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Pseudorabies Virus Glycoproteins gII and gp50 Are Essential for Virus Penetration

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Pseudorabies virus (PrV) glycoproteins gII and gp50 are major constituents of the viral envelope and targets of neutralizing monoclonal antibodies. Both are homologs of essential glycoproteins found in herpes simplex virus, gB (gII) and gD (gp50). We recently isolated a gII-negative PrV deletion mutant on complementing cell lines and established the essential character of gII for PrV replication (I. Rauh, F. Weiland, F. Fehler, G. Keil, and T. C. Mettenleiter, J. Virol. 65:621–631, 1991). In this report, we describe the isolation of a gp50-negative PrV mutant after constructing cell lines that constitutively express gp50 and phenotypically complement the gp50 defect. Analysis of the gp50$^-$ mutant proved that gp50 is essential for PrV replication. Further studies showed that both gII and gp50 are required for viral penetration into target cells. The penetration defect in the gII and gp50 deletion mutants could be overcome by experimental polyethylene glycol-induced membrane fusion. Surprisingly, whereas gII proved to be essential for both penetration and cell-cell spread of the virus, gp50 was required only for penetration and appeared dispensable for direct cell-cell spread.

In the genome of pseudorabies virus (PrV), genes coding for seven glycoproteins have been located to date. Homologous proteins can be found in other herpesviruses, e.g., in herpes simplex virus (HSV). Four of these glycoproteins, namely, gI, gp63, gIII, and gX, are dispensable for viral replication in vitro and are therefore designated as nonessential. The three PrV glycoproteins gII, gp50, and gH are homologous to the essential HSV glycoproteins gB, gD, and gH, respectively (24, 36, 40). All essential HSV glycoproteins found and characterized to date are involved in membrane fusion events during infection, virus penetration into target cells, and direct virus spread from infected cells to adjacent noninfected cells (4, 11, 15, 27, 41). This has been shown by using either complement-independent neutralizing monoclonal antibodies (MAbs) (17–19) or glycoprotein deletion mutants that were isolated after establishment of complementing cell lines (5, 27). To gain insight into the functions of essential PrV glycoproteins, we started by constructing cell lines that express glycoprotein gII. Glycoprotein gII is a major constituent of the PrV envelope and is represented by a disulfide-linked complex of two polypeptides of 67 and 58 kDa that are derived from a common 120-kDa precursor by proteolytic cleavage (28). Subsequent isolation and characterization of a gII-negative PrV mutant proved that gII is essential for PrV replication and that gII-negative PrV virions are noninfectious (38). In addition, it was shown that gII-negative PrV could be complemented by the homologous glycoprotein gI of bovine herpesvirus 1 (BHV-1) (38). This proved that gII(PrV) and gI(BHV-1) fulfill similar roles in their respective viruses. Whether gp50 and gH are also essential for PrV, as their counterparts are for HSV, has not been proven so far. Glycoprotein gp50 is one of the most potent immunogens of PrV (20, 29), and complement-independent neutralizing anti-gp50 antibodies can readily be obtained (9, 13, 45). Studies using partial neutralization with these antibodies indicated involvement of this glycoprotein in the viral penetration process (12). However, a detailed analysis of both gII and gp50 functions with appropriate virus mutants is still needed. We report here the establishment of gp50 *trans*-complementing cell lines and the isolation of a PrV mutant lacking gp50. Analysis of gII and gp50 deletion mutants showed that both glycoproteins are essential for PrV penetration into target cells. However, whereas gII, like its HSV counterpart gB, is strictly required for viral cell-cell spread, gp50, unlike gD(HSV), is essential only for initial penetration but not for cell-cell spread.

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MATERIALS AND METHODS

Viruses and cells. PrV strain Ka (23) was used as the parental strain in all experiments. Viruses were propagated on Madin-Darby bovine kidney (MDBK) cells. Cell line MT3 that constitutively expresses gII and complements the gII-negative PrV mutant has recently been described (38).

Plasmids. For construction of a gp50-expressing cell line, a plasmid containing a PrV genomic *Bam*HI-*Sal*I DNA fragment (Fig. 1, fragment I) was cleaved with *Bst*XI, which separates the gp50 gene from its putative 5′-terminal promoter elements (36). After blunt ending with T4 polymerase, *Bam*HI linkers (BRL, Eggenstein, Germany) were inserted and the plasmid was religated. Cleavage with *Bam*HI and *Sal*I then released a 552-bp fragment containing the 5′ part of the gp50 gene, which was cloned into pBR322. The complete gp50 gene was assembled by inserting a 3,346-bp *Sal*I-*Sph*I fragment (Fig. 2) into the *Sal*I site. Subsequent cleavage with *Bam*HI and *Dra*I excised a 2,433-bp fragment that contains the complete expression unit encompassing the genes encoding gp50 and gp63 (gp50-63) and the 3′-terminal mRNA processing signals (26, 36) (Fig. 2). This fragment was then cloned behind the mouse metallothionein promoter (3), giving rise to plasmid pMT50-63 (Fig. 2). gp50 expression was verified by immunofluorescence analyses in transient expression assays after transfection of pMT50-63 into MDBK cells and subsequent induction of the promoter with 100 μM $ZnSO_4$. For isolation of a gp50-negative PrV mutant, a 3.5-kb *Sal*I-*Bam*HI expression cassette containing the *Escherichia coli* β-galactosidase gene under control of the PrV glycoprotein gX promoter (32) was inserted into the *Sal*I

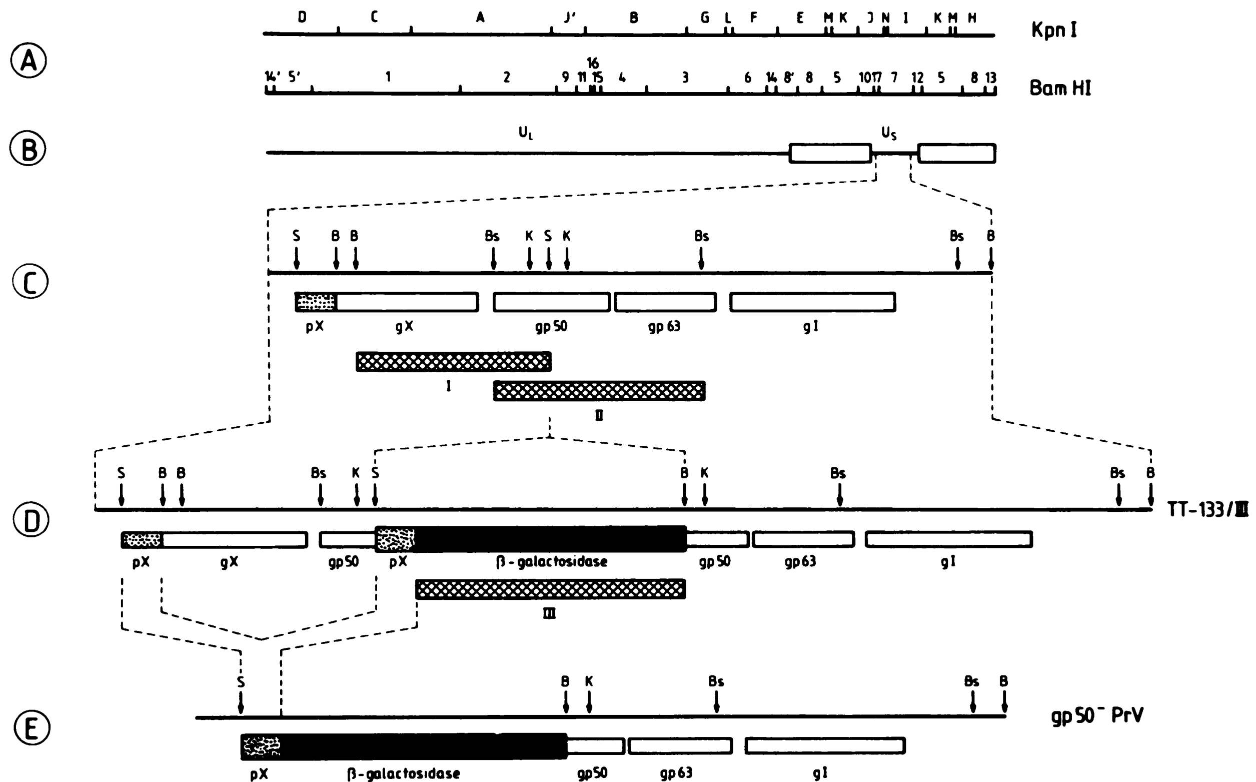

FIG. 1. Genotype of gp50⁻ PrV. *KpnI* and *BamHI* restriction maps (A) are shown in correlation to a diagrammatic representation of the PrV genome (B). U_L and U_S denote the long and short unique regions. The enlarged region (C) encompasses the genes coding for glycoproteins gX, gp50, gp63, and gI. After insertion of the β-galactosidase expression cassette (D), the resulting plasmid was used for cotransfection with PrV wild-type DNA that led to isolation of a β-galactosidase-expressing virus. The genotype of the gp50⁻ mutant is depicted (E), with the recombinational events that most likely led to its appearance. I, II, and III, hybridization probes (see Fig. 5); pX, gX promoter region. Cleavage sites: B, *BamHI*; Bs, *BstXI*; K, *KpnI*; S, *SalI*.

site in the middle of the gp50 gene to produce plasmid TT133/III. As can be seen in Fig. 1D, in this clone, the *BamHI* site at the 3′ end of the β-galactosidase cassette insert had inadvertently been restored. Transient expression assays showed the capability of this clone to express functional β-galactosidase.

Isolation of gp50-expressing cell lines. To establish stably transformed gp50-expressing cell lines, MDBK cells were cotransfected with plasmids pMT50-63 and pSV2-neo by the calcium phosphate precipitation method (16, 42). Selection for G418-resistant colonies was performed as described previously (38). Cells were tested for gp50 expression in immunofluorescence analyses with a gp50-specific MAb (kindly provided by N. Visser, Boxmeer, The Netherlands). Two cell lines, MT50-3 and MT50-5, exhibiting different fluorescence intensities were selected for further studies.

Isolation of gp50⁻ PrV. Plasmid TT133/III was cotransfected with PrV wild-type DNA into MT50-3 cells. After a complete cytopathic effect had been induced, the supernatant was harvested and clarified and the virus progeny was plated onto MT50-3 cells in serial dilutions. When plaques became visible after 2 days, the cells were overlaid with agarose medium containing 300 μg of Bluo-Gal (BRL) (31), a chromogenic substrate for β-galactosidase, per ml. Blue-staining plaques were picked and purified three times on MT50-3 cells until all plaques stained blue under a Bluo-Gal overlay.

DNA analyses. For analysis of virion DNA of the gp50⁻ PrV, MT50-3 cells were infected with the gp50⁻ mutant at a multiplicity of infection of 5. After overnight incubation, when the cytopathic effect was complete, the supernatant was harvested and clarified and virions were purified by centrifugation through a 30% sucrose cushion. DNA was extracted as described previously (1). To investigate the amounts of gp50 gene copies in the two cell lines, whole-cell DNA was isolated by phenol extraction and analyzed.

^{35}S labeling of viral proteins. For analysis of gp50 expression in the cell lines, cells were labeled for 24 h in medium depleted of methionine and supplemented by 50 μCi of [^{35}S]methionine per ml. Cell lysis and immunoprecipitation were performed as described previously (28). PrV virions were radioactively labeled with [^{35}S]methionine and purified as described previously (38).

PEG-induced virus penetration. Either complementing MT3 and MT50-3 or noncomplementing MDBK cells in six-well plates were incubated with the respective viruses for 1 h at 37°C. Afterwards, the inoculum was removed and the cells were treated with polyethylene glycol (PEG) (41) according to the following protocol. The monolayer was covered briefly with 2 ml of a solution made up of 16 g of PEG 8000 and 12 ml of Dulbecco's minimum essential medium (DMEM) (PEG was dissolved and sterilized by autoclaving and cooled to approximately 60°C before being mixed with the medium). The PEG solution was decanted immediately, and the monolayer was washed once with 2 ml of a solution of 8 g of PEG 8000 and 18 ml of DMEM, once with 2 ml of

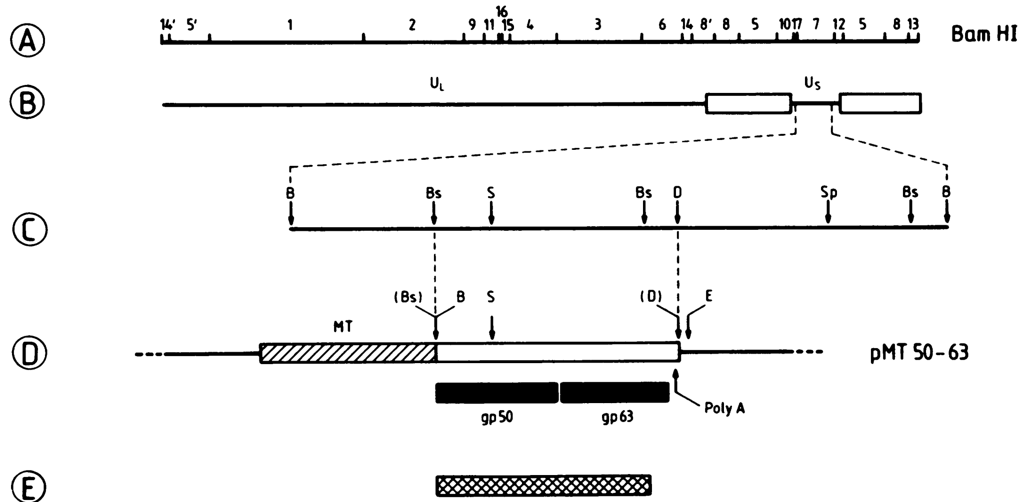

FIG. 2. Construction of plasmid for establishment of cell lines. Below a *Bam*HI restriction fragment map (A), a schematic diagram of the PrV genome (B) is shown. U_L and U_S denote the long and short unique regions, respectively. The enlarged *Bam*HI fragment 7 (C) encompasses the genes encoding gp50 and gp63. After conversion of the leftmost *Bst*XI to a *Bam*HI site, a *Bam*HI-*Dra*I fragment was excised and cloned behind the mouse metallothionein promoter (MT) (D), creating plasmid pMT50-63. It contains the gp50 and gp63 genes, including 3′ mRNA processing signals such as the polyadenylation consensus sequence (Poly A). Location of the hybridization probe used for Fig. 4 is indicated (E). Restriction sites: B, *Bam*HI; Bs, *Bst*XI; D, *Dra*I; E, *Eco*RI; S, *Sal*I; Sp, *Sph*I. (Bs) and (D) indicate inactivation of cleavage sites during cloning.

a solution of 8 g of PEG 8000 and 42 ml of DMEM, and three times with DMEM supplemented with 10% fetal calf serum. Thereafter, the monolayer was incubated for 2.5 h at 37°C with DMEM–10% fetal calf serum. Then the medium was removed and the cells were overlaid with 0.75% methylcellulose in DMEM–10% fetal calf serum.

RESULTS

Isolation of gp50-expressing MDBK cell lines. To isolate a PrV mutant deficient in glycoprotein gp50 expression, we first established cell lines that were able to complement the gp50 defect in the mutant in *trans*. This approach was chosen since the gp50-homologous glycoprotein gD in HSV had previously been shown to be essential for virus growth (27). After cotransfection of MDBK cells with plasmid pSV2-neo conferring resistance to the antibiotic G418 and plasmid pMT50-63 (Fig. 2) containing the viral gp50-gp63 expression unit (26) under control of the mouse metallothionein promoter, G418-resistant colonies were isolated and analyzed. Immunofluorescence studies showed that individual cell clones exhibited different fluorescence intensities, indicating different gp50 expression levels (data not shown). Two clones, MT50-3 exhibiting weak fluorescence and MT50-5 showing strong fluorescence, were selected for further studies. The amount of gp50 expressed in both lines is shown in Fig. 3 after radioimmunoprecipitation of [^{35}S]methionine-labeled cell extracts with a gp50-specific MAb. It is evident that both lines do express authentic gp50 (Fig. 3A, lanes 2 through 5) compared with gp50 precipitated from ^{35}S-labeled PrV-infected cell extracts (Fig. 3A, lane 1). Also visible is the dramatically different expression level of line MT50-3 (Fig. 3A, lanes 4 and 5) compared with that of line MT50-5 (Fig. 3A, lanes 2 and 3). Induction by $ZnSO_4$ (Fig. 3A, lanes 2 and 4) had only a slight effect on gp50 expression. A similar finding has previously been described for MDBK cell lines expressing glycoprotein gII(PrV) under metallothionein promoter control (38). We conclude that both cell lines MT50-3 and MT50-5 express authentic gp50 in a constitutive fashion but at different levels.

For analysis of whether the different expression levels are due to differences in the number of gp50-63 gene copies inserted into the cellular genome, whole-cell DNA was isolated and cleaved with *Bam*HI-*Eco*RI and the fragments

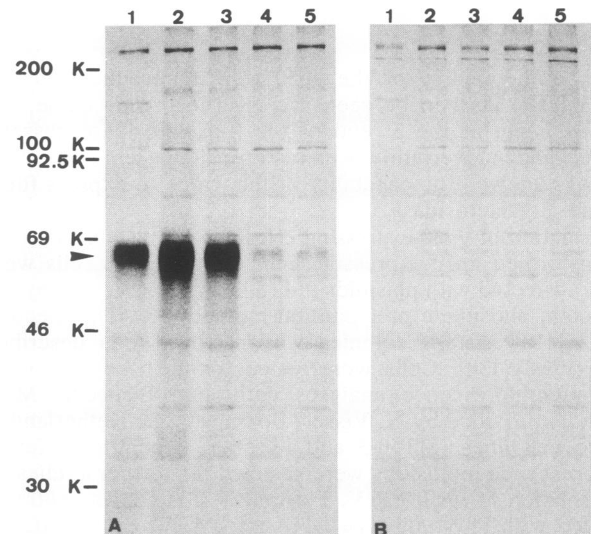

FIG. 3. Protein analysis of gp50-expressing cell lines. Recombinant cell lines MT50-5 (lanes 2 and 3) and MT50-3 (lanes 4 and 5) were labeled with [^{35}S]methionine with (lanes 2 and 4) or without (lanes 3 and 5) induction by $ZnSO_4$. Lysates were then immunoprecipitated with either anti-gp50 MAb MCA50-1 (A) (kindly provided by M. Wathen, Kalamazoo, Mich.) (45) or a non-PrV-specific control serum and separated by SDS-10% PAGE (B). In lanes 1, ^{35}S-labeled PrV-infected MDBK cells were assayed. In this gel system, gp50 migrates with an apparent molecular size of approximately 60 kDa, as has already been noted by others (36). Molecular size markers are indicated on the left.

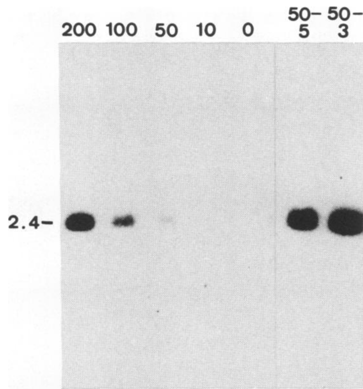

FIG. 4. Genome analysis of gp50-expressing cell lines. DNA was extracted from cell lines MT50-5 and MT50-3 and cleaved with *Bam*HI-*Eco*RI to release the gp50-63 insert of 2.4 kb. For comparison, MDBK cell DNA was mixed with the indicated genome copies of plasmid pMT50-63 and cleaved. After gel electrophoresis and Southern transfer, hybridization was performed with a labeled probe specific for gp50-63 (Fig. 2).

were separated in a 0.8% agarose gel. After the fragments were transferred to nitrocellulose, the filter was probed with a labeled gp50-63-specific hybridization probe (Fig. 2). As can be seen in Fig. 4, both cell lines contained approximately equal numbers of gp50 gene copies, which amounted to more than 200 per cell. A similar high copy number had also been found for the gII-expressing cell line MT3 (38). Since no significant difference between MT50-3 and MT50-5 in the gp50 gene copy number could be found, the difference in expression probably reflects different transcriptional activity. Both MT50-3 and MT50-5 cells also contain the gp63 gene, which is situated immediately behind the gp50 gene, both being transcribed from viral DNA into a single structurally bicistronic RNA (26). Since antibodies against gp63 were not available to us, it is presently unknown whether these cell lines also synthesize gp63.

Isolation and characterization of a gp50⁻ PrV mutant. After cotransfection of PrV wild-type DNA and plasmid TT133/III into MT50-3 cells, among viral progeny β-galactosidase-positive plaques were picked and plaque purified three times until all plaques stained blue under a Bluo-Gal overlay. This virus mutant was further analyzed.

Virion DNA was prepared and cleaved with either *Bam*HI or *Kpn*I, and the resulting fragments were separated in a 0.8% agarose gel (Fig. 5; for location of fragments, see Fig. 1). Comparison of wild-type PrV DNA and gp50⁻ mutant DNA revealed lack of *Bam*HI fragment 7 in the mutant DNA fragment pattern (Fig. 5A, lanes 1 and 2). This finding is consistent with integration of the expression cassette into the *Sal*I site of the gp50 gene which resides in *Bam*HI fragment 7 and concomitant introduction of a *Bam*HI site at the end of the β-galactosidase gene. However, cleavage with *Kpn*I yielded an unexpected pattern in mutant virus DNA, which showed an absence of fragment *Kpn*J (which in wild-type DNA comigrates with *Kpn*J') (Fig. 5A, lanes 3 and 4). Hybridization with the *Bst*XI fragment encompassing the gp50 and gp63 genes clearly showed *Bam*HI fragment 7 and both *Kpn* fragments I and J in wild-type PrV DNA (Fig. 5C, lanes 1 and 3). Longer exposure also revealed the small 0.4-kb *Kpn*I fragment originating from the body of the gp50 gene (data not shown). In DNA from the gp50⁻ mutant, only one *Bam*HI fragment of approximately 4.6 kb migrating slightly faster than wild-type *Bam*HI-8 was recognized by the hybridization probe instead of two expected fragments of 4.6 and 5.5 kb (Fig. 5C, lane 2). After *Kpn*I cleavage, mutant virus DNA yielded only one signal at the position of *Kpn*I fragment I (Fig. 5C, lane 4). A labeled β-galactosidase-specific probe correctly recognized only mutant virus DNA and not wild-type DNA (Fig. 5D). However, fragment sizes were again not as expected. To explain this, we reasoned that introduction of the *Bam*HI-*Sal*I cassette led to the

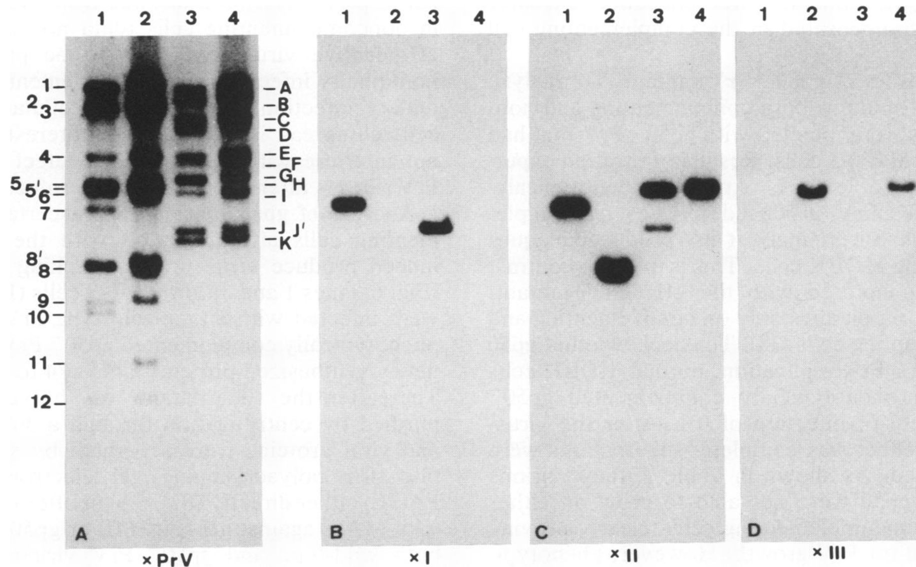

FIG. 5. Southern analysis of gp50⁻ PrV DNA. Virion DNA of either wild-type PrV (lanes 1 and 3) or gp50⁻ PrV (lanes 2 and 4) was extracted and cleaved with *Bam*HI (lanes 1 and 2) or *Kpn*I (lanes 3 and 4). After electrophoresis and transfer, replica filters were hybridized with labeled PrV DNA (A) and labeled probes (as indicated in Fig. 1). Hybridization with probe I representing a *Bam*HI-*Sal*I subfragment of *Bam*HI-7 (B), with a *Bst*XI fragment originating from *Bam*HI-7 (probe II) (C), and with the β-galactosidase-specific probe III (D) is shown. Numbers on the left relate to positions of BamHI fragments, and capital letters relate to positions of *Kpn*I fragments from wild-type PrV DNA.

TABLE 1. Titers of wild-type and gp50⁻ PrV on different host cells[a]

Cell line	Titer of:		
	Wild type (MDBK)	gp50⁻ (MT50-3)	gp50⁻ (MDBK)
MDBK	1.4×10^6	5.0×10^5	0[b]
MT50-3	1.2×10^6	4.0×10^5	0[b]
MT50-5	2.0×10^5	2.0×10^4	0[b]

[a] The PrV wild type or gp50 deletion mutant was grown on MDBK or MT50-3 cells as indicated in parentheses, and the supernatants were titrated on MDBK, MT50-3, and MT50-5 monolayers. Titers given represent the averages from three independent experiments.
[b] No plaques were found in the lowest dilution tested (10^{-1}).

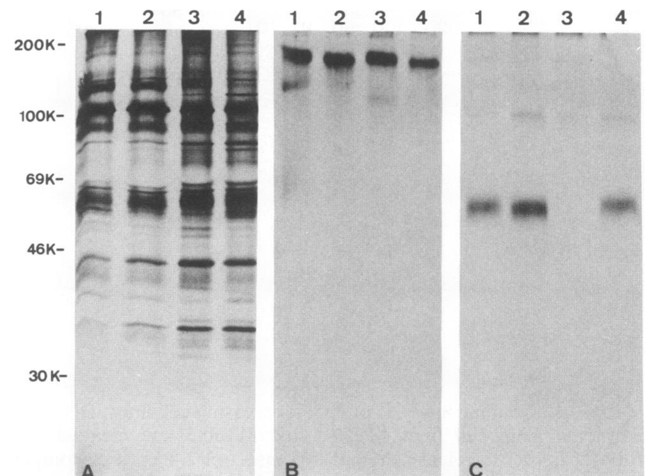

FIG. 6. Analysis of gp50⁻ virions. Noncomplementing MDBK cells (lanes 1 and 3) and complementing MT50-3 cells (lanes 2 and 4) were infected with either wild-type PrV (lanes 1 and 2) or phenotypically complemented gp50⁻ PrV (lanes 3 and 4) and labeled with [^{35}S]methionine. Virions were purified and lysed, and the proteins were separated either directly (A) or after immunoprecipitation with either the anti-gII(PrV) MAb 5/14 (B) (kindly provided by H.-J. Rziha, Tübingen, Germany) (28) or the anti-gp50(PrV) MAb MCA 50-1 (C) by SDS–10% PAGE. Proteins were visualized after fluorography. The positions of molecular size marker proteins are indicated on the left. Electrophoresis was performed under reducing (A) or nonreducing (B and C) conditions. Under nonreducing conditions, the disulfide-linked gII complex migrates with an apparent molecular size of 155 kDa.

creation of a direct repeat element consisting of the gX promoter sequences, which are present in their proper location in front of the gX gene and also in front of the expression cassette inserted into the gp50 gene (Fig. 1). Recombination between these repeated elements would lead to a deletion of the intervening sequences encompassing the gX gene and the 5′ part of the gp50 gene (Fig. 1). We tested this by hybridization with labeled *Bam*HI-*Sal*I fragment 7B (probe I in Fig. 1 and 5B). As hypothesized, this probe did not recognize gp50⁻ mutant DNA (Fig. 5B, lanes 2 and 4) but hybridized to wild-type DNA (Fig. 5B, lanes 1 and 3).

We interpret our results, taken together, in the following way. Because of the insertion of the gX–β-galactosidase expression cassette into the *Sal*I site in the middle of the gp50 gene, a direct repetition of sequences from the gX promoter region was created. The repeated sequences bracket the body of the gX gene and the 5′ part of the gp50 gene. Recombination led to deletion of the intervening sequences and one copy of the repeat. The deletion then encompasses all of *Bam*HI-*Sal*I fragment 7B, which is therefore missing in the resulting mutant. The mutant is viable since gX is nonessential for viral replication and since the gp50 defect is compensated in the complementing cell line.

Growth characteristics of gp50⁻ PrV mutant. To analyze growth of the gp50⁻ mutant, both complementing and noncomplementing cells were infected with gp50⁻ PrV that had been propagated on MT50-3 cells. Results of titration experiments are shown in Table 1. As expected, phenotypically complemented gp50⁻ PrV produced plaques on complementing MT50-3 cells. Surprisingly, it also produced plaques on noncomplementing MDBK cells. This is in sharp contrast to the situation encountered with the gII⁻ PrV mutant, which was able to form plaques only on complementing and not on noncomplementing cells (38). To check whether gp50 is indeed essential for PrV replication, normal MDBK cells were infected with phenotypically complemented gp50⁻ virus at a multiplicity of infection of 0.1. After the virus-induced cytopathic effect was complete, supernatants were harvested and titrated. As shown in Table 1, these virions supposedly lacking gp50 were not able to grow on either complementing or noncomplementing cells, clearly proving that gp50 is essential for PrV growth. However, phenotypically complemented gp50⁻ virus can produce plaques on and is also able to induce a complete cytopathic effect in noncomplementing cell cultures infected at a low multiplicity of infection. This indicates that although gp50 is essential for virus penetration, it is dispensable for cell-cell fusion events, which is in contrast to the situation found with gII⁻ mutants. It follows that gp50-negative virus stock can be prepared after low-multiplicity infection of noncomplementing cells. This serves to dilute the input gp50 from the phenotypically complemented mutant, which in high-multiplicity infections led to partial retention of infectivity even after one passage in noncomplementing cells (data not shown). In contrast, gII-negative virus stock has to be prepared by a high-multiplicity infection of noncomplementing cells, since secondary infection by either extracellular progeny or direct cell-cell spread is not possible. Interestingly, input gII did not interfere with the observed defect of the gII-negative PrV virions after high-multiplicity infections (38).

Analysis of gp50⁻ virions. To ascertain that noncomplementing cells after infection with the gp50⁻ mutant did indeed produce virus progeny lacking gp50, MDBK cells (Fig. 6, lanes 1 and 3), or MT50-3 cells (Fig. 6, lanes 2 and 4) were infected with either wild-type PrV (lanes 1 and 2) or phenotypically complemented gp50⁻ PrV (lanes 3 and 4) and newly synthesized proteins labeled 4 to 24 h after infection. Thereafter the supernatant was harvested, virions were purified by centrifugation through a 30% sucrose cushion, and viral proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) either directly (Fig. 6A) or after immunoprecipitation with MAbs against gII (Fig. 6B) or gp50 (Fig. 6C). Whereas both wild-type and gp50⁻ PrV virions contained similar amounts of glycoprotein gII (Fig. 6B) independent of the cell line used for propagation, gp50 could be demonstrated only in PrV wild-type-infected cells (Fig. 6C, lanes 1 and 2) and gp50⁻-infected complementing MT50-3 cells (Fig. 6C, lane 4). No gp50 was detectable after infection of noncomple-

TABLE 2. PEG-induced plaque formation[a]

Cell line	PFU of wild type			PFU of gp50⁻ mutant			PFU of gII⁻ mutant		
	+PEG	−PEG	Ratio[b]	+PEG	−PEG	Ratio	+PEG	−PEG	Ratio
MDBK	2×10^6	3×10^6	0.6	4×10^2[c]	<10[d]	>40	<10	<10	
				1×10^3	<10	>100	<10	<10	
MT50-3	2×10^6	4×10^6	0.5	1.4×10^4	4.6×10^2	30	ND[e]	ND	
	4×10^5	1.2×10^6	0.3	2.7×10^4	3×10^2	90			
MT3	2×10^6	4×10^6	0.5	ND	ND		6.6×10^3	4×10^2	17
	1×10^6	1.8×10^6	0.6				7.5×10^3	1×10^2	75

[a] Wild-type or phenotypically complemented gp50⁻ or gII⁻ PrV was grown on noncomplementing cells and supernatants were used to infect MDBK, MT50-3, and MT3 cells. After PEG-induced fusion, monolayers were incubated under agarose for 2 days before plaques were counted.
[b] Ratio of PFU values with and without PEG treatment.
[c] Data from two independent experiments with virus stocks different from those for Table 1 are shown. To quantitate exactly the increase in viral titer after PEG-induced membrane fusion, we used virus stocks that on MT50-3 and MT3 cells showed the appearance of a limited number of plaques even without PEG treatment, most likely produced by rescued virions.
[d] <10, no plaques found in lowest dilution tested (10^{-1}).
[e] ND, not done.

menting MDBK cells with the phenotypically complemented gp50⁻ mutant (Fig. 6C, lane 3). This result shows that virus particles of either wild-type or gp50⁻ PrV were produced in both noncomplementing and complementing cells. However, gp50⁻ progeny from noncomplementing cells lacked glycoprotein gp50 and consequently was noninfectious (Table 1). The absence of any detectable gp50 in Fig. 6C, lane 3, also proves that the gp50⁻ virus stocks used for inoculation were not significantly contaminated with revertants that had been rescued after propagation on complementing MT50-3 cells by the resident gp50 genes.

PEG-induced infectivity of gp50⁻ and gII⁻ PrV mutants. Having established that both gII (38) and gp50 are essential for PrV replication, we further elucidated the step in which infection is blocked when either of the two glycoproteins is absent from PrV virions. It had already been reported that the homologous HSV glycoproteins gB and gD are required for viral penetration (27, 41). A block in penetration might be overcome by experimental induction of fusion between the viral and cellular membranes by PEG (41). Complementing gII-expressing MT3 or gp50-expressing MT50-3 cells were therefore infected for 1 h at 37°C with serial dilutions of the respective virus mutant. After this period, the monolayer was washed and treated with PEG. Two days after PEG treatment, plaques were counted. As shown in Table 2, PEG treatment led to a significant increase in virus infectivity, which varied between 17- and >100-fold in independent experiments. PEG treatment after adsorption of wild-type PrV had, in contrast, a slight negative effect on infectivity. These results clearly show that in both gp50⁻ and gII⁻ PrV mutants, virus penetration is blocked and that therefore gp50 and gII are essential for viral penetration. When the PEG fusion assay was performed on noncomplementing cells, gII-negative virions did not show any increase in plaque production. However, after PEG-induced membrane fusion, gp50-negative virions were still able to produce plaques also on noncomplementing cells. This again demonstrates that whereas both gp50 and gII are essential for viral penetration, gp50 is dispensable for cell-cell spread.

Inhibition of infection by MT50-5 cells. It has previously been reported that cells expressing either HSV gD (7, 22) or the gD-homologous glycoproteins of PrV (gp50) (35) or BHV-1 (gIV) (8) become less susceptible to infection by either of these viruses. We did not find a similar resistance against infection in cell line MT50-3, which expresses low but obviously complementing levels of gp50. However, when cell line MT50-5 expressing high levels of gp50 was analyzed, a high degree of resistance to infection by either wild-type PrV or phenotypically complemented gp50⁻ PrV was found. As shown in Table 1, MT50-5 cells were able to block infectivity of both wild-type and complemented gp50⁻ PrV by 85 and 95%, respectively. The constitutively gII-expressing cell line MT3 (38) did not exhibit any resistance to infection (unpublished results). The degree of resistance to infection by cell lines expressing gp50 appears therefore to be directly correlated with the amount of gp50 expressed. This block could, however, also be overcome by PEG-induced membrane fusion (data not shown), a finding which points to interference at the penetration level.

DISCUSSION

Using viral deletion mutants propagated on complementing cell lines, we demonstrate here that both PrV glycoproteins gII and gp50 are essential for virus penetration into host cells. PrV glycoprotein gp50 had already been indirectly implicated in viral entry by the use of MAbs (46) and penetration analyses of partially neutralized virus populations (12). Similarly, the respective homologous proteins in HSV, gB and gD, are also essential for penetration (4, 5, 21, 27). It appears, therefore, that these sequence-homologous glycoproteins perform similar functions in the life cycles of the respective viruses. Functional complementation of a gII deletion mutant of PrV by the gB-homologous gI(BHV-1) has already been shown (38). Regarding the essential role gII(PrV) plays in penetration and cell-cell fusion, it can be concluded that gI is involved in the same aspects in the BHV-1 replicative cycle and that in the phenotypically complemented gII⁻ PrV mutant it complements both the penetration and the cell-cell fusion functions of gII(PrV). That gI(BHV-1) might play a role in cell fusion is also indicated by the spontaneous fusion activity of some gI(BHV-1)-expressing cell lines (14).

An unexpected finding was that whereas HSV gD appears to be required for both viral entry and direct cell-cell spread (27), the gD homolog of PrV, gp50, is required only for penetration and is dispensable for cell-cell spread. Similar results have also been found for a gp50⁻ PrV mutant

obtained by different mutagenesis technology based on a different virus strain (34). With regard to glycoprotein functions in alphaherpesviruses, this result has some generally interesting aspects. From analysis of the complete genomic sequence, it has been postulated that the alphaherpesvirus varicella-zoster virus does not specify a gD-homologous glycoprotein (10, 30). Since gD appears nonexistent in varicella-zoster virus, its functions are either not necessary in this virus-host system or executed by other (glyco)proteins. On the other hand, HSV gD is required for both penetration and cell-cell fusion (27). In considering the results presented here, PrV gp50 might represent a functional intermediate since it is essential for virus entry, as is HSV gD, but is dispensable for cell-cell spread. This also indicates that penetration and cell-cell spread, although both involve membrane fusion events, are clearly distinct processes that can be separated from each other by using the gp50$^-$ mutant. We therefore hypothesize that glycoprotein gD of HSV probably performs two functions, one in penetration and one in cell-cell fusion. Since PrV gp50 is essential only for penetration, its cell-cell fusion function either is not required or is performed by a different (glyco)protein. From this, it can be predicted that PrV gp50 will not be able to fully complement gD-negative HSV mutants because of its missing cell-cell spread function. Indeed, a lack of complementation with transiently expressed gp50 and a gD-negative HSV mutant has been found (33). On the other hand, gD (HSV) might be able to complement gp50$^-$ PrV mutants since it has the capacity to function in both penetration and cell-cell spread. Studies to test this prediction are under way.

Comparison of cell lines MT50-3 and MT50-5 showed different levels of expression of gp50. The amount of gp50 in MT50-5 cells, as seen after radioimmunoprecipitation, is at least 50-fold higher than that in line MT50-3. Since both cell lines contain approximately equal numbers of gp50 gene copies, the difference might be accounted for by different transcriptional activity. However, even the low level of gp50 present in MT50-3 cells is sufficient for efficient complementation of the gp50$^-$ mutant, whereas it appears inadequate for gp50-mediated inhibition of infection by either PrV wild type or the phenotypically complemented gp50$^-$ mutant. In contrast, the level of gp50 expression seen in MT50-5 cells led to an increased resistance of the cells against infection, blocking infectivity of both wild-type and phenotypically complemented gp50$^-$ PrV by about 90%. This shows that low gp50 levels that did not induce resistance against infection are nevertheless sufficient for complete functional complementation of a gp50$^-$ PrV mutant.

After construction of the gp50$^-$ PrV mutant, we found that this virus also lacked most of the gX gene because of a spontaneous deletion. This deletion most likely occurred as a consequence of the creation of a 417-bp direct repeat by introduction of a second gX promoter element. The resulting deletion mutant that lacks not only the 5′ part of the gp50 gene but also most of the gX gene is viable, since the gp50 defect can be complemented in the gp50-expressing cell lines and since gX constitutes a nonessential glycoprotein. Since no function for gX has been found either in vitro (31) or in vivo (43), we do not expect a lack of this glycoprotein to interfere with the results and conclusions in this paper. In addition, gX has not been found in virions but is secreted in large amounts by infected cells (39). It has therefore been characterized as being nonstructural (2). It appears highly unlikely that a protein that is not present in the virion plays any role in the initial steps of virus infection analyzed here. Our results, however, indicate that for expression of foreign genes, it might be advantageous to use a promoter from a different herpesvirus so as not to inadvertently construct deletion-prone viral mutants. First results show that, e.g., some BHV promoters are correctly used in a PrV background (25).

The gp50-expressing cell lines described above were constructed in a way that retained the proposed genomic PrV expression unit encompassing the genes coding for gp50 and gp63 and for the 3′-terminal mRNA processing signals (26). It has been reported that during virus infection both genes are transcribed into a single mRNA that starts in front of the gp50 gene, where promoterlike elements have been found (36), and ends after the gp63 gene, where 3′ processing signals can be demonstrated. However, it is presently still unclear whether both proteins are translated from this structurally bicistronic mRNA. In our cell lines, gp50 is constitutively expressed. Since anti-gp63 MAbs were not available to us, we did not test for gp63 expression in these cells. However, experiments are in progress to analyze transcription and to check whether gp63 in addition to gp50 can be found. It should be noted that gp63 is a minor glycoprotein in PrV virions and infected cells, and it has also been shown to be nonessential for viral replication in vitro (37). Neither cell line MT50-3 (expressing low levels of gp50) nor MT50-5 (expressing high levels of gp50) showed any sign of cytotoxicity of the expressed glycoprotein. This is in contrast to results obtained with the gD homolog gIV(BHV-1) in which expression has been found to be harmful to the cells (8). We also did not observe spontaneous cell-cell fusion (6, 44) in our cell lines despite the high level of gp50 present in MT50-5 cells both internally and at the plasma membrane, as analyzed by immunofluorescence (data not shown). Perhaps the essential cell-cell fusion function of gD that is missing in gp50 accounts for this difference.

In conclusion, our results demonstrate that both the gB-homologous glycoprotein gII(PrV) and the gD-homologous gp50(PrV) are essential for virus penetration, as are their HSV counterparts. However, whereas gD(HSV) appears essential also for cell-cell spread of the virus, gp50(PrV) can be dispensed with for that function. Our studies show that these membrane fusion events are distinct processes that can be separated by using the gp50$^-$ PrV mutant.

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