Pseudorabies Virus Protein Homologous to Herpes Simplex Virus Type 1 ICP18.5 Is Necessary for Capsid Maturation

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In pseudorabies virus (PrV), an open reading frame that partially overlaps the gene for the essential glycoprotein gII has been shown to encode a protein homologous to the ICP18.5 polypeptide of herpes simplex virus type 1 (N. Pederson and L. Enquist, *Nucleic Acids Res.* 17:3597, 1989). To study the function of this protein during the viral replicative cycle, a PrV mutant which carries a β -galactosidase expression cassette interrupting the ICP18.5(PrV) gene was constructed. This mutant could be propagated only on cell lines that were able to provide ICP18.5(PrV) *in trans* after transformation with a corresponding genomic PrV DNA fragment. Detailed analysis showed that inactivation of the ICP18.5(PrV) gene did not impair infection of noncomplementing cells, nor did it impair early or late gene expression, as shown by immunoprecipitation of glycoproteins gII, gIII, and gp50. Surface localization of glycoproteins as demonstrated by fluorescence-activated cell sorting analyses was also not affected. Southern blot hybridizations, however, showed that cleavage of replicative concatemeric viral DNA did not occur in noncomplementing cells infected by the ICP18.5 mutant PrV. In addition, electron microscopic analysis revealed an accumulation of empty capsids in the nucleus of mutant-infected noncomplementing cells. We conclude that the ICP18.5(PrV) protein is necessary for viral replication and plays an essential role in the process of mature capsid formation.

Gene blocks containing highly conserved open reading frames have been identified after complete sequence information for an increasing number of herpesvirus genomes has become available. One of these conserved blocks encompasses genes encoding proteins homologous to herpes simplex virus type 1 (HSV-1) glycoprotein gB, a protein designated as ICP18.5, ICP8, and the DNA polymerase. Homologs to these genes have been found in every herpesvirus analyzed in this respect so far (2, 3, 6, 9, 11, 16, 18, 19, 24, 27, 36, 40, 48, 49). Whereas functions for the DNA polymerase and the DNA-binding protein ICP8 have been analyzed in detail (5, 8, 10, 14, 29, 45) and the role of glycoprotein gB in membrane fusion events during viral infection has been demonstrated (7), the role of ICP18.5 during viral infection is still not well understood. Analysis of temperature-sensitive HSV-1 mutants indicated that mutant *ts1203*, whose lesion had been mapped to the ICP18.5 (U_L28) gene, at the nonpermissive temperature exhibits a defect in cleavage and encapsidation of high-molecular-weight viral DNA (1). However, a detailed analysis of a genetically engineered specific viral mutant has not yet been performed. In addition, the role that the highly conserved ICP18.5 homologs play in other viruses has not been studied.

In pseudorabies virus (PrV), the ICP18.5 homolog has been shown to constitute a 79-kDa protein expressed from a gene that overlaps by its 44 3'-terminal codons the gene encoding the essential glycoprotein gII (31, 32). During construction and isolation of PrV mutants deficient in synthesis of glycoprotein gII (homolog of HSV-1 gB), we observed that we were unable to isolate on gII-expressing complementing cells a viral mutant that in addition to a deletion in the gII gene also lacked the 3'-terminal 31 codons of the gene encoding ICP18.5(PrV). We report here that this mutant could indeed be purified on a cell line that expressed ICP18.5(PrV) as well as gII. In addition, we isolated a PrV

β -galactosidase (β -Gal) insertion mutant in the ICP18.5 gene and showed the essential character of ICP18.5 for PrV replication. After infection of noncomplementing cells, the ICP18.5 PrV mutant is blocked during mature capsid formation at or prior to cleavage and encapsidation of replicated concatemeric viral DNA.

MATERIALS AND METHODS

Viruses and cells. All virus mutants described in this report were derived from PrV strain Ka (23). They were propagated on Vero cells or derivatives thereof. Construction of plasmids containing deletion $\Delta 1$ or $\Delta 2$ in the gII gene with a concomitant insertion of *Bam*HI linkers and a gX- β -Gal expression cassette has been described elsewhere (35). Isolation and characterization of a PrV gII⁻ mutant carrying deletion $\Delta 2$ has been reported previously (35). Genotypes and phenotypes of viral mutants used are compiled in Table 1.

Transfection and blue plaque screening. Plasmid DNA was cotransfected with viral genomic DNA into Vero cells by the calcium phosphate coprecipitation technique (17). After complete cytopathic effect had been induced, viral progeny was harvested and plated onto complementing 19-11 cells. After 2 days under methylcellulose medium, when plaques were clearly visible, the methylcellulose medium was removed and the monolayers were overlaid with medium containing 1% agarose and 500 μ g of Blue-Gal (Bethesda Research Laboratories, Eggenstein, Germany) per ml (28). After further incubation at 37°C for 24 h, blue-staining plaques appeared. They were collected by aspiration, replated, and plaque purified three times.

Southern blot hybridization. Genomic viral DNA was extracted from virions after centrifugation of supernatants harvested from infected cells 24 h postinfection (p.i.) at 22,000 rpm for 2 h in a Beckman SW28 rotor. Whole cellular DNA was obtained after trypsinization of infected cells at 12 h p.i., after which cells were collected by low-speed centrif-

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TABLE 1. Characteristics of viral mutants

Mutant	Mutation	Gene(s) affected
6321	$\Delta 1/\beta$ -Gal ^a	gII, ICP18.5
4112	$\Delta 2/\beta$ -Gal	gII
332-31	β -Gal	ICP18.5

^a β -Gal denotes insertion of the gX- β -Gal cassette. Deletions $\Delta 1$ and $\Delta 2$ are indicated in Fig. 1.

ugation. After lysis and pronase digestion, DNA was phenol extracted, precipitated with ethanol, and pelleted by centrifugation. Restriction enzyme digestions were performed by using conditions recommended by the supplier (Bethesda Research Laboratories) in 20- μ l assays containing 2 μ g of DNA. Gel electrophoresis and transfer to nitrocellulose membranes were done according to standard procedures (42). Hybridization probes were labeled by nick translation (nick translation kit; Amersham-Buchler, Braunschweig, Germany). Hybridization and washing conditions were as described previously (41).

Radioimmunoprecipitation. Cells were infected at a multiplicity of infection (MOI) of 2 and labeled in medium containing 100 μ Ci of [³⁵S]methionine per ml from 2 to 24 h p.i. Thereafter, cells were lysed and processed for immunoprecipitation as described. Antibodies used were anti-gII monoclonal antibody (MAb) 5/14 (26), anti-gIII MAb M1 (20), and anti-gp50 MAb MCA50-1 (46). A polyclonal mouse serum directed against a bacterial MS2-polymerase-ICP18.5(PrV) fusion protein (13, 43) was generously provided by H.-J. Rziha, Tübingen, Germany.

Fluorescence-activated cell sorting analyses. Cells were infected at an MOI of 2 for 1 h at 4°C. Thereafter, the inoculum was removed and the cell monolayer was overlaid with prewarmed (37°C) medium. Immediately after the temperature shift and at 4 and 8 h thereafter, cells were removed from the plate by incubation for 10 min with 0.02% EDTA in phosphate-buffered saline without divalent cations. Surface expression of glycoproteins gI, gII, and gIII was analyzed in a fluorescence-activated cell sorter (FACStar Plus; Becton Dickinson, Mountain View, Calif.), using MAbs 3/6 (anti-gI [26]), 5/14 (26), and M1 (20), respectively. As a control antibody, MAb 11/295/33 directed against the porcine lymphocytic CD8 surface molecule was used (22).

Electron microscopy. Vero cells were infected at an MOI of 5 with wild-type PrV and mutant 332-31, respectively, and fixed 15 h p.i. with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Cells were gently scraped off the plate, collected by centrifugation, and preembedded in 4% agarose. Cell pellets were postfixed with 1% osmium tetroxide, dehydrated in acetone, and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined with a Zeiss 109 electron microscope (Zeiss, Oberkochen, Germany).

RESULTS

Construction of an ICP18.5(PrV)-expressing cell line. We previously reported on the isolation of a Vero cell line inducibly expressing glycoprotein gII(PrV), designated N7, which was able to complement a PrV mutant lacking approximately two-thirds from the middle of the gII gene ($\Delta 2$; Fig. 1) (34, 35). However, a PrV mutant containing a larger deletion encompassing nearly all of the gII gene ($\Delta 1$; Fig. 1) could not be isolated on N7 cells. We reasoned that the failure to isolate this mutant was due to an impairment of

ICP18.5 expression, since deletion $\Delta 1$ also removed the 3'-terminal 31 codons of the ICP18.5 gene. To test this assumption, cells were cotransfected with plasmids pSV2-neo, conferring resistance to the antibiotic G418 (44), and pFBT-19, containing the genomic *KpnI* fragment C of PrV. In *KpnI* fragment C, among others, the complete genes for ICP18.5 and gII are located. Transformed cells were selected with G418, and single colonies were isolated. They were then analyzed for the ability to complement the gII- $\Delta 2$ deletion mutant. One positive clone, 19-11, was selected for further experiments.

Isolation of a gII⁻ PrV mutant containing deletion $\Delta 1$. After cotransfection of wild-type PrV DNA and a plasmid containing a PrV DNA fragment with deletion $\Delta 1$ and insertion of a β -Gal expression cassette (35), viral progeny was tested on 19-11 cells for the appearance of blue plaques after staining with Blueo-Gal. Blue-staining plaques were identified, and they could be purified to homogeneity. One plaque isolate, mutant 6321, was analyzed further for presence of the correct mutation. To this end, viral DNA was isolated and after cleavage with *Bam*HI separated in an 0.8% agarose gel. As can be seen in Fig. 2A, compared with wild-type PrV (lanes 1), fragment *Bam*HI-1 appeared smaller, and an additional fragment migrating between *Bam*HI-4 and -5 appeared in DNA from mutant 6321 (lanes 3). A similar pattern was observed after cleavage of DNA from the gII⁻ PrV mutant 4112 containing deletion $\Delta 2$ (lanes 2). Cleavage of *Bam*HI fragment 1 into two subfragments was due to the insertion of a *Bam*HI cleavage site prior to introduction of the β -Gal expression cassette after deletion of gII sequences $\Delta 1$ and $\Delta 2$. However, the smaller fragment migrated faster in mutant 6321 DNA, since deletion $\Delta 1$ encompassing 2,540 bp eliminated more sequences from this fragment than did deletion $\Delta 2$ of 1,395 bp. Hybridization with labeled *Bam*HI fragment 1 highlights this result (Fig. 2B). A 765-bp probe from the 5' terminus of the gII gene, as expected, recognized DNA from the gII $\Delta 2$ mutant but failed to hybridize to mutant 6321 containing deletion $\Delta 1$ (Fig. 2D; lanes 2 and 3). Hybridization with probes detecting ICP18.5 (Fig. 2C) and β -Gal sequences (Fig. 2E) confirmed correct presence of deletion $\Delta 1$ and concomitant insertion of the β -Gal cassette in mutant 6321.

Construction and isolation of a specific ICP18.5(PrV) mutant. In mutant 6321, both the gene encoding gII and the gene encoding ICP18.5 were impaired by deletion $\Delta 1$. To isolate a PrV mutant that lacked only ICP18.5, a *Stu*I subfragment of *Bam*HI fragment 1 that encompasses the 5' terminus of the gII gene and most of the gene encoding ICP18.5 (see Fig. 1) was blunt-end cloned into the *Sma*I site of a pBR322 derivative lacking *Bam*HI, *Sal*I, and *Sph*I sites (27a). The unique *Sph*I site in the ICP18.5 gene was then converted to a *Bam*HI site, using *Bam*HI linkers (New England Biolabs, Schwalbach, Germany) followed by introduction of a *Sal*I-*Bam*HI gX- β -Gal expression cassette (28, 35) (Fig. 1), resulting in plasmid TT-332/IV-2. Transcriptional orientation of the inserted marker gene paralleled that of the ICP18.5 and gII genes. After cotransfection of viral genomic DNA with plasmid TT-332/IV-2 into 19-11 cells, virus progeny was screened for the appearance of blue plaques after Blueo-Gal staining. Blue plaques were purified three times on 19-11 cells, and one isolate, 332-31, was used for further studies.

Analysis of viral DNA derived from mutant 332-31 showed correct integration of the β -Gal cassette into the ICP18.5 gene. *Bam*HI fragment 1, which acquired an additional *Bam*HI cleavage site as a result of the linker insertion, was cleaved into two fragments by *Bam*HI (Fig. 2A and B, lanes

FIG. 1. Diagram of PrV genomic regions relevant for isolation and characterization of viral mutants. (A) Schematic representation of the PrV genome. U_L and U_S denote the long and short unique parts of the viral DNA. Open rectangles represent inverted repeat regions. (B) *Bam*HI and *Kpn*I restriction fragment map. (C) Enlargement of the region encompassing the genes encoding gII and ICP18.5. Transcriptional direction is indicated by horizontal arrows. Relevant cleavage sites: Bs, *Bst*EII; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I. $\Delta 1$ and $\Delta 2$ denote deletions introduced into the gII gene concomitant with introduction of a *Bam*HI site and integration of the β -Gal expression cassette (28). (D) Insertion of the β -Gal expression cassette into the ICP18.5(PrV) gene. B, *Bam*HI; (Sa), *Sal*I site that was lost during cloning. Shaded areas with roman numerals show positions of hybridization probes used for Fig. 2.

4). The smaller of these fragments hybridized as expected with probe III originating from the 5'-terminal part of the gII gene, which was absent in mutant 6321 containing deletion $\Delta 1$ (Fig. 2D, lanes 3 and 4). Labeled probe II consisting of the *Stu*I fragment used for β -Gal insertion recognized both subfragments of *Bam*HI-1, as expected (Fig. 2C, lane 4). The β -Gal-specific hybridization highlighted corresponding fragments in mutants 4112 (Fig. 2E, lane 2), 6321 (lane 3), and 332-31 (lane 4). In conclusion, we were able to isolate a PrV mutant containing a β -Gal expression cassette interrupting the ICP18.5(PrV) gene.

Propagation of PrV mutants on complementing and non-complementing cells. To assay the importance of ICP18.5 expression for PrV replication, mutants deficient in gII (4112), gII and ICP18.5 (6321), or only ICP18.5 (332-31) after propagation on 19-11 cells were titrated on normal Vero cells or gII-expressing N7 or gII- and ICP18.5-expressing 19-11 cells in comparison with wild-type PrV. As is evident in Fig. 3, none of the mutants was able to efficiently form plaques on normal Vero cells. The residual plaque-forming ability was most likely due to revertants rescued by the resident viral fragment from the complementing cell lines. This result reflects the essential character of gII(PrV) for viral replication and also indicates that ICP18.5 constitutes an essential

PrV protein. On N7 cells that provide only gII in *trans*, the gII $\Delta 2$ mutant 4112 is able to efficiently form plaques, whereas neither the ICP18.5 mutant 332-31 nor the $\Delta 1$ mutant 6321 showed plaque formation. On cell line 19-11, which is able to provide both proteins in *trans*, all viral mutants were capable of efficient plaque formation. Wild-type PrV was able to efficiently form plaques on all cells. In conclusion, we show that mutants with an impairment in ICP18.5 expression are unable to form plaques on cells lacking ICP18.5 complementation, demonstrating that the presence of ICP18.5 is essential for PrV plaque formation. In the following experiments, we focused on the specific ICP18.5(PrV) mutant 332-31.

Absence of ICP18.5 in mutant 332-31-infected cells. To analyze ICP18.5(PrV) expression in mutant 332-31-infected cells, either normal Vero cells (Fig. 4, lanes 1 and 3) or ICP18.5-expressing 19-11 cells (lanes 2 and 4) were infected at an MOI of 2 with mutant 332-31 (lanes 1 and 2) or wild-type PrV (lanes 3 and 4). From 2 until 24 h p.i., proteins were labeled with [35 S]methionine. Cell lysates were then immunoprecipitated with either a mouse antiserum directed against a bacterial ICP18.5 fusion protein (Fig. 4A) or negative control serum (Fig. 4B). Figure 4 shows the result of a fluorography of immunoprecipitates after electrophore-

FIG. 2. Southern blot hybridization of wild-type and mutant PrV DNAs. Virion DNA of either wild-type PrV (lanes 1) or of mutant 4112 (lanes 2), 6321 (lanes 3), or 332-31 (lanes 4) was cleaved with *Bam*HI, and the resulting fragments were separated in a 0.8% agarose gel. (A) Ethidium bromide-stained gel; (B to E) results after hybridization with labeled genomic fragment *Bam*HI-1 (B), after hybridization with a *Stu*I fragment encompassing most of the ICP18.5 gene and the 5' terminus of the *g*II gene (C), after hybridization with a *Sma*I fragment from the 5'-terminal part of the *g*II gene (D), and after hybridization with a β -Gal-specific probe (E). Roman numerals identify hybridization probes as indicated in Fig. 1.

sis in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels under reducing conditions. Whereas ICP18.5 is present in all wild-type PrV-infected cells (Fig. 4A, lanes 3 and 4) and also in 19-11 cells infected by mutant 332-31 (Fig. 4A, lane 2), albeit in lesser amounts, it is absent from normal Vero cells infected with mutant 332-31 (Fig. 4A, lane 1). Other proteins were nonspecifically precipitated in an identical fashion from all PrV-infected cells (Fig. 4A and B).

Growth kinetics of ICP18.5 mutant 332-31 on complementing and noncomplementing cells. To further analyze the effect of lack of ICP18.5, complementing 19-11 or normal Vero cells were infected at an MOI of 1 with either wild-type PrV

or ICP18.5 mutant 332-31. Supernatants taken at different times p.i. were titrated on 19-11 cells. As shown in Fig. 5, mutant 332-31 was able to produce infectious virions only on 19-11 cells, whereas on noncomplementing cells, infectious progeny was not observed. In contrast, wild-type PrV replicated equally well on normal Vero and 19-11 cells. It is also evident that replication of mutant 332-31 on 19-11 cells was less efficient than wild-type PrV replication on these cells, indicating that the defect due to lack of ICP18.5 expression was not fully compensated for in the complementing cell line.

Expression of glycoproteins is not impaired in mutant

FIG. 3. Titration of viral mutants on complementing and noncomplementing cell lines. Wild-type PrV (▨), mutant 6321 (▩), 4112 (□), or 332-31 (▧) was propagated on 19-11 cells and titrated on either Vero, *g*II-expressing N7, or *g*II- and ICP18.5-expressing 19-11 cells. Titers in PFU per milliliter are indicated.

FIG. 4. Lack of ICP18.5 in mutant 332-31-infected noncomplementing cells. Normal Vero cells (lanes 1 and 3) or complementing 19-11 cells (lanes 2 and 4) were infected at an MOI of 2 with mutant 332-31 (lanes 1 and 2) or wild-type PrV (lanes 3 and 4). ^{35}S -labeled proteins were precipitated by using either an anti-ICP18.5(PrV) serum (A) or a negative control serum (B). The arrow indicates the position of ICP18.5(PrV).

332-31-infected cells. To further characterize the defect associated with lack of ICP18.5 in PrV, normal Vero cells (Fig. 6, lanes 1 and 3) or 19-11 cells (lanes 2 and 4) were infected with mutant 332-31 (lanes 1 and 2) or wild-type PrV (lanes 3 and 4) at an MOI of 2, and proteins were labeled with [^{35}S]methionine. After radioimmunoprecipitation with MAbs directed against gII (Fig. 6A), gIII (Fig. 6B), or gp50 (Fig. 6C), precipitates were separated under nonreducing conditions in SDS-10% polyacrylamide gels and visualized by fluorography. As is evident in Fig. 6, no difference in expression of any of these glycoproteins could be observed, regardless of the virus (wild type or mutant 332-31) or cell line (normal Vero or 19-11) used. The anti-gII MAb precipitated the 155-kDa glycoprotein complex gII and its 110- and 120-kDa precursor proteins (20, 26). The anti-gIII MAb recognized mature ca. 90-kDa gIII (37) as well as a protein of ca. 65 kDa which most likely represents an intracellular breakdown product of gIII that accumulates as a result of the long labeling period of 22 h. The anti-gp50 MAb precipitated gp50, which under our conditions migrated with a molecular weight of ca. 60,000.

Whereas the gII gene has been shown to be regulated as an early gene (4, 38), the gene encoding gIII has been described as a true late gene (4, 37). The gp50 gene is most likely regulated as an early-late gene (4). We therefore conclude that expression of glycoprotein genes belonging to different kinetic classes is not affected in mutant 332-31-infected noncomplementing cells.

Surface localization of glycoproteins is not affected in mutant 332-31-infected cells. It was previously reported that cells infected by ICP18.5 mutants of HSV-1 exhibited a relative resistance to immune-mediated cytolysis, which was attributed to a failure of glycoprotein translocation to the cellular surface and proper presentation to the immune system (30, 33). To test this in our mutant, complementing 19-11 or normal Vero cells were infected with mutant 332-31 at an MOI of 1. Immediately after 1 h of adsorption at 4°C followed by a temperature shift to 37°C and at 4 and 8 h thereafter, the cells were harvested and reacted with anti-

FIG. 5. Growth of ICP18.5 mutant 332-31 in complementing or noncomplementing cells. Normal Vero cells (open symbols) or complementing 19-11 cells (closed symbols) were infected at an MOI of 1 with either wild-type PrV (triangles) or mutant 332-31 (circles). At the indicated times after infection, supernatants were titrated on 19-11 cells. Viral titers in PFU per milliliter are indicated.

bodies against gI, gII, and gIII or a control MAb (22). After incubation with a second fluorescein-conjugated antibody, cells were analyzed in a fluorescence-activated cell sorter for surface expression of glycoproteins. Figure 7 shows that no

FIG. 6. Glycoprotein expression in mutant 332-31-infected complementing and noncomplementing cells. Normal Vero cells (lanes 1 and 3) or complementing 19-11 cells (lanes 2 and 4) were infected with either mutant 332-31 (lanes 1 and 2) or wild-type PrV (lanes 3 and 4), and proteins were labeled with [^{35}S]methionine. Cell lysates were then immunoprecipitated with MAbs directed against gII (A), gIII (B), or gp50 (C) or a negative control MAb (D). Precipitates were separated in nonreducing SDS-10% polyacrylamide gels and subjected to fluorography.

FIG. 7. Surface expression of viral glycoproteins in mutant 332-31-infected complementing or noncomplementing cells. Complementing 19-11 (columns A) or normal (columns B) Vero cells were infected at an MOI of 1 with mutant 332-31. Immediately after a 1-h adsorption period at 4°C (0 h) and at 4 and 8 h after a temperature shift to 37°C, surface expression of glycoproteins was analyzed in a fluorescence-activated cell sorter after incubation of cells with anti-gI, anti-gII, or anti-gIII MAbs followed by incubation with a fluorescein-conjugated second antibody (solid lines). Dotted lines show results from assays using a control antibody (22). Indicated are the relative number of cells (y axis) and fluorescence intensity (x axis).

significant difference was observed between complementing (columns A) and noncomplementing (columns B) cells infected by ICP18.5 mutant 332-31. Both kinetics and amount of surface expression of the glycoproteins were similar, demonstrating that lack of ICP18.5 did not impair surface localization of glycoproteins. The weak positive signal observed with anti-gI and anti-gIII antibodies at 0 h most likely represents input virions that are attached at the outside of the cells after the adsorption period in the cold, where attachment occurs but penetration is prevented.

Cleavage of concatemeric replicative viral DNA is impaired in mutant 332-31-infected cells. It was previously established that a temperature-sensitive HSV mutant with a lesion in ICP18.5 exhibited a defect in cleavage of high-molecular-weight concatemeric DNA (1). To test for this phenotype, normal Vero or 19-11 cells were infected with either wild-type PrV or mutant 332-31 at an MOI of 1. At 12 h p.i., cells were harvested and total cellular DNA was extracted. After cleavage with *Bam*HI and agarose gel electrophoresis, fragments were transferred to nitrocellulose and probed with either labeled PrV virion DNA (Fig. 8A) or cloned PrV fragment *Bam*HI-13 (Fig. 8B), which constitutes a terminal genomic fragment (see Fig. 1). Hybridization with labeled PrV virion DNA (Fig. 8A) showed the presence of viral *Bam*HI fragments in DNA from wild-type-infected (Fig. 8A, lanes 1 and 3) and mutant 332-31-infected (Fig. 8A, lanes 2 and 4) Vero (Fig. 8A, lanes 1 and 2) or 19-11 (Fig. 8A, lanes 3 and 4) cells. The smaller fragment *Bam*HI-1 and the presence of an additional fragment migrating above *Bam*HI-4 is clearly visible in DNA from mutant 332-31-infected cells (Fig. 8A, lanes 2 and 4; compare with Fig. 2). Labeled *Bam*HI-13 detected both fragments 13 and 8' in wild-type-infected Vero cells (Fig. 8B, lane 1) and wild-type- and mutant 332-31-infected 19-11 cells (Fig. 8B, lanes 3 and 4) as a result of the presence in both fragments of homologous sequences derived from the inverted repeat regions (see Fig. 1). However, the terminal fragment 13 could not be found in mutant 332-31-infected Vero cells (Fig. 8B, lane 2).

FIG. 8. Defect in cleavage of concatemeric DNA after infection of noncomplementing cells by mutant 332-31. Normal Vero cells (lanes 1 and 2) or complementing 19-11 cells (lanes 3 and 4) were infected with either wild-type PrV (lanes 1 and 3) or mutant 332-31 (lanes 2 and 4) at an MOI of 1. Twelve hours p.i., cells were harvested and cellular DNA was isolated, cleaved with *Bam*HI, and electrophoresed in a 0.8% agarose gel. After transfer to nitrocellulose filters, the DNA was probed with either labeled total PrV DNA (A) or terminal fragment *Bam*HI-13 (B). Hybridization with *Bam*HI-13 revealed signals corresponding to fragments 8' and 13, which share homologous sequences since they are both derived from the inverted repeat regions, and the junction fragment composed of *Bam*HI-13 and -14', which is derived from head-to-tail concatemeric DNA. It is evident that terminal fragment 13 is missing in normal Vero cells infected by mutant 332-31 (B, lane 2). For locations of fragments, see Fig. 1.

FIG. 9. Electron microscopic examination of wild-type- or mutant 332-31-infected noncomplementing cells. Normal Vero cells infected with wild-type PrV (A) or mutant 332-31 (B) were fixed 15 h p.i., embedded in araldite, and after thin sectioning and staining analyzed with an electron microscope. $\triangleright$, an empty type I capsid with electron-lucent core structure; $\blacktriangleright$, an empty type II capsid without inner structure; $\rightarrow$, a full capsid; $\rightarrow$, a complete enveloped virion in the perinuclear space. In the upper right corner in each micrograph, the nuclear membrane is visible. The bar represents 200 nm.

The junction fragment containing *Bam*HI-13 and -14', which is derived from head-to-tail concatemeric replicative DNA and which comigrates with *Bam*HI-11 in Fig. 8A, is absent in wild-type PrV-infected Vero cells (Fig. 8B, lane 1), indicating efficient cleavage of concatemeric viral replicative DNA. In contrast, the junction fragment is present in DNA from mutant 332-31-infected Vero cells (Fig. 8B, lane 2). Together, these results show that cleavage of concatemeric DNA was impaired in ICP18.5(PrV) mutant-infected Vero cells.

The reason for the incomplete cleavage of both wild-type PrV and mutant 332-31 DNA after infection of 19-11 cells (Fig. 8B, lanes 3 and 4), as indicated by the presence of the junction fragment, is presently unclear. However, it is interesting to note that cleavage of concatemeric DNA in mutant 332-31-infected 19-11 cells (Fig. 8B, lane 4) appears to be not as efficient as after wild-type PrV infection (Fig. 8B, lane 3), as judged from the stronger signal from the

junction fragment and the weaker *Bam*HI-13 signal in mutant 332-31-infected cell DNA.

Formation of DNA-containing mature capsids is blocked in mutant 332-31-infected cells. Since cleavage of concatemeric DNA and encapsidation into newly formed capsids appear to be linked in herpesviruses (12, 25, 50), we also electron microscopically analyzed Vero cells infected by mutant 332-31 or wild-type PrV. In wild-type-infected Vero cells (Fig. 9A), three different maturation stages of intranuclear capsids (empty type I capsids with inner core-like structure [Fig. 10A], empty coreless capsids [type II; Fig. 10B], and full capsids [Fig. 10C]) as well as complete enveloped extranuclear virions were observed. For an overall picture, see Fig. 9A. In contrast, in mutant 332-31-infected Vero cells, an intranuclear accumulation of empty capsids was noticed (Fig. 9B). Quantitation of the result is presented in Fig. 10. In wild-type PrV-infected Vero cells, 42% of the total number of capsids represented empty type I capsids,

FIG. 10. Quantitation of electron microscopy results. Beneath electron micrographs of representative empty capsids of types I (A) and II (B) and of full capsids (C), percentages of the respective particles among 1,000 capsids counted in the nuclei of different wild-type PrV- or ICP18.5 mutant 332-31-infected normal Vero cells are indicated. The bar represents 50 nm.

31% represented empty type II capsids, and 27% represented full capsids. In contrast, in mutant 332-31-infected Vero cells, 94% of the capsids were of the empty type I variety whereas 6% appeared as empty type II capsids. Neither full capsids nor extranuclear complete particles were observed in mutant 332-31-infected Vero cells. It appears, therefore, that mutant 332-31 genomes did not become packaged into capsids in normal Vero cells.

DISCUSSION

The U_{L28} gene encoding ICP18.5 in HSV-1 is highly conserved among herpesviruses that have been analyzed in this respect (2, 3, 6, 9, 11, 16, 18, 19, 24, 27, 36, 40, 48, 49). Homology between the deduced ICP18.5 proteins of HSV-1 and PrV amounts to 56%, whereas ICP18.5(PrV) is 49% identical to the homologous varicella-zoster virus gene 30 product (31). This high degree of sequence conservation is indicative of an important function of the ICP18.5-homologous proteins in replication of the respective viruses.

The gene for the PrV homolog of HSV-1 ICP18.5, here designated ICP18.5(PrV), overlaps with its 3'-terminal 44 codons the 5'-terminal part of the gene encoding the essential glycoprotein gII (31). Recent studies identified a 79-kDa ICP18.5 protein in PrV-infected cells by using an antiserum directed against a bacterial Cro-ICP18.5 fusion protein (32). Therefore, presence of the ICP18.5(PrV) gene and gene product is well established. However, because of the lack of respective virus mutants, no functional analysis of the role of ICP18.5(PrV) during viral infection had been performed so far.

We show here that ICP18.5(PrV) constitutes a polypeptide whose expression is essential for viral replication, since mutants with impaired ICP18.5 expression are unable to productively replicate on noncomplementing cells. However, they can be complemented by transformed cells that express the ICP18.5(PrV) gene upon PrV infection. Further analysis proved that after infection of noncomplementing cells, the ICP18.5(PrV) mutant is blocked late in infection in the process of formation of mature capsids. Whereas cleavage of concatemeric replicative DNA and packaging into capsids were readily observed in wild-type PrV-infected

cells (Fig. 9A), both processes did not occur in normal cells after ICP18.5(PrV) mutant infection (Fig. 8B and 9B). Since replication and capsid formation in herpesviruses occur in the nucleus (39), a nuclear localization of ICP18.5(PrV) needs to be postulated. In fact, in immunofluorescence analyses and immunoprecipitation after subcellular fractionation using a polyclonal anti-ICP18.5(PrV) antiserum, it has been shown that after synthesis in the cytoplasm, ICP18.5(PrV) becomes associated with the nucleus (32).

Whereas both ICP18.5 mutants 6321 and 332-31 were clearly dependent for productive replication upon complementing ICP18.5-expressing cells, it was evident from the growth analysis that cell line 19-11 did not fully complement the defect in ICP18.5 mutant 332-31. Both growth kinetics and final titers were lower than those of wild-type PrV grown on either 19-11 or normal Vero cells. This finding coincided with a reduced expression of ICP18.5(PrV), as detected by radioimmunoprecipitation and a lower efficiency of cleavage of concatemeric DNA (Fig. 8). These observations again link the observed defect with the ICP18.5 mutation and the growth properties of the viral mutant.

In Vero cells infected by wild-type PrV, two types of intranuclear empty capsids were observed. Whereas one contained an internal core-like structure (type I; 42%), the other did not show this internal structure (type II; 31%). In addition, full capsids were observed (27%). Similar distinct types of empty capsids, with and without electron-lucent cores, have also been demonstrated in studies on the morphogenesis of the betaherpesvirus murine cytomegalovirus, and it has been postulated that capsids with electron-lucent cores precede coreless capsids (47). Alternatively, the coreless capsids may represent dead-end products of capsid formation (15). In noncomplementing cells infected with the ICP18.5(PrV) mutant, the only capsid structures found were intranuclear empty capsids. The overwhelming number of capsids found were of the type I variety (94%), with only few empty capsids of type II (6%). Whether these type II-like capsids really represented empty type II capsids or whether they in fact are type I capsids with the core-like structures lying outside the plane of the section is presently unclear. It therefore appears possible that the defect in the ICP18.5(PrV) mutant impairs a step in capsid maturation

that precedes cleavage and encapsidation of replicated genomic viral DNA. Detailed analyses to clarify this point are presently under way.

Our results for ICP18.5(PrV) are in agreement with previous studies of a temperature-sensitive HSV-1 mutant which failed to synthesize functional ICP18.5 at the nonpermissive temperature (1). This phenotype led to a deficiency in cleavage of concatemeric DNA and formation of DNA-containing capsids. The high degree of sequence conservation between these ICP18.5-homologous proteins is therefore reflected in a common function of both ICP18.5(HSV) and ICP18.5(PrV) during viral replication. On the basis of these results, it is reasonable to speculate that the ICP18.5 homologs of other herpesviruses also play a role in the formation of DNA-containing capsids.

Two mutants in ICP18.5 homologs obtained by different mutagenizing procedures and selections based on either HSV-1 (1) or PrV (this report) have now been isolated. The mutants exhibit similar phenotypes under conditions in which the effect of the mutation can be analyzed. It therefore appears certain that the observed defects are indeed attributable to a functional impairment of ICP18.5. Previously, it was postulated after analysis of HSV-1 mutants that supposedly also contained mutations in ICP18.5 that this polypeptide could play a role in transport of viral glycoproteins to the cell surface (30, 33). This function was deduced from the fact that cells infected by the temperature-sensitive viral mutant *tsZ47* (otherwise designated *icr ts78*) that was supposed to contain a lesion in ICP18.5 were resistant to immune cytolysis by polyclonal antibodies against gB and expressed reduced amounts of viral glycoproteins on the cell surface (30). However, others showed that a different ICP18.5 HSV mutant with a temperature-sensitive phenotype (*ts8*) did not yield similar results (21). Our ICP18.5(PrV) mutant did not exhibit a defect in either glycoprotein expression or localization of glycoproteins to the cellular surface, which would argue against an involvement of ICP18.5 in these processes. A likely explanation of this discrepancy would be the presence of other, hitherto unspecified defects in *tsZ47* (30).

Whereas the ICP18.5 mutants of HSV-1 and PrV exhibit a replication block during capsid formation at or prior to cleavage and encapsidation of concatemeric viral DNA, the exact role that the respective ICP18.5 proteins play is not known. ICP18.5 may be a protein necessary for the cleavage reaction. Alternatively, its presence might predispose preformed capsids for uptake of viral DNA, perhaps by mediating the conversion of empty type I capsids into empty type II capsids. Since the processes of cleavage and encapsidation have so far not been separated (12, 25, 50), this issue remains to be clarified further.

In conclusion, our results show that ICP18.5 represents an essential PrV protein that is necessary for formation of mature DNA-containing capsids. The defect in the ICP18.5(PrV) mutant can be complemented *in trans* by ICP18.5 expressed from the resident gene in a transformed cell line. The virus mutant described represents the first genetically engineered herpesviral ICP18.5 mutant that exhibits its mutant phenotype at the normal growth temperature. Availability of this mutant should help in the analysis at the molecular level of the exact functional role that ICP18.5 plays in the process of mature capsid formation.

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