The UL4 gene of pseudorabies virus encodes a minor infected-cell protein that is dispensable for virus replication

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Although homologues of the open reading frame (ORF) UL4 of herpes simplex virus 1 (Human herpesvirus 1) have been found in the genomes of all hitherto-analysed alphaherpesviruses, little is known about their function. In a project to analyse systematically, in an isogenic and standardized assay system, the gene products of the alphaherpesvirus pseudorabies virus (PrV; Suid herpesvirus 1), the PrV UL4 gene product was identified using a monospecific rabbit antiserum prepared against a bacterial fusion protein. Western blot and immunofluorescence analyses revealed that the 146 codon UL4 ORF of PrV was translated into a nuclear 15 kDa protein which was detectable from 6 h after infection of rabbit kidney cells, but was not found in purified virus particles. For functional analysis, a UL4-negative virus recombinant (PrV-ΔUL4F) was generated by mutagenesis of an infectious full-length clone of the PrV genome in E. coli. PrV-ΔUL4F was replication-competent in rabbit kidney cells, and plaque formation was not affected by the mutation. However, maximum virus titres of PrV-ΔUL4F were decreased about fivefold compared with wild-type PrV, and electron microscopy of infected cells demonstrated an impairment of release of mature virions. This growth defect of PrV-ΔUL4F could be corrected completely by propagation in UL4-expressing cells. Correlating with the inconspicuous in vitro phenotype, neurovirulence of PrV-ΔUL4F was also not affected significantly. Thus, UL4 encodes a non-structural protein of PrV that enhances virion formation but is not essential for PrV replication in vitro or in vivo.

INTRODUCTION

Pseudorabies virus (PrV; Suid herpesvirus 1) is the causative agent of Aujeszky’s disease in pigs and of fatal neurological disorders in many other mammalian species (Mettenleiter, 2000). PrV has been classified as a member of the genus Varicellovirus within the subfamily Alphaherpesvirinae of the Herpesviridae (Davison et al., 2005). It possesses a class D genome of about 143 kbp consisting of long and short unique regions (UL1, UL2) and inverted repeat sequences (IRs, TRs) which flank the US region (Klupp et al., 2004; Roizman & Pellet, 2001). The genome contains 72 open reading frames (ORFs), which exhibit homologies to genes of other mammalian and avian alphaherpesviruses including herpes simplex virus 1 (HSV-1; Human herpesvirus 1) (McGeoch et al., 1988), varicella-zoster virus (Human herpesvirus 3) (Davison & Scott, 1986), Equid herpesvirus 1 (Telford et al., 1992) and Marek’s disease virus (Gallid herpesvirus 2) (Tulman et al., 2000). Since the gene arrangement also proved to be widely collinear, the designations originally introduced for HSV-1 were adopted for PrV. Several genes and gene clusters are further conserved throughout alpha-, beta- and gammaherpesviruses, which may indicate that they encode proteins that are important for fundamental steps of the virus replication cycle (Roizman & Pellet, 2001).

The UL5 gene, which encodes a component of the helicase-primase complex required for viral DNA replication (Crute et al., 1989), is conserved throughout the herpesviruses, whereas the downstream UL4 gene is restricted to the subfamily Alphaherpesvirinae, and the adjacent UL3-5 gene of PrV (Dean & Cheung, 1993) possesses homologues in other members of the genus Varicellovirus, but not in the genus Simplexvirus. Nevertheless, the UL3-5 protein of PrV has been shown to play an important role during secondary envelopment of virus particles in the cytoplasm of infected cells (Fuchs et al., 1996).

The function of UL4 is largely unknown, and only the corresponding protein products of HSV-1 (Eide et al., 1998) and HSV-2 (Yamada et al., 1999; Yamada et al., 1998) have been identified. Both have been classified as true late (?2) gene products that localize in the cytoplasm and in small nuclear structures of infected cells (Eide et al., 1998; Jahedi et al., 1999; Yamada et al., 1998). For HSV-1, a colocalization of intranuclear UL4 protein with the immediate-early protein ICP22 has been observed, but it remained unclear whether there is a
functional relation between the two proteins (Jahedi et al., 1999). Conflicting results were obtained with respect to virion incorporation of the UL4 gene products. Whereas the HSV-2 UL4 protein could be detected in mature virus particles, as well as in capsidless light (L) particles (Yamada et al., 1998), the UL4 gene product of HSV-1 is considered a non-structural protein (Eide et al., 1998).

To test the biological relevance of UL4, HSV-1 recombinants containing truncated genes have been generated and characterized in cell culture and in the mouse model (Jun et al., 1998). These studies indicated that UL4 is a non-essential gene, since only minor differences between mutant and wild-type virus were observed with respect to virus titres, pathogenesis and establishment and reactivation of latent infections of the central nervous system.

Whereas the amino acid sequences of the UL4 proteins of HSV-1 and HSV-2 exhibit a considerable identity of 76 %, sequence identity to the corresponding gene products of other alphaherpesviruses is much lower (Jun et al., 1998). For example, the deduced UL4 protein of PrV shares only 23 % identical amino acid residues with the HSV-1 gene product (Dean & Cheung, 1994) and, therefore, functional equivalence is not certain. So far, the only evidence for expression of PrV UL4 has been the detection of a 0.9 k b RNA transcript that starts upstream of the predicted initiation codon and which is 3'-coterminal with the UL5 mRNA (Dean & Cheung, 1994).

During the last few years, we have identified and functionally analysed numerous PrV-encoded proteins (reviewed by Mettenleiter, 2004) using monospecific rabbit antisera raised against bacterial fusion proteins and virus recombinants generated by mutagenesis of a bacterial artificial chromosome (BAC) containing the entire PrV genome in E. coli (Kopp et al., 2003). In the present study, these techniques were applied to the UL4 gene. The respective
protein could be detected and localized in infected cells and a UL4-null mutant of PrV was isolated. The growth properties of this mutant were investigated in vitro and in vivo.

**METHODS**

**Viruses and cells.** The genome of PrV strain Kaplan (PrV-Ka; Kaplan & Vatter, 1959) has been cloned as a BAC in *E. coli* (Kopp et al., 2003). The resulting pPrV-ΔgB was used for construction of a UL4-negative virus mutant as described in this study. Viruses were propagated on rabbit kidney (RK13) cells in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Invitrogen). To permit trans-complementation of the PrV mutant, a UL4-expressing cell line was established after calcium phosphate-mediated transfection (Graham & van der Eb, 1973) of RK13 cells with the expression plasmid pCDNA-UL4 (see below), which contained a neomycin/geneticin resistance gene. Two days after transfection, the cells were trypsinized, diluted in medium containing 500 μg ampicillin ml⁻¹ (Invitrogen), and seeded into 96-well microtitre plates. Resistant cell clones were used for UL4 expression by Western blot analyses with a monospecific antiserum (see below) and one stable cell line (RK13-UL4) was propagated further.

**Construction of plasmids.** A 2390 bp SalI fragment containing the UL4, UL3-5 and UL3 genes of PrV was cloned into vector pUC19 (New England Biolabs). To remove unwanted restriction sites, the insert of the obtained plasmid pUC-S2.4 (Fig. 1b) was shortened to 894 bp by double-digestion with AgeI and EcoRI and religation. For prokaryotic expression of UL4, the resulting plasmid pUC-S2.4AE was cleaved with SalI and StyI and religated. The insert was then recloned as a 605 bp HindIII–EcoRI fragment into pGEX-4T-1 (Amersham), which had been digested with BamHI and EcoRI. In pGEX-UL4 (Fig. 1c), the complete UL4 ORF, preceded by eight normally unexpressed codons, was fused in-frame to the glutathione S-transferase (GST) gene. For generation of a eukaryotic UL4 expression plasmid, the insert of pUC-S2.4AE was shortened by double-digestion with SphI and StyI, followed by religation, and recloned as a 607 bp HindIII–EcoRI fragment into the similarly cleaved vector pcDNA3 (Invitrogen). From pcDNA-UL4 (Fig. 1c), the UL4 gene could be transcribed and translated in vitro under control of the T7 promoter (TNT coupled reticulocyte lysate system; Promega). Furthermore, the human cytomegalovirus immediate-early promoter–enhancer complex (Phcmv-ie) permitted transient or stable expression in transfected RK13 cells (see above). Plasmid pUC-UL4KF (Fig. 1b) was derived from pUC-S2.4AE by deletion of a 434 bp StyI–EcoNI fragment containing UL4 codons 1–139 and concomitant substitution by a 1258 bp BstI fragment of pKD13 (Datsenko & Wanner, 2000), which contained a kanamycin-resistance gene flanked by Flp recombinase recognition target sites (FRT). In all cloning experiments, incompatible fragment ends were blunt-ended with Klenow polymerase prior to ligation.

**Preparation of a UL4-specific antiserum and Western blot analyses.** After transformation of *E. coli* XL-1 Blue MRF’ (Stratagene) with plasmid pGEX-UL4, expression of an approx. 43 kDa GST–UL4 fusion protein could be induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The fusion protein was isolated and used for immunization of a rabbit as described previously (Fuchs et al., 2002). Sera collected before and after four intramuscular applications of 100 μg protein at 4-week intervals were analysed. For Western blot analyses, RK13 cells were infected with PrV-Ka at a multiplicity of 5 p.f.u. per cell and incubated at 37°C for 1–18 h. Samples of infected and non-infected RK13 cells, RK13-UL4 cells and PrV virions were prepared as described previously (Dietz et al., 2000), separated by discontinuous SDS-PAGE (Laemmli, 1970) and electrotransferred to nitrocellulose membranes (Transblot SD cell; Bio-Rad) and blocked with 0.5% gelatin in 0.1% tris-buffered saline (TBS). After blocking, samples of infected and non-infected RK13 cells, RK13-UL4 cells and PrV virions were prepared as described previously (Dietz et al., 2000), separated by discontinuous SDS-PAGE (Laemmli, 1970) and electrotransferred to nitrocellulose membranes (Transblot SD cell; Bio-Rad) and blocked with 0.5% gelatin in 0.1% tris-buffered saline (TBS). After blocking, blots were incubated with monospecific antiserum against the UL4 gene product (a), the UL34 protein (b) or gH (c). Binding of peroxidase-conjugated secondary antibodies was detected by chemiluminescence. Molecular masses of marker proteins are indicated.

![Fig. 2. Identification of the UL4 protein in infected and stably transfected cells.](image-url)
anti-rabbit antibodies (Dianova) and visualized by chemiluminescence in TBS-T. Bound antibody was detected with peroxidase-conjugated (gH) of PrV (Klupp & Mettenleiter, 1999) at dilutions of 1 : 50 000.

Expression kinetics of the UL4 protein. RK13 cells were infected with PrV-Ka at a multiplicity of 5 p.f.u. per cell of wild-type PrV-Ka or PrV-AUL4F and incubated on ice for 1 h. After addition of prewarmed medium, incubation was continued at 37 °C for 1 h. Non-penetrated virus was then inactivated by low-pH treatment (Mettenleiter, 1989). At 1, 4, 8, 12, 24, 36 and 48 h after the temperature shift, cells were scraped into the medium and lysed by freezing (−70 °C) and thawing (37 °C). Progeny virus titres were determined by plaque assays in RK13-UL4 cells, which were overlaid with MEM containing 5 % FCS and 6 % methyl cellulose 1−1. After 2 days, cells were fixed for 1 h with 2 % formaldehyde and stained for 15 min with 1 % crystal violet in 50 % ethanol. For determination of one-step growth kinetics, the mean virus titres and standard deviations of six parallel experiments were calculated. Furthermore, the diameters of 30 plaques per virus in RK13 and RK13-UL4 cells were measured. Mean plaque sizes and standard deviations were calculated.

Indirect immunofluorescence tests and confocal microscopy. RK13 cells were grown on coverslips and infected with PrV-Ka or PrV-AUL4F at a multiplicity of about 0-001 p.f.u. per cell. Parallel coverslips were transfected (Graham & van der Eb, 1973) with pcDNA-UL4 or the empty expression vector pcDNA3. After 20 h, all cells were fixed with acetone for 20 min at −20 °C, dried and blocked for 1 h with 10 % FCS in PBS. Subsequently, the cells were incubated with the anti-UL4 rabbit serum and a gC-specific monoclonal antibody (Klupp & Mettenleiter, 1999) at dilutions of 1 : 100 in blocking buffer and binding reactions were detected by Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 647-conjugated anti-rabbit secondary antibodies (Invitrogen) at dilutions of 1 : 1000 in PBS for 1 h each. After either incubation step, the slides were washed repeatedly with PBS and finally preserved and counterstained with a 9 : 1 mixture of glycerol and PBS, containing 25 mg 1,4-diazabicyclooctane ml−1 and 1 g propidium iodide ml−1. Fluorescence was analysed in a confocal laser scanning microscope (LSM 510; Zeiss).

Electron microscopy. RK13 or RK13-UL4 cells were infected with 1 p.f.u. per cell of PrV-AUL4F or PrV-Ka. After 1 h on ice and an additional hour at 37 °C, the inoculum was replaced by fresh medium and incubation was continued for 13 h at 37 °C. Fixation and embedding were done as described by Klupp et al. (2000) and counterstained ultrathin sections were analysed in an electron microscope (Tecnai 12; Philips).

In vivo studies. To determine the relevance of UL4 for virulence and neuronal spread of PrV, ten 8-week-old CD1 mice each were infected by bilateral intranasal instillation of 105 p.f.u. of either PrV-AUL4F or PrV-Ka. The animals were observed three times a day and mean survival times were determined. Single animals were necropsied 24, 48 and 72 h after infection (p.i.) and viral spread in the trigeminal circuit was analysed by immunohistochemistry of paraffin sections of the brains as described previously (Klopfleisch et al., 2004).

Bio-Rad). Blots were blocked with 5 % low-fat milk in Tris-buffered saline (TBS-T; 150 mM NaCl, 10 mM Tris/HCl, pH 8.0, 0.25 % Tween 20) and incubated for 1 h with rabbit antisera against the UL4 gene product (a), the UL34 protein (b) or gH (c). Molecular masses of marker proteins are indicated.

**Fig. 3.** Expression kinetics of the UL4 protein. RK13 cells were infected with PrV-Ka at a multiplicity of 5 p.f.u. per cell and incubated at 37 °C for 0–18 h. Lysates of infected and non-infected cells (N) and purified virions (V) were separated by SDS-PAGE.

Western blots were incubated with monospecific antisera against the UL4 gene product (a), the UL34 protein (b) or gH (c). Molecular masses of marker proteins are indicated.

Construction of UL4-negative PrV. A UL4 deletion mutant was generated by Red recombinase-mediated mutagenesis of pPrV-ΔgB (Datko & Wanner, 2000; Kopp et al., 2003) with the insert fragment of pUC-ΔUL4KF (Fig. 1b). For this purpose, the insert was amplified by PCR using vector-specific primers M13/pUC (−47) and M13/pUC reverse (−48) (New England Biolabs) and Pfx DNA polymerase (Invitrogen). Bacteria containing pPrV-ΔgB were transformed with the PCR product and kanamycin-resistant clones were isolated. Subsequently, the resistance gene was removed by transformation with helper plasmid pCP20 (Cherepanov & Wackernagel, 1995), which provided the FRT site-specific Flp recombinase. Finally, the mini F-plasmid vector sequences were removed and the essential gB gene was restored by cotransfection (Graham & van der Eb, 1973) of RK13 cells with mutagenized BAC DNA and plasmid pUC-B1BclI (Kopp et al., 2003). A single plaque isolate of the virus progeny was further propagated, characterized by genome analyses (data not shown) and designated PrV-AUL4F (Fig. 1b).
RESULTS AND DISCUSSION

Identification of the UL4 protein of PrV

The complete UL4 ORF of PrV was expressed in a bacterial fusion protein, which was used for immunization of a rabbit. In Western blot analyses of RK13 cells which were transiently or stably transfected with the eukaryotic expression plasmid pcDNA-UL4 (Fig. 1c), the antiserum detected an abundant 15 kDa protein (Fig. 2a). This reactivity was specific, since the 15 kDa protein was not found in non-transfected RK13 cells (Fig. 2a) and was also not detected by the preimmune serum (not shown). The apparent mass of the protein matches the calculated molecular mass of 15.8 kDa of the 145 amino acid UL4 gene product as deduced from the DNA sequence (Dean & Cheung, 1994; Klupp et al., 2004). UL4 gene products of about 15 kDa were also found after in vitro transcription and translation of pcDNA-UL4 (not shown) and in cells infected with wild-type PrV-Ka (Fig. 2a). However, the amount of UL4 protein found in PrV-infected cells appeared significantly smaller than that expressed in RK13-UL4 cells (Fig. 2a). These findings indicate that the UL4 protein of PrV is expressed at low levels in infected cells and is not extensively processed after translation. Downstream of the first initiation codon, the UL4 sequence indicates the presence of a second putative TATA signal and of a matching in-frame ATG codon at position 37 of the ORF (Klupp et al., 2004). However, neither in plasmid-transfected nor in PrV-infected cells was a second UL4 gene product corresponding to the predicted 11.8 kDa protein detectable. This finding is in agreement with previous RNA analyses, in which only one UL4-specific transcript, starting upstream of the first ATG codon, could be identified (Dean & Cheung, 1994). As controls, blots were analysed with antisera against the UL34 (Fig. 2b) and gH (Fig. 2c) proteins.
In kinetic studies, the UL4 protein was first detected 6 h after PrV infection and was present at almost constant levels from 7 to 18 h p.i. (Fig. 3a). A long exposure of the Western blot was required for detection of the protein and, therefore, unspecific reactions of the antiserum with other viral and cellular gene products also became visible. Correlating with these results, the UL4 mRNA of PrV has been previously identified as a low-abundance 9 kb transcript that is detectable from 6 h p.i. (Dean & Cheung, 1994). Thus, the UL4 gene of PrV, like its HSV-1 and HSV-2 homologues (Eide et al., 1998; Yamada et al., 1998), is apparently expressed with late (\( \gamma \)) kinetics. However, unlike most other late proteins of herpesviruses, the PrV UL4 gene product was not detectable in purified virus particles (Fig. 3a). In agreement with earlier results, the primary envelope protein encoded by UL34 (Klupp et al., 2000) was also not found in mature virions (Fig. 3b), whereas tegument and envelope proteins including gH (Klupp & Mettenleiter, 1999) were present (Fig. 3c). Thus, the UL4 protein of PrV is apparently a non-structural protein, similar to the homologous gene product of HSV-1 (Eide et al., 1998). In contrast, the UL4 protein of HSV-2 has been described as a virion component, which is presumably located in the tegument layer, since it was also detected in capsidless L-particles (Yamada et al., 1998). It remains to be elucidated whether the localizations of the UL4 protein in these closely related alphaherpesviruses are really different; it is conceivable that the analysed HSV-2 particles were contaminated with cell remnants or that the amount of UL4 protein incorporated into HSV-1 and PrV particles is too small for detection by the available antisera.

By immunofluorescence studies of infected cells, the UL4 proteins of HSV-1 and HSV-2 have been localized both in the cytoplasm and in the nuclei, where they were shown to accumulate in dense nuclear bodies (Eide et al., 1998; Jahedi et al., 1999; Yamada et al., 1998). In similar analyses of PrV-infected cells, the reaction of the UL4-specific antiserum was relatively weak, but also revealed a predominantly nuclear localization of the target protein (Fig. 4a; blue fluorescence). The specificity of this speckled nuclear fluorescence could be demonstrated by its absence from cells infected with a UL4-negative PrV mutant (Fig. 4b). Unlike the UL4 gene product, the envelope glycoprotein gC of PrV was detected in the cytoplasm and along the plasma membrane of infected cells (Fig. 4a, b; green fluorescence). However, in RK13 cells transfected with pcDNA-UL4 (Fig. 1c), an exclusively cytoplasmic reactivity of the anti-UL4 serum was observed (Fig. 4c), which was not found in cells transfected with control plasmids (Fig. 4d). A similar cytoplasmic localization of the homologous UL4 protein of HSV-2 has been found after transfection of African green monkey kidney cells with an expression plasmid (Yamada et al., 1998). Thus, transport of the alphaherpesvirus UL4 proteins to the nucleus may be dependent on interaction(s) with other viral gene products. Nevertheless, a function of the UL4 protein in the cytoplasm is also conceivable.

**Isolation and in vitro characterization of a UL4-negative PrV mutant**

For functional analysis, the UL4 gene was deleted by mutagenesis of a previously described BAC clone of the PrV genome (Kopp et al., 2003). The resulting PrV- \( \Delta UL4F \) lacked the complete UL4 ORF except for the last six codons (Fig. 1b) and contains instead a 36 bp insertion of an FRT site as a remnant from the targeted manipulation in *E. coli*. Genome analyses of PrV- \( \Delta UL4F \) confirmed the expected deletion (results not shown) and, in Western blot analyses of infected cells, the UL4 protein was not detectable (Fig. 2a). In contrast, expression of the UL34 protein and gH were not affected (Fig. 2b, c). PrV- \( \Delta UL4F \) was isolated and further propagated in non-complementing RK13 cells, which demonstrated that UL4 is dispensable for in vitro replication of PrV. Plaque assays did not provide evidence for a role of UL4 in cell-to-cell spread of PrV, since plaque diameters of PrV- \( \Delta UL4F \) in RK13 cells were similar to those of the parental wild-type strain PrV-Ka (Fig. 5). One-step growth studies revealed similar replication kinetics of wild-type and mutant viruses. However, the maximum titres of
PrV-ΔUL4F were about fivefold lower than those of PrV-Ka (Fig. 6a). This minor growth defect could be fully corrected by propagation in UL4-expressing RK-UL4 cells (Fig. 6b), demonstrating that it was indeed caused by the UL4 deletion.

To elucidate possible reasons for the observed, albeit slight growth defect, RK13 and trans-complementing RK13-UL4 cells were infected with PrV-ΔUL4F, fixed 14 h after infection and analysed by electron microscopy. In non-complementing cells, all stages of herpesvirus morphogenesis were detectable, including empty and genome-containing capsids in the nucleus (Fig. 7a), primary enveloped particles in the perinuclear space (Fig. 7b), naked and enveloped nucleocapsids in the cytoplasm (Fig. 7a, c, d) and mature virions in the extracellular space (Fig. 7a, d). Although no particular step of viral egress was apparently blocked, larger accumulations of predominantly enveloped PrV-ΔUL4F particles were found in the cytoplasm of RK13 cells than in RK13-UL4 cells (Fig. 7e) or in cells infected

Fig. 7. Electron microscopy of infected cells. RK13 (a–d, f) or RK13-UL4 cells (e) were fixed and stained 14 h after infection with PrV-ΔUL4F (a–e) or PrV-Ka (f) at a multiplicity of 1 p.f.u. per cell. Investigation of ultrathin sections revealed nucleocapsid formation in the nucleus (a), nuclear egress (b), secondary envelopment of cytoplasmic nucleocapsids (c, d) and extracellular virions (a, d) of PrV-ΔUL4F in non-complementing cells. However, the studies indicate an accumulation of enveloped particles in the cytoplasm when compared with the situation found in UL4-expressing cells (e) or cells infected with wild-type PrV (f). Bars, 2 μm (a, e, f), 500 nm (b, c, d) or 300 nm (enlarged insets in a and f).
with PrV-Ka under similar conditions (Fig. 7f). In representative sections of RK13 and RK13-UL4 cells (Fig. 7a, e), the total numbers of intracytoplasmic PrV-AUL4F capsids were 176 and 22, respectively, and 87 % (153) and 14 % (3) of them were enveloped in Golgi-derived vesicles. In the cytoplasm of RK13 cells infected with wild-type PrV-Ka (Fig. 7e), 43 of 100 virus particles were enveloped, which was also a considerably lower portion than found with PrV-AUL4F in non-complementing cells. Thus, a possible function of the UL4 protein might be an enhancement of virus release.

**Influence of UL4 on virulence of PrV in vivo**

It appeared conceivable that UL4, despite its dispensability in cell culture, might play a more relevant role in vivo. Therefore, virulence and pathogenesis of PrV-AUL4F after intranasal infection of mice was analysed. In these model animals, as well as in many other mammals, PrV has been shown to be highly neuroinvasive and to induce a fatal neurological disorder (Enquist, 2002; Mettenleiter, 2003). In mice infected with PrV-AUL4F or wild-type PrV-Ka, the first clinical symptoms (depression, anorexia) could be observed after about 24 h. Subsequently, all animals showed increasing excitation and convulsions, heavy dyspnoea and extensive scratching of the facial and nasal skin, thereby causing severe haemorrhagic dermal erosions (‘mad itch’ syndrome). After 24 h, viral antigen could be detected by immunohistochemistry in clusters of respiratory epithelial cells of the nasal mucosa and in a few first-order neurons in the trigeminal ganglia of animals infected with either virus. At 48 h p.i., infection of second-order neurons in the spinal trigeminal nuclei was observed in both groups. At the time of death, the number of infected cells was increased further, but no infection of third-order cortical neurons could be detected. Thus, the only effect of UL4 deletion was a slight prolongation of the mean survival time to 54 h, compared with 50 h after infection with PrV-Ka. This delay correlates with the moderately reduced replication efficiency of PrV-AUL4F in cell culture, and does not indicate a specific role of UL4 during neuroinvasion or transneuronal spread of PrV. Up to now, evidence for such tissue-specific functions has been found only for the non-essential proteins encoded by the UL37, US8 (gE) and US9 genes of PrV (Babic et al., 1996; Brideau et al., 2000; Klopflleisch et al., 2004). In contrast, like PrV-AUL4F, most other gene deletion mutants of PrV, whose in vitro replication properties were not substantially impaired, also did not exhibit a pronounced inhibition of neurovirulence in rodents (Klopflleisch et al., 2006). However, most of these mutants, including PrV-AUL4F, remain to be tested in pigs, the natural host of PrV.

Besides PrV, HSV-1 is the only alphaherpesvirus of which UL4 mutants have been described to date (Eide et al., 1998; Jun et al., 1998). However, unlike PrV-AUL4F, these recombinants retained significant portions of the respective ORF which could still have allowed the expression of truncated UL4 proteins containing the N-terminal 40 or 60 amino acids, respectively. These HSV-1 mutants exhibited only minor replication defects in vitro, and pathogenesis as well as latent infections in mice were also not significantly affected (Jun et al., 1998). However, it could not be excluded that the retained UL4 fragments of the HSV-1 recombinants were expressed and partly functional and that deletion of the entire gene would lead to a more pronounced phenotype. Thus, PrV-AUL4F is strictly speaking the first complete UL4 deletion mutant, which unequivocally demonstrates the non-essential character of a member of the UL4 gene family.

Because of the inconspicuous phenotypes of all UL4 mutants analysed so far, the precise function of this gene remains enigmatic. The observation that deletion of the UL4 gene of PrV did not affect plaque formation but led to a detectable titre reduction indicates a role during a very late step of virus morphogenesis which is not required for cell-to-cell spread, but only for release of infectious particles from the cell. Therefore, it appears unlikely that the UL4 protein of PrV functions during DNA replication and encapsidation or nuclear egress of capsids, although the UL4 proteins of PrV, HSV-1 and -2 were at least partly localized in the nucleus of infected cells (Eide et al., 1998; Jahedi et al., 1999; Yamada et al., 1998). Since no detectable amounts of the UL4 protein are incorporated into PrV or HSV-1 virions, a direct involvement in tegumentation and subsequent envelopment of cytoplasmic nucleocapsids is also hardly conceivable. Thus, most likely, the UL4 protein modulates host-cell functions in a manner that permits increased or prolonged production or release of infectious virions.

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