Negative regulation of herpes simplex virus type 1 ICP4 promoter by IE180 protein of pseudorabies virus

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Recombinant pseudorabies viruses (PRVs) gIS8 and N1aHTK were constructed by the insertion of a chimeric gene (\(z_4-TK\)) from herpes simplex virus type 1 (HSV-1) into wild-type PRV. HSV-1 TK expression by these recombinant viruses resulted in enhanced sensitivity to ganciclovir, compared to that of the wild-type PRV, and was similar to the sensitivity shown by HSV-1. Infection with gIS8 or N1aHTK recombinant viruses led to expression of HSV-1 TK mRNA as an immediate–early (IE) gene, observed by downregulation of the HSV-1 \(z_4\) promoter. This negative regulation was due to a PRV IE protein, IE180. IE180, however, does not have all the regulatory functions of the infected-cell protein ICP4, as it does not restore the growth of ICP4-deficient HSV-1 mutants.

During lytic infection of alphaherpesviruses, viral genes are expressed in three temporally ordered tiers, designated immediate–early (IE) or \(a\) genes, early or \(b\) genes and late or \(c\) genes (Roizman & Knipe, 2001). The five HSV-1 \(a\) genes, \(a_0, a_4, a_22, a_27\) and \(a_47\), encode infected-cell proteins (ICPs) 0, 4, 22, 27 and 47, respectively. ICP4 is the major regulatory protein and affects viral gene expression both positively and negatively, including its own regulation (Leopardi et al., 1995). These HSV-1 \(a\) genes are transactivated by the structural protein VP16 (Weir, 2001).

Pseudorabies virus (PRV) has only one IE gene (IE180), which encodes a protein of 180 kDa (IE180; Ihara et al., 1983). IE180 has a high level of similarity to the IE proteins of other alphaherpesviruses, such as ICP4 [herpes simplex virus type 1 (HSV-1)], IE40 [varicella-zoster virus (VZV)], IE1 (equid herpesvirus 1) and p180 (bovine herpesvirus 1) (Vlcek et al., 1989). These proteins have been divided into five collinear regions based on their predicted amino acid sequences, with a high level of similarity in regions 2 and 4 and little similarity in regions 1, 3 and 5 (Cheung, 1989; Wu & Wilcox, 1991). The IE180 protein has a DNA-binding domain that binds specifically to the consensus sequence of the alphaherpesviruses’ transcription initiation site (Wu & Wilcox, 1991), implying that it has a role in the negative regulation of its own gene.

The purpose of this study was firstly to construct and characterize recombinant PRV by using the HSV-1 \(z_4-\)TK gene as a selection marker, and then to use the recombinants to study the role of the PRV IE180 protein in HSV-1 \(z_4\) promoter regulation and in the possible complementation of ICP4-deficient HSV-1.

Two PRV recombinants, gIS8 and N1aHTK, were constructed by insertion of the HSV-1 chimeric gene \(z_4-\)TK (Post et al., 1981; Poffenberger et al., 1983), which is included in the 3-6 kbp PvuII fragment. This fragment has the \(z_4\) gene promoter–regulatory region juxtaposed in appropriate transcriptional orientation to the HSV-1 \(TK\) gene; it is used widely for genetic manipulation of HSV-1 (Roizman & Jenkins, 1985) to supply TK activity to recombinant viruses (Post et al., 1981). The gIS8 virus was obtained by insertion of the \(z_4-\)TK gene into \(Nco-I-Nco\) sites in the PRV BamHI-7 fragment (Fig. 1a), which also includes \(gI, gE\) and US9 (Fernández et al., 1999). This insertion produced an increase in mobility in the PRV BamHI-7 fragment compared to that of the BamHI-6 fragment (Fig. 1b and c, lanes 3 and 4). The N1aHTK recombinant was made by insertion of the \(z_4-\)TK gene into the \(Bgl-II\) site at codon 264 of the PRV TK gene sequence, which is located in the BamHI-11 fragment (Fig. 1a). \(z_4-\)TK insertion produced a mobility increase in the PRV BamHI-11 fragment, compared to that of the BamHI-7 fragment (Fig. 1b and c, lanes 5 and 6).

Functional consequences of the incorporation of the \(z_4-\)TK chimeric gene into gIS8 and N1aHTK recombinant viruses were studied by HSV-1 TK expression analysis. BHK (TK−) cells were infected with wild-type viruses (PRV NIA 3 or HSV-1 F), the TK-deficient PRV mutant (ATK5) (Prieto et al., 1991) or the recombinant viruses (N1aHTK and gIS8). Cell extracts were then assayed for TK activity. No TK induction was seen with the TK mutant virus ATK5, but similarly high activity levels were observed for wild-type PRV and recombinants N1aHTK and gIS8 (Table 1).
Table 1. TK activity levels and inhibition of infection with PRV recombinants by GCV

TK activity level was assayed indirectly in BHK (TK−) cells by 3H-labelled thymidine incorporation into DNA for 24 h (Prieto et al., 1991). Drug sensitivity of PRV strains NIA 3, N1aHTK and gIS8 and HSV-1 strain F was tested by TCDI 50 reduction in Vero cells in the presence of 10 μg drug ml−1. Virus concentration was determined 3 days post-inoculation and expressed as TCDI 50 ml−1. The n-fold reduction was calculated by dividing virus titre without drug by virus titre in the presence of drug.

<table>
<thead>
<tr>
<th>Virus (strain)</th>
<th>TK activity (c.p.m.)</th>
<th>GCV (μg ml−1)</th>
<th>Virus concentration (TCDI50 ml−1)</th>
<th>n-fold virus titre reduction</th>
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<tbody>
<tr>
<td>PRV:</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NIA 3</td>
<td>53 067</td>
<td>0</td>
<td>4·20 × 10⁴</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0·84 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>N1aHTK</td>
<td>44 594</td>
<td>0</td>
<td>0·84 × 10⁴</td>
<td>13·3 × 10²</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0·63 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>gIS8</td>
<td>38 945</td>
<td>0</td>
<td>2·9 × 10⁴</td>
<td>4·6 × 10²</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>6·3 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>HSV-1 (F)</td>
<td>29 669</td>
<td>0</td>
<td>2·9 × 10⁴</td>
<td>4·6 × 10²</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>6·3 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>PRV (ATK5)</td>
<td>541</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
One consequence of HSV-1 TK expression in the PRV recombinant viruses was a distinct sensitivity to nucleoside analogues compared to that of the wild-type PRV, which expressed its own TK activity. Herpesvirus TK enzymes show individual differences in their ability to phosphorylate nucleoside analogues (Kit, 1985). The sensitivity of gIS8 and N1aHTK recombinant viruses to inhibition by late nucleoside analogues (Kit, 1985). The sensitivity of HSV-1 TK expression in the PRV recombinants could also be used as vaccine vectors in genetic manipulation studies, using GCV as a non-mutagenic selection factor (Gododeu et al., 1992). gIS8 is of particular interest as it is defective in glycoprotein E, which is used as a vaccine marker in the control of Aujeszky’s disease (van Oirschot et al., 1990). These PRV recombinants could also be used to generate oncolytic viruses in human gene therapy (Boldogkoi & Nogradi, 2003). The increased GCV sensitivity compared to wild-type would confer additional biosafety in the event of replication outside tumour regions, as occurs with HSV-1 (Stanziale & Fong, 2003).

Detection of TK activity in the recombinant viruses correlated with HSV-1 TK gene mRNA expression. By using Northern blot analysis, we studied expression of IE mRNA (α-RNA) and early mRNA (β-RNA) in recombinant viruses by using cycloheximide and phosphonoacetic acid (PAA) treatment, respectively, and late RNA in the absence of inhibitors (Fig. 1d). Cytoplasmic RNA (Camacho & Tabarés, 1996) was fractionated on a 1% formaldehyde agarose gel, blotted onto nitrocellulose and hybridized [according to Cistué & Tabarés (1992)] with 32P-labelled 0.3 kbp TK DNA corresponding to the ScaI–PstI fragment, nt 447–748 of the coding sequence (McKnight, 1980). HSV-1 TK mRNA was detected as a transcript of approximately 1.3 kbp (McKnight, 1980). TK behaved as an early gene in HSV-1, its natural infection context, as it was expressed in the presence of PAA (Fig. 1d, lane C) and in the absence of cycloheximide or PAA (Fig. 1d, lane D). In contrast, HSV-1 TK mRNA derived from the α4–TK chimeric gene was expressed as an IE transcript in recombinant PRVs N1aHTK and gIS8, showing overaccumulation in the presence of cycloheximide (Fig. 1d, lanes E and H) and was inhibited strongly in early or late infection phases (Fig. 1d, lanes F, G, I and J). The HSV-1 α4 promoter thus behaves as an IE promoter in the PRV recombinant genomic context, with strong inhibition of expression in the early and late phases, implying homologous regulation of the HSV-1 promoter by PRV proteins.

Autorepression of gene expression by ICP4 involves physical interaction between the protein and DNA (Michael & Roizman, 1993). As the ICP4 and IE180 proteins share extensive identity in the domains termed regions 2 and 4, autoregulation of HSV-1 ICP4 and PRV IE180 gene transcription may occur through a similar mechanism. Region 2 domains of both ICP4 and IE180 expressed in Escherichia coli bind to DNA sequences that overlap the transcription start site in their respective gene promoters (Wu & Wilcox, 1991). Our results suggest that the high binding affinity observed in vitro of PRV IE180 domain 2 to the DNA sequences that flank the HSV-1 ICP4 promoter transcription start site (Wu & Wilcox, 1991) may be functional in vivo and could be implicated in negative regulation of the HSV-1 ICP4 promoter of PRV recombinants.

As autorepression of ICP4 on its own promoter is affected by binding-site orientation and its distance from the TATA box (Leopardi et al., 1995), it was unclear whether the similarity of IE180 and ICP4 domain 2 regions and their target DNA sequences would be reflected by functional equivalence. To determine whether IE180, the unique PRV IE protein, is responsible for negative regulation of the HSV-1 α4 promoter, we performed transfection experiments in 293T cells with pRTKC, which encodes the chimeric α4–TK gene, either alone or together with a plasmid that encodes the PRV IE180 protein (pE180). As a control for positive regulation of the HSV-1 α4 promoter, we cotransfected pRTKC with pVP16, which encodes HSV-1 VP16 protein. The pE180 plasmid was constructed by insertion of the BamHI-8 fragment, which contains the PRV IE180 coding region, into pCDNA3 (Invitrogen). Plasmid pVP16 consists of an EcorV–FokI fragment that contains the HSV-1 VP16 coding region, inserted into the vector pCDNA 3.1HisA (Invitrogen). Plasmid pRTKC was obtained by cloning the Sall–KpnI fragment from pHSV-1-TK into the Sall–KpnI site in the vector pRE4 (Invitrogen) without the RSV promoter. pHSV-1-TK contains α4–TK in the pUC18 vector.

To ensure that all DNA, RNA and protein expression measurements could be compared directly, experiments were performed with the same transfection cultures of 293T–pRTKC cells, either alone or cotransfected with plasmid pEGFP, pE180 or pVP16. 293T cells (9 x 106) were plated in a 150 cm2 flask 18 h before transfection and treated with 25 μM chloroquine (Sigma) in 6 ml medium, 5 min before transfection (Abad et al., 2002). Plasmid construct (60 μg) in 3 ml 204 mM CaCl2 was mixed with 3 ml 2x HBS (274 mM NaCl, 50 mM HEPES, 1.5 mM Na2HPO4, pH 7). Medium was changed after 8 h. DNA, RNA and IE180 and VP16 proteins were analysed at 48 h post-transfection. Cellular DNA was isolated by SDS–protease treatment (Tabarés, 1987); RNA was isolated with the Total Isolation system (Promega). RNA synthesis from transfected cells was analysed by RT-PCR. PCR was carried out with primers HTK5A (5′-ACTCGGTTTATATAGACGG-3′) and HTK6 (5′-ATGAGGCCCCAGGACGCGCAG-3′) for the HSV-1 TK gene, IE180-S (5′-CTTCAGGCCAGTCTGCGGGG-3′) and IE180-AS (5′-GCGGGAGAGATCTCCTCGG-3′) for the IE180 gene, VP16-S (5′-CGCGCATGTTACCAGTGTGCGG-3′) and VP16-AS
Fig. 2. Regulation of HSV-1 α4 promoter and non-complementation of ICP4-deficient mutants by PRV IE180 protein. (a) PCR amplification of the HSV-1 TK gene [panel (i)] and hybridization [panel (ii)] with sequenced TK PCR product from 293T cell DNA, either untransfected (lane 1) or transfected with plasmid pEGFP–N1 (lane 2), plasmid pRTKC (lane 3), plasmids pRTKC plus pE180 (lane 4) or plasmids pRTKC plus pVP16 (lane 5). RT-PCR amplification of HSV-1 TK mRNA [panel (iii)] and hybridization [panel (iv)] with sequenced TK PCR product from 293T cell RNA, either untransfected (lane 1) or transfected with plasmid pRTKC (lane 3), plasmids pRTKC plus pE180 (lane 4) or plasmids pRTKC plus pVP16 (lane 5). Immunodetection of IE180 in 293T cells transfected with plasmids pRTKC plus pE180 [panel (v), lane 4] and of VP16 in 293T cells transfected with plasmids pRTKC plus pVP16 [panel (v), lane 5] by monospecific antibodies and Western blotting of cell lysates, analysed by 12 % SDS-PAGE (Tabarers et al., 1980) and electrotransferred onto a nitrocellulose membrane (Hybond; Amersham Biosciences); bands were visualized by using the ECL detection system (Amersham Biosciences). Monospecific IE180 rabbit antiserum [against an N-terminal fragment, aa 1–615 of the IE180 gene as expressed by pRSET B (Invitrogen) in E. coli (Domingo et al., 2003) as antigen reagent] and monospecific VP16 antiserum (Clontech) were used. (b) PCR amplification of GAPDH [panel (i)] and hybridization [panel (ii)] with sequenced GAPDH PCR product from 293T cell DNA, either untransfected (lane 1) or transfected with plasmid pRTKC (lane 3), plasmids pRTKC plus pE180 (lane 4) or plasmids pRTKC plus pVP16 (lane 5). RT-PCR amplification of GAPDH mRNA [panel (iii)] and hybridization [panel (iv)] with sequenced GAPDH PCR product from 293T cell RNA, either untransfected (lane 1) or transfected with plasmid pEGFP–N1 (lane 2), plasmid pRTKC (lane 3), plasmids pRTKC plus pE180 (lane 4) or plasmids pRTKC plus pVP16 (lane 5). In all PCR and RT-PCR experiments (a, b), a negative control without DNA or RNA (respectively) (lane 6) was included. (c) DNA from HSV-1 strain F [panel (i)], 293T–IE180 transfection cultures infected with G2 mutant [panel (ii)], 293T–pEGFP with G2 mutant [panel (iii)], 293T–IE180 with d120 mutant [panel (iv)], 293T–pEGFP with d120 mutant [panel (v)], 293T–IE180 with strain F [panel (vi)] or 293T–pEGFP with strain F [panel (vii)] were hybridized with 32P-labelled SacI–NaeI TK DNA. DNA from the pRGAPDH-1 plasmid (Piechaczyk et al., 1984) [panel (vii)], 293T–IE180 transfection cultures infected with G2 mutant [panel (vii)], 293T–pEGFP with G2 mutant [panel (vii)], 293T–IE180 with d120 mutant [panel (vii)], 293T–pEGFP with d120 mutant [panel (vii)], 293T–IE180 with strain F [panel (vii)] or 293T–pEGFP with strain F [panel (vii)] was hybridized with 32P-labelled PstI pRGAPDH-1 DNA. The viral DNA was detected by dot-blot hybridization (Tabarés, 1987) and the expression of IE180 in 293T was detected by Western blot analysis.
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REFERENCES


Domingo, C., Gadea, I., Pardeiro, M. & other authors (2003). Immunological properties of a DNA plasmid encoding a chimeric protein of herpes simplex virus type 2 glycoprotein B and glycoprotein D. Vaccine 21, 3565–3574.


Leopardi, R., Michael, N. & Roizman, B. (1995). Repression of the herpes simplex virus 1 α4 gene by its gene product (ICP4) within the context of the viral genome is conditioned by the distance and stereoaxial alignment of the ICP4 DNA binding site relative to the TATA box. J Virol 69, 3042–3048.


Michael, N. & Roizman, B. (1993). Repression of the herpes simplex virus 1 α4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. Proc Natl Acad Sci U S A 90, 2286–2290.


