Deletion of the UL21 gene in Pseudorabies virus results in the formation of DNA-deprived capsids: an electron microscopy study

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Original article

Deletion of the UL21 gene in Pseudorabies virus results in the formation of DNA-deprived capsids: an electron microscopy study

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Abstract – We studied the morphogenesis of three pseudorabies virus mutants lacking parts of the gene homologous to the UL21 gene of the herpes simplex virus type 1. The mutants were examined in an SK-6 cell-line, in an SK-6 cell-line expressing the UL21 gene product, in porcine lung alveolar macrophages (PLAM) and in porcine nasal mucosa explants. Although on SK-6 cells and PLAM, the virus-assembly and egress of mutant virus M155, lacking almost the entire UL21 gene, was similar to that of the rescued PRV mutant, M155 producing virions containing little or no DNA (A-type particles). Virus mutants M133 and M134 (lacking 23 and 232 amino acids respectively) produced more C-type particles. In SK-6 cells stably expressing the UL21-encoded protein, all mutants produced C-type particles. All mutants produced C-type particles in nasal mucosa explants, indicating that the UL 21-gene product is not essential for virus production in porcine tissue. These results support and extend previous work that indicated a role for the UL21 encoded protein in the packaging of newly replicated viral DNA.

electron microscopy / morphology / pseudorabies virus / UL21 gene

Résumé – La délétion du gène UL21 du virus de la maladie d'Aujeszky résulte dans la formation de capsides dépourvues d'ADN : étude en microscopie électronique. Nous avons étudié la morphogenèse de trois mutants du virus de la maladie d'Aujeszky (VMA) comportant des délétions dans l'homologue du gène UL21 du virus de l'herpes simplex de type 1. Les mutants ont été examinés dans une lignée cellulaire SK-6, dans une lignée SK-6 exprimant le produit du gène UL21, dans des

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macrophages alvéolaires de poumon de porc (MAPP), et dans des explants de muqueuse nasale de porc. Bien que dans les cellules SK-6 et les MAPP l'assemblage et la sortie du virus mutant M155, à qui il manquait la presque totalité du gène UL21, étaient similaires à celles du mutant du VMA récupéré, ce mutant produisait des virions contenant peu ou pas du tout d'ADN (particules de type A). Les virus mutants M133 et M134 (à qui il manquait 23 et 232 acides aminés respectivement) produisaient plus de particules de type C. Dans les lignées cellulaires SK-6 exprimant de manière stable la protéine codée par UL21, tous les mutants ont produit des particules de type C. De même, tous les mutants ont produit des particules de type C dans les explants de muqueuse nasale, indiquant que le produit du gène UL21 n'est pas essentiel pour la production de virus dans les tissus porcins. Ces résultats sont en accord avec les travaux précédents et renforcent l'idée que la protéine codée par UL21 joue un rôle dans l'empaquetage de l'ADN viral nouvellement répliqué.

microscopie électronique / morphologie / virus de la maladie d'Aujeszky / gène UL21

1. INTRODUCTION

Pseudorabies virus (PRV; synonyms suid herpesvirus type 1 and Aujeszky's disease virus), a member of the Alphaherpesvirinae subfamily, causes economically important disease of pigs. Pigs are the natural host of PRV, although many other animal species can be infected, usually with a fatal outcome [29]. Like all herpesviruses, PRV has an envelope surrounding a capsid of approximately 100 nm in diameter, which contains the double stranded DNA [14].

During packaging, concatameric viral DNA is cleaved and subsequently packaged into the preformed capsids [20, 26] yielding capsids with electron dense cores [16, 17, 20, 22, 25, 28]. According to Booy et al. [5] viral DNA is packed as locally ordered, liquid-crystalline, parallel packings of DNA duplexes. Next, the capsids adhere at virally induced patches in the inner nuclear membrane, and become enveloped by a budding process at the inner nuclear membrane. Complete virions appear in the perinuclear space, followed by cytoplasmic transport and budding at the outer nuclear membrane [20].

Based on biochemical data, de Wind et al. [6] suggested that the UL21 gene product has a regulatory or accessory role in the processing of viral DNA. In this process that is linked to encapsidation, concatemers of viral DNA are cleaved to unit-length linear DNA molecules. De Wind et al. [6] showed that a UL21 deletion mutant hardly produced any processed (1.1 kbp) viral DNA. A mutant virus containing part of the UL21 gene produced an intermediate amount of processed (1.1 kbp) viral DNA [6].

The UL21 gene is situated in the *Bam*HI fragment 4 (Fig. 1). According to Lomniczi et al. [13] *Bam*HI fragment 4 appears to encode only four genes, all of which are involved in nucleocapsid assembly. Baines et al. [2] reported that the UL21 gene is dispensable for growth in cells, however they found a 3- to 5-fold lower virus yield in human embryonic lung cells, compared to Vero cells.

To study the effect of (partial or total) UL21 deletion on the morphogenesis and virus-host cell interaction, we investigated three UL21 deletion mutants and a rescued PRV mutant on the SK-6 cell-line, porcine lung alveolar macrophages (PLAM), and porcine nasal mucosa.

2. MATERIALS AND METHODS

2.1. Mutants

We tested PRV UL21 mutants M133 (B35), M134 (B9), M155 (B59) and M156 [6] (Fig.1). M133 has an oligonucleotide insertion at the C-terminus at amino acid position 500, lacking a minor part of the UL21 gene. M134 has an oligonucleotide

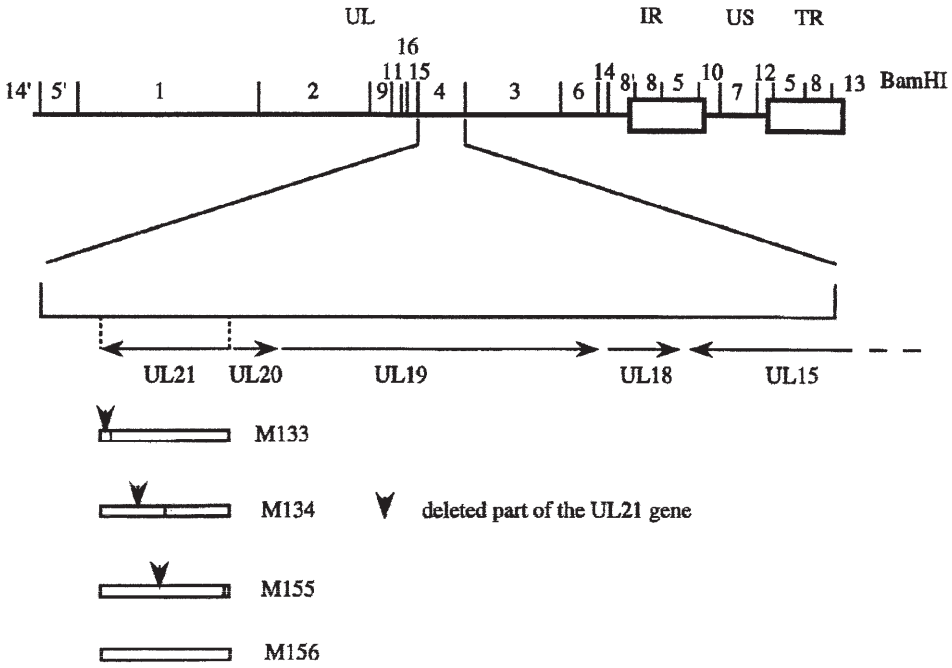

Figure 1. Physical map of the PRV genome (upper line). Open rectangles represent the internal (I_R) and terminal (T_R) repeat sequences which divide the genome into the U_L and U_S regions. B*am*HI fragment 4 is enlarged to show the genes and their transcriptional directions (arrows) from [6, 11].

insertion at amino acid 291, and lacks an intermediate part of the UL21 gene. M155 has an oligonucleotide insertion at amino acid 4 and lacks almost the entire UL21 gene. M156 was generated by marker rescue of M155 after cotransfection of a cloned 3.1 kb subcloned *Eco*RI-*Bam*HI fragment from insertion mutant B33 [7] and contains all 523 amino acids. The virulence of these mutants is 262 ± 25, 145 ± 4, 54 ± 8 hours to death for M133, M134 and M156 respectively [6, 7].

2.2. Cells

Mutants were tested in SK-6 cell-line cultures [10] expressing the UL21 protein (SK-6-69), PLAM and porcine nasal mucosa explants.

The SK-6-69 cell-line was generated by stably transfecting SK-6 cells with vector pRC/CMV (Invitrogen, Groningen, the Netherlands) expressing the UL21 gene under control of the immediate early promoter of the cytomegalovirus.

PLAM were obtained from the lungs of 6-week-old SPF pigs from the herd of the ID-Lelystad (Institute for Animal Science and Health, the Netherlands) [27].

Nasal mucosa explants were collected from the same animals [16].

2.3. Experimental protocol

SK-6, SK-6-69, and PLAM cell cultures were grown on carbon-coated coverslips (9×35 mm) in 35 mm plastic macro plates

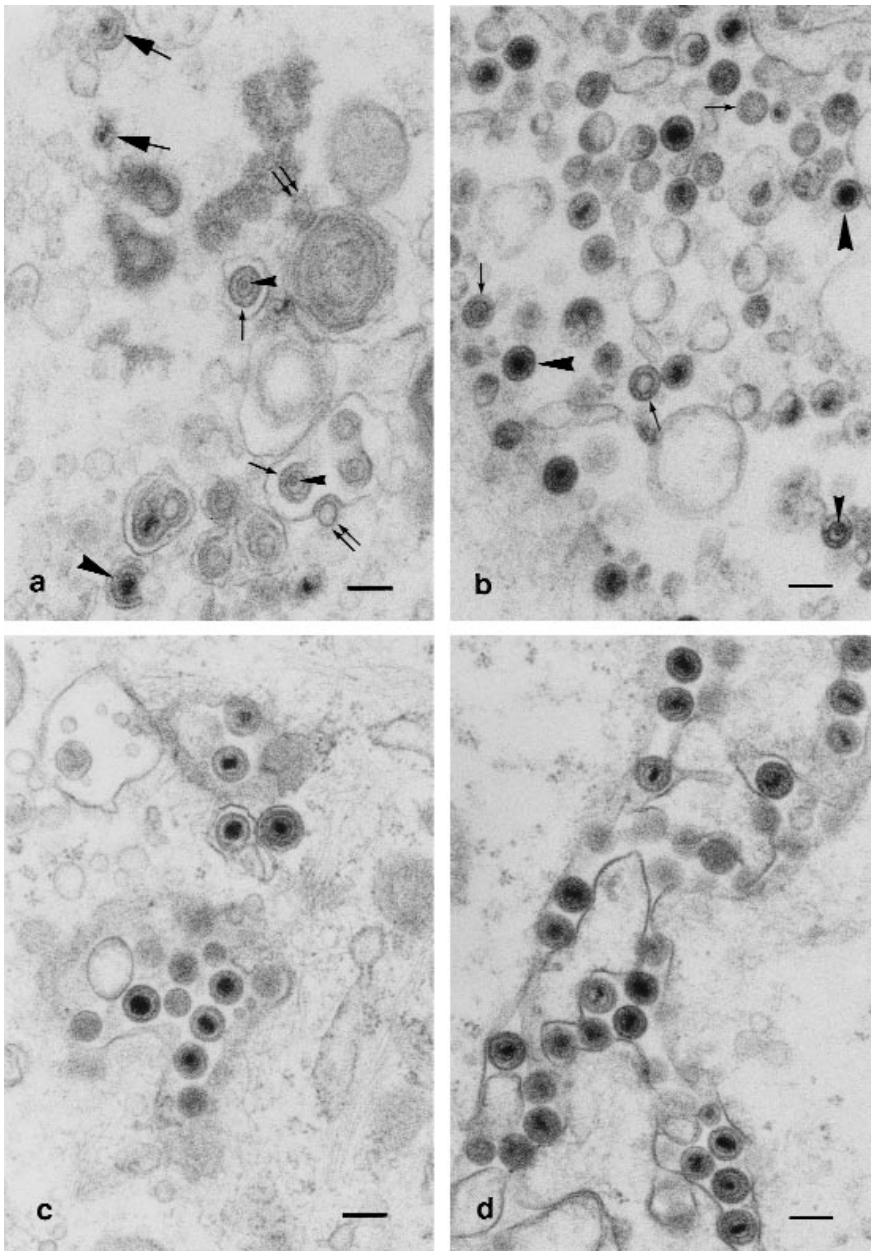

Figure 2. Electron micrographs of SK-6 cells, 16 hours after infection with the mutant PRV-M155, lacking a major part of the UL21 gene (a and b) or the rescued strain M156 (c and d).
(a) Empty (arrows) and incompletely DNA-filled virus particles (bold arrows) in the cytoplasm. In empty virus particles the spherical structure is clearly visible (arrowhead). Empty capsids bud easily at membranes of the smooth endoplasmic reticulum (double arrows). (b) In the extracellular space, empty, incompletely DNA-filled and completely DNA-filled virus particles are present. (c) Normal, completely DNA-filled virus particles in the cytoplasm and (d) in the extracellular space. Bar represents 200 nm.

(Corning Costar Europe, Badhoevedorp, the Netherlands) containing Earle minimal essential medium with 10% foetal calf serum and antibiotics as described previously [18]. At 9 and 16 hours post-infection (h.p.i.) with mutant virus strains (multiplicity of infection 1), the cultures were fixed with a cold fixative, containing osmium tetroxide and glutaraldehyde, by microwave irradiation, dehydrated and embedded in resin according to Wagenaar et al. [24].

Nasal mucosa explants were collected and treated as described previously [18]. Because PRV propagates in explants slower than in cell-lines, we studied the morphogenesis and viral infiltration at 24 h.p.i. and 48 h.p.i.

Nasal mucosa explants were processed according to Wagenaar et al. [24] as described above.

2.4. Morphometry

In the nucleus of the cell, viral capsids are assembled and subsequently filled with viral DNA. Next the nucleocapsids appear in the cytoplasm. Once in the cytoplasm, the nucleocapsids are in an end-state and filling with viral DNA is not possible anymore. After examining at least 100 infected cells to assess that the observed morphogenesis was generally identical, 25 cells were randomly assigned. In these cells, the extracellular virus structures and those in the cytoplasm were counted and expressed as a percentage of the total virus structures.

3. RESULTS

In SK-6 cells and in PLAM, viral capsids readily became enveloped at membrane structures and were detected in the extracellular space, irrespective of their DNA contents (Fig. 2). In the nuclei of infected cells, empty capsids and capsids completely or partially filled with DNA were present in equal numbers. The morphogenesis of the mutant strains M155, M133, and M134 differed from the rescued mutant M156.

Table I. Number of virus particles in the cytoplasm adhering to the cell surface of infected SK-6 cells, porcine lung alveolar macrophages (PLAM), SK-6-69 cells and epithelial cells of porcine nasal mucosa explants (n = 25).

Cell culture	Virus type (deletion size in no. of amino acids)			
	M133 (23)	M134 (232)	M155 (519)	M156 (0)
SK-6				
Number of virus particles	415	341	1058	318
Number of empty capsids (%)	18 (4)	63 (18)	581 (55)	2 (<1)
PLAM				
Number of virus particles	264	176	168	217
Number of empty capsids (%)	9 (3)	41 (23)	83 (49)	1 (<1)
SK-6-69				
Number of virus particles	901	954	1178	1247
Number of empty capsids (%)	8 (<1)	5 (<1)	10 (<1)	8 (<1)
Nasal mucosa explants				
Number of virus particles	384	616	369	490
Number of empty capsids (%)	3 (<1)	4 (<1)	1 (<1)	4 (<1)

Mutant strain M155, lacking 519 of the 523 amino acids, mainly showed A-particles (empty capsids), B-particles (incompletely DNA-filled particles) and only a few completely DNA-filled C-particles. In contrast, the rescued strain M156, containing all 523 amino acids, produced mainly C-particles. Mutant strain M133, lacking 23 of the 523 amino acids, showed a pattern that only slightly differed from M156; most progeny virus particles were C-particles, but some were B-particles or A-particles.

Mutant strain M134, lacking 232 of 523 amino acids, produced an intermediate phenotype.

In SK-6-69 cells, which express the UL21 protein constitutively, the morphogenesis of all mutant strains was similar to the rescued strain M156 (Tab. I). Thus, the morphogenesis of UL21 negative mutants was normalised by expression of the UL21 gene in SK-6 cells.

In epithelial cells, tissue macrophages, and fibroblasts of the nasal mucosa explant cultures infected with UL21 mutants, we did not observe the aberrant morphogenesis that was present in SK-6 cells and PLAM, but all mutants (M133, M134, and M155) showed a wild-type-like morphogenesis like the rescued strain (M156) (Tab. I). At 24 h.p.i. all mutant strains had infected epithelial cells and had passed the basal lamina. The virus spread into the stroma and the number of epithelial cells that contained viral antigens was similar for M133, M134, M155 and the rescued strain M156. At 48 h.p.i., the infection had invaded slightly deeper into the stroma (data not shown).

4. DISCUSSION

Biochemical data provided by de Wind et al. [6] and Gielkens and Peeters [8] showed that the UL21 gene product has a regulatory or accessory role in processing of viral DNA or in packaging of viral DNA. To provide morphological evidence for these options we studied the morphogenesis of the PRV UL21 mutants mentioned by de Wind et al. [6]. To assess the influence of UL21 we compared the mutants with the rescued mutant M156.

The SK-6-69 cell cultures were used as a second method to confirm the role of UL21. To obtain more information about the role of UL21 gene products in the natural host, we studied all strains in porcine nasal mucosa explant cultures, because they closely mimic the in vivo situation [16, 25].

After infection of SK-6 and PLAM with virus M155, lacking almost the entire UL21 gene, we mainly detected virus particles devoid of DNA outside of the nucleus. Interestingly, progressive inactivation of the UL21 gene correlated with decreased numbers of extranuclear C-particles. This morphological study corroborates biochemical data [6] showing that PRV-UL21 is involved in the cleavage of DNA and causes differences in DNA contents of the virus. Our observation that most of the virions are devoid of DNA agrees with the fact that the concatameric viral DNA is not efficiently processed into truncated viral DNA [6]. Capsid formation proceeds at a normal rate, resulting in a relative abundance of empty capsids (A-particles). The envelopment of capsids at cellular membranes efficiently produces empty enveloped virions. Empty enveloped virions have also been observed in studies using herpesviruses with deletions in other genes i.e. UL33, UL28, UL15, UL12 and UL6 [1, 3, 4, 15, 19, 21, 23]. Ward and Roizman [26] and Haarr and Skulstad [9] reviewed herpesvirus genes that are involved in processing and/or packaging. However, they did not mention the function of UL21. The UL21 gene product of both pseudorabies virus and herpes simplex virus type 1 was shown to be a capsid protein (reviewed in [14]).

In epithelial cells, tissue macrophages, and fibroblasts of the nasal mucosa explant cultures infected with UL21 mutants, we

did not observe the aberrant morphogenesis that was present in SK-6 cells and PLAM. Instead, we found a morphogenesis of the wild-type virus. This morphogenesis was completely identical to previous results of morphogenesis studies in porcine nasal mucosa explants after the wild-type PRV infection [25].

Probably the combination of cells in nasal explants complements the lack of the UL21-encoded protein. Thus, the marked decrease in viral replication of the UL21 negative virus, as observed by de Wind et al. [6] and Klupp et al. [12], is a feature of the mutant in isolated cells in culture.

A similar phenomenon was described by Baines et al. [2] when they compared viral replication of UL21 negative mutants in Vero cells (poor viral replication) with that in human embryonic lung cells (good viral replication).

The mutants tested in a single-step infection of SK-6 cells showed differences in plaque-morphology and titre. The mutant M155 (lacking almost the entire UL21 gene) replicated very poorly in SK-6 cells, yielding very tiny plaques [6]. Mutant M134 (lacking 232 aa of the UL21 gene), showed a 10-fold reduction in titre compared with the rescued mutant [6]. In contrast, mutant M133 (lacking 23 amino acids of the UL21 gene), showed the same titre and plaque size as the rescued mutant (data not shown).

De Wind et al. [6] demonstrated by cell-fractionation that the UL21 encoded protein in PRV is partially localised in the virion and in the nucleus of the cell and that UL21 is involved in cleavage of the viral DNA.

In conclusion, our results support the notion that the protein encoded by the UL21 gene also has a function in the packaging of viral DNA into the capsid.

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