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Properties of an equine herpesvirus 1 mutant devoid of the internal inverted repeat sequence of the genomic short region

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ABSTRACT

The 150 kbp genome of equine herpesvirus-1 (EHV-1) is composed of a unique long (UL) region and a unique short (Us) segment, which is flanked by identical internal and terminal repeat (IR and TR) sequences of 12.7 kbp. We constructed an EHV-1 lacking the entire IR (vL11ΔIR) and showed that the IR is dispensable for EHV-1 replication but that the vL11ΔIR exhibits a smaller plaque size and delayed growth kinetics. Western blot analyses of cells infected with vL11ΔIR showed that the synthesis of viral proteins encoded by the immediate-early, early, and late genes was reduced at immediate-early and early times, but by late stages of replication reached wild type levels. Intranasal infection of CBA mice revealed that the vL11ΔIR was significantly attenuated as mice infected with the vL11ΔIR showed a reduced lung viral titer and greater ability to survive infection compared to mice infected with parental or revertant virus.
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Introduction

Equine herpesvirus-1 (EHV-1) is a member of Genus *Varicellovirus* within the *Alphaherpesvirinae* subfamily. Its genome of 150 kbp (Telford et al., 1992) is comprised of a unique long (UL) region covalently linked to a short (S) region that is organized as a unique short segment (Us) bracketed by a pair of identical internal repeat (IR) and terminal repeat (TR) sequences (Henry et al., 1981; Ruyechan et al., 1982; Whalley et al., 1981). Each inverted repeat harbors six genes (IR1 to IR6) and a portion of the Us1 (gene 68) gene. The IR1 gene encodes the sole IE protein that governs early and some late gene expression and downregulates its own expression (Caughman et al., 1985; Harty et al., 1990; Smith et al., 1992, 1993). The early IR2 gene is located within the *IE* (*IR1*) ORF and generates the IR2 protein (IR2P) that strongly downregulates expression of all genes as a potent negative regulator (Kim et al., 2006). The IR3 gene, unique to EHV-1, is *trans*-activated by the IE protein (IEP), EICP0 protein (EICP0P) and IR4 protein (IR4P) and produces a non-coding 1 kb late transcript (Ahn et al., 2007; Holden et al., 1992a) that downregulates expression of the IE gene in a luciferase reporter system (Ahn et al., 2010). The early regulatory IR4P cooperates with the IEP to enhance expression of early and late viral genes (Holden et al., 1995) and comprises the major portion of the IR4/UL5 hybrid protein encoded by defective interfering particles (DIP) that can cause persistent EHV-1 infection (Chen et al., 1996, 1999; Ebner et al., 2008; Ebner and O'Callaghan, 2006). The IR5 gene encodes a late 236 amino acid protein that exhibits homology to the ORF64 protein of varicella-zoster virus and the Us10 protein of herpes simplex virus 1 (Holden et al., 1992b), the latter being a tegument phosphoprotein that copurifies with the nuclear matrix (Yamada et al., 1997). The IR6 early gene unique to EHV-1 and its close relative EHV-4 encodes a 33 kDa phosphoprotein that functions in nuclear egress and viral cell-to-cell spread (Breeden et al., 1992; O'Callaghan et al., 1994; Osterrieder et al., 1998) and is a major determinant of virulence (Osterrieder et al., 1996b). Lastly, 631 bp of the 3′ end of the EHV-1 Us1 ORF (a homolog of HSV-1 Us2) extend into the IR, and the Us1 and IR6 transcripts are 3′ coterminal (Breeden et al., 1992).

Manipulation of gene(s) within either inverted repeat segment has been shown to be elusive because alterations of sequences within one repeat are repaired by homologous recombination events involving identical sequences within the other inverted repeat (Ahn et al., 2010; Boldogkoi et al., 1998). We conducted experiments to ascertain if one set of the six diploid genes in the inverted repeat may be dispensable for EHV-1 replication so that the EHV-1 genome may be simplified for genetic manipulation of these six genes in the S region. Here we present findings that an EHV-1 lacking the entire 12.7 kbp inverted repeat was capable of replication both in cell culture and in the murine model of EHV-1 infection, but exhibited delayed growth

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kinetics, decreased virulence *in vivo*, and delayed protein expression of representative viral genes as compared to the parental EHV-1.

Results

The 12.7 kbp IR sequence of EHV-1 is dispensable for replication

The presence of six diploid genes within the identical IR and TR segments (Henry et al., 1981; Ruyechan et al., 1982; Whalley et al., 1981) of the EHV-1 genome suggested the possibility that one repeat segment may be dispensable for viral replication without altering the biological properties of the virus. Thus, we deleted the entire IR of the EHV-1 genome using GalK technology (Ahn et al., 2010; Rudolph et al., 2002; Warming et al., 2005), and characterized vL11ΔIR reconstituted from the recombinant BAC in cell culture. As shown in Fig. 1A, 12,715 bp of the EHV-1 genome that includes the entire IR and an additional 1 bp of the UL sequence were deleted. The removal of the entire IR also resulted in deletion of 631 bp of the Us1 gene (gene 68) that extends into the IR as shown in Fig. 1A (Breeden et al., 1992). Replacement of the entire IR with the GalK marker was confirmed by PCR amplification of two junction regions between the GalK marker and the EHV-1 genomic sequences at the UL terminus and the start of the Us segment (Fig. 1A). PCR analyses indicated that the expected sizes of amplicons were observed from pL11ΔIR-GalK (Fig. 1B, lanes 1 and 3), but not from pRacL11 (Fig. 1B, lanes 2 and 4). Removal of the GalK marker from pL11ΔIR-GalK was confirmed by PCR amplification of the GalK marker flanking sequence (Fig. 1C, lanes 2 and 3) and DNA sequence analysis of PCR amplicons (data not shown). Replacement of the entire IR with the GalK marker from pL11ΔIR-GalK was confirmed by the PCR amplification of IR junction sequences and DNA sequence analysis (data not shown). Primer sets specific to U_L (or Us) and IR sequence amplified the expected sizes of PCR amplicons from pL11ΔIRR (Fig. 1D, lanes 1 and 3), but not from pL11ΔIR-GalK (Fig. 1D, lanes 2 and 4). Deletion and recovery of the IR were further examined by BamHI digestion and Southern blot analyses. The BamHI digestion pattern showed that an additional band of approximately 10 kb in size was observed in the case of pL11ΔIR-GalK (Fig. 2A, lane 2), but pL11ΔIR lacking the 1.2 kb GalK marker showed an approximate 8.8 kb fragment instead of a 10 kb fragment (Fig. 2A, lane 3). The pL11ΔIRR showed a BamHI digestion pattern identical to that of pRacL11 (Fig. 2A, lanes 1 and 4).

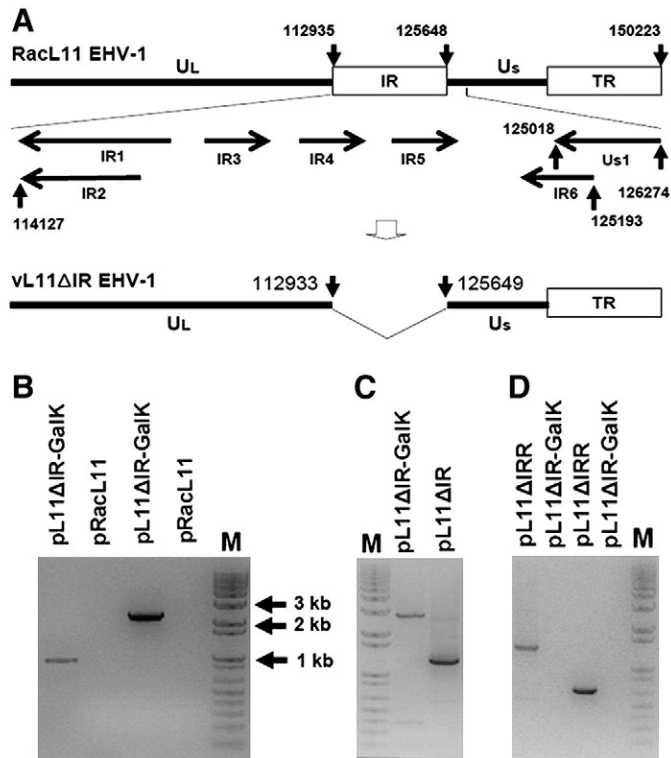

Fig. 1. EHV-1 RacL11 genomic structure and deletion of the 12.7 kbp IR segment confirmed by PCR analysis. (A) RacL11 EHV-1 genomic structure based on the DNA sequence of the Ab4 EHV-1 (Telford et al., 1992). IR and TR are the internal repeat and terminal repeat segments, respectively. UL is the unique long region, and Us is the unique short segment within the Short region. The entire IR within sequences 112934 bp to 125649 bp was removed by GalK positive selection followed by GalK counter selection as described in Materials and methods. (B) PCR confirmation of the insertion of the GalK marker. PCR with primer sets specific to the GalK marker flanking sequences detected the predicted 1 kb (lane 1) and 2.4 kb (lane 3) fragments, respectively, from pL11ΔIR-GalK, but not from pRacL11. M = size markers. (C) PCR confirmation of the removal of the GalK marker from pL11ΔIR-GalK. PCR with a primer set specific to IR flanking sequences detected the predicted 2.7 kb fragment (lane 2) from pL11ΔIR-GalK and the predicted 1.4 kb fragment (lane 3) from pL11ΔIR. (D) PCR confirmation of the restored IR in pL11ΔIRR. PCR with primer sets specific to IR and U_L junction region or to the IR and U_S junction region detected the predicted sizes of amplicons from pL11ΔIRR (Fig. 1D, lanes 1 and 3), but not from pL11ΔIR-GalK (Fig. 1D, lanes 2 and 4).

To confirm that the pL11ΔIR-GalK harbored the GalK marker in the proper location, that pL11ΔIR lacks the GalK marker, and that the GalK marker from pL11ΔIR-GalK was replaced with the entire IR sequence, Southern blot analyses were performed using BamHI digested BAC DNAs (pRacL11, pL11ΔIR-GalK, pL11ΔIR, and pL11ΔIRR) and a radiolabelled GalK marker PCR fragment as the probe. These analyses showed that the GalK marker probe bound only to one fragment of the BamHI digested pL11ΔIR-GalK DNA (Fig. 2B, lane 2), but not to any band of pRacL11 used as the control (Fig. 2B, lane 1), pL11ΔIR (Fig. 2B, lane 3), or pL11ΔIRR (Fig. 2B, lane 4), indicating that the entire IR of the pRacL11 was correctly replaced with the GalK marker, that the GalK marker was removed in the final pL11ΔIR, and that the entire IR was restored in pL11ΔIRR. Once the deletion and restoration of the entire IR were confirmed, the recombinant vL11ΔIR and vL11ΔIRR viruses were generated by cotransfection of pL11ΔIR (or pL11ΔIRR) DNA and a plasmid containing the EHV-1 Us4 gene (gene 71) (Rudolph et al., 2002). Successful reconstitution of vL11ΔIR cloned DNA indicated that the IR deletion virus was replication competent, but plaque assays showed that the plaque areas of vL11ΔIR were significantly reduced compared to those of parental RacL11 and vL11ΔIRR ($p < 0.0001$, Figs. 3A and B). To exclude the possibility that the entire IR was restored by the TR segment during serial virus passage in RK13 cells, the IR flanking region of the vL11ΔIR genome was PCR-amplified by a primer set specific for the IR flanking sequences. PCR amplicons of the same size were generated from

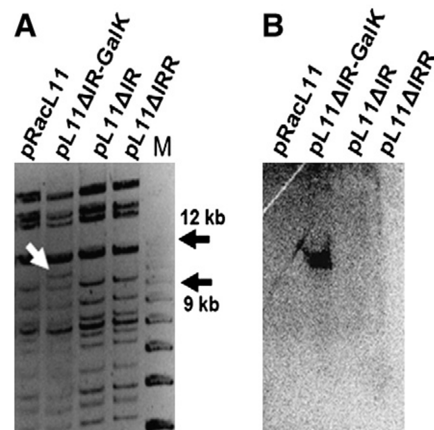

Fig. 2. BamHI digestion patterns and Southern blot analysis to document construction of ΔIR EHV-1. (A) BamHI digested pRacL11, pL11ΔIR-GalK, pL11ΔIR, and pL11ΔIRR DNAs were separated on a 0.8% agarose gel. Black arrows indicate marker sizes. M = size markers. (B) Southern blot analysis. BamHI digested pRacL11, pL11ΔIR-GalK, pL11ΔIR, and pL11ΔIRR DNAs separated on a 0.8% agarose gel were transferred onto a membrane, and the presence and absence of GalK marker in EHV-1 BAC DNAs were examined by Southern blot using a probe specific to the GalK marker as described in Materials and methods.

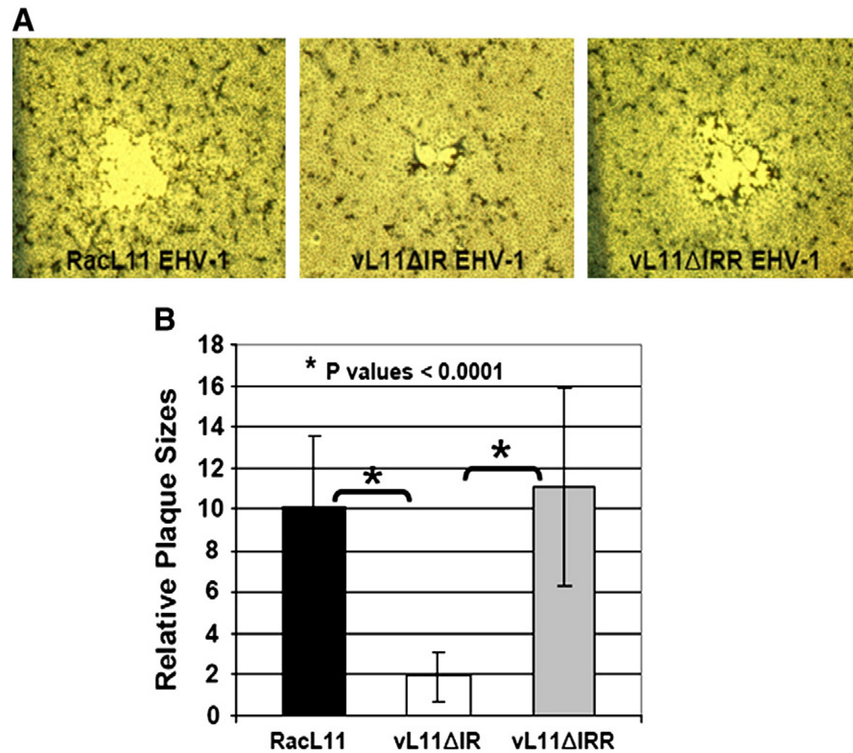

Fig. 3. Plaque morphology and relative plaque size of parental RacL11 EHV-1, vL11ΔIR, and vL11ΔIRR. (A) Representative plaque morphology in RK13 cells of parental virus, the IR-deleted vL11ΔIR, and the IR-restored vL11ΔIRR. (B) Relative plaque size. The plaque sizes were measured by using the ImageJ software program (http://www.rebweb.nih.gov/ij/). Bars represent means of 60 plaques of each virus; error bars represent standard deviations.

both pL11ΔIR (Fig. 4A, lane 1) and DNA derived from vL11ΔIR-infected RK13 cells (Fig. 4A, lane 3). However, no amplicon was detected in DNA prepared from pRacL11 (Fig. 4A, lane 2) or from RacL11-infected RK13 cell DNA (Fig. 4A, lane 4), indicating that the IR segment was not repaired by recombination events with TR sequences during vL11ΔIR replication in RK13 cells. To address whether the IR sequences restored in the revertant virus were functionally similar to parental virus with respect to gene expression, synthesis of the IEP was examined in the various viruses. IEP expression levels of both parental RacL11 and vL11ΔIRR viruses were similar at immediate-early, early, and late times of replication (Fig. 4B), results that indicated that the IR was completely restored in vL11ΔIRR.

Cellular tropism and growth kinetics of vL11ΔIR

Even though the IR was not essential for EHV-1 replication, there remained the possibility that the cellular tropism of vL11ΔIR may differ from that of the parental virus. Our recent studies had revealed that a mutant EHV-1 in which both copies of the IR4 gene were absent

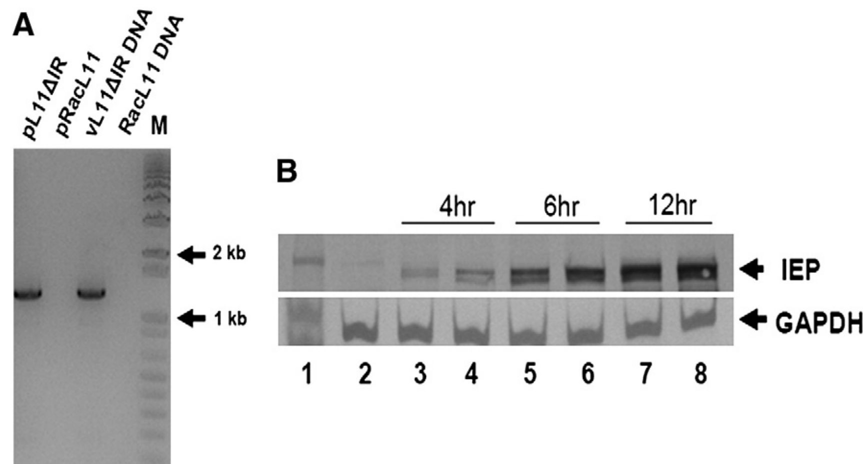

Fig. 4. Characterization of the vL11ΔIR genome and IE protein expression in cells infected with vL11ΔIRR. (A) Confirmation of the absence of the GalK marker in the vL11ΔIR genome. PCR amplification with a primer set specific to IR flanking sequences was performed as described in Materials and methods. Lane 1, 2, 3, and 4 indicate the DNA templates of pL11ΔIR DNA, DNA of RK13 cells infected with vL11ΔIR, pRacL11 DNA, and DNA of RK13 cells infected with RacL11, respectively. (B) Comparison of the IEP expression in RK13 cells infected with RacL11 EHV-1 (lanes 3, 5, and 7) or vL11ΔIRR (lanes 4, 6, and 8). Detection of the IEP was performed as described in Materials and methods. Lanes 1 and 2 indicate marker and mock-infected cells, respectively.

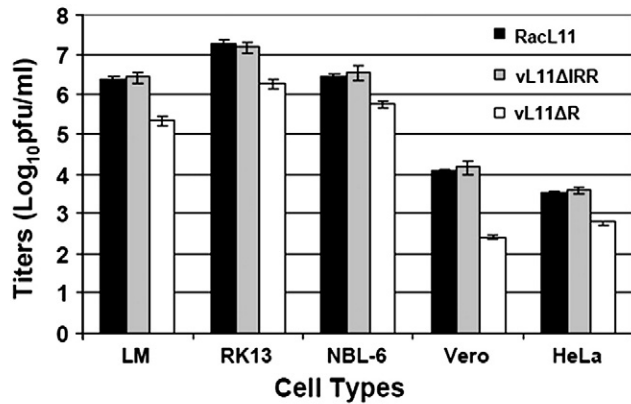

Fig. 5. Tropism of vL11ΔIR in various cell types. Monolayer cultures of each of the five cell types were infected with vL11ΔIR or RacL11 EHV-1 at a moi of 1. After a 2 h virus attachment at 37 °C, the infected cells were washed with PBS followed by adding equal volumes of growth medium. At 72 hpi, samples were harvested and titered by plaque assay as described in Materials and methods. Error bars indicate standard deviations.

was capable of replication in equine NBL-6 cells, but, unlike its parent virus, was not capable of replication in mouse, rabbit, monkey, or human cells (Breitenbach et al., 2009). These observations suggested that the deletion of the entire IR may affect the biological properties of EHV-1. Investigation of the cellular tropism and replication of EHV-1 showed that vL11ΔIR like the parental RacL11 was capable of replicating in all five cell types tested but the vL11ΔIR replicated with significantly reduced titers when compared with parental virus and the revertant virus in all cell types examined (all p values were <0.05; Fig. 5). The growth kinetics of vL11ΔIR was analyzed in RK13 cells by examining intracellular and extracellular viral titers at various times after infection (Figs. 6A and B). Overall, growth of the vL11ΔIR was impaired as compared to that of the RacL11 as its replication exhibited a lag in reaching maximal titer. Both viruses reached maximal titers at 18 to 24 h post infection, but the titer of the RacL11 and vL11ΔIRR exceeded that of the vL11ΔIR by more than one log.

To examine whether the delayed growth of vL11ΔIR is due to an impaired ability of the mutant virus in entry/penetration, cell-associated viral DNA was quantified by real time PCR after the parental virus, vL11ΔIR, and the revertant virus were incubated with RK cells for a 2 h attachment period at 4 °C followed by a 30-minute incubation period at 37 °C. Comparison of Ct values of the RacL11, vL11ΔIR, and vL11ΔIRR DNAs revealed no significant difference (Fig. 6C). All p values were greater than $P=0.50$, suggesting that the delayed growth of vL11ΔIR is due to a reduced rate of replication rather than impaired virus entry/penetration.

Protein expression of the IE and representative early and late genes was delayed in vL11ΔIR-infected cells

The change of phenotype and the delayed growth kinetics of vL11ΔIR suggested that the deletion of the IR may affect viral gene regulation such that proteins encoded by IR genes would be decreased in cells infected with the IR deleted virus. Therefore, we compared protein expression of the IE and representative early (IR4, EICP0, and UL5), and late (gD) genes in cells infected with wt EHV-1 and the IR deleted virus. The IE protein (IEP) was detected at 4 hpi in cells infected with either virus (Fig. 7A, lanes 3 and 4), but the amount of the IEP was significantly greater in cells infected with parental RacL11 EHV-1 until 6 hpi (Fig. 7A lanes 5 and 6). However, by late times of infection, the amount of the IEP was similar in cells infected with either virus (Fig. 7A lanes 7 and 8). In the case of the EHV-1 early gene products, a similar pattern was observed at early times after infection

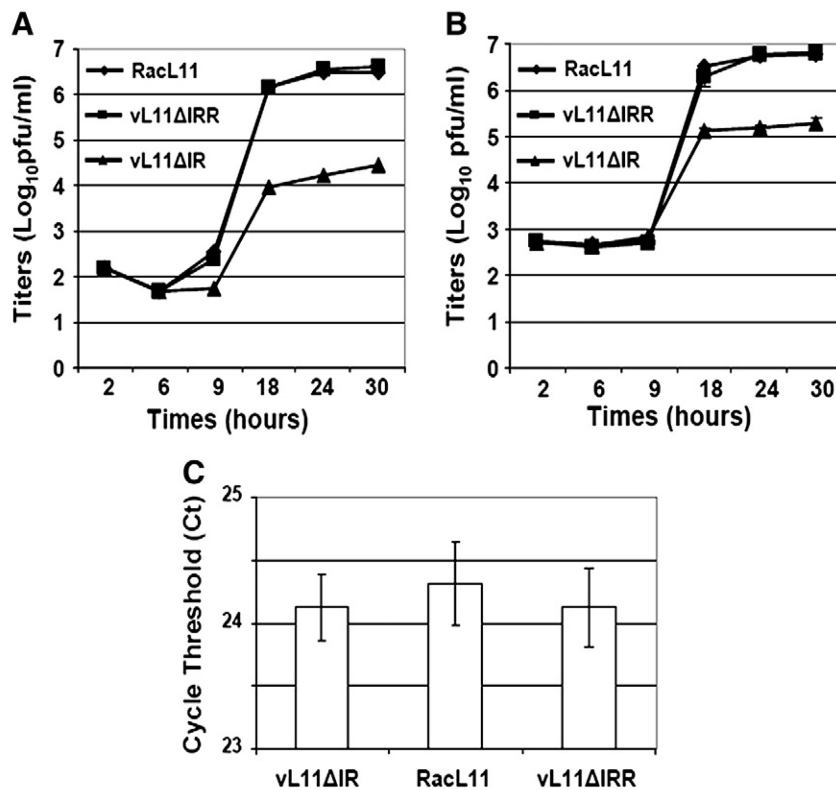

Fig. 6. Growth kinetics of parental, vL11ΔIR and vL11ΔIRR, and quantitative real time PCR. RK13 cells were infected with the respective virus at an moi of 0.2, and intracellular and extracellular viruses were harvested at the indicated times post infection and titered as described in Materials and methods. (A) Intracellular viral titer. (B) Extracellular viral titer. (C) Quantitative real time PCR. RK13 cells were infected with RacL11, vL11ΔIR and vL11ΔIRR at an moi of 10 followed by incubation at 4 °C for 2 h and at 37 °C for 30 min, and PBS washing. Total DNAs were extracted from virus infected RK13 cells, and the relative number of viral genomes was quantified as described in Materials and methods. Error bars indicate standard deviations. P values were $P=0.54$ for vL11ΔIR and RacL11 and $P=0.56$ for vL11ΔIR and vL11ΔIRR.

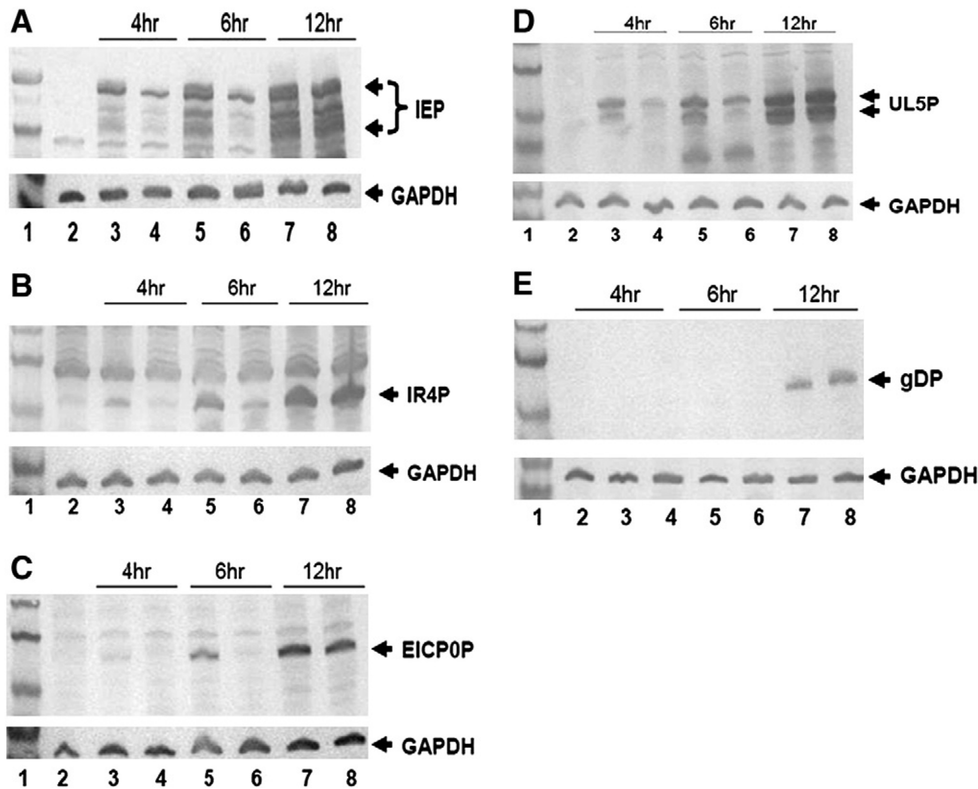

Fig. 7. Comparison of the expression of viral immediate-early, early, and late proteins in RK13 cells infected with RacL11 EHV-1 or vL11ΔIR by western blot analyses. Lane 1: protein markers; lane 2: mock-infected RK13 cells; lanes 3, 5 and 7: RacL11-infected RK13 cells; and lanes 4, 6 and 8: vL11ΔIR-infected RK13 cells. GAPDH was used to normalize protein loading. RK13 cells were infected with RacL11 EHV-1 or vL11ΔIR at a moi of 5, whole cell lysates were prepared, and viral proteins were detected as described in Materials and methods. Detection of (A) the immediate-early protein; (B) the early IR4 protein; (C) the early EICP0 protein; (D) the early UL5 protein; and (E) the late glycoprotein D.

(4 and 6 hpi), and there was reduced synthesis of the early viral proteins in cells infected with the IR deleted virus. However, by late times (12 hpi), the amounts of early proteins were similar in cells infected with either parental or IR-deleted virus. The pattern of delayed early protein synthesis is shown for the early regulatory proteins IR4P (Fig. 7B), EICP0P (Fig. 7C) and UL5P (Fig. 7D). Lastly, the synthesis of a late EHV-1 gene product, glycoprotein D, was also reduced in cells infected with the vL11ΔIR when compared to cells infected with parental virus (Fig. 7E). Therefore, these experiments showed that there was an overall delay in EHV-1 protein synthesis in cells infected with a virus mutant that harbored only one copy of the short region 12.7 kbp repeat sequence.

vL11ΔIR EHV-1 exhibited decreased virulence in CBA mice

Finally, experiments were carried out to determine if the deletion of the IR affected virulence in the well-characterized CBA mouse model of EHV-1 pathogenesis (Frampton et al., 2002; O'Callaghan and Osterrieder, 2008; Osterrieder et al., 1996b; Smith et al., 2005; von Einem et al., 2004). CBA mice infected intranasally with RacL11, vL11ΔIR, or vL11ΔIRR showed clinical signs of huddling, ruffled fur, lethargy, and significant loss of body weight from 2 dpi whereas mock-infected control mice continued to gain weight and showed no clinical signs throughout the observation period (Fig. 8A). Mice infected with either RacL11, vL11ΔIR, or vL11ΔIRR lost 20% or more of total body weight by 3 dpi. Overall comparison of body weight loss of the three mouse groups infected with RacL11, vL11ΔIR, or vL11ΔIRR showed there was no significant difference ($p>0.8$). Mortality was observed in all groups of mice, and 100% (9 of 9), 11% (1 of 9), and 89% (8 of 9) of mice infected with parental EHV-1, IR-deleted virus, and IR-restored virus, respectively, succumbed to infection. Differences in the virulence among RacL11, vL11ΔIR and vL11ΔIRR were examined by monitoring the percent survival as shown in Fig. 8B. Survival curve comparisons showed that survival following infection with the RacL11 (or vL11ΔIRR) and vL11ΔIR was significantly different ($p<0.008$), indicating that the deletion of the IR led to decreased virulence of EHV-1 in this animal model. Lung virus titers of mice necropsied at various days post infection were approximately 10-fold higher in the case of mice infected with the parental virus and vL11ΔIRR virus when compared to those of animals infected with the vL11ΔIR (Fig. 8C). Similarly, high virus titers were seen in the case of lungs of mice that had succumbed to infection with wt and IR-restored EHV-1 (Fig. 8D). Overall, the animal studies revealed that absence of the IR sequence attenuated EHV-1 virulence in the mouse, and also reduced the ability of the mutant virus to replicate in the lung to high titers.

Discussion

Sequence arrangement of the EHV-1 genome (Henry et al., 1981; O'Callaghan and Osterrieder, 2008; Ruyechan et al., 1982; Whalley et al., 2007) is classified as group D of herpesviruses that have sequences at one terminus which are repeated in an inverted orientation internally (Roizman, 1996; Roizman and Pellet, 2001). Such a structure is observed for the genomes of several members of the *Alphaherpesvirinae* subfamily, including human herpesvirus 3 (varicella-zoster virus), bovine herpesvirus 1, suid herpesvirus 1, gallid herpesvirus 1, equine herpesvirus 3, and equine herpesvirus 4 (Roizman, 1996; Roizman and Pellet, 2001). Herpesviruses are currently being engineered such that they may be considered for use as gene therapy vectors and development of recombinant vaccines (Rosas et al., 2008; Srinivasan et al., 2008; Yokoyama et al., 2009). Manipulation of the genome, such as the introduction or deletion of gene(s), can be carried out by homologous recombination utilizing full-length infectious genomes established as BACs in *E. coli* (Rudolph et al., 2002; Tischer et al., 2006). In herpesvirus genomes,

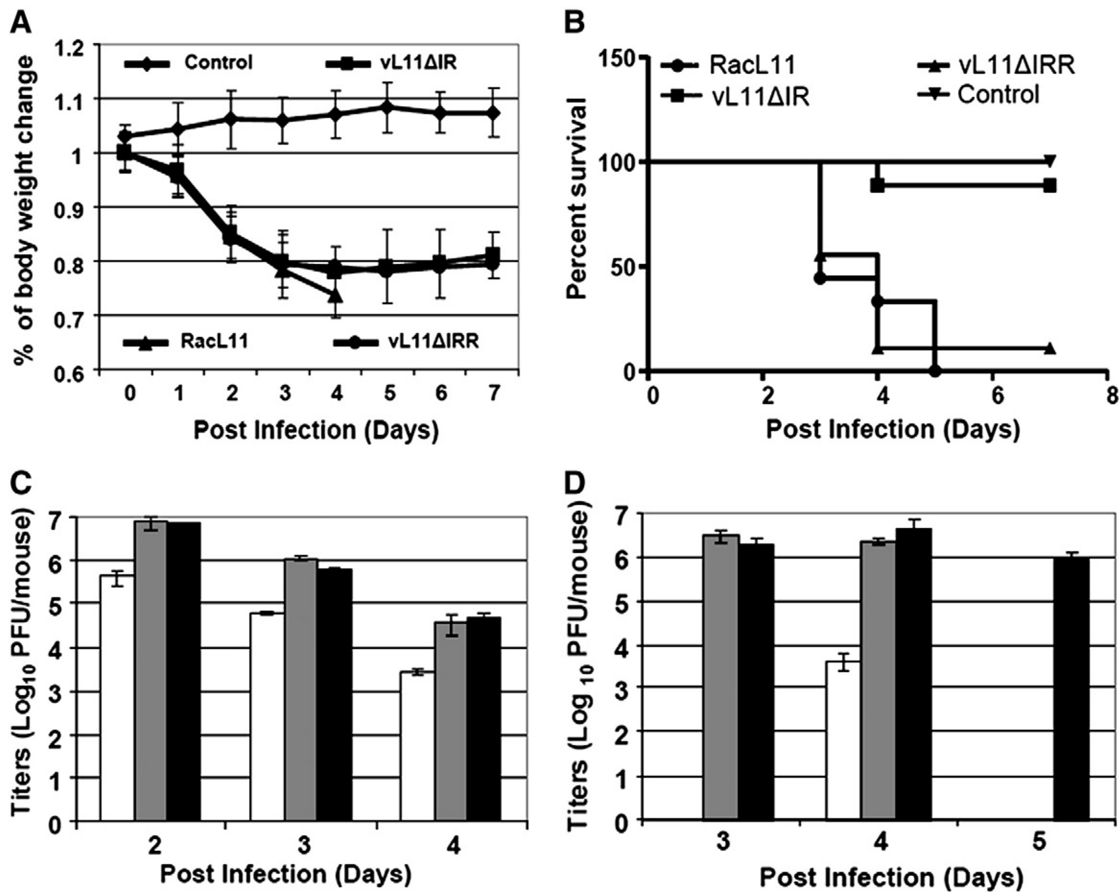

Fig. 8. Percentage change in body weight and percent survival of mock infected mice and mice infected with RacL11, vL11ΔIR, or vL11ΔIRR, and EHV-1 titers of mouse lungs. Mice were intranasally inoculated with sterile medium as control or infected with 1×10^6 PFU of RacL11 EHV-1, vL11ΔIR, or vL11ΔIRR, and total virus was isolated from mouse lungs as described in Materials and methods. Body weight was measured daily, and the Student's-t test was used to compare measurements of body weight between groups. Error bars indicate standard deviations. (A) Percentage change in body weight of control CBA mice (n = 5) or mice infected with RacL11 (n = 9), vL11ΔIR (n = 9), or vL11ΔIRR (n = 9). (B) Percent survival of mock infected mice (n = 5), and mice infected with RacL11 EHV-1 (n = 9), vL11ΔIR (n = 9), or vL11ΔIRR (n = 9). (C) Viral titers from lungs of live mice infected with RacL11 EHV-1 (n = 3, black bars), vL11ΔIR (n = 3, empty bars), or vL11ΔIRR (n = 3, gray bars) at days 2, 3, and 4 post infection. (D) Viral titers of lungs from mice that succumbed to infection with RacL11 (black bars), vL11ΔIR (empty bars), or vL11ΔIRR (gray bars). The number of mice that succumbed at each day during 3 dpi to 5 dpi are n = 4 (bar 1), n = 5 (bar 2), n = 1 (bar 3), n = 4 (bar 4), n = 1 (bar 5), and n = 3 (bar 6).

the presence of repeat sequences makes manipulation of some genes difficult, because deletion of a diploid gene may be rescued by the same gene located in the other repeat (Ahn et al., 2010; Boldogkoi et al., 1998). Therefore, it would be preferable to manipulate viruses such that the viral genome presents a simplifier structure but retains the ability to replicate and its major biological properties.

Here, we deleted the entire IR from the EHV-1 genome and characterized the biological properties of the resulting virus in cell culture and a small animal model. Deletion of the EHV-1 ~13 kbp IR revealed that one repeat is dispensable for virus replication, suggesting that construction of such a deleted virus is also possible for related herpesviruses with a genome that can assume one of two isomeric conformations. In addition, such a deletion mutant may be employed to accommodate the insertion and expression of foreign gene(s) that total to at least 13 kbp. The findings that the vL11ΔIR showed reduced plaque size and delayed growth in RK13 cells clearly suggest that the deletion of sequences including the genes within the IR affects the biological properties of EHV-1 in cell culture. Previous studies showed that the EHV-1 Us4, Us6, Us7, UL6, UL10, and UL20 gene products are involved in cell-to-cell spread of virus and virus egress (Frampton et al., 2002; Guggemoos et al., 2006; Neubauer and Osterrieder, 2004; Osterrieder et al., 1996a; von Einem et al., 2004), but none of these genes is located within sequences deleted here. The smaller plaque size and delayed growth of vL11ΔIR as compared to the parent virus may be explained by the roles of EHV-1 major regulatory genes IE and IR4 that are located within both inverted repeats (Grundy et al., 1989; Holden et al., 1994; Telford et al., 1992). The IEP as the essential sole immediate-early gene product *trans*-activates early and some late genes independently or synergistically with the IR4P (Garko-Buczynski et al., 1998; Holden et al., 1995; Smith et al., 1992). Therefore, loss of one copy of the diploid IE and IR4 genes could delay the progress of the EHV-1 gene expression program and result in reduced plaque size and delayed growth kinetics, possibly because protein levels are reduced. Indeed, there was a delay in the accumulation of viral gene products of all three classes in cells infected with the IR mutant virus (Fig. 7).

It has been known that EHV-1 exhibits a broad host range and replicates in a variety of cell types (O'Callaghan and Osterrieder, 2008; Trapp et al., 2005). Whalley et al. showed that EHV-4, although closely related to EHV-1, has very limited cellular tropism that could be broadened when the EHV-4 gD gene was replaced with the EHV-1 homolog (Whalley et al., 2007). That the tropism of vL11ΔIR was identical to that of the parental virus in the five cell types tested was interesting because our recent studies with an EHV-1 mutant deleted of both copies of the IR4 gene showed a major change in its tropism as compared to that of the wt EHV-1 (Breitenbach et al., 2009). Thus, a single copy of this auxiliary regulatory gene was sufficient for vL11ΔIR to replicate in the five cell types.

The virulence of EHV-1 in the CBA mouse model is well characterized by body weight loss and a significant mortality rate due to a massive inflammatory reaction in the lung mediated by the induction of cytokine/chemokine responses (Frampton et al., 2002;

O'Callaghan and Osterrieder, 2008; Smith et al., 2005). Previous studies showed that the gp2 protein encoded by the Us4 gene is a major EHV-1 virulence factor and that mutation of this gene attenuated the virus but did not inhibit virus replication in the murine lung (Frampton et al., 2002; Smith et al., 2005). However, deletion of both copies of the IR4 regulatory gene abolished EHV-1 virulence in the CBA mouse because the ΔIR4 mutant failed to replicate in the mouse lung (Breitenbach et al., 2009). Our finding that the vL11ΔIR was less virulent than the parental virus as judged by overall mortality was attributed to the inability of this ΔIR mutant to replicate to high titers in the murine lung. Whereas the EHV-1 mutant virus lacking both copies of the IR4 gene was completely avirulent (Breitenbach et al., 2009), the ΔIR virus that harbors and expresses one copy of the IR4 gene and one copy of the IR6 gene, a known determinant of virulence (Osterrieder et al., 1996b), could replicate in the mouse respiratory system and elicit a fatal outcome in a small percentage of the animals.

As noted (Roizman, 1996; Roizman and Pellet, 2001), herpesvirus genomes are classified into groups A to F with regard to their structural properties such as the number and location of repeat and inverted sequences and ability to exist in one, two or four isomeric arrangements. Herpesviruses such as EHV-1 (Henry et al., 1981; O'Callaghan and Osterrieder, 2008; Whalley et al., 2007) with genomes of group D have a fixed long region covalently linked to a short genomic region comprised of a pair of inverted repeat sequences that bracket the unique short segment. To our knowledge, this paper is the first report that describes a mutant herpesvirus of group D that lacks the entire internal inverted repeat and has as a consequence a genome that can only exist as a single isomer. Previous reports on the organization of the genome of herpes simplex virus 1 mutants by Roizman et al. (Jenkins and Roizman, 1986; Poffenberger and Roizman, 1985; Poffenberger et al., 1983) and a recent report (Sauer et al., 2010) describing a genomic mutant of human cytomegalovirus showed that these group E genomes that normally are composed as equal amounts of four isomers can exist in a single isomeric arrangement that fails to invert. These mutant forms of the genomes of HSV-1 and CMV and now EHV-1 that fail to undergo isomerization are capable of replication in cell culture with an efficiency similar to that of wild type virus. However, the v11LΔIR mutant exhibited a significant reduction in pathogenicity in the mouse, and these findings are similar to the situation with the noninverting HSV-1 mutant that was found to be profoundly attenuated *in vivo* (Jenkins et al., 1996; Jenkins and Martin, 1990).

Since the vL11ΔIR has reduced virulence *in vivo*, it would be of interest to ascertain if this mutant virus has the ability to spread to the central nervous system and/or establish latency in the natural host. Whatever its biological phenotype may be in the horse *in vivo*, the simplified genomic structure of the vL11ΔIR should enhance the manipulation of the six genes in the inverted repeat of the EHV-1 genome. Also, this virus with a truncated genome of ~137 kbp may prove to be a useful tool for delivery of foreign genes and/or development of a recombinant vaccine to combat EHV-1 infections in the equine.

Materials and methods

Cell culture and viruses

Mouse L-M, rabbit RK13, equine NBL-6, monkey Vero, and human HeLa cells used for viral propagation were maintained with Eagle's minimal medium supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, nonessential amino acids, and 5% (or 10%) fetal bovine serum. The pathogenic RacL11 EHV-1 strain (RacL11) was used as the parental virus in our studies (Ahn et al., 2007; Ahn et al., 2010; Breitenbach et al., 2009).

Construction of plasmids

PCR products were amplified using Accuprime *pfx* polymerase (Invitrogen, Carlsbad, CA), pRacL11 EHV-1 BAC (pRacL11) template, and appropriate primers. To construct the IR-deleted EHV-1, GalK BAC technology was used as previously described (Ahn et al., 2010; Warming et al., 2005). pRacL11 (Rudolph et al., 2002) was transformed into SW106 *E. coli* (Warming et al., 2005). The purified PCR product of the GalK marker harboring the EHV-1 IR flanking sequences (primers, 5′ ccg ggc cat atc tgg tca agg gtc acg ggc ccg cgc ccg aga gag agc ctg gcc cct gtt gac aat taa tca tcg gca 3′/5′ aca ccg tag tgg gtg agt gtg ggt ttt cca aac ata gct cga att cat tag ttc agc act gtc ctg ctc ctt 3′) was transfected into SW106 cells containing pRacL11. Positive colonies were selected on Gal positive selection agar plates (Warming et al., 2005) and confirmed by PCR amplification (left flanking region primers, 5′ atg atc ccg cag tta cag cct aca aac tgg 3′/5′ tag cac acc taa cct cct gag tgt gag cg 3′; right flanking region primers, 5′ agt tga tgg ata ggc gag cat ctc aaa caa g 3′/5′ tga aac atc tgc aac tgc gta aca aca gct tcg g 3′) of EHV-1 IR flanking regions (named pL11ΔIR-GalK). To remove the GalK marker from the intermediate, counter selection was performed as previously described (Ahn et al., 2010; Warming et al., 2005). Both flanking regions of the IR were combined by multiple rounds of PCR amplification (left flanking region primers, 5′ tag cac acc taa cct cct gag tgt gag cg 3′/5′ aga tgt ata tct gcc agg ctc tct ctc ggg cg 3′; right flanking region primers, 5′ aga tat aca tct act aat gaa ttc gag cta tgt ttg g 3′/5′ ttc tct ttg gat ggt ata aga caa tcg tcg 3′; combined flanking region primers, 5′ tag cac acc taa cct cct gag tgt gag cg 3′/5′ ttc tct ttg gat ggt ata aga caa tcg tcg 3′). Purified PCR amplification products of the IR flanking region were transfected into SW106 cells containing pL11ΔIR-GalK, and positive colonies were selected on the Gal counter selection plates as described (Ahn et al., 2010; Warming et al., 2005). To generate the revertant virus recovering the entire IR sequence, plasmid (pAYC177-XbaI I/B1:harboring the entire IR sequence and IR flanking sequences of the EHV-1 genome) (Ahn et al., 2007) was electroplated into SW106 cells containing pL11ΔIR-GalK (named pL11ΔIRR), and positive colonies were selected on the Gal counter selection plates as described (Ahn et al., 2010; Warming et al., 2005). The identity of the resulting final BAC clone named pL11ΔIR and pL11ΔIRR was confirmed by PCR targeting the flanking sequences of the IR-deleted BAC (primers, 5′ aca cat tga gtc ctt tct act ctc ctc ctc gg 3′/5′ ttc tct ttg gat ggt ata aga caa tcg tcg 3′) and the flanking region of the revertant clone in which the IR had been restored (primers, 5′ ccg ttt gaa tgc gat tgg tgg g 3′/5′ gcg ttg tat cta gca gcc cac g 3′.and 5 aga gta ggc gtt cca tcc acg 3′/5′ gac cct acc aaa ggc gtg tag g 3′). The deletion and restoration of the entire IR was ultimately verified by sequence analysis of amplified PCR amplicons, BamHI digestion, and Southern blot analysis.

Generation of recombinant EHV-1 from cloned BAC DNA and DNA isolation from virus-infected RK13 cells

Purified pL11ΔIR DNA or pL11ΔIRR DNA and a plasmid DNA containing the EHV-1 Us4 gene (gene 71) (Rudolph et al., 2002); 40) were co-transfected into RK-13 cells by using the BD CalPhos Mammalian Transfection Kit (Clontech, Mountain View, CA) according to the manufacturer's directions. At three days post transfection (dpt), supernatants were harvested from DNA transfected RK13 cells, and virus reconstitution was examined by plaque assay. EHV-1 plaques lacking green fluorescence (suggesting replacement of the gene encoding green fluorescent protein (GFP) with the *EUs4* sequence) were isolated by three rounds of plaque purification, and the resulting viruses were named vL11ΔIR or vL11ΔIRR. Viruses were propagated in RK13 or NBL-6 cells, and titered according to standard procedures (Perdue et al., 1974). The deletion or restoration of the entire IR in the respective viruses was confirmed by the PCR amplification of the IR-flanking regions using virus-infected RK13

cell DNA as a template and primers (5′ ttc tct ttg gat ggt ata aga caa tcg tcg 3′, 5′ aca cat tga gtc ctt tct act ctc ctc ctc gg 3′). DNA from EHV-1-infected RK13 cells was prepared by using DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions, and used for PCR as a template.

Southern and western blot analyses

To confirm the insertion of the GalK marker into pRacL11, the removal of the GalK marker from pL11ΔIR-GalK, and the replacement of the GalK marker from pL11ΔIR-GalK with the entire IR sequences, BamHI digested pRacL11, pL11ΔIR-GalK, pL11ΔIR, and pL11ΔIRR were separated on a 0.8% agarose gel and transferred onto a positively charged nylon membrane (Ambion, Austin, TX) by using a semi-dry electroblotter (Bio-Rad Laboratories, Hercules, CA). After DNA transfer, the membrane was placed on blot paper saturated with 0.5 M NaOH for 15 min, briefly washed with 2×SSC, and incubated at 80 °C for 1 h. The PCR amplicon of the GalK marker (primers, 5′ cct gtt gac aat taa tca tcg gca tag 3′/5′ act gtc ctg ctc ctt gtg atg g 3′) was end-labeled with [γ-^{32}P]ATP (New England Nuclear Corporation, Boston, MA) and T4 polynucleotide kinase (Promega, Madison, WI) according to the manufacturer's directions. Radiolabeled probe was denaturated by adding 1/10 volume of 3 M NaOH, incubated for 10 min at room temperature, and then neutralized by adding an equal volume of 1 M Tris–HCl (pH 7). Prehybridization, hybridization, and washing were performed using a NorthernMax Kit (Ambion, Austin, TX) followed by autoradiography using a phosphorimage screen and the molecular imager FX system (Bio-Rad Laboratories). For protein detection, RK13 cells were infected with parental RacL11 virus or vL11ΔIR at a multiplicity of infection (moi) of 5, and cells were harvested at 4, 6, and 12 h post infection (hpi). Whole cell lysates of virus-infected cells were separated by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose membrane (Ambion) by using a semi-dry electroblotter (Bio-Rad Laboratories). The IE, early (E; IR4, EICP0, UL5), and late (L; gD) proteins were detected by using monospecific rabbit polyclonal antibodies (Bowles et al., 1997; Caughman et al., 1995; Flowers and O'Callaghan, 1992; Holden et al., 1994; Zhao et al., 1995) as primary antibodies and anti-rabbit IgG[Fc]-alkaline phosphatase conjugate (Promega) as the secondary antibody. Proteins were visualized by incubating the membrane containing blotted proteins in an AP conjugate substrate (AP conjugate substrate kit, Bio-Rad Laboratories) according to the manufacturer's directions.

Plaque morphology, growth kinetics, and cell tropism

For the plaque assays, RK13 cell monolayers were infected with serial 10-fold dilutions of the respective viruses and overlaid with medium containing 1.5% methylcellulose at 2 h after infection. At 4 days post infection (dpi), plaques were fixed with 10% formalin, stained with 0.5% crystal violet, and counted (Perdue et al., 1974). Plaque sizes were measured by using the ImageJ software program (http://www.rebweb.nih.gov/ij/). For single step growth kinetics, RK13 cells in 25 mm flasks were infected at an moi of 0.2 with the respective viruses. After 1 h of viral attachment at 4 °C, cells were washed with PBS, 4 ml of growth medium was added, and viruses were harvested at designated time points. To determine intracellular viral titer, virus infected cells were washed with PBS followed by adding 4 ml of growth medium, and freeze and thaw cycle, and the virus was titered described above. To determine extracellular viral titer, supernatants were used. To determine the cellular tropism, five cell types (L-M, RK13, NBL-6, Vero, and HeLa cells) were infected at an moi of 1 with mutant, revertant, or parental viruses. After virus attachment for 1 h at 4 °C, the virus-infected cells were washed with PBS followed by adding normal growth medium, and total viral titers were examined at 3 dpi.

Quantitative real time (RT)-PCR

To compare the number of viruses attached to the host cells, quantitative real time PCR assays were performed using the DNAs from virus infected cells as the template, the EHV-1 UL3 ORF region specific primer set (5′ ttt gaa ttc gcc acc atg ggg gcc tgc tgc tcc tct ag 3′/5′ tta tgt aca att cag acc gta tat ggt gtt ttg c 3′), rabbit GAPDH gene specific primer set (GeneBank:L2396.1; 5′ cat gtt tgt gat ggg cgt gaa cca 3′/5′ taa gca gtt ggt ggt gca gga t 3′), and iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's directions. Confluent RK13 cells in the 6 well plates were infected with RacL11, vL11ΔIR, or vL11ΔIRR at an moi of 10. Virus infected RK13 cells were incubated at 4 °C for 2 h and then 37 °C for 30 min followed by washing virus infected RK13 cells with PBS. Total DNAs including cellular and viral DNAs were prepared from virus infected RK13 cells by using DNeasy Blood & Tissue kit (Qiagen Inc. Valencia, CA), and used as the template for quantitative RT-PCR. Cycle threshold (Ct) values to detect the viral genome were normalized by using Ct values of housekeeping GAPDH gene amplification.

Animal experiments

Animal experiments were conducted as described previously (Ahn et al., 2010; Frampton et al., 2002; Osterrieder et al., 1996b; von Einem et al., 2004). Groups of 4-week-old CBA female mice (n = 12/group) were inoculated intranasally with sterile medium (mock infection) or 1×10^6 pfu of vL11ΔIR, vL11ΔIRR or RacL11. Mice were observed daily and weighed from prior to inoculation, and weights were compared. Virus isolation from the lungs of mice infected with vL11ΔIR, vL11ΔIRR, or RacL11 (n = 3/group) at 2, 3, and 4 dpi for live mice and at the time of death for dead mice was performed by using silica beads and BeadBeater (BioSpec Products, Inc., Bartlesville, OK) according to the manufacturer's directions, and viral titers were determined as described above. For statistical analyses, two-tailed Student's-*t* test was performed by using the Excel software program (Microsoft Corporation, Redman, WA). Virulence as judged by percent survival data was determined by the Log-rank (Mantel–Cox) test using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

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