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

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(Received 18 July 2000; accepted 9 October 2000)

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

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, concatamers 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 BamHI fragment 4 (Fig. 1). According to Lomniczi et al. [13] BamHI 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...
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 EcoRI-BamHI 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

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**Figure 1.** Physical map of the PRV genome (upper line). Open rectangles represent the internal (I<sub>R</sub>) and terminal (T<sub>R</sub>) repeat sequences which divide the genome into the U<sub>L</sub> and U<sub>S</sub> regions. BamHI fragment 4 is enlarged to show the genes and their transcriptional directions (arrows) from [6, 11].
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.
UL21 gene in Pseudorabies virus
(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).

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

REFERENCES


[13] Lomniczi B., Watanabe S., Ben-Porat T., Kaplan A.S., Genome location and identification of func-


