

Pseudorabies Virus Avirulent Strains Fail to Express a Major Glycoprotein

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The unique short (U_s) region of the pseudorabies virus (PRV) genome, which displays high transcriptional activity during the late phase of infection and has been found to code for glycoproteins, is partially deleted in the genomes of three vaccine strains (A57, Norden, and NIA-4). This deletion is located in the *SalI* subfragment 7A of *BamHI* fragment 7. To identify possible viral gene products involved in PRV virulence, we investigated the transcriptional and translational pattern of the deleted part of the U_s region. Northern blots demonstrated that one major RNA species (3.8 kilobases) transcribed from fragment 7A was missing in the vaccine strains, whereas other transcripts were altered. Radioimmunoprecipitation of *in vivo*-labeled PRV glycoproteins and of *in vitro*-translated polypeptides with hyperimmune serum and monoclonal antibodies indicated a lack of glycoprotein gI. Hybrid-selection experiments with subcloned DNA fragments confirmed the absence of gI and of a 40,000-molecular-weight polypeptide. We suggest that both viral proteins are involved in the expression of PRV virulence.

Pseudorabies virus (PRV), the causative agent of Aujeszky's disease, can infect a wide range of animals. Broadly speaking, however, only swine and, more specifically, only older animals can survive a PRV infection concomitant with the establishment of latency. These carrier pigs act as a potent virus reservoir. The increasing prevalence of Aujeszky's disease, especially in Europe, requires the evaluation of attenuated PRV strains used to induce immunity from serious clinical symptoms after subsequent infection with wild-type PRV. Analysis of attenuated strains should provide more insight into the molecular basis of PRV virulence and thus should lead to the development of more defined vaccines. Lomniczi et al. (14) and Hermann et al. (9) have shown that two avirulent virus strains, Bartha and Norden, both display a similar deletion in the unique short

involved in virulence. The U_s region is very actively transcribed during the late phase of PRV infection (14, 16), and three glycoproteins, including glycoprotein I (gI), have been shown to be encoded on this region (16, 17, 26).

We cloned the *SalI* subfragments 7A and 7B (molecular weights, 3.1×10^6 and 1.3×10^6 , respectively) of *BamHI* fragment 7 of the U_s region (26) and used these nick-translated clones for Southern blot hybridizations (18, 21) with the DNA of either purified virions or virus-infected cells of the vaccine strains (4, 15; map location, Fig. 1). These experiments confirmed that the DNA deletion of all three vaccine strains tested, A57 (a Bartha derivative; obtained from J. Jakubik, Tübingen), NIA-4 (obtained from J. B. McFerran, Belfast, Northern Ireland), and Norden (Norden Laboratories, Lincoln, Nebr.; obtained from D. Burger,

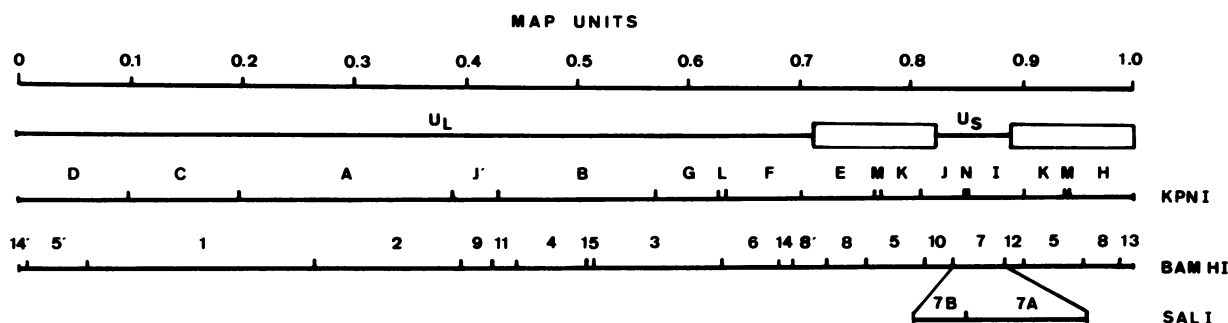


FIG. 1. Genomic map of PRV Phylaxia. Boxes represent inverted repeat regions dividing the genome into the U_L and U_s regions.

(U_s) region of the viral genome. Furthermore, Berns et al. (3) recently reported that the substitution of the repeat and U_s regions of a virulent PRV strain by the repeat and partially deleted U_s regions of a vaccine strain resulted in markedly reduced virulence of the recombinant. It is therefore likely that this part of the viral genome specifies the functions

Pullman, Wash.), is localized in *SalI* subfragment 7A.

To compare the transcriptional patterns of wild-type PRV strain Phylaxia and the vaccine strains, RNA classes encoded by *SalI* subfragments 7A and 7B were examined by Northern blot hybridization. For this purpose, virus-infected MDBK cells (Madin-Darby bovine kidney cells; multiplicity of infection, 20 PFU per cell) were harvested after showing a pronounced cytopathogenic effect and lysed; the RNA was

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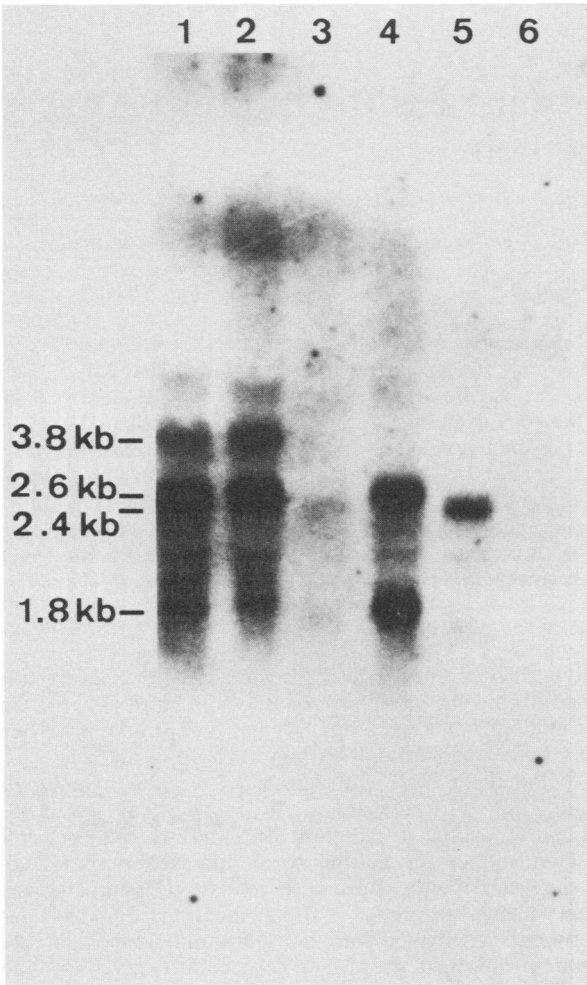


FIG. 2. Late RNA classes encoded in *SalI* subfragment 7A of wild-type and vaccine strains. Whole-cell RNA from late infected cells (15 μ g per lane) was separated on 1% agarose gels after denaturation with glyoxal. Results obtained with wild-type strain Phylaxia before (lane 1) and after (lane 2) passage in vivo and with the vaccine strains A57 (lane 3), Norden (lane 4), and NIA-4 (lane 5) as well as with RNA from mock-infected cells (lane 6) are shown after hybridization with nick-translated *SalI* subfragment 7A. The RNA sizes are indicated in kilobases. Identical amounts of whole-cell RNA were separated in each lane; however, the content of virus-specific messages differed between the Phylaxia RNA and A57 RNA preparations.

isolated by centrifugation through a CsCl cushion as described previously (7, 16). Whole-cell RNA (15 μ g per lane) was denatured with glyoxal, electrophoresed in agarose gels (5), and transferred to nitrocellulose filters (24). The filters were incubated in boiling 20 mM Tris hydrochloride (pH 8.0) for 10 min and hybridized as recently described (16).

After RNA derived from cells infected with wild-type or vaccine virus was probed with nick-translated fragment 7B, the transcriptional pattern of all PRV strains was essentially identical (data not shown). In contrast, hybridization with radioactive fragment 7A revealed some striking differences. All three vaccine strains lacked a major 3.8-kilobase (kb) wild-type transcript (Fig. 2). Instead of the 2.6-kb RNA of wild-type Phylaxia (Fig. 2, lanes 1 and 2) and strain Norden (Fig. 2, lane 4), a 2.4-kb RNA was found in strains NIA-4 (Fig. 2, lane 5) and A57 (Fig. 2, lane 3). A third major 7A

transcript, 1.8 kb in size, appeared unaltered in strains Norden and A57 but was present in a smaller form (1.7 kb) and in smaller quantities in strain NIA-4. However, from these Northern blot data we cannot differentiate whether the 2.4-kb RNA of strains NIA-4 and A57 and the 2.6-kb RNA of strain Norden represent a truncated form of the 3.8-kb RNA with a loss of the 2.6-kb wild-type transcript. Since we have previously suggested that the 3.8-kb mRNA represents the gI-specific transcript (16) and have now demonstrated the lack of this RNA in the vaccine strains, these strains were further analyzed for the expression of gI.

The occurrence of gI in virions was tested by radioimmunoprecipitation with pig hyperimmune serum (27; obtained from V. Ohlinger, Tübingen) of viral proteins labeled with [3 H]glucosamine (15). gI was present in the virulent strain Phylaxia (Fig. 3, lane 2) but was not detectable in the vaccine strains NIA-4 (Fig. 3, lane 4) or A57 (Fig. 3, lane 6). The same results were obtained with different monoclonal antibodies directed against gI (8, 15; former designation, gA) in both radioimmunoprecipitation and Western blotting of

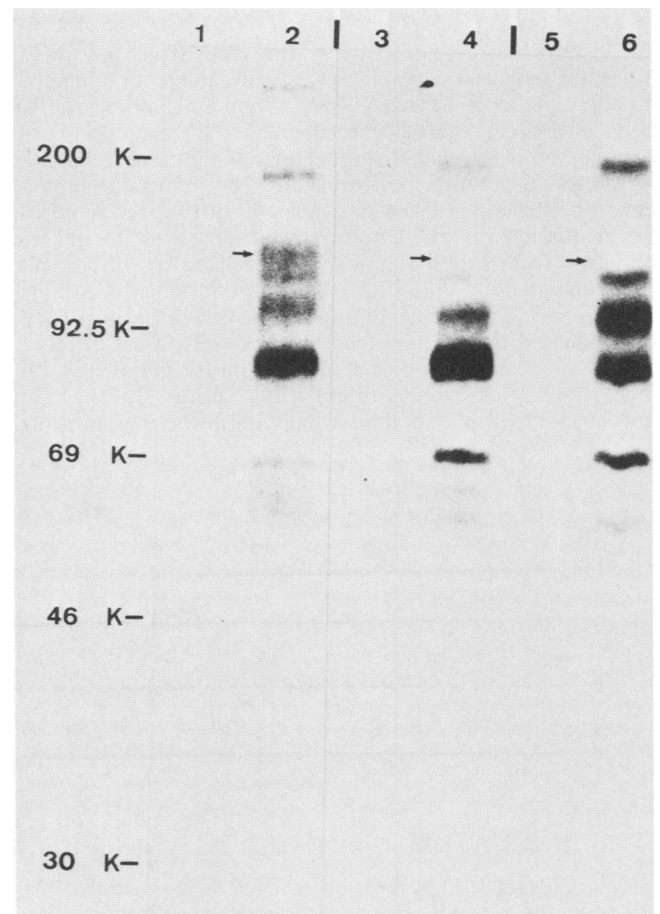


FIG. 3. Immunoprecipitation of glycoproteins of PRV strains Phylaxia (lanes 1 and 2), NIA-4 (lanes 3 and 4), and A57 (lanes 5 and 6). Infected MDBK cells were labeled with [3 H]glucosamine overnight and lysed, and the proteins were subsequently precipitated with either pig normal serum (lanes 1, 3, and 5) or pig hyperimmune serum (lanes 2, 4, and 6). The immunoprecipitates were separated on a 10% SDS-polyacrylamide gel. The position of gI is marked by an arrow; molecular weight markers are indicated on the left.

purified virions and in situ enzyme-linked immunosorbent assays on plaque-titrated viruses (10; data not shown).

To investigate the expression of the nonglycosylated precursor of gI, pgI, late RNA was isolated from infected cells and used to direct cell-free protein synthesis in a rabbit reticulocyte lysate (New England Nuclear Corp., Boston, Mass.). Radioimmunoprecipitation with the monoclonal antibody 3/6, which specifically precipitates nonglycosylated pgI (16), or with a control monoclonal antibody, 3dB11, directed against a late murine cytomegalovirus protein (unpublished data; gift from U. Koszinowski, Tübingen) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were performed as described previously (11, 12, 15, 16). The results of these experiments are shown in Fig. 4. None of the three vaccine strains, Norden (Fig. 4, lane 1), NIA-4 (Fig. 4, lane 2), or A57 (Fig. 4, lane 3), produced late RNA which could be translated into a protein precipitable by the monoclonal antibody, whereas pgI was readily detected after precipitation of the translation products from the virulent PRV strain Phylaxia (Fig. 4, lane 5). Even prolonged incubation of the in vitro-translated proteins in the presence of the antibody and overexposure of the autoradiograms did not result in the appearance of pgI (data not shown).

The possibility that certain epitopes of this glycoprotein are missing or altered in the vaccine strains, thus preventing its recognition by the monoclonal antibody, was tested by translation in vitro of RNA which was hybrid selected on the *SalI* subfragments. In addition, it was expected that these analyses would demonstrate whether the expression of other genes is affected by the DNA deletion. To this end, total late RNA isolated from infected cells was selected on nitrocellulose filters which contained either *SalI* subfragment 7A or 7B in 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.8)–400 mM NaCl–65% (vol/vol) formamide for 3 h at 56°C. After thorough washing in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS and subsequently in 2 mM EDTA (pH 7.4) at 60°C, the bound RNA was eluted in boiling distilled water, snap frozen in liquid nitrogen, and ethanol precipitated after the addition of 50 µg of yeast tRNA per ml (1, 16).

Selected RNA was used to direct in vitro translation (Fig. 5). The results obtained with Phylaxia RNA (Fig. 5A, lanes 1 and 2) or A57 RNA (Fig. 5A, lanes 3 and 4) are shown after hybrid selection with fragment 7A (Fig. 5A, lanes 1 and 3) or 7B (Fig. 5A, lanes 2 and 4). The fragment 7A-selected Phylaxia RNA codes for polypeptides with molecular weights of 80,000 (80K polypeptide) (representing the pgI), 40,000 and 27,000, whereas only the 27K polypeptide was translated in vitro after corresponding selection of A57 RNA. Neither the 80K pgI nor the 40K protein nor other translation products could be found. We therefore conclude that gI expression and expression of the 40K protein is abolished in the vaccine strain A57. Three fragment 7B-encoded proteins (65K, 60K, and 38K proteins) were found in both the virulent strain Phylaxia and the avirulent strain A57 (Fig. 5A, lanes 2 and 4). Analyses of the two other vaccine strains yielded similar results. Results of in vitro translation with RNA from strain Norden (Fig. 5B, lanes 1 and 2) or strain NIA-4 (Fig. 5B, lanes 3 and 4) are shown after hybrid selection with fragment 7A (Fig. 5B, lanes 1 and 3) or fragment 7B (Fig. 5B, lanes 2 and 4). All proteins encoded in fragment 7B were present, although the 38K protein was visible only after overexposure of the autoradiogram. Selection with fragment 7A revealed that NIA-4 closely resembles A57. The pgI and the 40K protein were missing, whereas the 27K polypeptide was expressed (Fig.

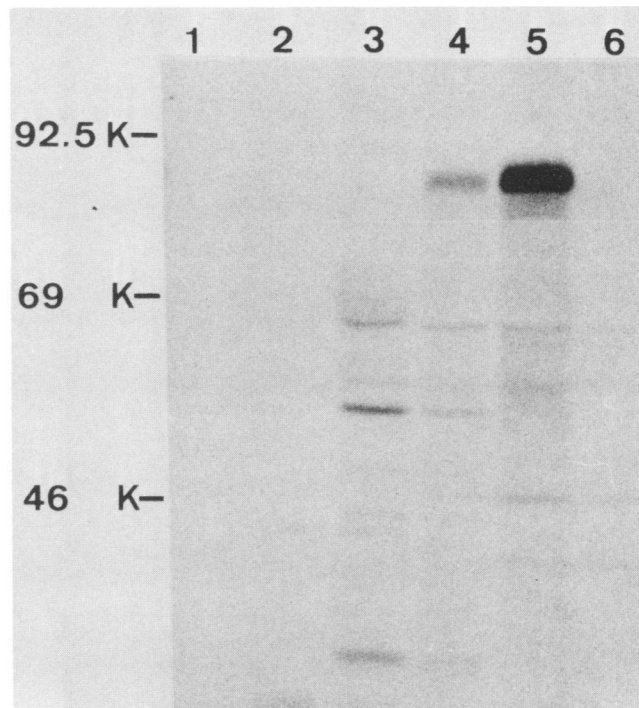


FIG. 4. Lack of pgI in PRV vaccine strains. Late RNA of the vaccine strains Norden (lane 1), NIA-4 (lane 2), and A57 (lane 3) and of two virulent PRV strains (lanes 4 and 5) was translated in vitro, and synthesized proteins were precipitated with the monoclonal antibody 3/6. A precipitation with a control monoclonal antibody is shown in lane 6. The precipitates were separated on 10% SDS-polyacrylamide gels. Molecular weight markers are indicated on the left.

5B, lane 3). No in vitro-translatable RNA from Norden-infected cells, was selected with fragment 7A (Fig. 5B, lane 1). These results prove that the precursor polypeptides of gI are not expressed in any of the vaccine strains tested. These data are in good agreement with our previous mapping studies which localized the gI gene in that part of the genome (16).

The hybrid-selection experiments allowed the identification of two further polypeptides (40K and 27K) of the virulent strain Phylaxia encoded by fragment 7A. Examination of the three vaccine strains revealed that the 40K polypeptide, whose function during PRV infection is unknown, was consistently absent, whereas the 27K protein could only be detected in strains A57 and NIA-4. The absence of the 27K protein in the Norden vaccine strain might be explained by the generation of a fusion fragment between truncated *BamHI* fragment 7 and *BamHI* fragment 12 (13). Loss of this *BamHI* cleavage site might result in the inactivation of the 27K protein gene.

Although only the 3.8-kb RNA was missing in strain Norden as seen in the Northern blot hybridization, no translation products could be detected on fragment 7A-selected RNA from Norden-infected cells. We suggest that the deletion in strain Norden does not exactly match the deletion in the other vaccine strains as indicated by the generation of the *BamHI* 7-12 fusion fragment. This in turn might lead to minor deletions of the other mRNAs, which seem to be unaltered in the Northern blot hybridization, which abolish their function in the translation reaction.

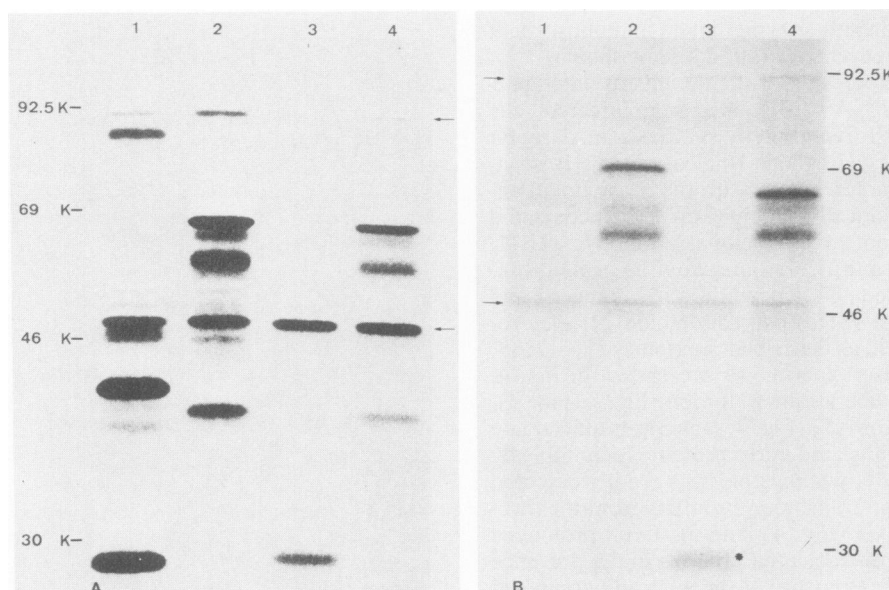


FIG. 5. Identification of proteins not expressed in the vaccine strains. Late RNA of virulent PRV strain Phylaxia (panel A, lanes 1 and 2) and vaccine strains A57 (panel A, lanes 3 and 4), Norden (panel B, lanes 1 and 2), and NIA-4 (panel B, lanes 3 and 4) was hybrid selected, and the resulting *in vitro* translation products were separated on 10% SDS-polyacrylamide gels. Selection was performed with the *Sall* fragments subclones 7A (lanes 1 and 3) and 7B (lanes 2 and 4). Molecular weight markers for each panel are indicated on the left. Protein bands which appear to be independent of added mRNA are marked by arrows.

Alternatively, the 2.6-kb RNA of strain Norden might be a truncated 3.8-kb transcript as already mentioned.

A further notable difference was observed with respect to the three polypeptides encoded by fragment 7B (65K, 60K, and 38K) which are present in all virus strains. Whereas in strains A57, NIA-4, Phylaxia, and a variety of other PRV strains (data not shown) the most prominent protein has a molecular weight of 65,000, in strain Norden it was slightly larger (69,000; Fig. 5B, lane 2). The 65K protein and the 69K protein were recognized after *in vitro* translation by an antiserum specific for another PRV glycoprotein (gX). This glycoprotein accumulates in the medium of infected cells (2), and the coding region thereof has recently been shown to be localized in fragment 7B (17). The larger pgX, therefore, seems to be characteristic of the vaccine strain Norden.

From the data presented we tentatively conclude that the expression of another PRV glycoprotein (gp50) also encoded by fragment 7B (26) is not impaired in the vaccine strains. Therefore, of the PRV glycoproteins mapped up to now (16, 17, 19, 26), only gI is directly affected by the DNA deletion.

By *in vivo* recombinant selection, Thompson et al. (25) and Rösen et al. (20) have localized herpes simplex virus type 1 genes associated with neurovirulence in the unique long (U_L) region adjacent to the internal inverted repeat. Using a similar approach, Lomniczi et al. (14) and Berns et al. (3) reported that genes residing in the repeat or the U_S region or both are necessary for PRV virulence. In both cases, however, no distinct gene product(s) were shown to be involved. So far, only thymidine kinase is suspected to play a role in the expression of virulence in both herpes simplex virus type 1 (6, 22) and PRV (23). We now report on a correlation between the lack of a herpesviral glycoprotein and avirulence.

In the virulence test system of Lomniczi et al. (14), i.e., PRV replication in chick brain, restoration of the U_S region of the vaccine strain Bartha, as demonstrated by restriction endonuclease cleavage, did not restore virulence completely but provided the virus with a limited ability to replicate in

chick brain. However, the correct transcription and translation of the U_S -encoded genes after restoration was not shown. Our monoclonal antibodies now allow the specific detection of gI expression, and further experiments should demonstrate if the correct transcription and translation of the genes in the U_S region and especially the gI gene is sufficient to restore PRV virulence.

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