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Glycoprotein H of Pseudorabies Virus Is Essential for Entry and Cell-to-Cell Spread of the Virus

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To study the function of the envelope glycoprotein gH of pseudorabies virus, a gH null mutant was constructed. A premature translation termination codon was introduced in the gH gene by linker insertion mutagenesis, and a mutant virus was rescued by using a cell line that expresses the wild-type protein. Mutant virus isolated from complementing cells was unable to form plaques on noncomplementing cells, indicating that gH is essential in the life cycle of the virus. Immunological staining and electron microscopy showed that the mutant virus produced noninfectious progeny and was unable to spread from infected to uninfected cells by cell-cell fusion. Thus, similar to gH of herpes simplex virus, gH of pseudorabies virus is required for entry and cell-to-cell spread.

Entry of herpesviruses into host cells results from fusion of the viral envelope with the cellular plasma membrane. This process is mediated by a number of glycoproteins that are located in the virus envelope (33). Pseudorabies virus (PRV) encodes at least six glycoproteins, designated gI, gII, gIII, gp50, gp63, and gX, that, with the exception of gX, are located in the virus envelope as well as the plasma membrane of infected cells (24). By analogy to herpes simplex virus (HSV) and other herpesviruses, another glycoprotein, designated gH (4), is probably also a component of the envelope of PRV. However, since monospecific sera against gH of PRV are not available, the presence of gH in infected cells or in the virus envelope has not yet been confirmed. By using a temperature-sensitive gH mutant of HSV, Desai et al. (6) showed that gH is not transported to the cellular plasma membrane and is not incorporated into the virion envelope at the nonpermissive temperature, resulting in the production of noninfectious progeny virus. This finding indicated that gH is required for virus entry. Since neutralizing antibodies against HSV gH prevent infection but not adsorption of the virus, gH is probably involved in penetration (11, 25). The observation that neutralizing monoclonal antibodies against HSV gH inhibit plaque formation even when added after the virus has entered the cell (12, 19) indicates that gH is also involved in cell-to-cell spread. The fact that a gene encoding gH is present in all herpesviruses examined to date (5, 12, 14, 17, 19, 21, 22, 26, 27, 29) indicates that it fulfills an important function in the life cycle of the virus and suggests that this function is highly conserved. However, differences in the functions of essential proteins may arise during evolution of these viruses. For instance, we and others have shown that the homologous

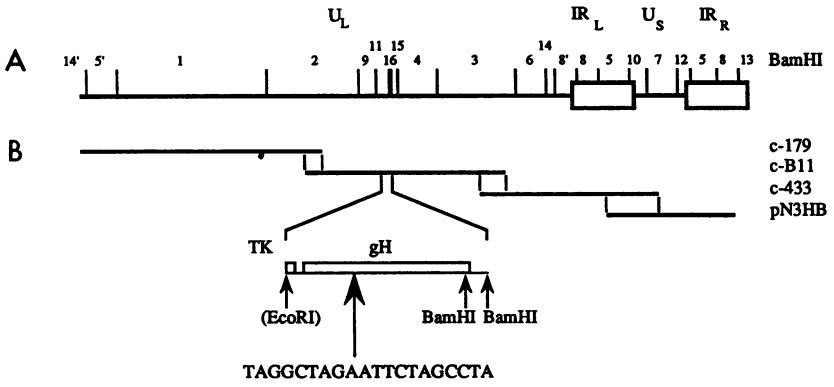

FIG. 1. (A) Physical map of the PRV genome. Open rectangles represent the left and right inverted repeats (IR_L and IR_R), which divide the genome in a unique long (U_L) and a unique short (U_S) region. Positions of the restriction fragments generated by *Bam*HI are shown and numbered according to size. (B) Locations of the subgenomic fragments that were used for the regeneration of an intact viral genome by means of overlap recombination. These fragments were excised from the cosmid clones c-179, c-B11, and c-443 and the plasmid clone pN3HB, respectively (34). The enlargement shows the location of the cloned *Eco*RI-*Bam*HI fragment that was sequenced (see Fig. 2) and used for the construction of stably transformed cell lines (see text). The arrow indicates the position of the self-complementary oligonucleotide 5′-TAGGCTAGAATTCTAGCCTA-3′ that was used for insertion of a premature translation termination codon and an *Eco*RI site in the gH gene of cosmid c-448, yielding cosmid c-B11.

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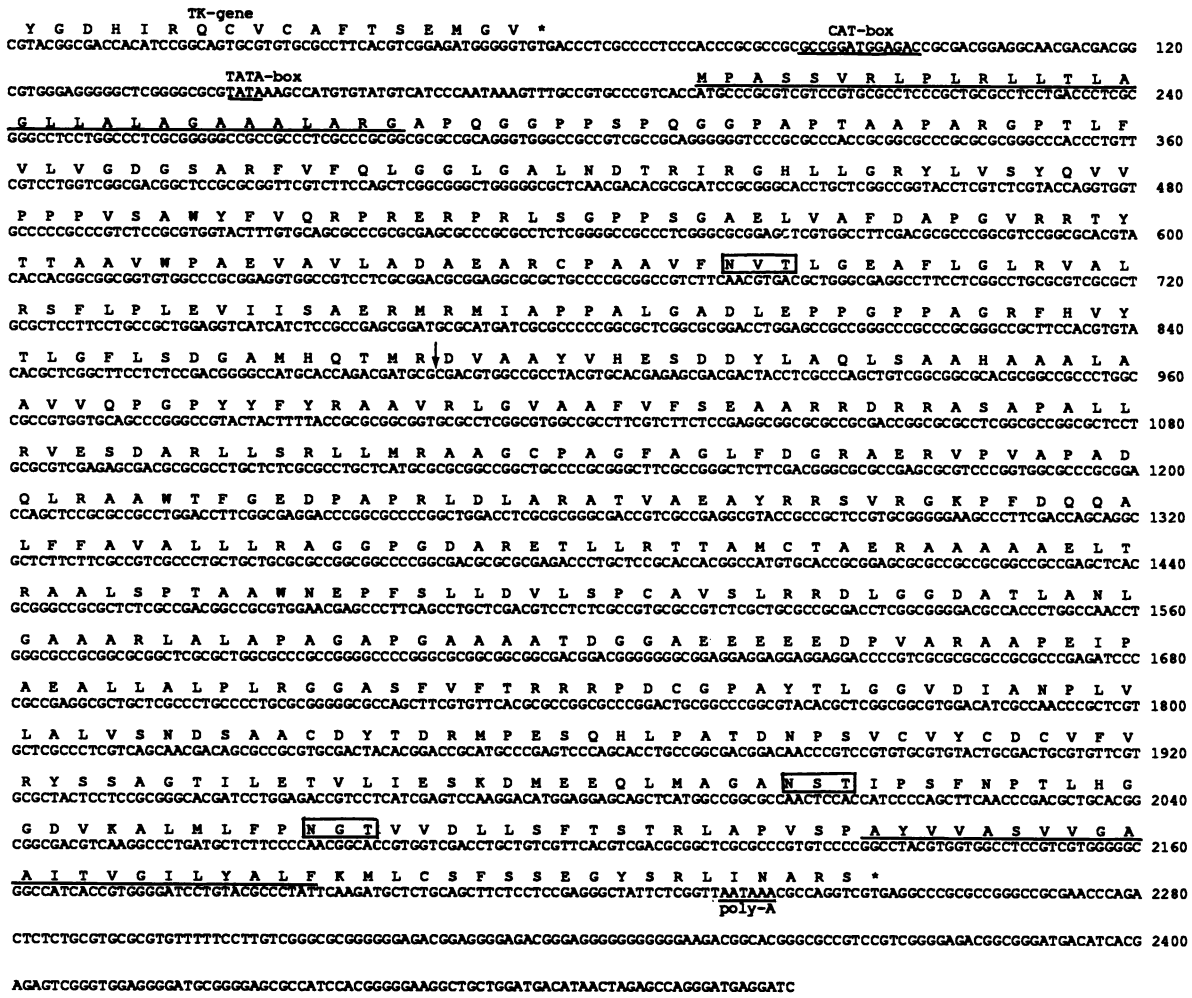

FIG. 2. Nucleotide sequence of the cloned *Eco*RI-*Bam*HI fragment (see Fig. 1). The amino acid sequence of the C-terminal end of the TK gene and the deduced amino acid sequence of the gH gene are shown. The locations of putative transcriptional control elements, i.e., the CAT box, TATA box, and polyadenylation signal AATAAA, are indicated. The predicted N-terminal signal sequence and C-terminal transmembrane region of gH are underlined. Potential N-linked glycosylation sites are boxed. The arrow indicates the position at which the mutagenic oligonucleotide (Fig. 1) was inserted into the gH gene (see Fig. 3).

envelope glycoproteins gp50 of PRV and gD of HSV are both required for penetration but differ with respect to their role in cell-to-cell spread (28, 31). These considerations prompted us to study the function of gH of PRV. To this end, a gH null mutant was constructed and its properties were examined.

Since we expected that, by analogy to gH of HSV, gH of PRV is essential for replication, we constructed a cell line that was able to complement a null mutant. The gene encoding gH of PRV is located downstream of the viral thymidine kinase (TK) gene (20, 29). Since we had at our disposal a cosmid clone in which a unique *Eco*RI site had been inserted at the 3′ end of the TK gene (Fig. 1), we cloned a 2.5-kb *Eco*RI-*Bam*HI fragment obtained after partial digestion of the cosmid DNA with *Bam*HI and complete digestion with *Eco*RI into pSP72 (Promega), yielding plasmid pSP72gH. A second plasmid, pGEM4ZgH, was constructed by subcloning the fragment as a *Bgl*II-*Xba*I fragment from pSP72gH into *Bam*HI-*Xba*I-digested pGEM4Z (Promega). To verify that the cloned fragment contained the complete gH gene, including its transcription regulation signals, we established its nucleotide sequence. Unidirectional deletions were generated by digestion and religation of plasmids pSP72gH and pGEM4ZgH by using restriction enzymes that cut once in the polylinker of the vector and in the cloned fragment. The resulting deletions were subcloned as *Eco*RI-*Hin*dIII fragments into the single-stranded DNA vectors M13mp18 and M13mp19 (23). The nucleotide sequence (Fig. 2) was established as described by Sanger et al. (32) by using a commercial sequencing kit containing 7-deaza-2′-deoxy-GTP (Pharmacia).

The sequence shows an overlap with the 3′ part of the TK gene (30) and contains an open reading frame representing the putative gH gene. The sequence contains a TATA box and a potential CAT box which represent the putative promoter of the gH gene (3). A potential polyadenylation signal, AATAAA (2), is located within the C-terminal part of the gH gene. The open reading frame is located from positions 194 to 2251, encoding a protein of 686 amino acids with a predicted molecular mass of 72 kDa. The protein has several features characteristic of a membrane protein, such as a hydrophobic N-terminal signal sequence, three potential

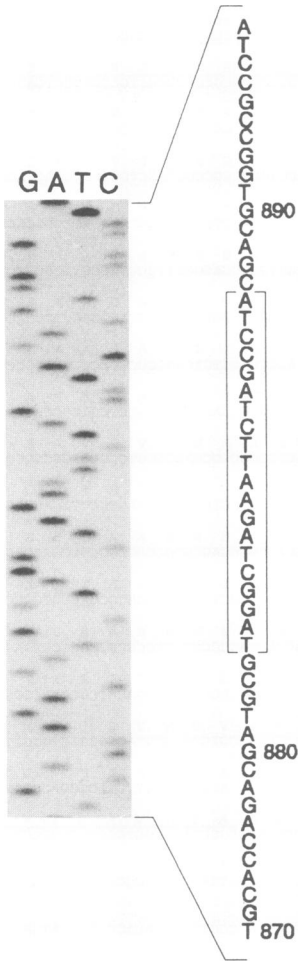

FIG. 3. Autoradiogram of a sequencing gel showing the position and sequence of the mutagenic oligonucleotide (see Fig. 1) in the gH gene of mutant virus H231. The oligonucleotide has been inserted between nucleotides 885 and 886 (Fig. 2).

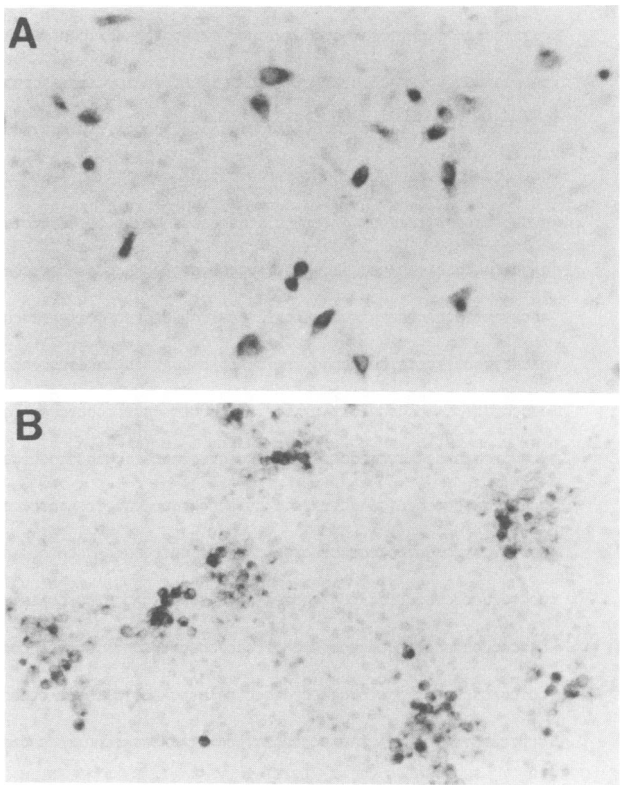

FIG. 4. Immunological staining of noncomplementing SK-6 cells (A) and complementing SKF12 cells (B) infected with the gH null mutant H231. Monolayers were infected with mutant virus H231, and after 1 h of adsorption, the monolayers were incubated for 21 h in Earle's minimal essential medium containing 1% methylcellulose and 2% fetal calf serum. Immunological staining was performed as described by Peeters et al. (28) by using a combination of rabbit anti-PRV serum and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Diagnostics Pasteur).

N-linked glycosylation sites, and a C-terminal hydrophobic transmembrane region (Fig. 2). During the establishment of this sequence, the nucleotide sequence of the gH gene of PRV strain Ka was published (21). Comparison of the sequences of the gH genes of PRV strains NIA-3 and Ka revealed that these genes are almost identical. Within the coding sequence of gH, only six nucleotide differences were noted. In four cases, this results in an altered amino acid sequence of the encoded protein. The differences are located at positions 304 (G to F, silent), 386 (C to T, R becomes W), 794 (G to T, A becomes S), 1626 (G to A, G becomes E), 1802 (C to A, L becomes I), and 1837 (C to T, silent). The gH protein of PRV shows significant homology to the gH proteins of other herpesviruses (5, 12, 14, 17, 19, 22, 26, 27). For a comparison of the gH proteins of PRV and other herpesviruses, see the report by Klupp and Mettenleiter (21).

Since it has been reported that constitutive expression of gH of HSV is detrimental to cells (12), we expected the same to be true for gH of PRV. Therefore, the gH gene was left under the control of its own promoter, since the promoters of PRV genes are subject to temporal regulation and are dependent on transactivation by the viral immediate-early protein during viral infection (1, 8). Thus, in cells containing the gH gene, expression of gH should take place only after infection by PRV. The gH gene was subcloned from pSP72gH as a *Bgl*II-*Xba*I fragment into *Bam*HI-*Xba*I-digested pEVhis10 (16, 28), yielding pEVhis10gH. Plasmid pEVhis10gH was used for the transfection of SK-6 cells (18) by using the Lipofectin reagent (GIBCO), followed by selection in Dulbecco's modification of minimal essential medium containing 5% fetal calf serum and 2.5 mM histidinol (Sigma). After 10 days, 96 colonies were picked. To identify cell lines expressing functional gH, the cells were used for the construction of a gH mutant by means of overlap recombination (34) (Fig. 1). In our collection of linker insertion mutants (7) prepared in cosmid c-448 (a derivative of c-27 [34] that contains flanking *Hin*dIII sites instead of *Eco*RI sites), we identified a cosmid, designated c-B11, in which the linker was inserted close to the *Pvu*II site in the putative gH gene (position 929; Fig. 2). Cosmid c-B11 was used together with the three overlapping wild-type PRV fragments c-179, c-443, and pN3HB for the construction of a gH mutant. As a control, all cell lines were transfected with the wild-type fragments c-179, c-27, c-443, and pN3HB. For transfection, 100 fmol of each DNA fragment was used and mixed with 100 μl of Lipofectin in 300 μl of water. After incubation for 15 min at room temperature, the mixture was added to 5 ml

of Optimem (GIBCO), and 50 μl was added to monolayers of SK-6 cells in 96-well culture disks. After incubation for 6 h at 37°C, 150 μl of complete medium was added. Three days after transfection, all cell lines transfected with the wild-type fragments yielded plaques, whereas only 1 of the 96 cell lines transfected with the set containing the mutagenized fragment yielded small plaques. The plaques produced by the putative gH mutant were picked and replated on SK-6 cells and on the cell line in which they were produced. Small plaques formed on the complementing cells, but no plaques were apparent on SK-6 cells. This result indicated that the virus had a mutant phenotype. The mutant virus was purified by using three rounds of plaque purification, and a virus stock was prepared. To verify that the oligonucleotide was present in the gH gene, viral DNA of the mutant was isolated, and a *Sac*I fragment containing part of the gH gene (positions 560 to 1433; Fig. 2) was cloned in M13mp19 and sequenced by using a synthetic primer (nucleotides 742 to 759; Fig. 2). This analysis (Fig. 3) showed that the mutagenic oligonucleotide was inserted between nucleotides 885 and 886, resulting in the formation of a premature termination codon in the gH gene. Because 231 amino acids of the truncated protein encoded by the mutant gH gene are identical to that of the wild-type protein, the mutant was designated H231. The complementing cell line was designated SKF12.

The stock of H231 virus was titrated on SKF12 cells and on noncomplementing SK-6 cells. On SKF12 cells, the virus titer was more than 1,000-fold higher than on SK-6 cells (2.8×10^7 and 4.5×10^2, respectively). This result confirmed the mutant phenotype of the virus. The observation that the plaques produced by the mutant virus on SKF12 cells were relatively small probably indicates that the amount of gH produced by these cells is relatively small. This could be due to the presence of a limited number of copies of the gH gene in SKF12 cells. The plaques produced on SK-6 cells were wild-type revertants that had arisen by homologous recombination of the viral gH gene with the cellular gH gene in the complementing cell line.

Although H231 did not produce plaques on SK6 cells, we noted that cells showed the rounding-up characteristic of infected cells when the monolayers were infected at a high multiplicity of infection. This finding suggested that infection of noncomplementing cells by the gH mutant still resulted in cell killing. When monolayers of H231-infected SK-6 or SKF12 cells were stained with an anti-PRV serum in an immunoperoxidase monolayer assay (28), we observed that only individual cells were stained in SK-6 monolayers, whereas small plaques were apparent in SKF12 monolayers (Fig. 4). This finding indicated that, in noncomplementing SK-6 cells, the virus was still able to express viral antigens but was unable to produce plaques. When monolayers of H231-infected SK-6 cells were examined after 24 h of infection by electron microscopy, we observed that individual cells were infected, again indicating that the virus was unable to spread from infected to uninfected cells. The mutant was still able to replicate and produce progeny, since virus particles were present in the cytoplasm and outside the cell (Fig. 5). The presence of extracellular progeny virions and the observation that neighboring cells were not infected indicated that the progeny virus produced by H231-infected SK-6 cells was noninfectious. When progeny virus was harvested from H231-infected SK-6 cells and plated on SKF12 and SK-6 cells, we obtained low but identical numbers of plaques on both cell lines. These plaques were produced by wild-type revertants, since they had a normal plaque size and had similar plating efficiencies after replating on SK-6 and SKF12 cells (data not shown). These observations confirmed that progeny virus of H231 produced by noncomplementing cells is noninfectious. We and others have previously shown that gp50 null mutants of PRV, which also produce noninfectious progeny, are still able to produce plaques by means of cell-cell fusion (28, 31). Therefore, the observation that H231 is unable to produce plaques indicates that gH is required not only for entry but also for cell-to-cell spread.

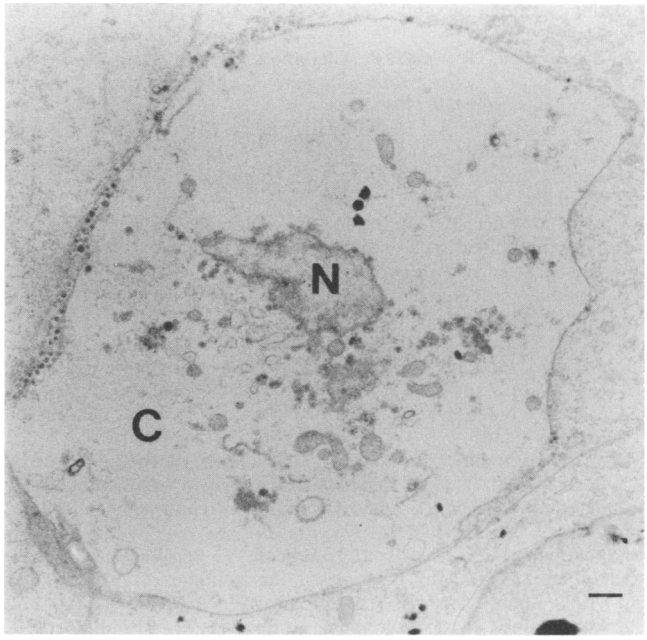

FIG. 5. Transmission electron micrograph of a thin section of SK-6 cells, 24 h after infection with the gH null mutant H231. The micrograph shows a single infected cell surrounded by uninfected cells. Note the presence of noninfectious progeny virus outside the cell. Abbreviations: N, nucleus; C, cytoplasm. Bar, 1 μm.

In this report, we have shown that gH of PRV is essential in the life cycle of the virus and is specifically involved in virus entry and cell-to-cell spread. By using different experimental approaches, others have reached similar conclusions about the function of gH of HSV. It has been shown that processing and transport of gH of HSV to the cell surface are dependent on some other viral protein(s) (9, 10, 13, 15). Whether this is also true for PRV remains to be established. However, in view of the protein's conserved function, this seems very probable. The use of a gH null virus may be an important step in identifying this accessory protein(s).

Nucleotide sequence accession number. The sequence data shown in Fig. 2 have been assigned EMBL accession number X61696.

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