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Deletion of the *UL4* gene sequence of equine herpesvirus 1 precludes the generation of defective interfering particles

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Abstract

Serial, high multiplicity passage of equine herpesvirus 1 (EHV-1) leads to the generation of defective interfering particles (DIP). EHV-1 DIP inhibit and interfere with the replication of standard EHV-1, establishing a state of persistent infection. These DIP package severely truncated and rearranged forms of the standard viral genome. Contained within the DIP genome are only three genes: *UL3*, *UL4*, and a unique hybrid gene (*Hyb*). The hybrid gene forms through a recombination event that fuses portions of the early regulatory *IR4* and *UL5* genes and is essential for DIP-mediated interference. The *UL4* gene is an early gene dispensable for lytic replication and inhibits viral and cellular gene expression. However, the contribution of the *UL4* gene during DIP-mediated persistent infection is unknown. Here, we describe the generation of a completely deleted *UL4* virus and its use to investigate the role of the *UL4* gene in the generation of the defective genome. Deletion of the *UL4* gene resulted in delayed virus growth at late times post-infection. Cells infected with a mutant EHV-1 that lacked expression of the *UL4* protein due to an inserted stop codon in the *UL4* gene produced defective particles, while cells infected with a mutant EHV-1 that had the complete *UL4* gene sequence deleted were unable to produce DIP. These data suggest that the *UL4* gene sequence, but not the *UL4* protein, is critical for the generation of defective interfering particles.

Keywords

Equine herpesvirus 1; *UL4* gene; Defective interfering particles; Persistent infection; Deletion mutant

Introduction

The first report of a defective interfering (DI) virus was more than 60 years ago when von Magnus [1] described interference after the formation of an “incomplete” influenza virus, though the term “DI particle” (DIP) was not introduced until 1970 [2]. At present, it is accepted that nearly all viruses are capable of producing defective interfering particles including animal, plant, and even fungal viruses irrespective of the type of nucleic acid being packaged, single- or double-stranded DNA or RNA [3, 4]. By definition, DIP must (1) contain a portion of the standard viral genome, (2) be composed of the normal structural proteins, (3) replicate only in the presence of the standard virus, and (4) specifically interfere with the propagation of the homologous standard virus [2]. The interference with standard

virus replication can allow for the establishment of DIP-mediated persistent infection. Though the great majority of defective particles impede the replication of helper virus, not all defective viruses are interfering. Defective Epstein-Barr virus (EBV) is unique in that the rearranged DNA transactivates latent EBV, enhancing rather than interfering with lytic replication [5, 6]. Interestingly, the defective EBV DNA was detected in oral epithelial lesions in patients afflicted with oral hairy leukoplakia [7].

It is well-established that high multiplicity passage of animal viruses leads to the generation of defective interfering particles [1–3, 8, 9]. Equine herpesvirus 1 (EHV-1), an alphaherpesvirus responsible for severe respiratory, neurological, and abortigenic disease in equines worldwide, was also demonstrated to generate DIP after serial, high multiplicity passage both in vitro and in vivo [10–12]. The EHV-1 DI particle packages a concatameric genome that lacks >95 % of the standard viral DNA sequences (~150 kbp) [13–15]. The DIP monomer (Fig. 1) ranges in size from 5.9 to 7.3 kbp and is comprised of sequences from three distinct regions within the standard genome: (1) the left terminus which includes the *UL3* and *UL4* genes and a 3' portion of the *UL5* gene, (2) the region between the unique long (*U_L*) and the internal inverted repeat (IR), and (3) an interior segment of the terminal IR that includes the 5' portion of the *IR4* gene and the origin of replication (ORI). Also present in the DIP genome are the cleavage-packaging signals. The exact events that lead to the generation of the DIP monomer and the inclusion of the various regions are still unclear, as is the variability in size. However, it is known that the sequences from the left terminus and IR are fused by homologous recombination that occurs at a conserved eight nucleotide sequence found within the two regions [16]. This recombination event gives rise to a unique hybrid gene (*Hyb*) that is translated into a protein that contains 196 amino acids from the amino-terminus of the *IR4* protein and the carboxy-terminal, 68 amino acids of the *UL5* protein. Previous studies demonstrated that the hybrid protein is responsible for altering viral gene expression during DIP-mediated persistent infection, and this inhibitory function is critical for the observed interference with standard viral replication, though not required for DIP production [17–19].

The *UL3* and *UL4* genes are conserved perfectly from the left terminus of the standard EHV-1 genome, and until recently, had no ascribed function(s). Both *UL3* and *UL4* are early genes not required for lytic replication, but they have inhibitory activity against a wide range of EHV-1 promoters [20, 21]. The role of either gene in DIP-mediated persistent infection is unknown, but the fact that they are preserved within the DIP genome indicates they may serve an essential function for the establishment or maintenance of persistence. In this study, we generated a mutant EHV-1 that lacked the entire *UL4* gene sequence and demonstrated that the physical presence of the *UL4* DNA, but not a functional *UL4* protein, was critical for the generation of DI particles. This suggests that the *UL4* DNA sequence helps stabilize or orient the standard viral genome in such a fashion that permits the homologous recombination that leads to the formation of the DIP genome.

Materials and methods

Cell culture, viruses, and DIP passage

Mouse L-M and rabbit RK13 cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with either 5 or 10 % fetal bovine serum at 37 °C in a 5% CO₂ incubator. Viruses used in this study included the wild-type (WT) RacL11, *UL4aa18stop* mutant virus that lacks *UL4* protein expression generated previously [20], and the Δ *UL4* and Δ *UL4Res* viruses that are discussed below. Defective interfering particles were generated by serially passaging the virus at high multiplicity in mouse L-M cells [11, 12, 15]. Wild-type, Δ *UL4*, Δ *UL4Res*, and *UL4aa18stop* mutant viruses were passaged every 24–48 h for up to 6 total passages.

Detection of the DIP hybrid gene

After DIP passage, the L-M cells were pelleted and DNA was isolated using DNA STAT-60 (Tel-Test, Friendswood, TX) following manufacturer's procedure. PCR analysis was used to detect the hybrid gene with primers 1 and 2 (Table 1) that spanned the site of recombination between the *IR4* and *UL5* genes. Primers 9 and 10 are specific for the IE gene and were used as a control for DNA quality. The PCR products were electrophoretically separated on a 0.8 % agarose gel and visualized after ethidium bromide staining.

Generation and confirmation of a completely *UL4*-deleted EHV-1

A RacL11 bacterial artificial chromosome (BAC) deleted for the entire *UL4* gene was generated through *galK* BAC recombineering [20, 22–24]. The starting $\Delta UL4(galK^+)$ BAC was generated previously [20] and contained the *galK* gene in place of the *UL4* gene. PCR splicing was utilized to completely delete the *UL4* open reading frame (ORF). This strategy used two PCR reactions to (1) generate separate flanking sequences and (2) join the flanking sections into a contiguous junction. The left flanking sequence used primers 3 and 4 (Table 1), while the right flanking sequence used primers 5 and 6 (Table 1). The second PCR reaction to join the flanking sequences into a contiguous junction fragment used primers 3 and 6 (Table 1). These last primers were also used to generate an authentic *UL4* gene revertant fragment using the parental RacL11 BAC [24]. The completely deleted *UL4* gene fragment ($\Delta UL4$) and authentic *UL4* gene revertant ($\Delta UL4Res$) PCR products were transformed into SW106 *E. coli* harboring the $\Delta UL4(galK^+)$ RacL11 BAC. Bacteria were plated onto negative selection plates containing glycerol and 2-de-oxygalactose. Resulting colonies were screened by PCR for the correct sequences between the *UL3* and *UL5* genes, as well as DNA sequence analysis. The *UL4*-deleted and *UL4*-restored BACs, isolated from the selected bacterial colonies, were examined by *Bam*H I restriction enzyme digestion and Southern blot analysis. Mutant and revertant RacL11 viruses were reconstituted from the BACs by transfecting RK13 cells with both the BAC and a plasmid containing the EU_s4 sequences that were removed during generation of the BAC [24], effectively replacing the green fluorescent protein (GFP) gene and the BAC sequences with authentic viral sequences that encode glycoprotein 2. Plaques lacking GFP expression were plaque purified for three rounds and then propagated to high titer on RK13 cells.

Southern and western blot analyses

Mutant and revertant RacL11 BACs were verified by *Bam*H I restriction enzyme digestion and then electrophoretically separated on a 0.6 % agarose gel. Digested DNA was transferred onto a positively-charged nylon membrane (Ambion, Austin, TX) using a semi-dry electroblotter (Bio-Rad Laboratories, Hercules, CA). The transferred DNA was hybridized with a fragment of the *UL4* gene that was a PCR product, amplified using primers 7 and 8 (Table 1), and end-labeled with [γ -³²P]ATP (New England Nuclear Corporation, Boston, MA) by T4 polynucleotide kinase (Promega) in ULTRAhyb ultrasensitive hybridization buffer (Ambion). Unbound radio-labeled probe was washed away with 2× SSC/0.1 % SDS followed by 0.1× SSC/0.1 % SDS. A final wash with 2× SSC was completed, then the membrane was wrapped in plastic, exposed to a phosphor screen, and scanned on the molecular imager FX system (Bio-Rad Laboratories). Western blot analyses were performed using protein lysates collected from RK13 cells infected with virus or mouse L-M cells passaged with virus preparations enriched with DI particles. The protein samples were separated on 10 % SDS polyacrylamide gels and transferred to nitrocellulose membranes using the semi-dry electroblotter. Membranes were blocked with either 1 or 5 % dry milk in PBS/0.3 % Tween-20 (PBST). Membranes were rinsed with PBST and incubated with EHV-1 specific primary antibodies as indicated, followed by goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (AP; Sigma, Saint Louis, MO). Tubulin was used as a loading control and was detected with anti- β -tubulin antibody

followed by goat anti-mouse secondary antibody (Sigma). Protein-antibody complexes were visualized using the AP color reagent (Bio-Rad Laboratories) following the manufacturer's instructions. Generation and characterization of the polyclonal, mono-specific antibodies to the UL3, UL4, and IR4 proteins were described previously [20, 25, 26].

Growth kinetics and plaque morphology

Rabbit kidney (RK13) cells were seeded into 60 mm dishes to 80 % confluency and infected at an MOI of 5. Virus was harvested from the supernatant at the indicated times post-infection and serially diluted to perform plaque assays on RK13 monolayers. Infected monolayers were incubated with medium containing 1.5 % methylcellulose, and plaques were enumerated after 3 days by fixing with 10 % formalin and staining with 0.5 % methylene blue [27]. Plaque area was measured by means of Image J software (NIH, <http://rsbweb.nih.gov/ij/>).

Results

Generation of *UL4*-deleted EHV-1

Previously, we reported the generation and characterization of a mutant EHV-1 that lacked expression of the UL4 protein due to the insertion of a stop codon in the UL4 ORF that corresponds to amino acid position 18 [20]. This mutant virus was named UL4aa18stop (Fig. 1) and was demonstrated to display similar replication and virulence as compared to wild-type EHV-1 in a mouse model of EHV-1 pathogenesis. The fact that *UL4* was dispensable for lytic replication provided no additional information pertaining to its role in DIP-mediated persistent infection. It was hypothesized that the UL4 protein was important for the establishment and/or maintenance of DIP-mediated persistence. However, it is conceivable that the UL4 DNA sequence itself is important due to the nature of the recombination event that occurs within 450 nucleotides of the *UL4* start codon to produce to the DIP genome. To distinguish between these possibilities, another mutant virus was generated, which lacked the complete *UL4* gene sequence. The *galK* BAC technology was used to delete the *UL4* gene. The *galK* gene was replaced with a DNA segment that joined the flanking regions around the *UL4* gene that include the *UL3* and *UL5* genes (Fig. 1). The resulting *UL4*-deleted BAC was termed Δ UL4. A revertant BAC was also created to restore the *UL4* gene (Δ UL4Res), and these BACs were characterized through *Bam*H I restriction enzyme digestion to affirm that the deletion of the *UL4* gene had no deleterious effects elsewhere in the genome (Fig. 2). As can be seen, the overall digestion patterns of the wild-type RacL11, Δ UL4, and Δ UL4Res BACs were similar with the exception of the second largest band, denoted by the asterisk (*). Deletion of the 603 nucleotides of the *UL4* gene caused the band to be shifted to a lower molecular weight that appeared to comigrate with the band below it. Southern blot analysis of the digested genomes revealed that the *UL4* gene was removed from the Δ UL4 BAC and restored in the Δ UL4Res BAC (Fig. 2). Infection of the RK13 cells recapitulated the smaller plaque phenotype observed previously with a virus that lacks UL4 protein expression (Fig. 2) [20].

Δ UL4 EHV-1 displayed delayed growth

Upon generating and verifying that the *UL4*-deleted EHV-1 was correct, its ability to replicate was assessed through a growth kinetics experiment. Wild-type, Δ UL4, and Δ UL4Res viruses were infected into RK13 cells, supernatant media were collected at the indicated times post-infection, and infectious virus was enumerated via plaque assay (Fig. 3). Wild-type and Δ UL4Res viruses grew with indistinguishable kinetics and to similar titer. However, the Δ UL4 virus appeared to have a delayed growth at late times post-infection resulting in slightly decreased viral titers. Though statistically significant, the delay in viral

growth exhibited by the Δ UL4 virus was not considered to be biologically significant to preclude its use in this study.

Cells infected with DI particles over-expressed the UL4 protein

The recombination event that produces the DIP genome also results in deletion of a tremendous amount of the standard viral genome [13, 14]. The DIP monomer is between 5.9 and 7.3 kbp in length and is replicated in a head-to-tail concatameric fashion such that it is packaged at a size comparable to that of the standard viral genome (~150 kbp). This reiterated nature of the DIP genome results in ~20 copies of each of the three genes being packaged per particle. Therefore, it is likely that expression from each reiterated gene would result in over-expression of the DIP proteins [28]. In fact, over-expression of proteins in cells infected with DI particles was also described for other herpesviruses including the ICP4 and ICP8 proteins of herpes simplex virus [29, 30] as well as some pseudorabies virus proteins expressed in cells rich in DIP [31]. Hence, the level of protein expression was determined for the IR4/UL5 hybrid, UL3 and UL4 proteins through western blot analysis. Protein lysates were collected from mock infected cells, cells infected with wild-type EHV-1, or cells that were passaged with DIP preparations. As shown in Fig. 4, the hybrid protein was not detected in either mock or wild-type infected cells, but was abundant in the cells infected with DIP preparations. It was reported previously that the hybrid protein migrates as a family of proteins between 36 and 42 kilodaltons [15]. When immunoblotted for either the UL3 or UL4 protein, it was observed that both proteins were over-expressed in the cells infected with DIP preparations as compared to cells infected with standard EHV-1. Densitometric analysis indicated that the levels of these proteins were up to 5-fold greater in the DIP infected cells than in wild-type infected cells (data not shown).

UL4 DNA sequence was required to produce DIP

With the observations that the UL4 protein was over-expressed in cells infected with DIP preparations and that the UL4 protein can inhibit gene expression, it is possible that the UL4 protein is important for the establishment and/ or maintenance of DIP-mediated persistent infection. The UL4 protein may play a critical role in inhibiting standard viral gene expression through a gene dosage effect, allowing for persistence. But, as mentioned before, it might also be possible that the physical presence and location of the *UL4* DNA sequence are required for the correct recombination to occur between the unique long (U_L) and terminal IR regions to produce the DIP genome. To ascertain whether it is the *UL4* DNA sequence or a functional UL4 protein that is required for DIP production, wild-type (WT) EHV-1, Δ UL4, Δ UL4Res, and UL4aa18-stop viruses were passaged in mouse L-M cells every 24–48 h for 6 total passages. From each passage, cells were collected and DNA was isolated. The DNA samples were assessed for the presence of the IR4/UL5 hybrid gene by means of PCR and primers (arrowheads, Fig. 1) that spanned the recombination site unique to the hybrid gene. As a positive control for the production of DIP and the hybrid gene, DNA was included from a virus preparation known to contain DI particles (DI.3 p3). PCR for the IE gene was also included as a control for DNA quality. Cells serially passaged with the WT EHV-1 were positive for the hybrid gene at passages 3, 5, and 6 (Fig. 5). When the Δ UL4Res virus was used, the hybrid gene was detected in passages 1, 5, and 6. Passaging the UL4aa18stop mutant virus that lacks UL4 protein production produced DI particles, as evidenced by the presence of the hybrid gene in passages 1 and 3. However, L-M cells infected with the Δ UL4 virus that lacks the complete *UL4* gene sequence were unable to produce any DIP, indicated by the absence of the hybrid gene from all six passages (Fig. 5). The IE gene was consistently detected in cells passaged with each of the viruses. The variability in detection of the hybrid gene was expected based on reports from others that repeated serial passage of virus leads to a coincidental cyclic production of standard virus and defective particles [32, 33]. These data demonstrated that a functional UL4 protein was

not required for the generation of defective interfering particles. Rather, the presence of the *UL4* DNA sequence was necessary for the production of DIP, suggesting that the physical presence of the *UL4* gene plays a role.

DIP production was further examined by western blot analysis for the hybrid protein (Fig. 6). Protein lysates were collected at passages 3 and 6 and immunoblotted with the IR4 antibody that recognizes the hybrid protein. The hybrid protein is distinguished from the IR4 protein based on size. The IR4 protein is 293 amino acids and migrates in a poly-acrylamide gel between 42 and 47 kDa, while the hybrid protein is 264 amino acids and migrates as a family of proteins between 36 and 42 kDa. The hybrid protein was detected in both passages from cells infected with WT, Δ UL4Res, and UL4aa18stop EHV-1, but not in cells infected with Δ UL4 (Fig. 6). These results further indicated that the *UL4* gene sequence was important for the generation of DIP, but that a functional UL4 protein was not essential.

Discussion

Previous studies concerning EHV-1 defective interfering particles demonstrated that the highly expressed IR4/UL5 hybrid protein was capable of altering gene expression and was critical for the ability of DIP to interfere with standard virus replication [17–19]. It was demonstrated that a recombination event resulted in the deletion of a tremendous amount of the standard genome and the formation of the unique hybrid gene. The site of recombination was identified as an eight nucleotide sequence that was located within the *UL5* gene that mapped near the left terminus of the genome and within the *IR4* gene located in the terminal inverted repeat of the standard viral genome [16]. There are more than 110 kbp separating the eight nucleotide sequences in the linear genome and more than 12 kbp between the sequences during replication of the genome when the termini are fused to form the concatamers before cleavage and packaging. It is intriguing that these eight nucleotide sequences, surrounded by otherwise very limited identity, are capable of promoting the recombination between such significantly separated regions of the genome. The minimum requirements for and the mechanism by which the recombination occurs have not been investigated and are still poorly understood.

In the present study, we reported findings that suggested the *UL4* DNA sequence was required for the production of DI particles. A mutant virus that lacked the complete *UL4* ORF was generated and characterized. Although Δ UL4 EHV-1 appeared to have delayed growth and a slightly decreased total titer at late times post-infection (Fig. 3), this mutant virus was employed alongside another mutant EHV-1 that was deficient in UL4 protein expression, while still retaining the *UL4* ORF (UL4aa18stop). These viruses as well as wild-type EHV-1 were passaged serially at high multiplicity in mouse L-M cells to examine the ability of each virus to generate DI particles. Through PCR and western blot analysis, it was determined that the hybrid gene and protein were detectable in serially passaged cells infected with wild-type, Δ UL4Res, and UL4aa18stop EHV-1. In contrast, neither the hybrid gene nor the hybrid protein was present in cells infected with the Δ UL4 EHV-1 (Figs. 5, 6). These results indicated that a functional UL4 protein was not required for the generation of DIP. Conversely, the production of DIP appeared to be dependent on the presence of the *UL4* DNA sequence. We cannot exclude the possibility that DIP still form in the absence of the *UL4* ORF, but the process may be so inefficient that it was undetected in our assays.

In the absence of the UL4 DNA sequence and the subsequent inability of the Δ UL4 virus to produce DIP, it was hypothesized that the Δ UL4 virus would replicate to very high titers. This should be a functional consequence because no defective particles would be produced to interfere with viral replication. However, this predicted outcome was not observed. When infectious viral titers from passage 6 were compared to those of the wild-type and Δ UL4Res

viruses, the Δ UL4 virus displayed a 1,000-fold decrease in infectious titer as determined by plaque assay (data not shown). Additionally, when standard viral genomic DNA was examined after 6 passages via quantitative real-time PCR, an approximately 23-fold decrease in the amount of standard Δ UL4 genomic DNA was observed (data not shown). The large disparity between the amount of viral genomic DNA and infectious titer might be explained by a defect in the switch from early to late gene expression in the absence of the UL4 protein. This possibility is the focus of ongoing work.

The specific function that the *UL4* DNA sequence affords to the generation of DI particles is not clear. The site of recombination located within the *UL5* gene is less than 450 nucleotides upstream of the *UL4* ORF. Therefore, it is possible that the *UL4* DNA sequence is important for stabilizing or orienting the left terminus of the standard genome to allow for recombination to occur. Secondary structures within the *UL4* DNA sequence may position the terminal inverted repeat in close proximity to the left terminus of the genome. This juxtapositioning would then allow for the eight nucleotide recombination sites to be aligned before the recombination event. Alignment through a third, non-participating sequence has been proposed as a model of recombination in other systems, whereby a sequence not directly involved in the recombination plays a role in positioning the participating sequences [34]. Mocarski and colleagues suggested this model might be responsible for the generation of a defective genome in herpes simplex virus [35]. Whether this is the mechanism by which the *UL4* DNA sequence participates in the generation of DI particles is yet to be determined. Extensive structural analysis would be required to determine whether *UL4* DNA secondary structures are involved, but it is an attractive explanation for the requirement of the physical presence of the *UL4* ORF in the production of the defective interfering particles.

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Fig. 1.

Schematic diagram detailing the structure and organization of the EHV-1 standard and mutant genomes as well as the genome of defective interfering particles. *UL4aa18stop* EHV-1 contains a stop codon (*) that corresponds to amino acid position 18 to prevent *UL4* protein expression. $\Delta UL4$ EHV-1 lacks the complete *UL4* ORF. *Black arrowheads* flank the site of recombination and indicate the position of PCR primers for the detection of the *IR4/UL5* hybrid gene (hybrid protein contains the 196 N-terminal amino acids of the *IR4* protein and the 68 C-terminal amino acids of the *UL5* protein). *STD* standard virus, *DIP* defective interfering particles, U_L unique long, *IR* inverted repeat, U_S , unique short, *CPS* cleavage-packaging signal, *ORI* origin of replication

Fig. 2.

BAC DNA was subjected to *Bam*HI restriction enzyme digestion (*upper left panel*) and Southern blot analysis (*lower left panel*) to verify that deletion and restoration of the *UL4* gene occurred correctly and did not introduce spurious recombination elsewhere in the genome. Asterisks (*) denote the shift in molecular weight of the band that contains the *UL4* DNA sequence. Southern blotting was completed using a radio-labeled probe specific for the *UL4* DNA sequence. Images of representative plaques (*upper right panel*) are included to demonstrate that each virus replicated. RacL11 parent virus (WT) and ΔUL4Res display similar plaque sizes, while ΔUL4 displayed a smaller plaque size (*lower right panel*). Error bars represent standard deviation. Statistical analysis was performed by means of a one-way ANOVA. *cl* clone; *WT* wild-type RacL11 parent virus

Fig. 3.

Single-step growth curves illustrating the efficiency of replication of wild-type, Δ UL4, and Δ UL4Res EHV-1. RK13 cells were infected at MOI 5. Wild-type and Δ UL4Res viruses replicated with indistinguishable kinetics, while the Δ UL4 virus had a delay in replication and decreased total titer at late times post-infection. *Error bars* represent standard deviation. Statistical analysis was performed on the geometric means of log-transformed data by means of a two-way ANOVA [27]

Fig. 4.

Western blot analysis was utilized to examine the protein levels of wild-type infected cells and cells passaged with DIP preparations. Cells were infected with equivalent infectious doses of virus and DIP preparations. DIP-encoded proteins are highly expressed in DIP infected cells compared to wild-type infected cells. The anti-IR4, anti-UL3, and anti-UL4 antibodies are described in "Materials and methods". Tubulin detected by anti- β -tubulin served as a loading control

Fig. 5. PCR analysis of DNA isolated from mouse L-M cells passaged with wild-type, Δ UL4, Δ UL4Res, and UL4aa18stop viruses at serial high multiplicity. The hybrid gene was detected as an indication of the generation of DIP denoted by an asterisk (*). All viruses were capable of producing DI particles with the exception of the Δ UL4 EHV-1 that lacks the *UL4* ORF. DI.3 p3 is the third passage of cells known to be persistently infected with DIP. The IE gene PCR product was included as a control to indicate that high quality DNA was used for the PCR reactions, as exemplified by consistent amplification

Fig. 6.

Western blot analysis was performed using protein lysates collected from mouse L-M cells that were passaged three or six times with wild-type, Δ UL4, Δ UL4Res, or UL4aa18stop EHV-1. Antibody to EHV-1 IR4 protein was used to detect the IR4/UL5 hybrid protein and is described in “Materials and methods”. Hybrid protein was detected in cells infected with all viruses, except for the Δ UL4 virus completely deleted of the *UL4* DNA sequence. β -tubulin serves as a loading control

Table 1

Primers used for PCR analyses

Primer	Name	Sequence	Position ^a
1	Hyb Fwd	CCTGCAGCTGCTGCCCCG	141349–141333
2	Hyb Rev	CGCTTGTCGCGTCTATCGCC	4559–4579
3	UL4 Left Fwd	CAGACCCAGCGATCCACGCACCGTCC	3348–3373
4	UL4 Left Rev	CTGCGTCTTGATACCCAGACCGTATATGGTG	3643–3628 ^b 4309–4295 ^c
5	UL4 Right Fwd	CACCATATACGGTCTGGGTATCAAGACGCAG	3628–3643 ^b 4295–4309 ^c
6	UL4 Right Rev	GGCAGTACCTGCAGCCTTGTATCGGCC	4543–4522
7	UL4 Probe Fwd	GGCCTGGGCAGAGTTGGCTGCCTGCC	3713–3738
8	UL4 Probe Rev	GCAGATCTTGCTCCCAGACCTGACC	3944–3920
9	IE Fwd	ACGACGATGAGATGGAGATG	118030–118049 ^d 145130–145111 ^e
10	IE Rev	ACAGCGATACCGAGACCTG	118143–118125 ^d 145017–145035 ^e

^aPosition refers to the nucleotide position within the EHV-1 genome (DNA Accession AY665713.1)^bLocation within the *UL3* gene^cLocation within the *UL5* gene^dLocation of the IE gene within the internal repeat^eLocation of the IE gene within the terminal repeat