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Pseudorabies Virus Envelope Glycoproteins gp50 and gII Are Essential for Virus Penetration, but Only gII Is Involved in Membrane Fusion

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To investigate the function of the envelope glycoproteins gp50 and gII of pseudorabies virus in the entry of the virus into cells, we used linker insertion mutagenesis to construct mutant viruses that are unable to express these proteins. In contrast to gD mutants of herpes simplex virus, gp50 mutants, isolated from complementing cells, were able to form plaques on noncomplementing cells. However, progeny virus released from these cells was noninfectious, although the virus was able to adsorb to cells. Thus, the virus requires gp50 to penetrate cells but does not require it in order to spread by cell fusion. This finding indicates that fusion of the virus envelope with the cell membrane is not identical to fusion of the cell membranes of infected and uninfected cells. In contrast to the gp50 mutants, the gII mutant was unable to produce plaques on noncomplementing cells. Examination by electron microscopy of cells infected by the gII mutant revealed that enveloped virus particles accumulated between the inner and outer nuclear membranes. Few noninfectious virus particles were released from the cell, and infected cells did not fuse with uninfected cells. These observations indicate that gII is involved in several membrane fusion events, such as (i) fusion of the viral envelope with the cell membrane during penetration, (ii) fusion of enveloped virus particles with the outer nuclear membrane during the release of nucleocapsids into the cytoplasm, and (iii) fusion of the cell membranes of infected and uninfected cells.

Pseudorabies virus (PRV) is a herpesvirus that causes Aujeszky's disease in pigs (2, 46). The genome of PRV consists of a linear double-stranded DNA molecule of approximately 150 kb comprising a unique short region flanked by inverted repeats and a unique long region (3). PRV synthesizes at least seven glycoproteins, designated gI (41, 50), gII (42, 55), gIII (56), gp50 (49, 65), gp63 (50), gX (54), and gH (47), which, with the exception of gX, are located in the virus envelope as well as the plasma membrane of the infected cell. Glycoproteins gI, gIII, gp63, and gX have been shown to be dispensable for viral growth in tissue culture, whereas gII, gp50, and gH are thought to be essential for virus replication, in analogy to their herpes simplex virus type 1 (HSV-1) homologs gB, gD, and gH, respectively (7, 17, 35).

The functions of the essential glycoproteins gB, gD, and gH of HSV-1 have been studied extensively in the past few years (7, 15, 17, 21, 23–25, 28, 31, 35, 36, 45, 59). Several of these studies showed that virus particles that were treated with neutralizing monoclonal antibodies, or virus mutants that lacked one of these glycoproteins, were unable to enter the target cells, despite their ability to adsorb to these cells. Furthermore, (over)expression in mammalian cells of gD and gB of HSV-1 and their homologs of other herpesviruses indicated that these proteins were also involved in cell fusion (9, 10, 12, 20, 30, 32, 39, 48, 63). These results indicate that these glycoproteins are essential for the penetration of the virus particle into the target cell and that they are involved in spreading of the virus by fusion of infected cells with noninfected cells.

Although gp50 and gII of PRV are homologous to the HSV proteins gD and gB, respectively, remarkable differences exist between the posttranslational processing of these proteins. Whereas gD contains three N-linked glycosylation sites that are all used (14), gp50 does not contain N-linked glycosylation sites but instead is extensively glycosylated by O-linked oligosaccharides (49). Glycoprotein gII of PRV consists of a complex of three glycoproteins, called gIIa, gIIb, and gIIc, which are covalently linked by disulfide bonds. It has been shown that gIIb and gIIc result from proteolytic cleavage of gIIa (68, 69). The gB homologs of several other herpesviruses are subject to similar proteolytic processing (6, 40, 44, 57, 60), with the notable exception of gB of HSV, which is not processed but exists as a dimer (11, 13, 18, 58). Whether these structural differences are related to differences in their biological functions remains to be determined. The observation that cell lines that constitutively express gp50 are able to interfere not only with PRV replication, but also, and even more efficiently, with HSV-1 replication (48) suggests that both proteins are, at least in part, functionally similar. Furthermore, the finding that gB homologs are highly conserved among herpesviruses suggests that these proteins have at least one important function in common. Recently, Rauh et al. (53) described the construction and properties of a PRV gII mutant. This mutant was unable to produce plaques on noncomplementing cell lines, indicating that, similar to gB of HSV-1, gII of PRV is essential in the life cycle of the virus.

To investigate the functions of the PRV proteins gp50 and gII in more detail, we have constructed mutant viruses that are unable to express functional gp50 or gII. To this end, we constructed cell lines that are able to express either gp50 or gII. These complementing cells were used for the generation of mutant viruses by means of overlap recombination (64), using PRV fragments that were mutagenized in the respec-

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tive genes by means of linker insertion (16). Analysis of the phenotypes of the resulting mutants indicated that gp50 is required in an early step of the penetration process, probably receptor binding, but is not required for spreading of the virus by means of cell fusion. Glycoprotein gII is involved in several membrane fusion events, probably by acting as a fusogen.

MATERIALS AND METHODS

Cells, viruses, and antibodies. The swine kidney cell line SK-6 (33) was routinely used for the propagation of virus. SK-6 cells were grown in Dulbecco's modification of Eagle's medium containing 5% fetal calf serum, glutamine (0.3 mg/ml), and the antibiotics penicillin (200 U/ml), streptomycin (0.2 mg/ml), and mycostatin (100 U/ml). SK-6 cells expressing gp50 or gII were grown in the same medium supplemented with 2.5 mM histidinol (Sigma). The wild-type PRV NIA-3 strain has been described previously (2). The gp50 mutant viruses R122 and R332 were grown on SK-6 cells to produce virus stocks lacking gp50 ($R122^-$ and $R332^-$) or on SK-6 cells expressing high levels of gp50 (G5 cells [see below]) to produce virus stocks containing gp50 ($R122^+$ and $R332^+$). The gII mutant virus B145 was grown on SK-6 cells expressing gII (10B2 cells [see below]). Rabbit antiserum against PRV (Bartha strain) and monoclonal antibodies specific for gp50 were prepared at this institute and were kindly provided by T. Kimman and K. Glazenburg. The gII-specific monoclonal antibody 75N10 was a generous gift of M. Eloit (19).

Plasmids, cloning procedures, and mutagenesis. All recombinant DNA techniques were performed by standard methods (38). Plasmid pEVhis14 (45a) was derived from pSV2his (27) by replacing the *Eco*RI-*Bam*HI fragment by a fragment containing the immediate-early enhancer-promoter of human cytomegalovirus (4), followed by a synthetic oligonucleotide containing stop codons in all three reading frames and a polyadenylation site. Plasmid pEVhis10 was derived from pEVhis14 by deleting a *Kpn*I fragment that contains the human cytomegalovirus enhancer-promoter. The gp50 gene of PRV was cloned as a *Bst*XI-*Stu*I fragment (lacking the gp50 promoter [see Fig. 1]) into the *Eco*RV site located downstream of the human cytomegalovirus promoter in pEVhis14, yielding plasmid pEVhis14gp50. The gII gene of PRV, obtained as an *Sph*I fragment after partial digestion of cosmid c-179 (see below), was cloned in pEVhis10, yielding pEVhis10gII. The construction and characterization of cosmids c-179, c-27, and c-443, which contain overlapping subgenomic PRV fragments, and plasmid pN3HB, which contains the *Hin*dIII B fragment of PRV in the *Hin*dIII site of a pBR322 derivative, has been described previously (64) (see Fig. 3). Inactivation of gp50 expression by means of linker insertion at two different positions in the gp50 gene of pN3HB (insertions R1 and 322) has been described previously (16). Insertion of the same mutagenic oligonucleotide in the *Sca*I site in the 5′ part of the gII gene of cosmid c-179 was accomplished in a similar way. The resulting cosmid was designated c-450.

Construction of cell lines that express gp50 or gII. SK-6 cells were transfected with plasmid pEVhis14gp50 by means of electroporation. SK-6 cells were harvested by trypsinization, washed once in phosphate-buffered saline (PBS) at room temperature, and resuspended at 2×10^7 cells per ml in ice-cold PBS. Ten micrograms of plasmid pEVhis14gp50 was added to 0.5 ml of cells which were kept at 0°C in a sterile disposable electroporation cuvette (inner electrode distance, 0.4 cm; Bio-Rad Laboratories), and a discharge of 1,000 V was delivered at a capacitance setting of 25 μF by using a Bio-Rad GenePulser. The cells were left at 0°C for 15 min, transferred to a 75-cm^2 flask containing 50 ml of medium, and incubated overnight. SK-6 cells were transfected with plasmid pEVhis10gII by using the Lipofectin reagent (Bethesda Research Laboratories). After overnight incubation, transfected cells were trypsinized and replated at several dilutions in 100-mm petri dishes in medium containing 2.5 mM histidinol. Medium was changed every 3 to 4 days until colonies were clearly visible (7 to 10 days). Individual colonies were picked and grown in microtiter culture plates (Greiner). Expression of gp50 was determined by an immunoperoxidase monolayer assay (see below). Clones obtained after transfection of SK-6 cells with pEVhis10gII were used for the construction of mutant virus by means of overlap recombination (see below), without prior testing for gII expression.

Construction of mutant viruses. Mutant viruses R122 and R332 were constructed by means of overlap recombination (64) in cells expressing gp50, by using three cosmids (c-179, c-27, and c-443) containing overlapping wild-type PRV sequences and the *Hin*dIII B fragments R1 or 322 (containing the mutagenic oligonucleotide at different positions in the gp50 gene [16; see Fig. 1 and 3]), respectively. The viral DNA fragments were released from the plasmids by *Eco*RI digestion (cosmids) or *Hin*dIII digestion (clones R1 and 322) and were not further separated from vector sequences. Transfection was performed by means of electroporation (see above) with the Bio-Rad GenePulser and Capacitance Extender at settings of 250 V and 960 μF, respectively. Cells were seeded in six-well plates, and after incubation for 3 h at 37°C the medium was replaced by Earle's minimal essential medium containing 2% fetal calf serum and 1% methylcellulose and incubated at 37°C until plaques appeared (2 to 3 days).

Mutant virus B145 was constructed by means of overlap recombination, using DNA fragments excised from c-27, c-443, pN3HB (wild-type fragments), and c-450 (containing the oligonucleotide inserted into the gII gene [see Fig. 1 and 3]). Transfection of 96 independent stably transformed cell lines was performed in microtiter plates by using the Lipofectin reagent. As a control, the same cell lines were transfected with the same set of fragments after the c-450 fragment had been replaced by the wild-type fragment of c-179 (see Fig. 3). Monolayers were overlaid with Earle's minimal essential medium containing 2% fetal calf serum and 1% methylcellulose and incubated at 37°C until plaques appeared (2 to 3 days).

Immunoperoxidase monolayer assay. Expression of viral antigens was determined by an immunoperoxidase monolayer assay (66). Cells were seeded in culture dishes and grown to near confluency. When appropriate, the cells were infected with virus and incubated until plaques appeared. The monolayers were washed with PBS, and the plates were dried for 60 min at 37°C and frozen for at least 60 min at −20°C. The monolayers were fixed with cold 4% (wt/vol) paraformaldehyde in PBS for 5 min at room temperature. After fixation, the plates were washed three times in PBS and incubated for 2 h at 37°C with the relevant antibody (either monoclonal antibodies or rabbit anti-PRV serum) in PBS containing 0.1% bovine serum albumin and 0.01% Tween 80. Horseradish peroxidase-conjugated goat anti-mouse antibody (Institut Pasteur) or goat anti-rabbit antibody which was diluted 1:1,000 in the same buffer was used in the second incubation for 1 h at 37°C. After each incuba-

tion the plates were washed three times with PBS–0.05% Tween 80. Peroxidase activity was visualized by the addition of 2 mg of 3-amino-9-ethylcarbazole (Sigma) per ml in 0.05 M sodium acetate (pH 5.0) containing 0.01% hydrogen peroxide.

Immunoprecipitations. SK-6 cells growing in 25-cm^2 flasks were infected with virus at a multiplicity of infection of 5 in medium containing dialyzed fetal calf serum and 1/20 of the original amount of cysteine. After adsorption for 1 h at 37°C, the inoculum was removed, 1 ml of the same medium containing 50 μCi of [^{35}S]cysteine (Amersham) was added, and the infected cells were incubated at 37°C for 15 h. Lysates were prepared from the infected cells and the culture supernatant (which was centrifuged at 4°C for 90 min at 85,000 × *g* in the Beckman type 40 rotor to remove virions) by using PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 mM phenylmethylsulfonyl fluoride (PBSTDS). Labeled gp50 was immunoprecipitated by adding 5 μl of monoclonal antibody G50N2 to 300 μl of lysate. The gII complex was immunoprecipitated by adding 10 μl of monoclonal antibody 75N10 to 50 to 100 μl of lysate. After incubation overnight at 4°C, antigen-antibody complexes were harvested by the addition of 40 to 200 μl of protein A-Sepharose CL-4B beads (Pharmacia) (40% [vol/vol] in PBSTDS). After incubation for 2 h at 4°C, the beads were washed four times with PBSTDS and resuspended in 15 μl of 3× sample buffer (6% sodium dodecyl sulfate, 15% 2-mercaptoethanol, 30% glycerol, 0.03% bromophenol blue, 188 mM Tris-hydrochloride [pH 6.8]). The samples were boiled for 5 min and loaded on an acrylamide-bisacrylamide (37:1) gel consisting of a 4% stacking gel and a 10% resolving gel and electrophoresed by using the buffer system described by Laemmli (34).

Purification of labeled virions and adsorption of virions to cells. SK-6 cells grown in medium containing 10% dialyzed fetal calf serum were infected with wild-type virus or the mutant viruses R122$^+$ and R332$^+$ at a multiplicity of infection of 1. After an adsorption period of 1 h, the inoculum was removed and medium containing 50 μCi of [^{3}H]thymidine (Amersham) per ml was added. After a labeling period of 20 h, the medium was removed and the infected monolayer was collected by scraping into 1 ml of 1 mM phosphate buffer (pH 7.5) with a rubber policeman. The medium, which contained a considerable number of detached infected cells, was centrifuged for 10 min at 2,900 × *g* and the pellet was added to the collected monolayer. The cells were disrupted by three freeze-thaw cycles, and the lysates were centrifuged at low speed to remove nuclei and cellular debris. The supernatant was combined with the virus pellet obtained after centrifugation of the medium at 4°C for 2 h at 85,000 × *g* in the Beckman type 40 rotor, and labeled virions were isolated from the resulting preparation by using dextran-T10 gradients (Pharmacia) as described previously (61). Pooled peak fractions containing labeled virions were diluted with 1 mM phosphate buffer (pH 7.5) and centrifuged at 4°C for 2 h at 120,000 × *g* in the Beckman SW28 rotor. Pelleted virions were resuspended gently in PBS containing 0.1% bovine serum albumin and stored at 4°C until use.

The rate of adsorption of labeled virions to SK-6 cells was measured at 37°C by the method of WuDunn and Spear (71). Confluent monolayers of SK-6 cells in 24-well culture dishes were preincubated for 15 min with 200 μl of PBS containing 1% fetal calf serum, 0.1% glucose, and 0.5% bovine serum albumin. The preincubation mixture was removed, and labeled virus in 100 μl of PBS containing 0.1% bovine serum albumin was added to the wells and incubated for various periods. The inoculum was removed, and the monolayers were washed twice with 1 ml of PBS. The monolayers were lysed by the addition of 200 μl of PBS containing 1% sodium dodecyl sulfate and 1% Triton X-100. Finally, the lysates were dissolved in 4 ml of ATOMLIGHT (New England Nuclear) and counted in a liquid scintillation counter.

Polyethylene glycol-induced virus-cell fusion. Virus was allowed to adsorb to SK-6 cells for 1 h at 37°C. Subsequently, the inoculum was removed and cells were washed three times with medium. Cells were exposed to polyethylene glycol 4000 (gas chromatography grade; Merck) for 60 s and washed as described previously (58). After incubation for 2 to 3 days in Earle's minimal essential medium containing 2% fetal calf serum and 1% methylcellulose, plaques were counted.

ELISA. Lysates of gp50-expressing cells were prepared by disrupting monolayers by means of two freeze-thaw cycles followed by incubation for 1 h at 37°C in PBS containing 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. Relative quantities of gp50 were determined by means of an optimized gp50-specific sandwich enzyme-linked immunosorbent assay (ELISA). ELISA plates (Dynatech 129A) were coated overnight at 37°C with 100 μl of monoclonal antibody G50N2 that was diluted 1:4,000 in 50 mM sodium carbonate buffer (pH 9.6). The plates were washed five times with PBS containing 0.05% Tween 80, and serial dilutions of the gp50 lysates (100 μl) were added to the wells and incubated for 1 h at 37°C. The plates were washed three times with PBS containing 0.05% Tween 80, and then 100 μl of horseradish peroxidase-conjugated monoclonal antibody G50N6 diluted 1:4,000 in PBS containing 4% horse serum, 0.36 M NaCl, and 0.05% Tween 80 was added. After incubation for 1 h at 37°C, the plates were again washed and incubated with 100 μl of 0.1 mg of 3,3′,5,5′-tetramethylbenzidine (Sigma) per ml in 0.11 M sodium acetate (pH 6.0) and 0.01% hydrogen peroxide. Color development was allowed to proceed for 15 min and was stopped by the addition of 100 μl of 2 N sulfuric acid. The optical density was measured with a Titertek Multiscan spectrophotometer.

Transmission electron microscopy. Cells were grown on carbon-coated coverslips and infected with the appropriate virus strain. After 1 h of adsorption the monolayers were washed with medium and further incubated. After 24 h of infection the monolayers were fixed and processed for electron-microscopic examination as described previously (51).

RESULTS

Construction of cell lines that express gp50. To study the function of gp50 of PRV, we wanted to construct a mutant virus in which expression of gp50 was abolished. Since previous attempts to construct such a mutant were unsuccessful (16), we assumed that gp50, like its HSV-1 homolog gD (35), is an essential protein in the replication cycle of PRV. Therefore, we constructed a cell line that was able to complement the genomic defect of the mutant virus. A *Bst*XI-*Stu*I fragment containing the gp50 gene of PRV (Fig. 1) was cloned in the expression vector pEVhis14 (45a). The latter plasmid contains the strong immediate-early gene promoter of human cytomegalovirus (4, 5, 22) in addition to the histidinol dehydrogenase gene, *hisD*, from *Salmonella typhimurium* under control of the simian virus 40 early promoter. The *hisD* gene can be used as a selective marker since cells expressing histidinol dehydrogenase are able to grow in medium containing histidinol (which is toxic to most

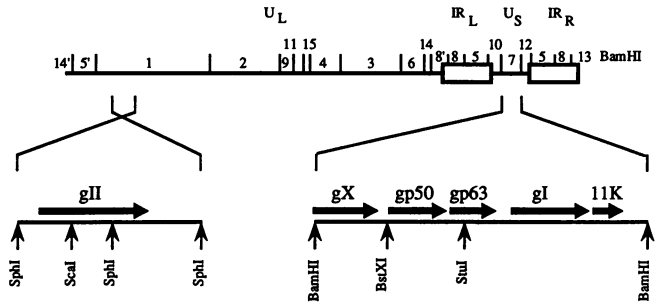

FIG. 1. Physical map of the PRV genome (upper line). Open rectangles represent the left and right inverted repeats (IR_L and IR_R), which divide the genome into a unique long (U_L) region and a unique short (U_S) region. The positions of the restriction fragments generated by *Bam*HI are shown and are numbered according to size. The lower part of the figure shows the locations of the gII and gp50 genes. Relevant restriction sites (see text) are indicated.

cells by the inhibition of histidyl-tRNA synthetase) by the oxidation of histidinol to histidine (27). SK-6 cells were transfected with plasmid pEVhis14gp50, and transformants able to grow in medium containing histidinol were isolated and screened for the expression of gp50 in an immunoperoxidase monolayer assay (see Materials and Methods). Seven cell lines were chosen, and the relative levels of gp50 expression were determined by means of a sandwich ELISA with two gp50-specific monoclonal antibodies that recognize nonoverlapping, conformation-dependent epitopes of gp50 (see Materials and Methods). The results indicated that the expression levels varied by a factor of 10 (data not shown). The cell line that showed the highest level of gp50 expression was designated G5 and was characterized in more detail by radioimmunoprecipitation. The results showed that gp50 expressed by G5 cells was similar in size to gp50 expressed by PRV-infected cells (Fig. 2). The amount of gp50 produced by G5 cells was somewhat smaller than that produced by infected cells. Immunological staining and immunofluorescence studies showed that in both G5 cells and PRV-infected cells, gp50 was present in the endoplasmic reticulum as well as in the cell membrane (data not shown). This indicated that in G5 cells, the protein was correctly processed and transported to the cell membrane and suggested that G5 cells would be suitable as complementing host cells for the isolation of gp50 mutants.

In accordance with earlier observations (48), we found that cells that expressed gp50 were partially resistant to PRV infection. Similar observations have been made with cells expressing gD of HSV-1 (9, 32) or gIV of bovine herpesvirus type 1 (12). In contrast to cells expressing gD of HSV-1 (10) or gIV of bovine herpesvirus type 1 (63), none of the cell lines that expressed gp50 showed any signs of spontaneous fusion.

Isolation of gp50 mutants of PRV. The gp50 gene is located in the unique short region of the PRV genome (Fig. 1). This region is completely present in plasmid pN3HB, which contains the 27-kb *Hin*dIII B fragment of wild-type PRV (NIA-3) (64; Fig. 3). For the insertional inactivation of the gp50 gene, a synthetic palindromic 20-mer oligonucleotide, 5′-TAGGCTAGAATTCTAGCCTA-3′, which contains an *Eco*RI recognition sequence (underlined) and TAG stop codons in all reading frames, was inserted at two different positions in the gp50 gene of pN3HB, yielding insertions R1 and 322. As a result, stop codons were introduced behind

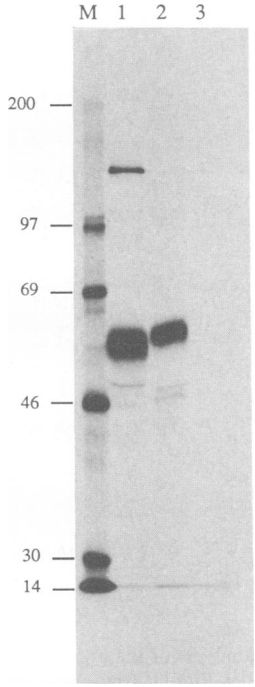

FIG. 2. Expression of gp50 by NIA-3 infected cells (lane 1), G5 cells (lane 2), and uninfected SK-6 cells (lane 3). Proteins were labeled with [^{35}S]cysteine, and gp50 was precipitated from the lysates by using monoclonal antibody G50N2. Precipitated proteins were analyzed by polyacrylamide gel electrophoresis. The sizes of the molecular mass markers (lane M) are given in kilodaltons.

amino acids 122 and 332 of gp50, respectively. Full details of the mutagenesis procedure have been previously described (16).

Mutant virus was constructed by means of overlap recombination (64) in the complementing G5 cell line. To this end, the cells were cotransfected with the mutagenized *Hin*dIII fragment R1 or 322 and three overlapping wild-type PRV fragments (derived from cosmids c-179, c-27, and c-443 [64]), which together make up the entire viral genome (Fig. 3). After transfection, plaques were obtained and the viruses were designated R122 and R332, respectively.

Construction of cell lines that are able to express gII. For the construction of a gII mutant of PRV we used a strategy

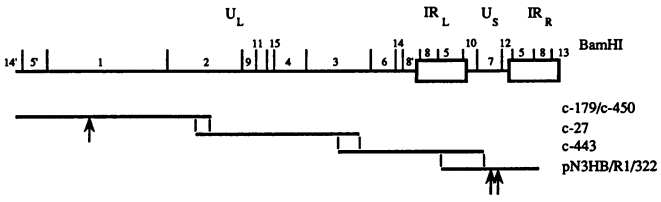

FIG. 3. Location of the overlapping subgenomic PRV fragments that were used for the reconstitution of an intact viral genome by means of overlap recombination (64). The upper line represents the PRV genome (cf. Fig. 1). Fragments c-179, c-27, c-443, and pN3HB are derived from the wild-type PRV strain, NIA-3. As a result of the insertion of a mutagenic oligonucleotide (see Materials and Methods), fragment c-450 contains a premature translation termination codon in the gII gene and fragments R1 and 322 contain premature termination codons in the gp50 gene. The positions of the termination codons are indicated by arrows.

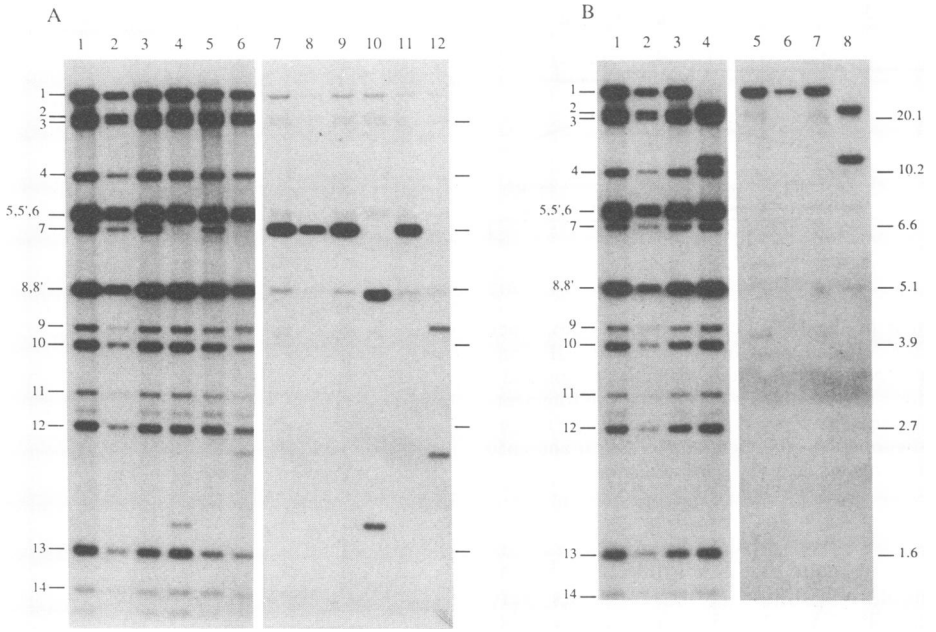

FIG. 4. Genomic analysis of the gp50 mutants R122 and R332 and the gII mutant B145. Viral DNA was isolated from infected cells and was digested with either *Bam*HI (odd-numbered lanes) or a combination of *Bam*HI and *Eco*RI (even-numbered lanes). The fragments were separated by agarose gel electrophoresis, transferred to GeneScreen Plus, and hybridized with ^{32}P-labeled probes. (A) DNA of strains NIA-3 (lanes 1, 2, 7, and 8), R122 (lanes 3, 4, 9, and 10), and R332 (lanes 5, 6, 11, and 12), hybridized with NIA-3 DNA (lanes 1 to 6) or with *Bam*HI-7 (lanes 7 to 12). (B) DNA of NIA-3 (lanes 1, 2, 5, and 6) and B145 (lanes 3, 4, 7, and 8) hybridized with NIA-3 DNA (lanes 1 to 4) or with *Bam*HI-1 (lanes 5 to 8). The molecular size markers are given in kilobase pairs.

similar to the one described above for gp50, since, by analogy of gB of HSV-1 (7, 8), gII of PRV is probably also an essential protein. We wanted to construct cell lines that allowed inducible expression of gII since it had been shown that constitutive expression of the gII homologs of other herpesviruses resulted in spontaneous cell fusion (1, 20). Therefore, we cloned the gII gene under the control of its natural promoter. Because the gII promoter is dependent on transactivation by the viral immediate-early protein (70), we expected that gII would be expressed only after PRV infection. The gII gene of PRV was obtained as an *Sph*I fragment after partial digestion of the subgenomic PRV DNA fragment present in cosmid c-179 (Fig. 1 and 3). The *Sph*I fragment, which contains the gII open reading frame including its promoter, was cloned in plasmid pEVhis10 (a derivative of pEVhis14 that lacks the human cytomegalovirus promoter), and the resulting plasmid was called pEVhis10gII. SK-6 cells were transfected with pEVhis10gII, and 96 histidinol-resistant colonies were isolated. Since we expected that uninfected cell lines harboring the gII gene did not express significant amounts of gII, the cells were used, without prior testing for gII expression, for the generation of a gII mutant by means of overlap recombination. In this way both the mutant virus and the cell lines able to complement the mutant could be identified in a single experiment (see below).

Isolation of a gII mutant of PRV. To inactivate the gII gene, we inserted the mutagenic oligonucleotide (see above) into the *Sca*I site of the gII gene (Fig. 1) in cosmid c-179, as described previously (16). As a result of the insertion of the oligonucleotide, a premature stop codon is introduced behind amino acid 145 in the gII sequence. The resulting cosmid, c-450, was used for the generation of a mutant virus by means of overlap recombination. To this end, the PRV fragment of c-450 was used, together with the overlapping wild-type fragments derived from c-27, c-443, and pN3HB (Fig. 3), for the cotransfection of cell lines that contained the gII gene. Of the 96 cell lines tested, 12 yielded plaques. When the complete set of overlapping wild-type fragments was used, all 96 cell lines yielded plaques. After three rounds of plaque purification a virus stock was prepared by using the cell line that yielded the highest virus titers. The putative gII mutant was called B145, and the complementing cell line was designated 10B2.

Genomic analysis of the gp50 and gII mutants. To verify that the oligonucleotide was inserted at the correct positions, we isolated viral DNA from the putative gp50 and gII mutants. The location of the mutagenic oligonucleotide could be easily mapped since it contained an *Eco*RI site and *Eco*RI sites are absent from the wild-type PRV genome. After digestion of the viral DNAs with either *Bam*HI (the map of *Bam*HI is given in Fig. 1 and 3) or *Bam*HI-*Eco*RI, the fragments were separated by agarose gel electrophoresis, blotted, and hybridized with labeled *Bam*HI-1 or *Bam*HI-7. As expected, *Bam*HI-7 (6.6 kb) of the gp50 mutants was cleaved by *Eco*RI, yielding fragments of 4.8 and 1.8 kb for R122 (Fig. 4A, lanes 4 and 10) and 4.45 and 2.15 kb for R332 (Fig. 4A, lanes 6 and 12); *Bam*HI-1 of the gII mutant B145 was cleaved by *Eco*RI, yielding two fragments of approximately 23 and 12 kb (Fig. 4B, lanes 4 and 8). These results indicated that the oligonucleotide was inserted at the correct positions both in the gp50 gene and in the gII gene.

gp50 mutants are able to form plaques on noncomplementing SK-6 cells. To analyze the phenotype of the gp50 mutants, R122 and R332 virus was isolated from complementing G5 cells and serial dilutions were replated on noncomplementing SK-6 cells. Surprisingly, both viruses formed plaques on these cells. Although the genomic analysis

TABLE 1. Effect of polyethylene glycol treatment on plaque production on SK-6 cells

Virus[a]	Titer (PFU/plaque)[b]	
	−PEG	+PEG
R122	0	2×10^4
R332	90	9×10^4
NIA-3	2×10^5	5×10^4

[a] Virus was isolated from noncomplementing SK-6 cells by resuspending individual plaques in medium.
[b] Titers were determined on SK-6 cells with or without polyethylene glycol (PEG) treatment, which was performed after 1 h of adsorption.

showed that the oligonucleotide was inserted at the correct positions in the gp50 gene (Fig. 4), we wanted to confirm that expression of gp50 was indeed abolished by premature termination of translation. Therefore, plaques produced on SK-6 cells by NIA-3, R122, and R332 were analyzed by immunological staining in an immunoperoxidase monolayer assay (see Materials and Methods), using either polyclonal anti-PRV serum or gp50-specific monoclonal antibodies. Both antisera reacted with NIA-3 plaques, but plaques from strains R122 and R332 failed to react with monoclonal antibodies against gp50 (results not shown). This indicated that expression of intact gp50 was impaired in cells infected with R122 and R332 and thus that R122 and R332 were true gp50 mutants. This conclusion was confirmed by radioimmunoprecipitation experiments (see below). Since these mutants are still able to form plaques on SK-6 cells, it can be concluded that gp50 is not required for viral DNA replication or for the transmission of the virus from infected to uninfected cells via fusion of cellular membranes.

Virus particles lacking gp50 are noninfectious. To determine whether infection of SK-6 cells by the gp50 mutants resulted in the formation and release of progeny virions, individual plaques of R122, R332, and NIA-3 were resuspended in medium and replated on SK-6 cells. The wild-type strain NIA-3 yielded 2×10^5 PFU; no plaques were produced by R122, and only a few plaques were produced by R332 (Table 1). The failure of R122 and R332 to efficiently form plaques on SK-6 cells suggested that these virus particles lacked gp50 and therefore were noninfectious. Alternatively, the formation of viral particles by SK-6 cells could be impaired by improper virus assembly or virus release because of the absence of functional gp50. To distinguish between these possibilities, 1 h after adsorption of the virus, the cells were treated with polyethylene glycol. Polyethylene glycol treatment results in fusion of the viral envelope with the cellular plasma membrane and has been shown to induce the penetration of noninfectious viral mutants into cells (58). Treatment with polyethylene glycol greatly increased the number of plaques formed by R122 and R332 (Table 1). These data indicated that viral particles were formed during replication of the gp50 mutants in noncomplementing SK-6 cells. The formation of viral particles was confirmed by electron microscopy (results not shown). We conclude that these virions are noninfectious because they lack gp50 and thus that gp50 is specifically required for the entry of PRV into the host cell. To distinguish between mutant viruses containing and lacking gp50, we shall refer to mutant virus isolated from complementing G5 cells as $R122^+$ and $R332^+$ and to mutant virus isolated from noncomplementing SK-6 cells as $R122^-$ and $R332^-$.

gp50 is required for penetration, not for adsorption. The

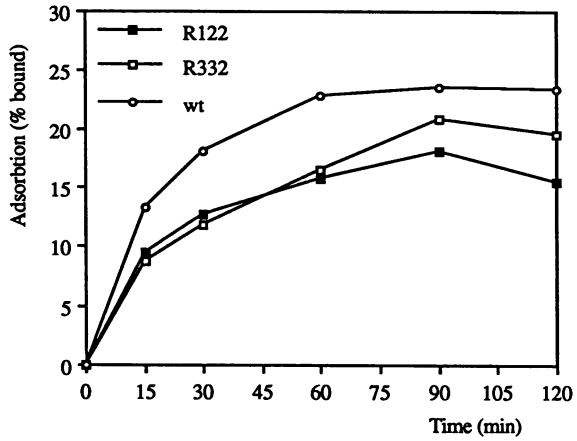

FIG. 5. Adsorption of labeled virus to SK-6 cells. Virus particles, labeled with [^{3}H]thymidine and purified on dextran gradients, were incubated with SK-6 cells for various times at 37°C. The cells were washed several times, and the amount of virus bound to the cells was determined by quantitating the amount of radioactivity associated with the cells. The mutant viruses R122 and R332 lack gp50 since they were grown on noncomplementing SK-6 cells. The results shown are the average of two experiments.

inability of virus lacking gp50 to initiate an infection might be due to impairment of either adsorption or penetration or both. To determine whether $R122^-$ and $R332^-$ virus was able to bind to SK-6 cells, we examined the adsorption of labeled virus particles to these cells as described by Wu-Dunn and Spear (71). SK-6 cells were incubated with [^{3}H]thymidine-labeled virions and washed, and the label associated with the cell monolayer was quantitated. Both $R122^-$ and $R332^-$ were able to bind to SK-6 cells, although the efficiency was somewhat reduced compared with that of the wild-type strain NIA-3 (Fig. 5). These results show that gp50 is not essential for the attachment of PRV to SK-6 cells, although it may contribute to some extent to the adsorption process.

Secretion of truncated gp50 by R332-infected cells. Although monoclonal antibodies against gp50 failed to react with plaques of R122 and R332 in an immunoperoxidase monolayer assay, we found that virus stocks of R332-infected SK-6 cells yielded high titers in a gp50 ELISA (data not shown). This apparent discrepancy could be explained by assuming that the truncated form of gp50 produced by R332 is secreted by the cell. This is a likely explanation since the truncated form of gp50 expressed by R332 lacks the putative transmembrane region which probably acts as a membrane anchor sequence (49). Secretion of truncated (glyco)proteins that lack their transmembrane sequence has been shown to occur in a number of cases, including the HSV-1 glycoproteins gD and gB (8, 29, 52, 62). To test whether truncated gp50 was secreted by $R332^+$-infected cells, we performed immunoprecipitations by using lysates prepared from the cellular fraction and from virus-free supernatant. The results showed that in wild-type-infected cells gp50 was present in the cellular fraction and was not detectable in virus-free supernatant (Fig. 6, lanes 1 and 2). In R332-infected cells, however, a smaller protein with an apparent molecular mass of about 50 kDa was readily precipitated from the virus-free supernatant (lane 4), whereas only a very small amount was present in the cellular fraction (lane 3). The size of this protein corresponded well to the size that was expected for the glycosylated form of the

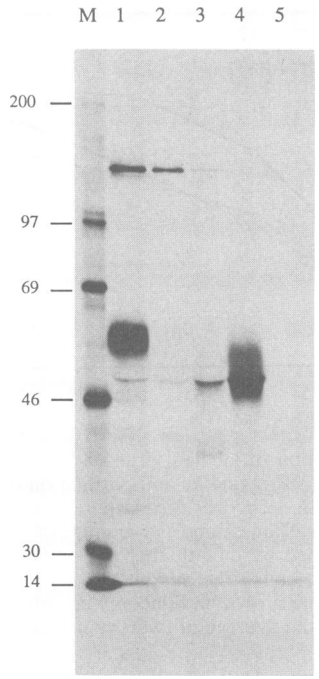

FIG. 6. Analysis of gp50 in the cellular fraction and in the virus-free supernatant of NIA-3-infected cells (lanes 1 and 2) and R332-infected cells (lanes 3 and 4). Cells were infected with virus, and the proteins were labeled with [^{35}S]cysteine for 16 h. Lysates were prepared from the cellular fraction and from the supernatant from which virus particles had been removed by centrifugation. gp50 was precipitated with monoclonal antibody G50N2 and analyzed by polyacrylamide gel electrophoresis. Lane 5 shows the cellular fraction of uninfected SK-6 cells. The sizes of the molecular mass markers (lane M) are given in kilodaltons.

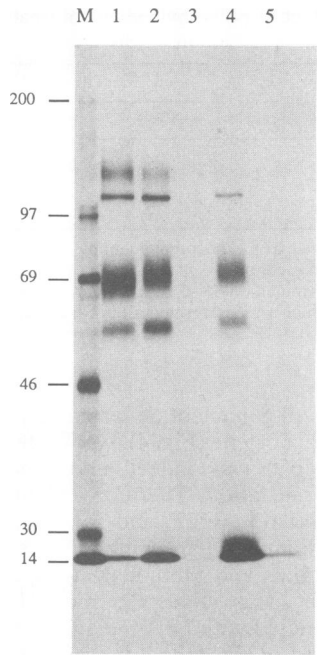

FIG. 7. Analysis of gII expression in uninfected SK-6 cells (lane 5) and 10B2 cells (lane 4) and in NIA-3-infected SK-6 cells (lane 1) or B145-infected SK-6 and 10B2 cells (lanes 3 and 2, respectively). [^{35}S]cysteine-labeled proteins were precipitated with monoclonal antibody 75N10 directed against gII and analyzed by polyacrylamide gel electrophoresis. The gel was run under denaturing conditions, resulting in the dissociation of the gII complex into three subunits (see text for details). For the precipitation of gII, 10 times less lysate was used from NIA-3-infected SK-6 cells than from the other samples. The sizes of the molecular mass markers (lane M) are given in kilodaltons.

332-amino-acid truncated protein. When the distribution of gp50 was examined by the gp50 ELISA, similar results were obtained (data not shown). From these results we conclude that the truncated form of gp50 expressed by mutant R332 is efficiently secreted. We could not detect the 122-amino-acid truncated form of gp50 in R122-infected cells either by radioimmunoprecipitation or by ELISA. The epitopes recognized by the monoclonal antibodies are probably not present in this truncated protein, or they do not have the correct conformation.

Phenotype of the gII mutant. To examine the phenotype of the putative gII mutant B145, we determined the virus titer on complementing 10B2 cells and noncomplementing SK-6 cells. On 10B2 cells the virus titer was more than 1,000-fold higher than on SK-6 cells (1.8×10^7 and 9.0×10^3, respectively); this confirmed the mutant phenotype of the virus. Immunological staining of the plaques by using a monoclonal antibody specific for gII showed that the plaques on 10B2 cells stained faintly whereas the plaques on SK-6 cells stained much more intensely (data not shown). These results suggested that the plaques on 10B2 cells were formed by gII mutants and that the plaques on SK-6 cells were formed by wild-type revertants which had arisen by recombination of the viral gII gene and the gII gene of the 10B2 cell line. After putative revertant plaques had been replated on 10B2 cells and SK-6 cells, both cell lines yielded the same number of plaques, indicating that they were formed by wild-type revertants (data not shown).

We noted that monolayers of SK-6 cells that had been infected with B145 virus at a high multiplicity of infection showed signs of cytopathogenic effect without actually forming plaques. Immunological staining of such monolayers showed the presence of a large number of single cells or small groups of cells that reacted positively with the anti-PRV antiserum but not with monoclonal antibodies specific for gII. This observation suggested that the gII mutant was still able to infect SK-6 cells and express viral proteins but, in contrast to gp50 mutants, was unable to form plaques.

Radioimmunoprecipitations were performed to examine the expression of gII in infected and uninfected 10B2 cells. The results showed that uninfected 10B2 cells constitutively expressed gII (Fig. 7, lane 4). Furthermore, infection of these cells with B145 resulted in only a modest increase in gII expression (lane 2). This indicated that the gII promoter was not completely dependent on transactivation when present in a context different from the viral genome and that transactivation in cell line 10B2 was rather inefficient. As expected, expression of gII could not be detected in B145-infected SK-6 cells (lane 3). The level of expression of gII by uninfected and B145-infected 10B2 cells was low in comparison with that by NIA-3-infected SK-6 cells (Fig. 7, compare lane 1 with lanes 2 and 4; note that 10 times less lysate was used in lane 1). This is probably due to the presence of a limited number of copies of the gII gene in the 10B2 cell line. The results furthermore showed that gII expressed by uninfected and B145-infected 10B2 cells is correctly processed, yielding the characteristic pattern of the gII complex which

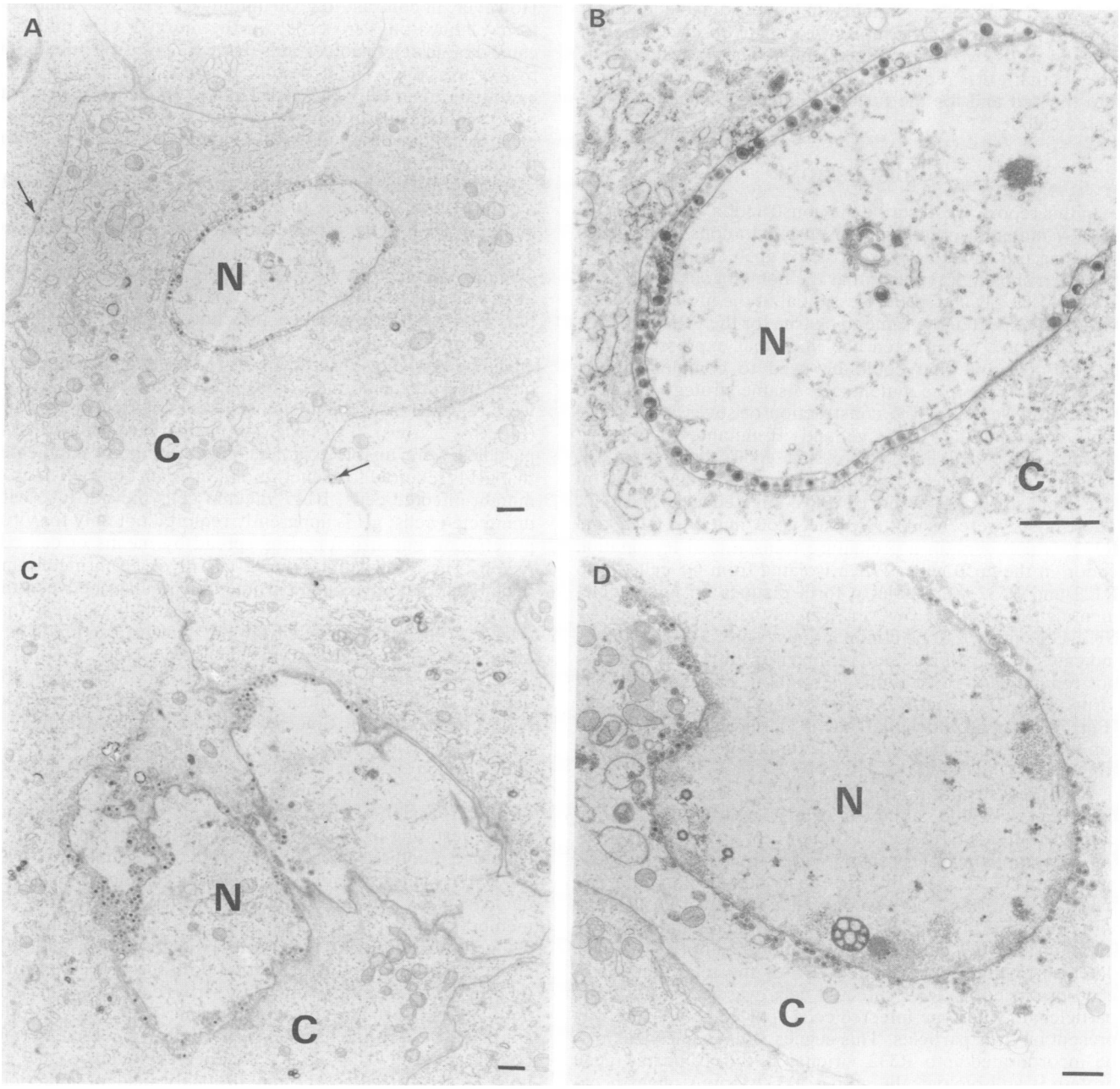

FIG. 8. Transmission electron micrographs of thin sections of SK-6 cells infected with the gII mutant B145. The micrographs were taken 24 h after infection and show the accumulation of enveloped virus particles between the inner and outer nuclear membranes. Note that few virions are present in the cytoplasm and outside of the cell (panel A, arrows). Panel B shows a detail of the cell shown in A. Abbreviations: N, nucleus; C, cytoplasm. Bar, 1 μm.

consists of proteins of 110 kDa (gIIa), 68 kDa (gIIb), and 55 kDa (gIIc) (55, 68). This indicates that no other viral proteins are required for the proteolytic processing of gII. In contrast to cells expressing the gII homologs of HSV-1 (1) and bovine herpesvirus type 1 (20), 10B2 cells did not show any signs of spontaneous fusion.

Infection of noncomplementing SK-6 cells by a gII mutant results in accumulation of enveloped virus particles between the inner and outer nuclear membranes. The fate of the gII mutant after infection of complementing and noncomplementing cells was studied by electron microscopy. When we examined infected monolayers of SK-6 cells 24 h postinfection, we observed that enveloped particles accumulated between the inner and outer nuclear membranes (Fig. 8). Viral particles were rare in the cytoplasm and outside of the cell. In complementing 10B2 cells, maturation and release of viral particles appeared to be normal (data not shown). These results indicated that the process that leads to the release of virus particles from the nucleus into the cytoplasm is specifically affected in the absence of gII. Nevertheless, some viral particles reached the cytoplasm and were eventually released from the cell (Fig. 8A, arrows). These parti-

cles were noninfectious since they were unable to enter neighboring cells. Furthermore, we did not observe fusion of infected cells with uninfected cells. These observations suggest that gII is required both for the penetration of virus into the cell and for the fusion of infected cells with uninfected cells.

DISCUSSION

In this report, we describe the construction and properties of PRV mutants containing nonsense mutations in the genes encoding the envelope glycoproteins gp50 and gII. Since it had been shown previously that the homologous proteins of HSV-1, i.e., gD and gB, are essential in the life cycle of the virus, we expected the same to be true for the PRV proteins. Therefore, we constructed cell lines that expressed either gp50 or gII and that could be used to complement the genomic defect of the mutants. The same strategy has been successfully used for the construction of several virus mutants, including the HSV-1 gD and gB mutants (8, 35).

One of the cell lines expressing gp50, designated G5, constitutively expressed gp50 that was indistinguishable in size from gp50 expressed by PRV-infected cells (Fig. 2). These cells were used to rescue gp50 mutants R122 and R332, which contain a premature translational termination codon in the gp50 gene. When isolated from G5 cells, both R122 and R332 were able to form plaques on noncomplementing SK-6 cells. However, progeny virions produced by SK-6 cells proved to be noninfectious (Table 1). This finding indicates that gp50 is essential for virus entry and that it is not required for replication, maturation, and egress of the virus. Since R122 and R332 were still able to adsorb to cells (Fig. 5), it can be concluded that gp50 is specifically required for penetration. In this respect the function of gp50 is similar to that of gD of HSV-1.

We observed that mutant virus R332 isolated from SK-6 cells was still able, although with a greatly reduced efficiency, to infect SK-6 cells (Table 1). Immunological staining and replating of progeny virus indicated that these plaques were formed by true gp50 mutants and not by wild-type revertants. We were unable to detect gp50 in the virus envelope of R332 virions by using radioimmunoprecipitations (data not shown). However, by using ELISA, we determined that approximately 1% of the total amount of gp50 produced by R332-infected cells was present in virus particles; in wild-type-infected cells approximately 40% was present in virus particles. This suggests that some gp50 may be incorporated into R332 virions. If this truncated gp50 protein is still biologically active, R332 virions might occasionally infect cells.

The observation that R122 and R332 virions that contain gp50 form plaques on noncomplementing SK-6 cells indicates that gp50 is not required for the transmission of the virus from infected to uninfected cells. Virus can be transmitted by infection of uninfected cells by progeny virions or by fusion of the infected cell with uninfected cells, or both. Because progeny virions of mutant R122 produced by SK-6 cells are noninfectious (Table 1), transmission of the virus must occur by cell-cell fusion. This implies that gp50 is not essential for cell-cell fusion. In this respect, gp50 differs from gD of HSV-1. Ligas and Johnson (35) have constructed an HSV-1 mutant, F-gDβ, that is unable to express gD. Whereas F-gDβ virions containing gD were able to infect noncomplementing Vero cells, F-gDβ virions lacking gD were not, although they were still able to adsorb to the cells. This indicated that gD is required for virus penetration. However, in contrast to gp50 mutants, F-gDβ was unable to form plaques on Vero cells. Furthermore, F-gDβ infection caused rapid cell fusion of cells that expressed gD but did not fuse Vero cells. These results indicate that gD plays an essential role in cell-cell fusion, in addition to virus penetration. Spontaneous fusion of cells that constitutively express gD also indicated that gD is involved in cell-cell fusion (10); this activity may, however, be cell type dependent (24).

In contrast to the gp50 mutants R122 and R332, the gII mutant B145 was unable to produce plaques on noncomplementing SK-6 cells. This demonstrates that gII plays an essential role in the life cycle of PRV. Immunological staining and electron microscopy (Fig. 8) showed that B145 virus was able to infect SK-6 cells and express viral antigens, but was unable to produce infectious progeny. Electron-microscopic examination of the infected cells indicated that enveloped progeny virus accumulated between the inner and outer nuclear membranes and that only few virus particles were released from the nucleus and eventually from the cell (Fig. 8A, arrows). The observation that extracellular virus particles were unable to infect neighboring cells indicates that gII is required for penetration. Since, in contrast to gp50 mutant-infected cells, B145-infected cells did not fuse with uninfected cells, gII is apparently required not only for virus penetration but also for virus spread by means of cell-cell fusion. The observations that gII is involved in membrane fusion and that enveloped particles accumulate between the inner and outer nuclear membranes strongly suggest that release of the virus particle from the nucleus also involves a membrane fusion event in which gII is involved. A model in which virus particles are released from the nucleus by deenvelopment at the outer nuclear membrane has recently been presented (67). Since deenvelopment is essentially a membrane fusion event, this would be in excellent agreement with our results. Together, these observations indicate that gII is involved in at least three types of membrane fusion: (i) fusion of the virus envelope with the cell membrane, resulting in virus penetration; (ii) fusion of the virus envelope, acquired from the inner nuclear membrane, with the outer nuclear membrane, resulting in release of naked nucleocapsids into the cytoplasm; and (iii) fusion of the cell membranes of infected and uninfected cells, resulting in cell-cell fusion. Since gII is proteolytically processed in the Golgi apparatus (68), it seems that both the processed form (in the cell membrane and the virus envelope) and the unprocessed form (in the endoplasmic reticulum and the contiguous outer nuclear membrane) of gII have membrane fusion activity. This is not inconceivable since the gII homologs of HSV-1 (11) and probably also of infectious laryngotracheitis virus (26) are not processed by proteolytic cleavage. Furthermore, it has been shown that cleavage of gB of bovine herpesvirus 1 is not necessary for infectivity or for its ability to fuse membranes (43).

While this work was being conducted, Rauh et al. (53) described the construction and properties of a PRV gII mutant. This mutant was obtained by replacing part of the gII gene by a β-galactosidase expression cassette by means of homologous recombination in a cell line that expressed gII. Our results regarding the biological properties of the gII mutant are largely consistent with the results of Rauh et al.

Since the glycoproteins that are present in the virus envelope are also present in the cell membrane of infected cells, it has been suggested that penetration of the virus by means of fusion of the viral envelope with the cell membrane is mechanistically similar to fusion of infected cells with uninfected cells (10, 35). However, our observation that

gp50 is required for penetration but not for cell fusion indicates that these processes are not identical. Our results suggest that gp50 itself is not a fusogen. As a working hypothesis, we assume that the activity of the fusogen(s) in the virus envelope is induced upon binding of gp50 to the cellular receptor. The activity of the fusogen(s) is not induced when gp50 is absent from the virus envelope (gp50 mutants lacking gp50 are unable to penetrate), but it is constitutive in the infected-cell membrane in the absence of gp50 (once inside the cell, gp50 mutants are able to form plaques by cell-cell fusion). This suggests that the conformation of the fusogen(s) in the virus envelope is different from that in the plasma membrane of infected cells. It is conceivable that in the virus particle the fusogen(s) interacts with a tegument protein(s) and that this interaction results in an inactive conformation. The activity of the fusogen(s) would be induced either after binding of gp50 to the receptor or by the absence of the interaction with the viral tegument proteins. The observations that gII and its homologs from other herpesviruses contain a relatively large cytoplasmic domain (26, 55) and that several mutations affecting the fusogenic activity of gB of HSV-1 are located in this domain (7) suggest that this protein may be involved in the proposed interaction. Since we and others (53) did not observe spontaneous fusion of cells that express gII, additional proteins are probably involved in this process. These proteins may include glycoprotein gH, which is essential for PRV infection (45b), and the PRV homologs of the membrane proteins encoded by the HSV-1 UL20 and UL53 genes (37).

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