Published in final edited form as:

J Virol Methods. 2008 December ; 154(1-2): 182–193. doi:10.1016/j.jviromet.2008.07.033.

Development of recombinant varicella-zoster viruses expressing luciferase fusion proteins for live in vivo imaging in human skin and dorsal root ganglia xenografts

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Abstract

Varicella-zoster virus (VZV) is a host specific human pathogen that has been studied using human xenografts in SCID mice. Live whole-animal imaging is an emerging technique to measure protein expression in vivo using luminescence. Currently, it has only been possible to determine VZV protein expression in xenografts postmortem. Therefore, to measure immediate early (IE63) and late (glycoprotein E [gE]) protein expression in vivo viruses expressing IE63 or gE as luciferase fusion proteins were generated. Viable recombinant viruses pOka-63-luciferase and pOka-63/70-luciferase, which had luciferase genes fused to ORF63 and its duplicate ORF70, or pOka-gE-CBR were recovered that expressed IE63 or gE as fusion proteins and generated luminescent plaques. In contrast to pOka-63/70-luciferase viruses, the luciferase gene was rapidly lost in vitro when fused to a single copy of ORF63 or ORF68. IE63 expression was successfully measured in human skin and dorsal root ganglia xenografts infected with the genomically stable pOka-63/70-luciferase viruses. The progress of VZV infection in dorsal root ganglia xenografts was delayed in valacyclovir treated mice but followed a similar trend in untreated mice when the antiviral was with-drawn 28 days post-inoculation. Thus, IE63-luciferase fusion proteins were effective for investigating VZV infection and antiviral activity in human xenografts.

Keywords

Varicella-zoster virus; Herpesvirus; Glycoprotein; Immediate early protein; SCIDhu; Valacyclovir

1. Introduction

Varicella-zoster virus (VZV), an alpha-herpesvirus, causes chicken pox (varicella) as a primary infection and shingles (zoster) upon reactivation from infected ganglia in humans (reviewed by Cohen et al., 2007). VZV has limited pathogenic potential in animals (reviewed by Myers and Connelly, 1992). This extreme host range restriction led to the development of human xenograft models in SCID mice to study VZV pathogenesis (Moffat et al., 1995; Zerboni et al., 2005). Studies of VZV are further complicated by the highly cell associated nature of VZV replication.

VZV has a 125 kbp double stranded DNA genome that encodes for at least 70 proteins from the 73 open reading frames (ORFs) that are currently recognized (reviewed by Cohen et al.,

2007). The linear genome of VZV has unique long (UL) and unique short (US) regions that are flanked by repeats. The internal and terminal repeats of the US region, IRS and TRS, contain the duplicate ORFs 62/71, 63/70 and 64/69 encoding the immediate early (IE) 62, IE63 and ORF64 proteins. The unique short region contains ORFs for a putative tegument phosphoprotein (ORF65), a serine/threonine kinase (ORF66) and the two glycoproteins I (gI; ORF67) and E (gE; ORF68). The remaining ORFs are encoded by the UL region of the VZV genome.

The immediate early proteins are the first to be transcribed in infected cells as they are factors important for the regulation of viral protein synthesis (reviewed by Cohen et al., 2007). IE63 is 278 amino acids in length and expressed as a modified protein of 45 kDa by SDS-PAGE (Debrus et al., 1995). An internal transcript of U_S1 (ICP22), U_S1.5, is the orthologue in herpes simplex virus 1 (HSV-1), the most closely related human pathogen to VZV in the alpha-herpesviruses (Baiker et al., 2004; Davison and McGeoch, 1986; Davison and Scott, 1986). IE63 was determined to be an essential protein by showing that the complete deletion of ORFs 63 and 70 from the VZV genome prevented recovery of recombinant virus in cell culture (Sommer et al., 2001). The partial deletion (90%; equivalent to amino acid residues 24–268) of ORFs 63 and 70 resulted in the recovery of virus but with an impaired growth phenotype and the lack of viral DNA persistence in sensory ganglia in a rat model (Cohen et al., 2004). The amino-terminal region (amino acids 1–142) of IE63 binds IE62 with residues ⁵⁹RL⁶⁰ critical for IE62 binding (Baiker et al., 2004; Lynch et al., 2002). Nuclear localization has been associated with residues in the carboxyl terminus (Stevenson et al., 1996). In addition, IE63 has been reported to displace basal cellular transcription factors by its interaction with TFIIF, TFIIE and RNA POL II and to have effects on gene transcription (Di Valentin et al., 2005; Habran et al., 2007). Thus, IE63 is a key regulatory protein of VZV replication. Therefore, the detection of IE63 expression as a luciferase fusion protein would provide a marker for early VZV replication.

The 623 amino acid transmembrane protein gE, similarly to IE63, is abundantly expressed and necessary for VZV infection (Mallory et al., 1997; Mo et al., 2002). This late protein becomes incorporated into the virion envelope and is a required component of cell-to-cell spread during infection (reviewed by Grose, 1990). The majority of ORF68, encoding gE, is within the unique short region of the VZV genome but the 3'-end of ORF68 traverses the TRS by 113 nucleotides and is thus duplicated in the IRS. VZV gE has a unique N-terminus with multiple functions in envelopment, cell-to-cell spread and egress (Berarducci et al., 2006). The cytoplasmic region of gE was demonstrated to have a localization signal (AYRV) for the *trans*-golgi network and a region for phosphorylation (SSTT) that regulates trafficking to the plasma membrane that caused altered VZV phenotypes when mutated as well as an endocytosis motif (YAGL) that was essential for virus recovery (Moffat et al., 2004; Olson and Grose, 1997). Therefore, mutations within the gE cytoplasmic tail influence its function and consequently the cell-to-cell spread of VZV. However, if these functions are not disrupted, the *in situ* detection of gE expression as a luciferase fusion protein would provide a marker for the later stages of VZV replication.

HSV spread and replication in mice has been successfully studied using live *in vivo* whole-animal imaging (Luker and Leib, 2005; Luker et al., 2003). These HSV viruses were constructed with a cassette that had luciferase genes driven from the UL29 and UL30 promoters and the OriL region (Luker et al., 2002). Recently, recombinant VZVs expressing luciferase genes have been constructed to study the ORF62/63 dual promoter and *in vivo* replication (Jones et al., 2006; Zhang et al., 2007). However, these viruses had the luciferase genes under the regulation of promoters at ectopic sites within the VZV genome. In the present study an alternative approach was used. In order to monitor the expression of proteins from their native sites in the VZV genome and as regulated by their native promoters, click beetle luciferase

genes were inserted in-frame with ORFs 63, 68 and 70 to generate IE63 or gE luciferase fusion proteins. Thus, the synthesis of IE63 and gE proteins could be assessed by the production of luminescence and enable the progress of infection to be studied in vitro and in vivo.

The present study had three objectives. Firstly, to establish whether the click beetle luciferase genes would be expressed, retain luciferase activity and remain within the VZV genome when placed in-frame with ORF63, both ORF63 and ORF70, or ORF68. Secondly, to use the IE63-luciferase recombinant viruses to study replication in vitro and in vivo in the human xenograft models for skin and dorsal root ganglia infection in SCIDhu mice. Thirdly, as an initial evaluation of the system for studying antiviral drugs, the effect of valacyclovir on VZV replication in dorsal root ganglia xenografts in vivo was assessed.

2. Materials and methods

2.1. Cell lines

Melanoma cells were propagated in culture medium (MEM supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), non-essential amino acids (100 μ M; Omega Scientific, Inc., Tarzana, CA), penicillin-G (100 units/ml; Omega Scientific, Inc., Tarzana, CA), streptomycin (100 units/ml; Omega Scientific, Inc., Tarzana, CA), amphotericin (0.5 mg/ml; Omega Scientific, Inc., Tarzana, CA)). Human embryonic lung fibroblasts (HELFs) were propagated in culture medium without non-essential amino acids.

2.2. Generation of IE63 and gE plus IE63- and gE-luciferase fusion expression vectors

All restriction endonucleases were obtained from New Bio-Labs, Inc., Ipswich, MA unless otherwise stated. Oligonucleotides were synthesized by Operon Biotechnologies, Inc., Huntsville, AL. ORF63 was amplified from the pvSpe23 cosmid and the click beetle luciferase genes from the Chroma-LucTM reporter vectors (Promega Biosciences, Inc., San Luis Obispo, CA) by PCR using AccuPrime (Invitrogen, Carlsbad, CA). Two click beetle luciferase genes were used throughout the study; one (CBG68) that emits luminescence at a green wavelength ($\lambda_{\max}$ 560 nm) and one (CBR) at a red wave-length ($\lambda_{\max}$ 610 nm). The 857 bp PCR product generated from pvSpe23 using oligonucleotides NcoI-5IE63 (5'-ttaccatgggcacatgttttgcacctcaccg-3')/3IE63-ApaLI (5'-taatctagagtgcacgcacatgagggggcggtat-3') was gel purified (QIAGEN, Inc., Valencia, CA) then digested with NcoI/ApaLI. The 1658 bp PCR product generated from the Chroma-LucTM reporter vectors using oligonucleotides ApaLI-5CBRLuc (5'-ttaccatggtgcacgtaaagcgtgagaaaaatgc-3')/3CBRLuc-PsiI 02 (5'-taatctagattataaagactaacccgcgccttcacc-3') or ApaLI-5CBG68Luc (5'-ttaccatggtgcacgtgaaacgcgaagaacg-3')/3CBG68Luc-PsiI 02 (5'-taatctagattataaagactagccgcagcttttcgagg-3') was gel purified then digested with ApaLI/XbaI. The digested 857 bp and 1658 bp PCR products were gel purified then ligated using T4 DNA ligase (Invitrogen, Carlsbad, CA) into the linearised control vector (Chroma-LucTM reporter vector) to generate IE63-CBR or IE63-CBG68. The NcoI site in ORF63 was abolished by a guanine to adenosine substitution at nucleotide 111,500 of the pOka genome (17,455 of the pvSpe23 cosmid).

A similar procedure was performed to construct the gE-CBR luciferase fusion construct. To abolish an NcoI site in the ORF68 gene, PCR was used to amplify ORF68 with oligonucleotides NcoI 5gE (5'-ttaccatggggacacgttaataaacc-3')/gE AseI (5'-aatattaatcgttccccgctatcgataccacggcctgattatacaccatgatgttcgtgtgc-3') and AseI_gE (5'-ttaattaatgcaacccacacaaatgtctctgc-3')/3gE_TRRVH_XbaI (5'-aattctagactagtgacacccgtctagtcgggtcttatctatatacacc-3') to generate PCR products of 329 bp and 1531 bp. The 329 bp PCR product was digested with NcoI/AseI and the 1531 bp PCR product was digested with AseI/XbaI then ligated into the control vector (Chroma-LucTM reporter

vector) to generate the control_gE construct. To generate a CBR luciferase gene with VZV specific sequence at the 3'-end of the luciferase gene was amplified by PCR using oligonucleotides CBR ApaI (5'-tttggcccggaaccaagtgg-3')/CBR VZVSwal XbaI (5'-aattctagatttaaatttacgctcgacgttgccccgggtcggtgatcaaccgcggccttcaccaac-3') to generate a 540 bp product. The control_CBR construct and the gel purified 540 bp PCR product were digested with ApaI/XbaI, gel purified then ligated to generate the CBR_VZVSwal construct. To generate the gE-CBR construct, control CBR, CBR_VZVSwal and control_gE were digested with AatII/ApaLI, AatII/NcoI and ApaLI/NcoI, respectively, to generate 229, 5041 and 1880 bp fragments that were ligated. All of the control constructs were sequenced using overlapping primers to ensure that the VZV ORFs and the luciferase genes were in-frame and did not have any unexpected nucleotide substitutions.

2.3. Generation of cosmids

The luciferase genes were inserted into the pvSpe23 cosmid in order to generate fusion proteins with luciferase following the last encoded amino acid of IE63 or gE (Fig. 1). To construct the pvSpe23-63-luciferase cosmids an EcoRV/XbaI fragment of pvSpe23 (107,876-112,815 bp of the pOka genome) was ligated into the pLITMUS28 vector to generate pLITMUS_Spe23-EcoRV-XbaI. The IE63-CBG68 and pLITMUS_Spe23-EcoRV-XbaI vectors were digested with NarI, PstI and XbaI. The 6041 bp XbaI/NarI and 1298 bp PstI/XbaI fragments from the pLITMUS_Spe23-EcoRV-XbaI plus the 2042 bp NarI/PstI fragment from IE63-CBG68 were ligated to generate the pLITMUS_Spe23-EcoRV-63-CBG68-XbaI vector. The two vectors pLITMUS_Spe23-EcoRV-IE63-CBG68-XbaI and pLITMUS_Spe23-EcoRI-AvrII were digested with EcoRV/XbaI and the 10,456 bp fragment from pLITMUS_Spe23-EcoRI-AvrII and the 6623 bp fragment from pLITMUS_Spe23-EcoRV-IE63-CBG68-XbaI were ligated to generate the pLITMUS_Spe23-EcoRI-IE63-CBG68-AvrII vector. The final step to generate the pvSpe23-63-CBG68 cosmid was to use the 14,014 bp BmtI/AvrII fragment from pLITMUS_Spe23-EcoRI-63-CBG68-AvrII and ligate it into the pvSpe23 cosmid. The same procedure was performed to generate pvSpe23-63-CBR except that the IE63-CBR construct was used. This inserted the luciferase gene between nucleotides 111,508 and 111,509 (ORF63) of the pOka genome.

To construct the pvSpe23-gE-CBR cosmid the EcoRI/AvrII (112,947-117,127 bp of the pOka genome) fragment from pvSpe23 was ligated into the control_gE-CBR construct to generate the gE-CBR-AvrII vector. In a separate ligation, the 14,407 bp Swal fragment of the pvSpe23 cosmid, which contains the SuperCos-1 vector, was inserted into the gE-CBR-AvrII vector to construct the Swal-gE-CBR-AvrII vector. The SpeI/AvrII 18,901 bp fragment from pvSpe23 and 20,685 bp fragment from Swal-gE-CBR-AvrII were ligated to generate the pvSpe23-gE-CBR cosmid. This inserted the luciferase gene between nucleotides 117,769 and 117,770 (ORF68) of the pOka genome.

To construct the pvSpe23-63/70-CBG68 cosmid, pLITMUS_Spe23_AvrII-AscI, a vector containing the 12,170 bp AvrII/AscI fragment from pvSpe23, was digested with either AvrII/BbvCI or AvrII/SwaI to generate fragments of 10,028 bp and 1355 bp. The pvSpe23-63-CBG68 cosmid was sequentially digested with BbvCI/EcoRI then with Swal to generate the 6797 bp BbvCI/SwaI fragment. The 1355, 6797 and 10,028 bp fragments were ligated together to generate the pLITMUS_Spe23_AvrII-70-CBG68-AscI vector. The pvSpe23-63/70-CBG68 cosmid was generated from the ligation of the 6870 bp AscI/AscI fragment and 20,534 bp AscI/AvrII fragments from pvSpe23-63-CBG68 and the 13,796 bp AvrII/AscI fragment from the pLITMUS_Spe23_AvrII-70-CBG68-AscI vector. The same procedure was used to generate the pvSpe23-63/70-CBR cosmid. This inserted the luciferase gene between nucleotides 111,508 and 111,509 (ORF63) and nucleotides 118,574 and 118,575 (ORF70) of the pOka genome.

All cosmids were sequenced directly to determine that the luciferase genes were in-frame and did not contain any unexpected nucleotide substitutions.

2.4. Generation of recombinant pOka, pOka-63-CBR, pOka-63/70-CBR, pOka-63-CBG68, pOka-63/70-CBG68 and pOka-gE-CBR viruses

Transfection of melanoma cells with the cosmids to generate recombinant pOka derived viruses was performed as described previously (Sato et al., 2003b). Briefly, the three cosmids Fsp13, Spe14, Pme2 (3 µg each) plus 1.5 µg of either the pvSpe23, pvSpe23-63-CBR, pvSpe23-63/70-CBR, pvSpe23-63-CBG68, pvSpe23-63/70-CBG68 or pvSpe23-gE-CBR cosmids were used to transfect melanoma cells using CaCl₂ followed by glycerol shock. Melanoma cells were passed near confluence until plaques were visible.

2.5. PCR of ORFs 63 (IE63) and 68 (gE) to determine the presence of the luciferase genes

DNA was extracted from cell lines using DNAzol (Invitrogen, Carlsbad, CA) or from tissue using proteinase K and phenol/ chloroform (Invitrogen, Carlsbad, CA). PCR was performed using VZV ORF specific oligonucleotides (ORF63-NcoI5IE63/ IE63001R (5'-gtccgatattccgcgtcg-3'); ORF63-CBG68-IE63001F (5'-gatgatggtggtgaagacg-3')/ CBG68luc003R (5'-tatgtttacgcagtcg-3'); ORF63-CBR-IE63001F/CBRluc003R (5'-gctggagagcagcaaa-cagc-3'); ORF68-NcoI5gE/gEseq002 (5'-tccaagtctcggtgtacc-3'); ORF68-CBR-gEseq003 (5'-catgcagataactacacc-3')/CBRluc003R) and recombinant Taq DNA polymerase (Invitrogen).

2.6. Western blot of luciferase fusion viruses infected melanoma cells

Cell lysates in radio immunoprecipitation buffer (RIPA: 50mM Tris [pH 8.0], 150mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS) were resolved using pre-cast SDS polyacrylamide gels (Bio-Rad). Proteins were transferred to Immobilon-P membranes (Millipore Biosciences, Temecula, CA) and blocked with 5% dried skimmed milk (rabbit antibodies) or 5% BSA (monoclonal antibodies). The primary antibodies used to detect VZV specific proteins were; IE4—rabbit polyclonal, IE62—rabbit polyclonal and IE63—IgG fraction of a rabbit polyclonal that were gifts from William Ruyechan, University of Buffalo, gE—either monoclonal antibody (mAb) 3B3 (a gift from Charles Grose, University of Iowa) or mAb MAB8612 (Millipore Biosciences, Temecula, CA). A monoclonal antibody to α -tubulin (Sigma—Aldrich Corp., St. Louis, MO) was used to ensure that equivalent protein levels were present in the lysates. Horse radish peroxidase conjugated antibodies to mouse or rabbit IgG (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were used and HRP activity detected using ECL plus (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.7. Confocal microscopy of luciferase fusion viruses in infected melanoma cells

Confocal microscopy of pOka, pOka-63-CBR, pOka-63/70-CBR, pOka-63-CBG68, pOka-63/70-CBG8 or pOka-gE-CBR infected melanoma cells was performed as described previously (Berarducci et al., 2006) using the primary antibodies to VZV proteins IE62(mAb H6), IE63 (rabbit polyclonal) and gE (mAb 3B3 or mAb MAB8612 (Millipore Biosciences, Temecula, CA)).

2.8. Replication and plaque size of luciferase fusion viruses in melanoma cells

Melanoma cells seeded at 10⁶ cells per well in 6-well titer plates were inoculated with Log¹⁰ 3.0 pfu of pOka or pOka-63-CBR, pOka-63/70-CBR, pOka-63-CBG68, pOka-63/70-CBG8 or pOka-gE-CBR viruses then titrated every 24 h. Titer plates were fixed with 4% paraformaldehyde then stained by immunohistochemistry. An IgG fraction of a high titer VZV-immune human serum was used followed by detection with streptavidin conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and alkaline

phosphatase conjugated avidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Enzyme activity was detected using a fast red substrate (0.1 M Tris [pH 8.0], 5 μ M Naphthol AS-Mx phosphate (Sigma), 80 μ M Fast Red TR (Sigma)). Images of the plaques were captured then the outline of each stained plaque was traced and the area (mm^2) was calculated using ImageJ (Abramoff et al., 2004).

2.9. Southern blot of VZV DNA

Southern blot was performed as described previously (Sato et al., 2003a). Either, 1 μ g of control DNA, cosmids pvSpe23, pvSpe23-63-CBR, pvSpe23-63-CBG68, pvSpe23-63/70-CBR or pvSpe23-63/70-CBG68, or 5 μ g genomic DNA was digested with 10IU EcoRI (Promega Biosciences, Inc., San Luis Obispo, CA). Probes to ORFs 63/70, CBR and CBG68 were generated by PCR using Accuprime Pfx (Invitrogen, Carlsbad, CA) with oligonucleotides NcoI5IE63/IE63001R (ORF63/70 probe), ApaLI-5CBR/CBRluc001R (CBR probe) and ApaLI-5CBG68/CBG68luc001R (CBG68 probe) and labeled using the gene images AlkPhos direct labeling and detection system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.10. Replication of luciferase fusion viruses in human tissue xenografts

Skin inoculation: SCID mice (CB-17^{scid/scid}) were implanted bilaterally with fetal human skin at least 5 weeks before inoculation of viruses as described previously (Moffat et al., 1995). At 10 and 21 days post-inoculation the implants were removed and homogenized for virus titration plus DNA and protein extraction. Dorsal root ganglia inoculation: SCID mice were implanted with fetal dorsal root ganglia under the left kidney capsule at least 4 weeks before inoculation of viruses as described previously (Zerboni et al., 2005). Virus-infected HELFs were used to inoculate xenografts in the SCID mice. Inocula were titrated on melanoma cells as described previously to determine virus titer. To assess the effect of valacyclovir on VZV replication in dorsal root ganglia valacyclovir was added to the drinking water (1 mg/ml) and taken ad libitum. Due to the anesthesia, mice did not start drinking the treated water for at least 2 h post-surgery.

2.11. In vivo imaging of human skin and dorsal root ganglia xenografts

The expression of IE63 was determined by measuring luminescence in vivo at 3-day intervals. Mice were administered 3mg p-luciferin (Caliper Life Sciences, Inc., Alameda, CA) intraperitoneally and imaged after 5 min using an IVIS Lumina imaging system. Luminescence values were calculated using the Living Image software (version 2.50.1; Caliper Life Sciences, Inc., Alameda, CA).

2.12. qRT-PCR of the late protein gB

To quantify transcripts for the late protein gB (ORF31) qRT-PCR was performed as described previously (Zerboni et al., 2005).

3. Results

3.1. Generation of pOka recombinants with IE63 and gE expressed as fusion proteins with click beetle luciferases

The click beetle luciferase genes, CBR and CBG68, were inserted into the pOka pvSpe23 cosmid in-frame with ORF68, encoding gE, or in-frame with ORF63 alone or both of the duplicate ORFs 63 and 70 that encode IE63 generating the five cosmids pvSpe23-63-CBR, pvSpe23-63-CBG68, pvSpe23-gE-CBR, pvSpe23-63/70-CBR and pvSpe23-63/70-CBG68. All cosmids yielded the expected DNA fragments upon digestion with either restriction endonuclease EcoRI, MscI or NcoI (data not shown).

Recombinant pOka viruses with IE63 and gE expressed as click beetle luciferase fusion proteins were recovered upon transfection of melanoma cells with the pvSpe23 luciferase cosmid along with the intact pvFsp13, pvSpe14 and pvPme2 cosmid. The expected PCR fragments were generated from DNA extracted from melanoma cells that were infected with pOka and the luciferase fusion viruses, which were designated pOka-63-CBG68, pOka-63-CBR, pOka-63/70-CBG68, pOka-63/70-CBR and pOka-gE-CBR. These PCR products were the same sizes as those amplified from the cosmid, pvSpe23-ORF63-CBG68, pvSpe23-ORF63-CBR, pvSpe23-ORF63/70-CBG68, pvSpe23-ORF63/70-CBR and pvSpe23-ORF68-CBR (data not shown).

3.2. Characteristics of the pOka-gE-luciferase and pOka-63-luciferase fusion viruses in vitro

Melanoma cells infected with pOka-gE-CBR produced a 140–160 kDa protein that reacted with the gE-specific monoclonal antibody, 3B3, which was in the range for the expected molecular mass of the gE-luciferase fusion protein (Fig. 2, lane B). For comparison, gE expressed in pOka-infected cells was 85–100 kDa, as expected for native gE (Fig. 2, lane A). As expected, IE63 was expressed as a 45 kDa protein in cells infected with the gE-luciferase recombinant and pOka. The replication kinetics of pOka-gE-CBR were equivalent to pOka in melanoma cells, with an approximately 100-fold increase in titer by day 3 that was maintained until day 6 post-inoculation (Fig. 3A). Luminescence was emitted by pOka-gE-CBR in melanoma cells and was associated only with virus plaques as determined by live imaging and immunohistochemistry (data not shown). The intracellular localization of gE as well as IE63 was similar for the gE-luciferase fusion virus and pOka by confocal microscopy (Fig. 4).

When only ORF63 was fused to the CBR luciferase gene, leaving the duplicate VZV gene, ORF70, in its native form, the pOka-63-CBR recombinant produced two proteins in melanoma cells that reacted with rabbit polyclonal anti-IE63 antibody; one had the expected molecular mass of 45 kDa for the native IE63 expressed from ORF70, and the second was 95 kDa, as predicted for the IE63-luciferase fusion protein (Fig. 2, lane C). Both native IE63 and the IE63-CBG68 fusion protein were also expressed in melanoma cells infected with pOka-63-CBG68 (data not shown). The IE63-luciferase recombinants produced gE of the expected molecular mass (85–100 kDa) (Fig. 2, lanes C). The replication of pOka-63-CBR and pOka-63-pOka did not differ from pOka or pOka-gE-CBR over 6 days in melanoma cells (Fig. 3A). Luminescence was emitted by the IE63-luciferase recombinants in melanoma cells and, as was observed with pOka-gE-CBR, it was associated only with virus plaques as determined by live imaging and immunohistochemistry (data not shown). The intracellular localization of gE in cells infected with pOka-63-CBR and pOka was similar (Fig. 4).

3.3. Characteristics of the pOka-63/70-luciferase fusion viruses in vitro

In contrast to the IE63-luciferase fusion viruses, only the 95 kDa fusion protein was detected by immunoblot analysis of melanoma cells infected with pOka-63/70-CBR and pOka-63/70-CBG68 (Fig. 5). The growth kinetics of these recombinants in melanoma cells was not significantly different from pOka over 6 days (Fig. 3B) and luminescence was detected in virus plaques by live imaging and immunohistochemistry (data not shown). Plaque sizes were slightly reduced for pOka-63/70-CBG68 (1.496 mm² SEM 0.066) and pOka-63/70-CBR (1.484 mm² SEM 0.058) when compared to pOka (1.962 mm² SEM 0.139). By confocal microscopy, IE63 in pOka-63/70-CBR infected cells appeared to be somewhat more cytoplasmic (Fig. 4). Therefore, cell fractionation and western blot experiments were done in order to determine whether this assessment could be substantiated. The relative levels of nuclear and cytoplasmic IE63/70-luciferase fusion proteins in cells infected with pOka-63/70-CBR and pOka-63/70-CBG68 were similar to native IE63 expressed by pOka at 48 h post-inoculation (Fig. 5). In addition, the expression of the IE4, IE62 and gE proteins was similar in melanoma cells infected with pOka-63/70-CBR, pOka-63/70-CBG68 and pOka. The lack

of α -tubulin in the nuclear fractions showed that the presence of IE63 or IE63-luciferase fusion protein was not a consequence of contamination from the cytoplasmic fraction. In addition, the intracellular localization of the major transactivator protein IE62, which binds to IE63 (Baiker et al., 2004; Lynch et al., 2002), appeared to be unaffected when the pOka-63/70-luciferase viruses were compared to pOka (Fig. 5). Thus, fusion of one or both copies of ORF63/70 with luciferase did not prevent IE63 localization to the nucleus.

3.4. Effects of passage on the pOka-63-luciferase and pOka-gE-luciferase fusion viruses in human fibroblasts in vitro

A loss of luciferase expression was observed in 82% (SEM 10.6) of plaques when the pOka-63-luciferase fusion viruses were transferred from melanoma cells to HELFs and passaged 4 times over 21 days. The presence of the expected DNA fragments (8.0 and 18.5 kbp) in viral genomic DNA extracted from HELFs infected with pOka-63-CBR and pOka-63-CBG68 was demonstrated by southern blot. In addition, DNA fragments (14.3 and 12.2 kbp) were also detected for pOka-63-luciferase with an inverted US region. This was in agreement with the DNA fragments generated from pOka-infected HELFs. The ORF63/70 probe annealed to the two expected DNA fragments of 8.0 and 16.8 kbp that were detected in cosmid-derived pOka, but also to two additional DNA fragments of 12.2 and 12.7 kbp that were not present in the pvSpe23 cosmid and likely a consequence of an inverted US region (Fig. 6, lanes 1 and A). However, DNA fragments consistent with native ORF63 (12.7 and 16.8 kbp) and a 9.7 kbp fragment of ORF63/70-luciferase were also detected in pOka-63-luciferase infected HELFs (Fig. 6, lanes B and D). Evidence of genomic rearrangement involving the duplicate ORF63 and ORF70 genes was not unexpected, based upon previous analyses of rOka Δ ORF63 and rOka Δ ORF70 mutants that had single copy gene deletions (Sommer et al., 2001).

While this result was anticipated, the passage of pOka-gE-CBR in HELFs for 4 passages (21 days) was also associated with loss of luciferase expression in 78% (SEM 7.6) of plaques. Southern blot analysis of an EcoRV endonuclease digest of DNA extracted from HELFs 4 days post-infection identified the presence of two DNA fragments (data not shown). The fragment sizes were 8.9 kbp, as was expected for ORF68-CBR, based on hybridization with the cosmid, pvSpe23-gE-CBR, and 7.3 kbp, which was consistent with the presence of native ORF68 without the fused luciferase gene. The CBR probe annealed only to the 8.9 kbp fragment. Upon further analysis, the loss of the luciferase gene from pOka-gE-CBR appears to be attributable to recombination between the small residual portion (113 bp) of the repeat region retained at the 3'-end of ORF68, which is located in the pOka genome at nucleotides 112,311-112,423 in the IRS and 117,660-117,772 in the TRS.

3.5. Effects of passage on the pOka-63/70-luciferase fusion viruses in human fibroblasts in vitro

In contrast to the pOka-63-CBR, pOka-63-CBG68 and pOka-gE-CBR viruses, passage of pOka-63/70-CBR and pOka-63/70-CBG68 in fibroblasts was not associated with a reduction in the percentage of plaques that had luminescence. The two expected DNA fragments (9.7 and 18.5 kbp) were generated from HELFs infected with pOka-63/70-CBR and pOka-63/70-CBG68 and two additional fragments (13.8 and 14.3 kbp) were also present (Fig. 6, lanes C and E). Each of the four fragments also annealed with the CBR and CBG68 probes in DNA from HELFs infected with pOka-63/70-CBR or pOka-63/70-CBG68, respectively, showing that the luciferase genes were fused to both ORF63 and ORF70. The ORF63/70-luciferase fusion genes in the pOka-63/70-CBR and pOka-63/70-CBG68 viruses were stable despite evidence for genomic rearrangements of the US region as determined by the appearance of the additional DNA fragments of 13.8 and 14.3 kbp. The luciferase genes remained in the genomes of the two viruses for at least 21 days of replication in vitro (data not shown).

3.6. Replication and whole-animal imaging of pOka-63/70-luciferase fusion viruses in human skin and dorsal root ganglia xenografts in vivo

pOka-63/70-CBR and pOka-63/70-CBG68 replicated in human skin xenografts with mean $\log_{10}$ titers of 1.3 and 1.7, respectively, at day 10 and 2.6 and 2.9 at day 21 post-inoculation (Table 1). The mean titer of pOka-63/70-CBG68, but not of pOka-63/70-CBR was significantly lower than pOka at day 10 (T -test, $p = 0.01$); no other significant differences were observed compared to pOka. In agreement with in vitro replication, luminescence was detected in 100% of plaques when pOka-63/70-CBR and pOka-63/70-CBG68 were recovered from skin xenografts at days 10 and 21 post-inoculation. Thus, the pOka-63/70-luciferase fusion viruses were pathogenic in human skin and the ORF63/70-luciferase genes were stable in the genomes of the two viruses after 21 days of replication, as confirmed by PCR (data not shown).

Luminescence was successfully detected in SCIDhu mice with skin xenografts that were inoculated with pOka-63/70-CBR and pOka-63/70-CBG68 but not pOka by whole-animal imaging for 21 days (Fig. 7A). Luminescence generated by the replication of both pOka-63/70-luciferase fusion viruses peaked at day 15. Of interest, the luminescence signal was higher in skin xenografts infected with pOka-63/70-CBR compared to pOka-63/70-CBG68 beginning at day 9, which was consistent with the difference in viral titers recovered at day 10 in skin xenografts inoculated with these recombinants (Table 1). The background luminescence in pOka-infected skin xenografts was not significantly above the baseline over the 21 day interval.

pOka-63/70-CBR was selected for evaluation in human dorsal root ganglia xenografts because its pathogenicity in skin xenografts did not differ from pOka. The luminescence generated by the CBR luciferase also has enhanced transmission compared to the CBG68 luciferase (Zhao et al., 2005). As expected, luminescence was detected, using whole-animal imaging for 56 days, in SCIDhu mice with dorsal root ganglia xenografts that were inoculated with pOka-63/60-CBR but not in those inoculated with pOka (Fig. 7B). The luminescence increased from day 3, the first time point measured post-inoculation, to a peak at day 15 post-inoculation. This was similar to the kinetics seen for replication of pOka-63/60-CBR in the human skin xenografts. After the peak in luminescence, a decline to a level that was significantly lower than that detected by day 3 was seen at 41 days post-inoculation. The decline in luminescence continued to levels equivalent to that of background luminescence associated with pOka infection at day 56.

3.7. The effect of valacyclovir on VZV infection in dorsal root ganglia xenografts

To assess whether this system could be used to evaluate antiviral drugs in vivo, dorsal root ganglia xenografts were inoculated with pOka-63/70-CBR and the mice were treated with valacyclovir for 28 days. IE63-luciferase expression increased in parallel in untreated and treated mice early after dorsal root ganglia inoculation, from days 3 to 9 (Fig. 7). These observations suggested that initial viral replication was not altered, probably due to the mice receiving the drug in the drinking water and because intake was limited until they recovered from the anesthesia given during the inoculation procedure. In addition, plasma concentrations of valacyclovir require an equilibration period to achieve a steady state. Subsequently, untreated mice showed a continued increase in the IE63-luciferase signal to day 15, which began to decline by day 18, followed by a substantial decrease over the period from 21 to 35 days and later. In our prior studies of the SCIDhu dorsal root ganglia model, this period corresponded to a transition to persistence, when infectious virus was not recovered followed by a decline and disappearance of viral protein synthesis. This included IE, early and late proteins, disappearance of late gene transcripts (gB) and a decline but persistence of low levels of ORF62 and ORF63 transcripts. The kinetics of pOka-63/70-CBR in dorsal root ganglia in the untreated mice were reproducible, as shown by comparison with the luminescence values from the first experiment (Fig. 7B and C).

In contrast, IE63-luciferase expression was sustained in treated mice at the peak level from day 15 through day 28, when valacyclovir treatment was withdrawn. At this point, IE63-luciferase expression began to decline, following a slope that paralleled that observed in the untreated mice, but was shifted, such that the values at day 42 were about equal to those at day 28 in untreated mice. As a result, valacyclovir treatment for 28 days produced an increase in the area under the luminescence–time curve from 71 (Luminescence [$\log_{10}$] days) for the untreated mice to 121 (Luminescence [$\log_{10}$] days) for the treated mice over 70 days. This trend suggested that events in viral pathogenesis of dorsal root ganglia infection progressed with similar kinetics to untreated mice upon withdrawal of valacyclovir. In the interval after peak expression was reached and valacyclovir treatment was continued, the tagged gene might have been expressed from residual pOka-63/70-CBR genomes in neural cells, whereas de novo synthesis of viral genomes was controlled. Removing the drug allowed active replication, followed by the transition to persistence. This transition also appears to be prolonged because transcripts from the late gene, ORF31 (gB), were detected in dorsal root ganglia of treated mice ($\log_{10}$ 4.3 copies/ng of human RNA) but were not detected above background levels in untreated mice at day 70 post-inoculation.

4. Discussion

The present study demonstrated that click beetle luciferase genes fused to VZV genes could be used to detect expression of IE proteins or late glycoproteins upon VZV infection in vitro and in vivo in SCIDhu mouse models of VZV pathogenesis. However, the location of the VZV-luciferase genes within the VZV genome was critical. The luciferase genes conjugated to the single copies of ORF63, encoding IE63, or ORF68, encoding gE, were eliminated from the genome, which was likely to be a consequence of genomic rearrangement and/or recombination (reviewed by Umene, 1999). Evidence for genomic recombination in VZV and the inversion of the US region has been reported previously (Kinchington et al., 1985; Sato et al., 2003a; Sommer et al., 2001; Straus et al., 1982). In the present study, the fragments generated by the EcoRI digest of DNA from the luciferase fusion viruses were those reported by Straus et al. (1982); A (16.7) and J (8.0) or E (12.7) and F (12.2) for the pOka virus. In agreement with previous studies (Kinchington et al., 1985; Straus et al., 1982), and based on our comparison to pOka, the ratio of EcoRI DNA fragments from the US, IRS and TRS regions of pOka-63/70-luciferase were equimolar. These results suggested that the VZV-luciferase genes had little effect on the usual pattern of inversion of the US region during VZV replication. Southern blot analysis of pOka-63-CBG68 and pOka-63-CBR showed that both of the luciferase gene sequences were eliminated from the VZV genome during replication. Evidence that pOka-63/70-luciferase genomes were generated from the pOka-63-luciferase constructs was obtained based on the appearance of the 9.7 kbp fragment, which was equivalent to the J fragment of Straus et al. (1982) with the addition of the luciferase gene sequence. To generate the 9.7 kbp fragment would require the insertion of the luciferase gene at the ORF70 site. Similar evidence of insertions of complete genes into the IRS and TRS was observed with ORF62 and ORF71 single deletion mutants (Sato et al., 2003a).

Of interest, the selective pressure caused by viral replication in cultured primary cells brought about loss of the luciferase genes in the gE (ORF68) fusion virus as well as the single copy IE63 fusion viruses. Since most of the ORF68 sequence is within the US region, the pOka-gE-luciferase virus was not expected to be as susceptible to reversion to wild type pOka. However, a 113 nucleotides fragment of ORF68 extends into the TRS region. The elimination of the luciferase gene sequences from pOka-gE-luciferase viruses appeared to be less efficient than from pOka-63-CBG68 and pOka-63-CBR. However, the loss of the CBR gene from pOka-gE-CBR suggests that the 113 nucleotides fragment in the TRS sequence was likely to permit repair of the VZV genome by homologous recombination. Selective pressure to maintain wild type gE is likely to be critical for VZV because, in contrast to other alphaherpesviruses, gE is

essential for replication (Berarducci et al., 2006; Mallory et al., 1997; Mo et al., 2002). In addition, amino acid motifs in the gE carboxyl terminus are required for its correct cellular trafficking and secondary envelopment; the endocytosis motif is essential for replication and trafficking motifs are important for the pathogenesis of VZV skin infection in vivo (Moffat et al., 2004; Olson and Grose, 1997). The fusion of the luciferase to gE at its C-terminus might have significantly reduced the viability of pOka-gE-luciferase viruses and forced genomic recombination to remove the exogenous gene.

In contrast to pOka-ORF63-luciferase and pOka-gE-luciferase viruses, insertion of the luciferase genes at duplicated ORF63/70 sites in both the IRS and TRS resulted in stable genomes that expressed fusion proteins with luciferase activity. IE63 is a vital protein for VZV replication that is likely to be involved in the regulation of viral gene transcription through interactions with viral and cellular transcription factors (Di Valentin et al., 2005; Sommer et al., 2001). IE63 must bind to IE62 to be functional, as shown previously by the lethal effects of the ⁵⁹RL⁶⁰ deletion in IE63, which demonstrated that these residues were critical for IE62 binding and VZV replication (Baiker et al., 2004). The replication of the pOka-63/70-luciferase viruses implied that the IE62/IE63 interaction was preserved. The IE63-luciferase fusion proteins appeared to localize with IE62 in the nucleus, suggesting the conjugation of click beetle luciferases to IE63 allowed normal intracellular trafficking and presumably did not interfere with IE63 binding to IE62.

Because the pOka-63/70-luciferase viruses retained genetic stability, it was feasible to use these recombinants to assess viral replication by in vivo imaging experiments in our SCIDhu mouse models of VZV pathogenesis. These models were developed to overcome the obstacles to studies of VZV infection in vivo that result from its highly restricted host range. The pOka-63/70-CBR luciferase virus retained pathogenicity in human skin and dorsal root ganglia xenografts compared to pOka. For both this virus and pOka-63/70-CBG68, as expected for a host specific pathogen, the luminescence remained localized to the skin and dorsal root ganglia xenografts, which was consistent with the recent report of thymus/liver and skin xenografts infected with VZV-BAC_{LUC} (Zhang et al., 2007). In contrast, HSV-1 is pathogenic in mice and in vivo imaging of mice infected with an HSV-1 recombinant expressing luciferase showed systemic spread of the virus from the inoculation site (Luker et al., 2002, 2003). As observed in previous studies with luciferase expressing viruses, the luminescence generated by the pOka-63/70-luciferase viruses in skin xenografts correlated well with the recovery of infectious virus and the viral titers, which were within the range found in previous studies (Berarducci et al., 2006; Besser et al., 2003; Che et al., 2006; Moffat et al., 1998). Previous in vivo imaging studies of herpesviruses have used luciferases expressed from ectopic sites, including the previous studies with VZV-BAC_{LUC} and the dual ORF62/63 promoter in skin xenografts (Burgos et al., 2006; Jones et al., 2006; Luker et al., 2002; Zhang et al., 2007). In contrast, the present study demonstrated the pattern of expression of the important viral regulatory protein, IE63, as controlled by its native promoter during VZV replication in vivo. It was hoped that comparisons of the kinetics of IE63 and late glycoprotein synthesis could be made in skin and dorsal root ganglia xenografts in vivo but the instability of the pOka-gE-CBR genome meant that data about gE expression based on luciferase detection was not reliable. The IE63 expression in dorsal root ganglia was consistent with our analyses of IE63 protein expression in dorsal root ganglia xenografts examined at intervals after inoculation with pOka, which show a cessation of IE63 protein synthesis despite persistence of transcripts at low levels (Zerboni et al., 2005, 2007). The purpose of the present study was to use IE63 as a marker for viral replication but alternative proteins could be used such as thymidine kinase that is dispensable in vitro or structural proteins such as the ORF23 protein. VP26, the HSV homologue of ORF23, has been used to study HSV incorporating GFP into the protein (Snyder et al., 2006). However, additional studies using luciferase fusion proteins to study VZV

replication would require comparison with pOka in the xenograft models in order to be sure that pathogenicity was not reduced.

The genetic stability of the pOka-63/70-luciferase virus enabled the evaluation of valacyclovir treatment on VZV replication in human dorsal root ganglia xenografts. Valacyclovir has high bioavailability as an oral drug and is used for treatment and prevention of VZV infections in healthy and high risk patients (Boeckh et al., 2006; Manuel et al., 2008; Soul-Lawton et al., 1995). In treated mice, IE63 expression determined from luminescence values, peaked at day 18 after infection and persisted at an almost constant level until the removal of the drug at day 28. This suggested that the tagged gene might be expressed from pOka-63/70-CBR genomes in neural cells in the presence of valacyclovir, whereas de novo genome synthesis was controlled. Upon withdrawal of valacyclovir, the pattern of IE63-luciferase expression mimicked that observed at earlier time points in the untreated mice. In a study of valacyclovir treatment of mice infected with HSV-1, the drug reduced luminescence values over a 9 day period (Luker et al., 2002). The present study differed in that luminescence was a consequence of VZV protein expression rather than expression of the luciferase gene from a non-native promoter in the HSV-1 genome. An important difference was that, unlike the murine model of HSV infection, the VZV pathogenesis experiments were performed in SCID mice. In the HSV-1 model, the combination of the antiviral drug and the adaptive immune response might allow more effective control of viral replication whereas SCID mice lack the capacity to mount an antiviral T cell response necessary to recognize and clear the infected cells. The fact that drug treatment slowed but did not eliminate VZV was unlikely to be the consequence of low levels of systemic acyclovir as the prodrug valacyclovir has good bioavailability. Although acyclovir levels were not measured, the approximate dose per mouse per day was 100mg/kg, which was comparable to doses that control herpesvirus infections in humans; the inhibitory doses (IC₅₀) of valacyclovir for VZV range from 0.12 to 10.8 µg/ml (O'Brien and Campoli-Richards, 1989). Failure of the drug to enter dorsal root ganglia xenografts was unlikely since the dorsal root ganglia are well vascularized and valacyclovir is known to achieve VZV inhibitory concentrations even in privileged sites, such as cerebrospinal fluid or the vitreous of the eye (Huynh et al., 2008; Lycke et al., 2003). Studies have shown that patients treated with valacyclovir, or the active compound acyclovir, have a reduced emergence of herpesvirus infections caused by HSV and VZV after solid organ or hematopoietic cell transplantation (Boeckh et al., 2006; Fiddian et al., 2002). However, upon removal of the drug, the frequency of herpesvirus reactivation occurred with a similar incidence to untreated patients (Boeckh et al., 2006; Manuel et al., 2008). The most severely immunocompromised patients who experience VZV reactivation also have a high incidence of relapse shortly after valacyclovir or acyclovir therapy is discontinued. These patients also fail to develop a VZV specific T cell response. Thus, the observation that VZV infection progressed in SCIDhu dorsal root ganglia when valacyclovir was discontinued is consistent with the clinical experience using anti-VZV drugs in patients who lack adaptive immunity.

Acknowledgements

This work was supported by NIH grants, AI053846, AI20459 and CA049605. We thank Barbara Berarducci for valuable scientific discussion.

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Fig. 1.

Construction of VZV recombinants expressing IE63 and gE as luciferase fusion proteins. (A) Schematic of the VZV genome. UL—unique long; US—unique short; TRL—terminal repeat long; IRL—internal repeat long; IRS—internal repeat short; TRS—terminal repeat short. (B) The four cosmids (genome fragments in nucleotides (nt)), pvFsp73 (nt 1–33,128), pvSpe14 (nt 21,796–61,868), pvPme2 (nt 53,756–96,035) and pvSpe23 (nt 94,055–125,123), used to generate recombinant pOka and the luciferase fusion viruses. (C) The location of ORF63 and ORF70 (IE63) and 68 (gE) in the pvSpe23 cosmid. (D–F) The luciferase gene was inserted at the 3'-termini of ORFs 63 (D), 63/70 (E) and 68 (F). Shaded boxes show ORFs. Transfection of these cosmids into melanoma cells yielded recombinant viruses designated pOka-63-CBG68, pOka-63-CBR, pOka-63/70-CBG68, pOka-63/70-CBR and pOka-gE-CBR.

Fig. 2.

Western blot of IE63- and gE-luciferase fusion proteins from infected melanoma cells. Cell lysates were prepared from melanoma cells infected with pOka (A), pOka-gE-CBR (B) and pOka-63-CBR (C). IE63 and IE63-CBR were detected with rabbit polyclonal anti-IE63. gE and gE-CBR were detected with monoclonal antibody 3B3. α -Tubulin was detected with a monoclonal antibody. UI—uninfected. Molecular masses (M_r) for the proteins were calculated from a standard curve derived from a protein marker.

Fig. 3. Replication *in vitro* of VZV recombinants expressing IE63 and gE as luciferase fusion proteins. Melanoma cells were infected with pOka or VZV recombinants and tested for replication kinetics over 6 days. (A) Replication of pOka, pOka-63-CBG68, pOka-63-CBR and pOka-gE-CBR. (B) Replication of pOka, pOka-63/70-CBR and pOka-63/70-CBG68. Standard error of the mean is shown on both graphs.

Fig. 4.

Cellular localization of IE63 and gE expressed as luciferase fusion proteins. Melanoma cells were infected with VZV recombinants, expressing gE (pOka-gE-CBR) or IE63 (pOka-63-CBR, pOka-63/70-CBR) as luciferase fusion proteins and examined by confocal microscopy at 24 h post-inoculation. IE63—rabbit polyclonal antibody to IE63, gE—monoclonal antibody MAB8612 and Nuclei—Hoescht 33342. The white bar on the merge panels indicates 50 μ m. Similar results were seen in melanoma cells infected with pOka-63-CBG68 and pOka-63/70-CBG68.

Fig. 5.

Western blot of nuclear and cytoplasmic expression of VZV proteins in cells infected with recombinants expressing IE63-luciferase fusion proteins. Nuclear (N) and cytoplasmic (C) fractions were recovered from melanoma cell lysates collected at 48 h post-inoculation with pOka, pOka-63/70-CBR or pOka-63/70-CBG68. IE4—rabbit polyclonal antibody to IE4, IE62—rabbit polyclonal antibody to IE62, IE63—rabbit polyclonal antibody to IE63, gE—monoclonal antibody 3B3 and α -tubulin—monoclonal antibody to α -tubulin. The anti α -tubulin antibody was used to control for the purity of the cytoplasmic and nuclear fractions. UI—uninfected. Molecular masses (M_r) for the proteins were calculated from a standard curve derived from a protein marker.

Fig. 6.

Southern blot detection of ORF63 and ORF70 plus the luciferase genes, CBR and CBG68 in cosmids and recombinant virus-infected cells. Southern blots were performed with cosmid or DNA extracted from HELFs infected with pOka or recombinants and digested with restriction endonuclease EcoRI. DNA probes to ORF63/70 or the CBR and CBG68 click beetle luciferases were used to detect the genes. Cosmid DNA: (A) pvSpe23, (B) pvSpe23-63-CBR, (C) pvSpe23-63/70-CBR, (D) pvSpe23-63-CBG68, (E) pvSpe23-63/70-CBG68. Viral DNA: (A) pOka, (B) pOka-63-CBR, (C) pOka-63/70-CBR, (D) pOka-63-CBG68, (E) pOka-63/70-CBG68. UI—uninfected HELFs. The white numbers mark the DNA fragments (kbp) that were

expected (1–16.8; 2–8.0; 3–18.5; 4–9.6) or generated from viral genomic reorganization (5–12.7/12.2; 6–14.3/13.8).

Fig. 7. Luminescence generated in vivo by IE63 expression in human tissue xenografts infected with pOka and pOka-63/70-luciferase viruses. Images of the infected mice in the figure were taken at 15 days post-inoculation. The intensity of the luminescence (photons/s/cm²) is indicated by the false colour scale. (A) (number of xenografts); 1—pOka ($n = 10$), 2—pOka-63/70-CBR ($n = 10$) and 3—pOka-63/70-CBG68 ($n = 10$). The graph shows the levels of luminescence generated by IE63-luciferase in skin xenografts for 21 days. Implants infected with pOka-63/70-CBR or pOka-63/70-CBG68 that did not generate luminescence were excluded from the graph with the exception of the pOka controls, which were used to determine the background levels of luminescence. (B) (number of xenografts); 1—pOka ($n = 4$) and 2—

pOka-63/70-CBR ($n = 4$). The graph shows the levels of luminescence generated by IE63-luciferase in dorsal root ganglia xenografts for 56 days. (C) Luminescence generated in vivo by IE63 expression in dorsal root ganglia xenografts infected with pOka-63/70-CBR with ($n = 8$) or without ($n = 10$) valacyclovir treatment. Valacyclovir was added to the drinking water (1 mg/ml) and taken ad libitum. Valacyclovir was removed at day 28 (black arrow head) and two mice from each group were euthanized for analysis. The open arrow head indicates that three mice from the treated group and three mice from the untreated group were removed from the data set as they received additional treatments. The grey arrow head indicates that three mice in the untreated group were euthanized. Corrected luminescence—luminescence values were normalized by subtracting the luminescence values emitted from dorsal root ganglia infected with wild type pOka from those infected with pOka-63/70-CBR. Standard error of the mean is shown on all graphs.

Titers and frequency of luminescent plaques for pOka and pOka-63/70-luciferase fusion viruses isolated from human skin xenografts at 10 and 21 days post-infection

Virus	Titer (log ₁₀ [SEM]) of inocula	Positive implants/implants		Mean titer (log ₁₀ [SEM]) at day		p-Value ^a at day		Frequency of luminescent plaques (%[SEM]) at day	
		10	21	10	21	10	21	10	21
pOka	4 [0.269]	2/4	4/6	2.4 [0.230]	3.5 [0.128]	–	–	N/A	N/A
pOka-63/70-CBR	5.7 [0.148]	4/5	5/6	1.7 [0.277]	2.9 [0.285]	0.125	0.098	100	100
pOka-63/70-CBG68	5.7 [0.135]	2/6	5/6	1.3 [0.262]	2.6 [0.599]	0.011	0.058	100	100

SEM—standard error of the mean. N/A—not applicable. N/C—not calculated.
^a p-Values are for *T*-tests to determine whether there were significant differences between wild type pOka and the luciferase fusion viruses.