

The UL49.5 Gene of Pseudorabies Virus Codes for an O-Glycosylated Structural Protein of the Viral Envelope

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Sequence analysis of *Bam*HI fragment 1 of the pseudorabies virus (PrV) genome identified a novel PrV gene located upstream of the UL50 gene encoding PrV dUTPase. The deduced protein product displayed homology to the product of the herpes simplex virus type 1 UL49.5 protein. The predicted PrV UL49.5 protein consists of 98 amino acids with a calculated molecular mass of 10,155 Da. It contains putative signal peptide and transmembrane domains but lacks a consensus sequence for N glycosylation. PrV UL49.5 was expressed as a fusion protein with glutathione *S*-transferase in *Escherichia coli*, and a rabbit antiserum was generated. In Western blots (immunoblots) of purified virions, the antiserum detected a protein with an apparent molecular mass of 14 kDa. After fractionation of the virions, the 14-kDa protein was detected in the envelope fraction. Localization of the UL49.5 protein in the viral envelope was confirmed by immunoelectron microscopy. The treatment of purified virions with glycosidases led to a reduction of the apparent molecular mass in Western blots by approximately 2 kDa following digestion with neuraminidase and *O*-glycosidase. Our results demonstrate that the PrV UL49.5 protein is an O-glycosylated structural component of the viral envelope. It represents the 10th PrV glycoprotein described. According to the unified nomenclature for alphaherpesvirus glycoproteins, we propose to designate it glycoprotein N (gN).

Herpesviruses are large enveloped viruses with a linear double-stranded DNA genome. Characteristically, herpes virions contain a large number of different glycoproteins in their envelopes, some of which are conserved in the subfamilies alpha-, beta-, and gammaherpesviruses. These structural glycoproteins play important roles in the interaction between virus and host both in mediating infection of target cells and in eliciting immune responses.

Among the alphaherpesviruses, herpes simplex virus type 1

(HSV-1) has been most extensively studied. It has been shown to specify at least 11 glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM (2, 24). With the exception of gJ, pseudorabies virus (PrV), also a member of the alphaherpesvirus subfamily, encodes homologs of all HSV glycoproteins identified so far (21). Recently, the gene encoding the PrV homolog of gM (UL10) has been sequenced and a protein product has been detected in purified virions (16). Of the 11 HSV glycoproteins, four are conserved in all mammalian her-

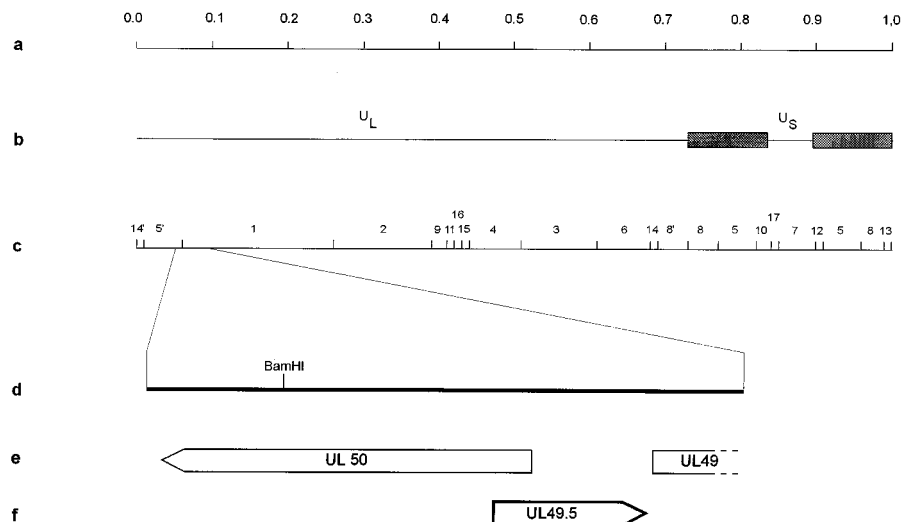


FIG. 1. Location of the PrV UL49.5 gene. (a) Map units. (b) Schematic representation of the PrV genome consisting of a long unique (U_L) segment and a short unique (U_S) segment which is bracketed by inverted repeats. (c) *Bam*HI restriction map. (d) Enlargement of the junction region between *Bam*HI fragments 5' and 1. (e) Locations of the PrV UL50 and UL49 genes. (f) Location of the PrV UL49.5 gene.

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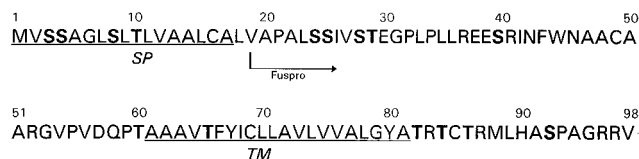


FIG. 2. Deduced amino acid sequence of the PrV UL49.5 polypeptide. Indicated are the hydrophobic putative signal peptide (SP) and transmembrane (TM) domains. Serine and threonine residues which might accept O-linked carbohydrates are printed in boldface type. An arrow indicates the part of the UL49.5 protein that was expressed as a fusion protein with GST in *E. coli*. It lacks the first 18 amino acids of the authentic UL49.5 protein.

pesviruses that have been investigated, namely, gB, gH, gL, and gM. Of these, gB, gH, and gL are essential for replication in tissue culture, which is indicative of their important function in the life cycle of the respective virus (21, 24).

When the complete sequence of the HSV-1 genome had been determined, it was predicted to contain 70 distinct genes (20). Further analysis of the coding potential revealed the existence of additional open reading frames (ORFs), UL26.5 (19), RL1 (8, 10), and UL49.5 (3, 4). UL49.5 of HSV is located close to the 5' end of UL50 in the same orientation as UL49. A γ 2 transcript has been mapped to this genomic region (map unit 0.701 [11]), but since there has been no evidence that the ORF was expressed, it has subsequently been ignored. The HSV-1 and HSV-2 UL49.5 ORFs encompass 91 and 87 codons, respectively, and the predicted protein products display characteristics of a membrane-spanning protein (3, 4). Homologs of HSV UL49.5 have been identified in all herpesvirus genomes analyzed so far, including the alphaherpesviruses HSV-1 and HSV-2, varicella-zoster virus, equine herpesvirus 1, and bovine herpesvirus 1 (BHV-1); the betaherpesvirus human cytomegalovirus; and the gammaherpesvirus Epstein-Barr virus (1, 4, 7, 9, 18, 20, 26). An epitope-tagged HSV-1 UL49.5 protein has been shown to be expressed in infected cells (3). However, no authentic gene product of any of the UL49.5 homologs has so far been characterized.

A comparison of the genomic sequences of alphaherpesviruses showed that their genes are arranged in a colinear fashion. This colinearity also applies to PrV, as far as can be determined from the sequence information available, with the exception that a large conserved gene block within the unique long segment is inverted compared with the sequences of HSV, varicella-zoster virus, and equine herpesvirus 1 (6). By sequence analysis of the region upstream of UL50 of PrV, we identified the PrV UL49.5 homolog.

Cloned *Bam*HI restriction fragments of PrV DNA were kindly provided by T. Ben-Porat (Nashville, Tenn.). Sequence determination was performed by the dideoxy chain termination method (23) with the Pharmacia T7 sequencing kit and [³⁵S]dATP (ICN, Meckenheim, Germany). Each strand was sequenced at least once with the regular set of nucleotides and once replacing dATP with c7-deaza-ATP and dGTP with c7-deaza-GTP. Sequences were analyzed with the Wisconsin Package (version 8, September 1994; Genetics Computer Group, Madison, Wis.).

By sequence analysis within a 2-kb *Bam*HI-*Sal*I subfragment of *Bam*HI fragment 1, which lies adjacent to the *Bam*HI 5' fragment (5), we identified a novel ORF in an orientation opposite to that of UL50 which encodes PrV dUTPase (13). This ORF consists of 294 nucleotides capable of encoding a polypeptide of 98 amino acids (GenBank accession number U38547). Its 5' end overlaps the 5' end of UL50 by 77 nucle-

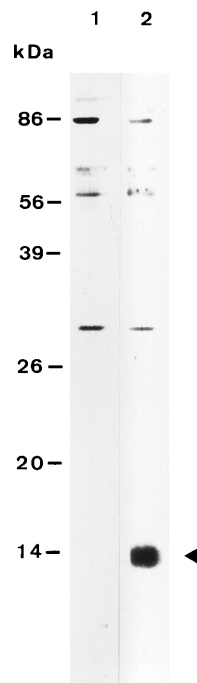


FIG. 3. Identification of the PrV UL49.5 protein as a component of virions. A Western blot of purified virions separated by SDS-13% PAGE (5 μ g of protein per lane) is shown. Lane 1 was incubated with preimmune serum, and lane 2 was incubated with the α -UL49.5 serum, both of which were diluted 1:200. The arrowhead indicates a protein of 14 kDa specifically detected by the α -UL49.5 serum.

otides (Fig. 1). A putative TATA box lies 76 nucleotides upstream of the initiation codon.

This genomic region is colinear with the homologous region of the HSV genome, and the deduced protein product of the

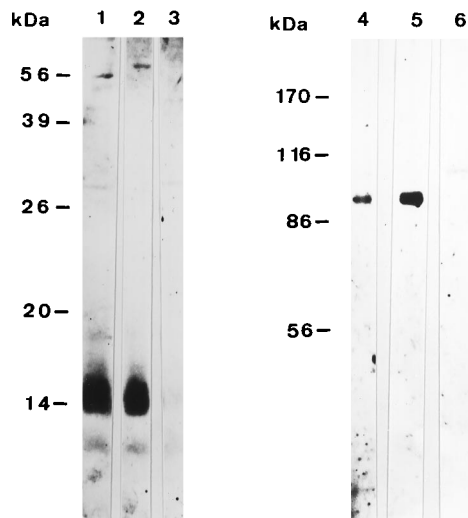


FIG. 4. Localization of the UL49.5 protein in the viral envelope. Western blots of fractionated envelopes and nucleocapsids of purified virions are shown. Lanes 1 and 4, whole virions; lanes 2 and 5, viral envelopes; lanes 3 and 6, nucleocapsids. Lanes 1 to 3 were incubated with the α -UL49.5 serum, and lanes 4 to 6 were incubated with a polyclonal antiserum against gH of PrV (15). The PrV UL49.5 protein is detected only in the whole virion sample and in the envelope fraction, as is gH.

newly identified ORF (Fig. 2) exhibits homology to the HSV-1 UL49.5 gene product and homologs of other herpesviruses (data not shown). The PrV UL49.5 homolog is most closely related to its BHV-1 counterpart (amino acid sequence identity, 39.3% [18]). A hydrophobicity plot of the deduced PrV UL49.5 polypeptide calculated with the programs "peptide-structure" and "pepplot" of the Wisconsin Package indicated that it has characteristics of a membrane protein (data not shown). A hydrophobic N terminus could serve as the signal sequence, and a second hydrophobic domain near the C terminus could function as membrane anchor (Fig. 2).

To characterize the putative protein product of UL49.5, a polyclonal rabbit antiserum was generated. A restriction fragment of 280 bp containing the UL49.5 ORF with the exception of the 5'-proximal 54 bp encoding the hydrophobic amino terminus (Fig. 2) was cloned into the procaryotic expression vector pGEX-4T (Pharmacia, Heidelberg, Germany). Genes or gene fragments inserted into pGEX are expressed as fusion proteins with glutathione *S*-transferase (GST), an enzyme of *Schistosoma japonicum* which is not present in *Escherichia coli*, under the control of the *lac* promoter. By cleavage with throm-

bin, the GST portion can be removed. Induction with IPTG (isopropyl- β -D-thiogalactopyranoside) led to overexpression of a fusion protein with the expected molecular mass of 34 kDa (a 26-kDa GST fusion part plus an 8-kDa UL49.5 fragment) as analyzed by sodium dodecyl sulfate-13% polyacrylamide gel electrophoresis (SDS-13% PAGE). The PrV UL49.5 fusion protein was purified by adsorption to glutathione-Sepharose beads via the GST part and elution with a glutathione solution. In Western blots (immunoblots), the expression product was recognized by an antiserum against GST. The eluted fusion protein was dialyzed extensively against phosphate-buffered saline (PBS) and injected into a rabbit. After three booster injections, an antiserum was obtained that detected the cleaved UL49.5 protein fragment of the GST-UL49.5 fusion protein in Western blots as an 8-kDa peptide, as expected (data not shown).

With the antiserum generated against the PrV UL49.5 fusion protein (α -UL49.5 serum) the UL49.5 protein was identified in Western blots of purified virions. PrV strain Ka was used as the prototypic wild-type strain (14) and propagated on pig kidney cells (PK15 or PSEK). Cells were infected at a

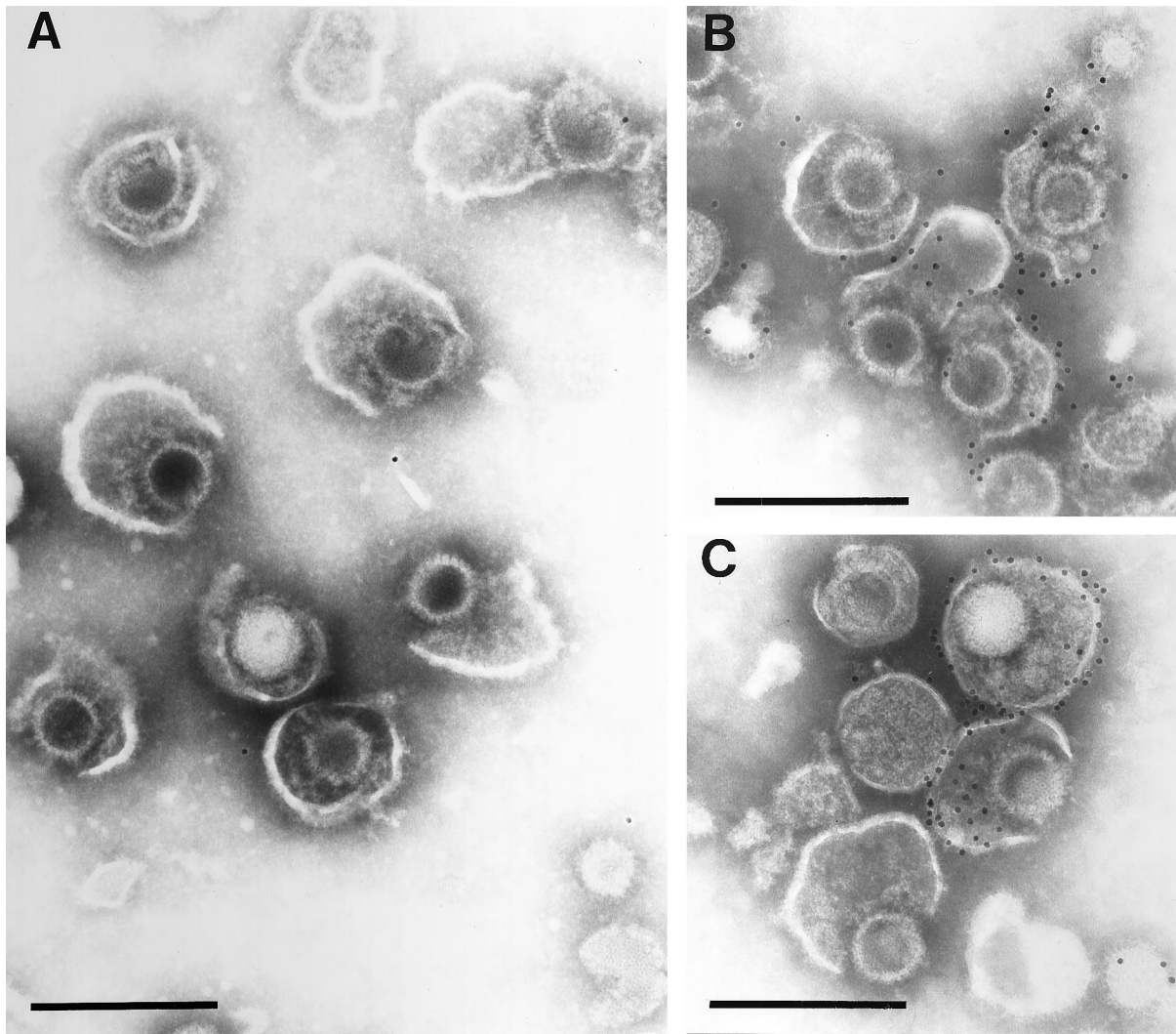


FIG. 5. Immunoelectron microscopy. Purified virions were incubated with either the preimmune serum (A), a control monoclonal antibody directed against gB (B), or the α -UL49.5 serum (C). The distribution of the gold label indicates specific binding of the anti-gB and α -UL49.5 antibodies to the envelopes of extracellular virions. Bars, 300 nm.

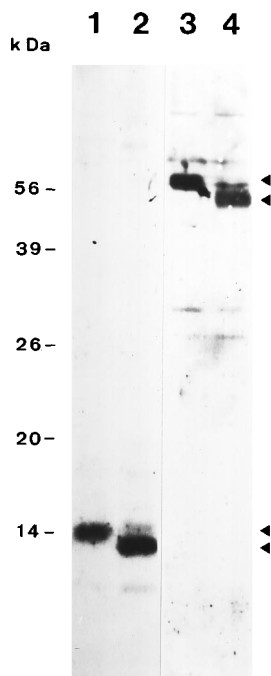


FIG. 6. The PrV UL49.5 protein is O glycosylated. Western blots of purified virions separated by SDS-13% PAGE and incubated with either the α -UL49.5 serum (lanes 1 and 2) or a monoclonal antibody against gD (lanes 3 and 4). Untreated virions (lanes 1 and 3) and virions treated with neuraminidase-O-glycosidase (lanes 2 and 4) were analyzed. Arrowheads indicate the mobility shifts of the UL49.5 protein (lanes 1 and 2) and of gD (lanes 3 and 4) after de-O-glycosylation.

multiplicity of infection of 1, and the supernatant was harvested after complete cytopathic effect was observed. Cell debris was sedimented by low-speed centrifugation ($800 \times g$, 10 min, 4°C), and virus was purified as previously described (17) by centrifugation on a discontinuous sucrose gradient. The protein concentration was determined by a modification of the method of Smith (25). Proteins of purified virions were separated by SDS-PAGE and analyzed in Western blots (Fig. 3). Whereas the preimmune serum did not exhibit any specific reactivity, the α -UL49.5 serum specifically detected a 14-kDa protein in lysates of purified virions (Fig. 3). This result demonstrates that PrV UL49.5 is expressed in infected cells, that its gene product has an apparent molecular mass of 14 kDa, and that it represents a structural component of PrV virions.

Since the deduced amino acid sequence of the PrV UL49.5 protein exhibits characteristics of a membrane protein (Fig. 2), we examined the location of the UL49.5 protein in the virion by two different approaches. Virions were fractionated into envelopes and nucleocapsids essentially as previously described (12). To purified virions, Triton X-100 was added to a final concentration of 1%. After incubation for 20 min at 45°C with occasional gentle shaking, the samples were centrifuged in a Beckman TLA45 rotor at $25,000 \text{ rpm}$ at 4°C for 30 min. The supernatant (envelope fraction) was collected, and the pellet (nucleocapsid fraction) was washed once with PBS. Fractions were separated by SDS-PAGE and examined by Western blotting with the α -UL49.5 serum (Fig. 4). As a control, a polyclonal antiserum against gH was used on the same preparations in parallel blots (15). The 14-kDa UL49.5 protein was detectable only in the envelope fraction, as was gH. The nucleocapsid fraction did not contain either the UL49.5 protein or gH as assayed by Western blotting.

The second approach employed immunoelectron microscopy for confirmation of the location of the UL49.5 protein in the virion. Purified virions diluted in PBS (0.5 mg of protein per ml) were adsorbed to Formvar- and carbon-coated 300-mesh copper grids for approximately 5 min at room temperature and subsequently floated on drops of either the preimmune serum or the α -UL49.5 serum for 45 min at room temperature. Antibodies were diluted in PBS-1% bovine serum albumine (BSA), pH 7.2. The grids were washed with PBS-BSA, bound antibodies were detected with colloidal gold-labelled goat anti-mouse immunoglobulin G or colloidal gold-labelled protein A (PAG_{10} ; BioCell, Cardiff, United Kingdom), and samples were negatively stained with 2% phosphotungstic acid (pH 7.4). The gold label was found almost exclusively on viral envelopes incubated with the α -UL49.5 serum (Fig. 5C). Preparations incubated with preimmune serum were essentially free of label (Fig. 5A). As a positive control, virions were labelled with a monoclonal antibody directed against PrV envelope glycoprotein gB (a generous gift from B. Klupp and E. Weiland) and gold-labelled goat anti-mouse IgG (GAM_{10} ; BioCell). Thus, two different assays demonstrated that the PrV UL49.5 protein is associated with the viral envelope.

The predicted molecular mass from the deduced amino acid sequence of UL49.5 is 10,155 Da. Cleavage of the putative signal sequence would reduce the molecular mass by approximately 2 kDa. However, by SDS-PAGE the apparent molecular mass of the mature protein amounts to 14 kDa. This discrepancy may indicate posttranslational modifications of the UL49.5 protein. The PrV UL49.5 protein does not specify consensus sequences for the addition of N-linked carbohydrates, but it contains eight serine and seven threonine residues which are potential sites for O glycosylation (Fig. 2). To assay whether the PrV UL49.5 protein is glycosylated, O-linked glycans were removed by enzymatic digestion. Purified virions (10 mg of protein) were incubated with 2 mU of *Vibrio cholerae* neuraminidase (Boehringer, Mannheim, Germany) in 50 mM sodium acetate (pH 5.2)-4 mM CaCl_2 for 1 h at 37°C . For subsequent O-glycosidase digestion, virions were pelleted at $125,000 \times g$ for 45 min at 4°C , resuspended in 20 mM Tris-phosphate (pH 7.4), and incubated for 16 h at 37°C with 1 mU of O-glycosidase (BSA free; Boehringer) as previously described (15). Samples were separated by SDS-PAGE, transferred onto nitrocellulose, and analyzed by Western blotting with the α -UL49.5 serum (Fig. 6). By neuraminidase-O-glycosidase treatment, the apparent molecular mass of the PrV UL49.5 protein was reduced by approximately 2 kDa (Fig. 6, lane 2) compared with that of authentic virion UL49.5 protein (Fig. 6, lane 1). As a control for the successful removal of O-linked glycans, samples from the same preparations were incubated with a monoclonal antibody against PrV glycoprotein D (generously provided by B. Klupp and E. Weiland), which is exclusively O glycosylated (Fig. 6, lane 3 [22]). O-Glycosidase treatment also reduced the apparent molecular mass of gD as determined by SDS-PAGE (Fig. 6, lane 4). Whether additional posttranslational modifications occur on the UL49.5 protein is unclear at present.

In summary, the translation product of the PrV UL49.5 gene represents a structural glycoprotein, the 10th PrV glycoprotein described. Since no homologous glycosylated gene product has been identified in other herpesviruses so far, according to the unified nomenclature of alphaherpesvirus glycoproteins we propose to designate it glycoprotein N (gN).

Concerning the function of gN in the viral replicative cycle, it will be of great interest to determine whether PrV UL49.5 is an essential gene, as is suggested for HSV-1 UL49.5 (3), or if

it is dispensable for replication in tissue culture, like the BHV-1 UL49.5 homolog (18). To this end, studies are under way to isolate a gN⁻ PrV mutant to subsequently investigate its function in virus replication. As a component of the viral envelope, gN might play a role either during infection of a target cell or during morphogenesis of progeny virions and their release from cells.

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