which is a Smal-HindIII fragment cloned into pCAT, and lacks the TAATGAGATTC motif. The correctly orientated recombinant was selected for further work. Plasmid pMC1, which was kindly provided by Dr R. Everett, contains the HSV-1 gene encoding VmW65 (Campbell et al., 1984). Plasmid pIE1CAT which contains the CAT gene under HSV-1 IE gene 1 control was also supplied by Dr R. Everett.

Cells. Baby hamster kidney (BHK) cells were grown in Eagle’s MEM (EMEM) containing 10% foetal calf serum (FCS). Equine dermal (ED) cells (ICN Flow Laboratories) were also grown on EMEM containing 10% FCS. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all media.

Virus. EHV-2 (isolate G9; Welch et al., 1992) and EHV-1 (isolate Ab4) were grown on ED cells of a low passage number. EHV-1 was irradiated for various times with u.v. light using a Hanovia Chromatolite bactericidal lamp with an output of 4·8 J/m²/s at a distance of 15 cm. Between 10 and 15 min was found to be the minimum time necessary to reduce the virus titre by a factor of 5 × 10² (S. Williamson, unpublished data; Notarianni & Preston, 1982).

Transfections and viral infections. Transfections were performed using calcium phosphate-precipitated plasmid DNA as described by Cordingly et al. (1983). Viral superinfections using u.v.-irradiated EHV-1 were performed as described by Campbell & Preston (1987). Infections were initiated 24 h after transfection. After incubation at 37 °C for 24 h the cells were harvested. Since EHV-2 grows as a cell-associated virus and is passaged as a stock of infected ED cells,
superinfections were performed by the addition of ED cells. Therefore the precise time and multiplicity of EHV-2 infection could not be determined. Cells were harvested after incubation at 37°C for 1 h, 24 h and 72 h. Non-infected ED cells were used for mock infections.

**CAT assays.** Cell extracts were prepared and assayed for CAT activity as described by Gorman et al. (1982). CAT assays were quantified by measuring the intensity of the spots obtained on autoradiographic film using a laser densitometer (Chromoscan 3, Joyce-Loeb). The amount of extract was varied to ensure that the linear response range of the assay was used. Protein concentrations of cell extracts were measured by the method of Bradford (1976) and used to calculate the stimulation of CAT activity.

**DNA sequencing.** DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (1977). Denatured plasmid pAA104 DNA was used as a template and pUC18-specific or overlapping custom oligonucleotides spanning both strands of the 1.8 kb insert (prepared using the Milligen/Biosearch Cyclone Plus DNA synthesizer) were used as primers. Primer sequences annealed to single-stranded plasmid DNA were extended using the T7 DNA polymerase sequencing kit from Pharmacia LKB. Sequencing reactions were run on 6% polyacrylamide wedge gels containing 8 M-urea using the $^2$ sequencing apparatus from Gibco BRL.

**Sequence analysis.** Analysis of overlapping sequence data for the 1.8 kb insert was performed using the DNA Star software which utilizes the algorithm of Wilbur & Lipman (1983) for the alignment of DNA sequences.

## Results

**Identification of cis-acting elements**

Grundy et al. (1989) sequenced the EHV-1 IE ORF; in addition, the same group (Harty et al., 1989) identified the mRNA initiation site. A subclone, pAA104, carrying this region as well as the upstream cis-acting sequences, has now been identified and sequenced (Fig. 1 and 2). The nucleotide sequence shows a 1879 bp HindIII fragment which contains one copy of the consensus motif TAATGARATTC (positions -630 to -620) which is also found upstream of other herpesvirus IE promoters (Preston et al., 1984; McKee et al., 1990). There is also one copy of the motif CCCGCC at position -680, a cis-acting element found in HSV-1 promoters (Preston et al., 1984). These sequences are downstream of the EcoRV site (−685) and therefore are contained within the pCATARVH construct which was used in these trans-activation studies. The consensus element CCCGCC was also identified at position −1088 and the motif CCAAT, important in positive regulation (Jones & Yamamoto, 1985), was present at positions −1597 and −1604. Potential TATA sequences are also present at positions −29, −638 and −1588. The presence of CCAAT at positions −1597 and −1604 and TATA at position −1588 have previously been cited by Yalamanchili & O'Callaghan (1990). These transcriptional control elements are located in the vicinity of the EHV-1 origin of replication which was identified by Baumann et al. (1989). There are two differences in the sequence of the Ab4 strain from the published sequence of the Kentucky A strain (at positions −1612 and −1587; see Fig. 2 legend) which was grown on mouse L-M cells (Baumann et al., 1989). The nature of the alterations does not affect the consensus 9 bp sequence CCGTTCGCA (positions −1612 to −1604) or the hypothetical stem-and-loop structure deduced for the EHV-1 origin of replication in the short region (Oris) (Baumann et al., 1989). The differences indicated above may have arisen due to repeated passage in non-equine cells.

**Functional activity of EHV-1 IE regulatory sequences**

After comparison of the deduced DNA sequence of strain Ab4 with that of Kentucky A (Harty et al., 1989), no differences were found in the region of the 5' mRNA initiation site. Consequently, plasmid pCAT1.8Hin containing sequences from +73 to −1806 inserted 5' to the CAT gene was constructed. The activity of this plasmid was compared with the negative and positive control plasmids pCAT and pIE1CAT, in which the HSV-1 IE1 control sequences have been inserted to direct the expression of CAT (Table 1). The activities of

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<th>Table 1. <strong>Transient expression of CAT activity</strong>* in BHK cells</th>
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<tr>
<td>Chimeric CAT plasmid</td>
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<tr>
<td>pE1ECAT</td>
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<tr>
<td>3.0 × 10^{-2} (4)</td>
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<td>pCAT.8Hin</td>
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<td>2.4 × 10^{-3} (2)</td>
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* CAT activity expressed as acetylation/μg protein (%).
† Number of independent determinations are shown in parenthesis.
‡ ND, Not done.
**Fig. 2.** DNA sequences in the upstream regulatory region of the EHV-1 IE gene. Restriction endonuclease sites relevant to the results are indicated. The mRNA start site is designated +1 by convention and possible TATA boxes are marked at positions -1588, -638 and -29 (o). A TAATGARATTC motif (*) is present at positions -630 to -620. A CCCGCC (x) motif is indicated at positions -1088 to -1082 and the consensus sequence CGTTCGCAC (+) which is found in HSV-1 and VZV origins of replication is located at positions -1612 to -1604. The CCAAT motifs present at positions -1597 and -1604 are marked (^). The nucleotide sequence of bases +78 to -362 and bases -1477 to -1682 has previously been cited by Grundy et al. (1989) and Yalamanchili & O'Callaghan (1990), respectively. Comparison of sequence data from the two strains (Ab4 and Kentucky A) showed that Kentucky A possesses an extra cytidine residue at position -1612 and has a thymidine substituted for an adenosine residue at position -1587.
these three plasmids were tested with and without cotransfected pMC1 which expresses HSV-1 VmW65. Dilutions of the cell extract prepared from these cotransfections showed that the upstream sequences of EHV-1 IE were slightly stronger in directing CAT expression than was HSV-1 IE. Cotransfection with pMC1 resulted in an approximately 1000-fold stimulation of CAT activity using pIE1CAT, whereas up to 1200-fold stimulation of CAT activity was observed using pCAT1.8Hin.

In order to identify the sequences involved in the response to VmW65, a preliminary study was performed using 5' deletions with convenient restriction sites within the 1-8 kb HindIII fragment (Fig. 1). The sites used were KpmI at position -994 and EcoRV at position -685, and the resulting plasmids pCATAKH and pCATARVH were cotransfected into BHK cells with pMC1. Results (Table 1) indicated that plasmids carrying deletions to -994 and -685 produced levels of CAT activity similar to those previously described for pCAT1.8Hin, i.e. >1000-fold stimulation of CAT expression after appropriate dilutions of the cell extract. However when plasmid pCATRV (which carries a possible splice donor site at position +113) was cotransfected with pMC1, a stimulation of CAT activity was not observed. Therefore these results suggest that sequences which respond to VmW65 are located within the HindIII–EcoRV fragment (+73 to -685), and that the proposed splice donor site affects the production of functional mRNA since the basal level of chloramphenicol acetylation using pCATRV is reduced.

The trans-activating factor(s) of EHV-1 and EHV-2

The above data clearly indicate that EHV-1 contains a conserved TAATGARATTC motif which would interact with the HSV-1 multiprotein complex. Therefore it is likely that EHV-1 possesses a protein that could perform the same role as VmW65. Consequently, the CAT activities of pCAT1.8Hin and pIE1CAT were tested with and without superinfection with u.v.-inactivated EHV-1. It is known that u.v. irradiation prevents synthesis of viral proteins by destroying the viral genome (Cordingley et al., 1983). Cell extracts prepared from superinfections showed that EHV-1 was able to direct CAT expression from these plasmids (Table 1). Appropriate dilutions of the cell extracts resulted in an approximately 500-fold stimulation of CAT activity using pIE1CAT and >1000-fold stimulation of CAT activity using pCAT1.8Hin. These results indicate that EHV-1 possesses a VmW65 equivalent which is capable of trans-activating HSV-1 and EHV-1 IE genes. Furthermore, results of CAT assays of pCATATAAT with and without superinfected u.v.-irradiated EHV-1 (Table 1), show that TAATGARATTC is likely to be the activation site of the trans-activation factor(s) of EHV-1.

To establish whether EHV-2 could trans-activate the IE genes of HSV-1 and EHV-1, the activity of pCAT1.8Hin and pIE1CAT was tested with and without superinfected EHV-2. It was not possible to u.v.-inactivate the highly cell-associated virus at this stage. Cell extracts were prepared after 1 h, 24 h or 72 h infection. Stimulation of CAT activity was not observed using cell extracts prepared after 1 h and 24 h infections. However, dilutions of cell extracts prepared 72 h post-infection resulted in an approximately 150-fold stimulation of CAT activity using pIE1CAT and a 300-fold stimulation of CAT activity using pCAT1.8Hin (Table 1). These results suggest that EHV-2 produces a protein during its temporal life cycle which is capable of trans-activating IE1 of HSV-1 and the IE gene of EHV-1. Currently, CAT activity of pCATATAAT with and without superinfected EHV-2 is being investigated in order to identify whether TAATGARATTC is the activation site of EHV-2 trans-activating factor(s).

Discussion

The EHV-1 IE gene possesses control sequences that mediate stimulation of expression by VmW65 within the region -685 to +73. This region possesses a TAATGARATTC consensus motif which is the binding site for the VmW65-multiprotein complex (O'Hare et al., 1988), as well as a TATA box and CCCGCC motif. Located further upstream is the Ori5 which contains the 9 bp consensus sequence CGTTCGCAC at position -1612 that has been identified in both HSV-1 and VZV origins of replication.

Using transient transfections and subsequent CAT assays, the EHV-1 IE gene upstream control region was found to be as effective as the HSV-1 IE control sequences in the stimulation of CAT expression. These results were not unexpected since the sequence data had identified a conserved TAATGARATTC motif which is the binding site for this trans-activating complex. The presence of the conserved motifs as well as the strong stimulation of transcriptional activation indicated that it was likely that EHV-1, like HSV-1, possessed a functional equivalent of VmW65.

Trans-activation studies using the u.v.-inactivated Ab4 isolate of EHV-1 showed a stimulation of CAT activity mediated by the control regions of the EHV-1 IE and HSV-1 IE1 genes. Therefore it is likely that EHV-1 possesses a counterpart of HSV-1 VmW65. This is in contrast to VZV and pseudorabies virus (PRV), which are herpesviruses that possess upstream IE regulatory sequences that can be stimulated by VmW65 but do not
possess a functional equivalent of this protein (McKee et al., 1990; Campbell & Preston, 1987). However, to compensate for the lack of this protein, Kozmik et al. (1991) have recently postulated that the regulation of the highly repetitive IE gene promoter of PRV may be exclusively under the control of cellular transcription factors. They suggest that since the cellular factor NF-\(\mu\)E1 binds to the CCAAT box the function of VmW65 may be mediated by this protein. Further work to identify the gene encoding the VmW65 equivalent is currently under way (A. Davison, personal communication). When this information is known, comparisons with the equivalent genes of HSV-1, -2 and VZV can be performed.

More intriguingly, EHV-2 is also able to trans-activate the IE genes of EHV-1 and HSV-1. The above findings indicate that EHV-2 possesses a protein or proteins that can stimulate EHV-1 and HSV-1 IE transcription. Cell extracts prepared from samples 72 h post-infection with EHV-2 showed a stimulation of CAT expression, unlike cell extracts prepared from samples 1 h and 24 h post-infection. However it is not possible to predict the temporal nature of the protein since the precise time of EHV-2 infection is not known. The exact role of the ubiquitous EHV-2 has yet to be determined. However, since latent EHV-1 is always detected in the presence of EHV-2 (Welch et al., 1992), the above findings may be significant in vivo where EHV-2 has been shown to be readily reactivated and could trigger stimulation of IE transcription in the latent EHV-1. Further work is being performed to identify the nature and the activation site of the EHV-2 trans-activating protein(s). Cellular factors such as Oct-1 and Oct-2 may also play an important role in vivo. Therefore it will be important to identify the distribution of these and other binding proteins in various equine tissues in order to begin to investigate the criteria that determine the sites and regulation of latency.

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References


Control of the EHV-1 IE gene


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