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Protocol

Construction and properties of a recombinant pseudorabies virus with tetracycline-regulated control of immediate-early gene expression

A.L. Muñoz, I. Gadea[1], L. Lerma, L. Varela, M. Torres, B. Martín, A. García-Culebras, F. Lim[2], E. Tabarés*

Departamento de Medicina Preventiva, Salud Pública y Microbiología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, E-28029 Madrid, Spain

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A study was carried out to determine whether altering the control of expression of the IE180 gene of pseudorabies virus (PRV), by replacing the IE180 promoter with the tetracycline-responsive promoter (Ptet), affects virus replication and virulence. This PRV-BT90 mutant virus was constructed by complementation and recombination in Hela Tet-Off cells. The virus yield produced by infection of Hela Tet-Off cells with PRV-BT90 was similar to that of the parental virus vBecker2. Viral replication of PRV-BT90 was reduced in Vero cells as reflected by a reduction of virus yield and plating efficiency compared to vBecker2. PRV-BT90 plaque formation in Hela Tet-Off cells was inhibited in the presence of doxycycline, whereas vBecker2 plaque formation was not affected. Subcutaneous infection of mice with the two viruses revealed a LD_{50} higher than 10^6 $TCID_{50}$ for the PRV-BT90 mutant virus while the LD_{50} was 178 $TCID_{50}$ for the vBecker2 parental virus.

1. Introduction

The order Herpesvirales is composed of three families, Herpesviridae, Alloherpesviridae and Malacoherpesviridae (Davison, 2010). In the Herpesviridae family there are three subfamilies, the alpha-, beta-, and gammaherpesvirinae. The alphaherpesvirinae includes herpes simplex virus types 1 and 2 (HSV1 and HSV2), varicella-zoster virus (VZV), pseudorabies virus (PRV), equine herpesvirus (EHV1), and bovine herpesvirus 1 (BHV1) (Pellet and Roizman, 2007). PRV causes neuropathological disease (Aujeszky's disease) in swine (Nauwynck et al., 2007; Paul et al., 2003), its natural host, however PRV can infect many animal species and is able to replicate in nearly any cell type in vitro included human cells (Fernández et al., 1999; Sawitzky, 1997). The PRV genome is a linear dsDNA molecule of 143 kbp, organized into a unique long (U_L) segment and an invertible unique short (U_S) segment bracketed by internal (IR) and terminal (TR) inverted repeat sequences (Klupp et al., 2004). The viral genome has been cloned in *Escherichia coli* as bacterial artificial chromosomes (Smith and Enquist, 1999, 2000).

Productive infection of cells by members of the *alphaherpesvirinae* subfamily is typified by a short reproductive cycle that can be subdivided into three temporally regulated phases, immediate-early (IE or α), early (E or β), and late (L or γ). Early gene expression, which is necessary for viral DNA replication, is dependent on the expression of the IE proteins. The L genes code mainly for components of the virion and require the replication of the viral DNA for their expression (Roizman et al., 2007; Weir, 2001). The PRV genome circularizes in the nucleus soon after infection as a result of the joint of the two ends. Replication then proceeds by a rolling-circle mechanism, the product DNA being cleaved and packaged into virions (Ben-Porat and Veach, 1980), with the *pac*1 and *pac*2 sequences provide the *cis* signal for correct cleavage and packaging (Rall et al., 1992).

Among the *alphaherpesvirinae* the number of α genes varies depending on the viral species; for example, HSV-1 has six α genes while PRV has only one (Mettenleiter, 2000; Roizman et al., 2007). This single PRV α gene (IE180) encodes the IE180 protein (180 kDa) (Ihara et al., 1983), which shows striking amino acid homology to other alphaherpesvirus IE proteins such as ICP4 of HSV1, IE40 of VZV, IE1 of EHV1, and p180 of BHV1 (Vlcek et al., 1989). IE180 is required in *trans* for the continuous transcription of later classes of viral genes and to shut off the synthesis of its own RNA (Ihara et al., 1983). PRV has two copies of its IE180 gene, one in each of the two inverted repeat regions (IE180-IR and IE180-TR) of the viral genome (Ihara et al., 1983).

The present study describes the construction and characterization of PRV BACs with deletions in the IE180 and pac sequences and the generation of PRV-BT90, an inducible recombinant virus expressing IE180 under control of the tetracycline-responsive promoter (Ptet). These PRV-BACs will be useful to develop packaging systems for helper virus-free amplicon vectors and as a model to

* Corresponding author. Tel.: +34 91 497 5440; fax: +34 91 497 5353.
E-mail address: enrique.tabares@uam.es (E. Tabarés).
[1] Servicio de Microbiología Médica, Fundación Jiménez Díaz, Madrid 28035, Spain.
[2] Centro de Biología Molecular Severo Ochoa, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain.

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study the function of individual PRV genes by genetic recombination. In addition, it was determined whether replacing the promoter of the IE180 gene with Ptet promoter influences virus replication and virulence.

2. Materials and methods

2.1. Viruses and cells

Vero cells (African green monkey kidney cells) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 5% foetal calf serum. HeLa Tet-Off cells (Clontech) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% foetal calf serum. The presence of the tetracycline-controlled transactivator (tTA) was demonstrated by induction of a tetO-PRV UL44 chimeric promoter (Muñoz et al., 2010). vBecker2 was generated by transfection of the pBecker2 plasmid (Smith and Enquist, 2000) into Hela Tet-Off cells. This virus showed high genetic stability in PK15 cells (Smith and Enquist, 2000).

2.2. Plasmid constructs

The pCLR-K plasmid was obtained by inserting the blunt-ended-EcoRI resistance fragment of pUC4k (Clontech) which encodes the kanamycin (Km) resistance gene, into the pCLS plasmid (17), which includes the PRV *BamH*I 14′ and 13 fragments after digestion with NaeI blunt-ending and religation. The removed NaeI fragment (1973 bp) includes the *pac*1–*pac*2 region of PRV (Rall et al., 1992).

The pOZPAC plasmid contains the Zeocin (Z) resistance gene of pGeasyZeo (see below), flanked by viral homologous sequences for recombination in PBAC70. The left-hand flanking homologous sequence consisted of nt 129,187–136,142 from the PRV genome (Klupp et al., 2004) which are included in the PRV *BamH*I 5 fragment, while the right-hand flanking homologous sequence consisted of covalently linked *Bam*HI terminal fragments 13 and 14′ (the 3.2 kbp ends of the genome containing the TR and UL junction) (DeMarchi et al., 1990). The gene conferring resistance to Zeocin was amplified from plasmid pDRIVE-CAG (InvivoGen) by PCR using oligos RZeo-S (5′-TACGCGTCCTGGCCTTTTGC-3′) and RZeo-AS (5′-ACGCGTGGCGTTACTATGG-3′) and cloned into pGEMTeasy (Promega), giving rise to the plasmid pGeasyZeo.

The pOKC plasmid contains the kanamycin (Km) resistance gene of pUC4K, flanked by viral homologous sequences for recombination into the BAC pBecker2. The left-hand flanking homologous sequence consisted of nt 129,187–131,139 from the PRV genome (Klupp et al., 2004), while the right-hand flanking homologous sequence right consisted of nt 1077–1337 from the PRV genome (Klupp et al., 2004). These regions are included in the PRV *BamH*I 5 and *BamH*I 14′ fragments, respectively (Fuchs et al., 2000; Rall et al., 1992).

The p8TcO plasmid contains the tetracycline (Tc) resistance of pBR322 flanked by viral homologous sequences for recombination in BAC 60. The left-hand flanking homologous sequence consisted of nt 97,918–100,814 from the PRV genome (Klupp et al., 2004), which are part of the PRV *BamH*I 8′ fragment and the right-hand flanking homologous sequence consisted of nt 129,187–130,947 from the PRV genome (Klupp et al., 2004), which includes ori_S (Fuchs et al., 2000). The p8T180-O plasmid was obtained by inserting the MluI-PvuII fragment of pAZT180 between the MluI and EcoRV sites of p8TcO. pAZT180 was obtained by inserting the HindIII fragment of pTRE180 in pAZDH, which includes a SV40 poly A signal with the addition of 229 bp of pBR332 including the *Eco*RI site in the pUC18 plasmid vector.

2.3. Recombinant PRV viruses

The PRV-BT90 recombinant virus was made by cotransfecting the *Pvu*II-*Ase*I fragment of p8T180-O (2 μg) together with PBAC90 DNA (2 μg) into Hela Tet-Off cells using lipofectamine 2000 (Invitrogen). The development of viral cytopathic effects was examined using a Nikon Eclipse TS100 microscope; positive foci of infection were detected 48 h after transfection.

2.4. In vitro viral growth in different cell types

In vitro growth of the vBecker2 and PRV-BT90 strains was determined in Vero cells and Hela Tet-Off cells. Cell monolayers (0.5 or 1×10^6 cells, respectively) were infected with the viral strains at a multiplicity of infection of 0.1. After an absorption period of 2 h at 37 °C, the cells were washed with PBS and incubated in 2.5 ml of medium for 72 h. The virus yield was determined in a total sonicated lysate of infected cells combined with culture supernatant. vBecker2 and PRV-BT90 were titrated on Hela Tet-Off cells. The assay was carried out on ninety-six-well microtiter plates seeded with 2×10^4 cells/well in 100 μl of growth medium. Fifty microliters of serial tenfold virus dilutions were added to the cells. Four wells per dilution were tested, and incubated at 37 °C for 3 days. The virus concentration was expressed in $TCDI_{50}$/ml. In addition, twelve-well (P12) microtiter plates seeded with 2×10^5 cells/well (Hela Tet-Off or Vero cells) were infected at 24 h with the same viral concentration of both viruses in three or four tenfold virus dilutions respectively and overlaid with 0.8% agarose medium. After incubation for 3 days at 37 °C, the infected cells were fixed with 4% formaldehyde and stained with cellular dye (0.2% crystal violet).

2.5. Homologous recombination in E. coli

GET recombination was carried out in *E. coli* DH10B by using the pGETrec plasmid (Orford et al., 2000). Recombination of pBecker2 with the pCLR-K *EcoR*I-*Sph*I fragment (2420 bp) resulted in PBAC51. Recombination of PBAC51 with the *Pvu*II fragment (3813 bp) of pOKC resulted in PBAC60. Recombination of PBAC60 with the *EcoR*I-*Xba*I fragment (6383 bp) of p8TcO resulted in PBAC70 and recombination of PBAC70 with the *Pvu*II fragment (11,120 bp) of pOZPAC resulted in PBAC90. The recombinant BACs were select in plates containing Tc, Km or Zeocin, analysed by restriction enzymes and characterized by Southern blot.

2.6. Virulence assay

Female Balb/c mice (4–6 weeks old) were inoculated subcutaneously (s.c.) with 0.1 ml of 10-fold dilutions of vBecker2 or PRV-BT90 viruses. Five mice were inoculated for each dilution, and the number of survivors after 15 days was recorded. The mean 50% lethal dose values were calculated. Mice were maintained and used in accordance with the Institutional Animal Care and Use Committee of Fundación Jímenez Díaz approved protocols (Domingo et al., 2003).

2.7. Isolation of viral and cellular DNA

Viral DNA was isolated and purified by NaI density gradient centrifugation of infected Vero cell extracts (Walboomers and Ter Schegget, 1976). Total DNA from infected cells was isolated by SDS-proteinase treatment (QF Amp DNA Micro, Qiagen).

2.8. Southern blot assays

Viral DNA was digested with restriction enzymes, electrophoresed on 0.75% agarose gels, and then transferred by blotting