JOURNAL OF VIROLOGY, Jan. 1987, p. 229–232
0022-538X/87/010229-04$02.00/0

Vol. 61, No. 1

Replication and Virulence of Pseudorabies Virus Mutants Lacking Glycoprotein gX

DARRELL R. THOMSEN, CARMINE C. MARCHIOLI, ROBERT J. YANCEY, JR., AND LEONARD E. POST*
The Upjohn Company, Kalamazoo, Michigan 49001

Received 26 June 1986/Accepted 6 October 1986

Pseudorabies virus (PRV) glycoprotein gX accumulates in the medium of infected cells. In an attempt to study the function of gX, two viruses were constructed that lacked a functional *gX* gene. One virus, PRVΔGX1, was derived by insertion of the herpes simplex virus thymidine kinase gene into the gX-coding region. The other virus, PRVΔGXTK$^-$, was derived by subsequent deletion of the inserted herpes simplex virus thymidine kinase gene. Both viruses replicated in cell cultures but produced no gX. Furthermore, PRVΔGX1 was capable of killing mice with a 50% lethal dose of less than 100 PFU.

Herpesviruses have large genomes that contain 50 to 100 genes. The large genetic potential may be necessary because of the complexity of the herpesvirus life cycle, involving infection of multiple cell types in a host and latency. When herpesviruses are grown in cell cultures, only those functions involved in viral genome replication and production of virus particles are likely to be essential. There are several examples of genes which are not essential for the growth of herpesviruses in cell cultures. The herpes simplex virus (HSV) glycoprotein gC is nonessential for growth in cultures (2, 8), and type 2 gC is also nonessential for the infection of mice (9). The pseudorabies virus (PRV) homolog of gC is nonessential for growth in cell cultures (20, 27). The thymidine kinase (TK) activities of both HSV (3) and PRV (21) are nonessential for growth in cultures but essential for virulence (4, 21, 22) and latency (23). The PRV *gI* (13) and HSV *gE* (12) genes, which are homologous (16), are also nonessential for growth in cell cultures.

We have studied a PRV glycoprotein we called gX (19), which was originally shown by Ben-Porat and Kaplan (1) to be excreted from PRV-infected cells. The gX sequence suggested that the primary translation product of gX is probably a membrane protein with the usually hydrophobic transmembrane and hydrophilic cytoplasmic domain structures. Recent evidence indicated a cellular form of gX with a higher molecular weight than that of the excreted form (L. M. Bennett, J. G. Timmins, D. R. Thomsen, and L. E. Post, Virology, in press). One of our goals in working with gX was to evaluate its possibilities as a subunit vaccine. The conclusions of numerous studies were that even in protocols by which animals raised high titers of anti-gX antibodies, no PRV-neutralizing activity was ever detected and that the animals were susceptible to lethal PRV infection (C. C. Marchioli, R. J. Yancey, and L. E. Post, unpublished data).

The ability of PRV to kill animals in the presence of high-titer anti-gX antibodies led us to ask whether gX is essential for the growth of PRV. We used a method originally devised to make deletions in nonessential genes in HSV (18). In this method, the parent virus is a TK$^-$ virus. PRV strain HR is an iododeoxyuridine-resistant mutant of PRV Aujeszky, obtained from H. E. Renis of The Upjohn Company. Virus was propagated in Vero cells, and viral DNA was purified by sodium iodide density gradient centrifugation as previously described (19).

* Corresponding author.

The first step of the gene inactivation procedure is construction of a plasmid in which the target gene is interrupted by the selectable *tk* gene. It was desirable to use a *tk* gene lacking homology with the *tk* gene of the target virus to avoid marker rescue of the *tk* mutation in the TK$^-$ target virus, which can occur with a higher frequency than the desired insertions (18). Therefore the *tk* gene chosen for insertion was the HSV type 1 (HSV-1) *tk* gene, which has little homology with PRV TK (10; M. Kit and S. Kit, U.S. patent 4,514,497, Dec., 1983). The *gX* gene was cloned on an *Xho*I/*Kpn*I fragment from PRV Rice (19), with *Eco*RI linkers added to the ends of the fragment in pPRXK4. Although the 5′ end of the *gX* mRNA has not been mapped, the DNA sequence of the *gX* gene is highly suggestive of where the gX promoter is located (19). An *Mst*I cleavage site was identified in the sequence 19 bases upstream from the translation initiation codon. A 600-base-pair *Eco*RI/*Mst*I fragment containing the gX promoter from pPRXK4 was isolated and cloned between the *Eco*RI and *Sma*I sites of pUC8 (26) to give pPGX1. Plasmid pPGX1 was digested with *Bam*HI, and the *Bam*HI/*Bgl*II fragment from plasmid pRB103 (17) containing the HSV-1 F *tk* gene was cloned into that *Bam*HI site to give plasmid pGXTK2. This plasmid contained the gX promoter fused to the HSV *tk* gene. To confirm that this produced a functional expression unit, pGXTK2 was shown to efficiently transform L TK$^-$ cells to a TK$^+$ phenotype. Furthermore, the TK activity in the transformed cells was induced threefold after superinfection with TK$^-$ PRV (data not shown). To complete the construction of the plasmid with the *gX* gene interrupted by the HSV *tk* gene, DNA coding for the C-terminal sequences of gX was added by the insertion of *Bam*HI-7 into the *Bam*HI site downstream from the *tk* gene in pGXTK2. The resulting plasmid, pGXTK3, is shown in Fig. 1A. This plasmid has DNA upstream from the *gX* gene, including the gX promoter and DNA for much of the *gX* gene, but lacks the initiation codon and coding sequences for the N-terminal 71 amino acids of the gX precursor.

The plasmid pGXTK3 has homology with PRV on both sides of a functional *tk* gene. The plasmid was cotransfected with PRV HR DNA into rabbit skin cells by the procedure described by Mocarski et al. (14). TK$^+$ recombinants were selected by plating onto 143 cells in hypoxanthine-aminopterin-thymidine medium as previously described for HSV (18). The recombinants were found to contain insertions of the HSV *tk* gene into the *gX* gene, as diagramed in

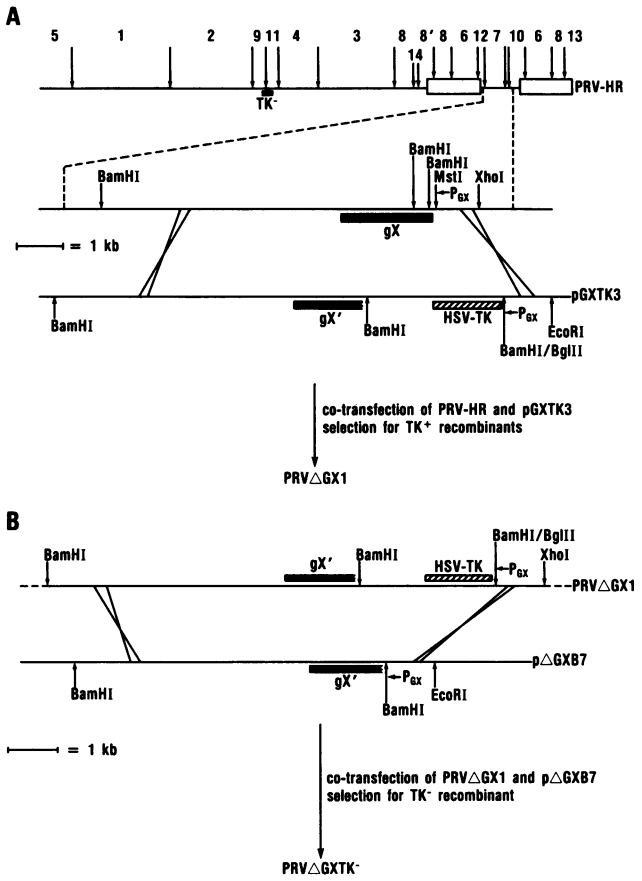

FIG. 1. Construction of gX⁻ PRV. (A) Construction of PRVΔGX1. A map of *Bam*HI cleavage sites on the PRV HR genome is shown with the position of the *tk* gene indicated. □, Inverted repeats. The region of the *gX* gene is expanded, including all of *Bam*HI-7. The structure of pGXTK3 is shown, along with an indication of the double-crossover event that led to PRVΔGX1. The structure of the gX region of PRVΔGX1 is exactly like that in pGXTK3. (B) Construction of PRVΔGXTK⁻. The upper line is the structure of PRVΔGX1 in the region of the inactivated *gX* gene. The structure of pΔGXB7 is shown, along with an indication of the double-crossover event that led to PRVΔGXTK⁻. The structure of the gX region of PRVΔGXTK⁻ is exactly like that in pΔGXB7. kb, Kilobase.

Fig. 1A. The resulting virus was named PRVΔGX1. A DNA gel demonstrated that the recombination event occurred (Fig. 2). The predicted effect of the recombination event on the *Bam*HI pattern was that *Bam*HI-10 should be 2.5 kilobases larger because of the fusion of *Bam*HI-10 with the HSV *tk* fragment. *Bam*HI-10 was no longer in the pattern from PRVΔGX1, and the larger fragment apparently comigrated with *Bam*HI-7, which was 2 molar in PRVΔGX1 (Fig. 2). An additional change in the restriction pattern of ΔGX1 from that of HR was that *Bam*HI-6 (identified by cleavage with *Hin*dIII; data not shown) apparently decreased electrophoretic mobility. This was probably unrelated to the recombination event in the *gX* gene region, since *Bam*HI-6 has been shown to be highly variable in size (6). Similarly, the decrease in size of *Bam*HI-12 was probably due to fragment size variation and not to the recombination event.

The final step of the insertion-deletion procedure was the deletion of the *tk* gene to produce the original parent virus with a simple deletion. This was accomplished by making a plasmid in which *Bam*HI-7 was inserted into the *Bam*HI site of pPGX1 to give plasmid pΔGXB7. This plasmid had the same PRV sequences deleted as pGXTK3 did but with no *tk* insertion. Plasmid pΔGXB7 was cotransfected with PRVΔGX1 DNA, and TK⁻ recombinants were selected with thymidine arabinoside (13). The recombinant was designated PRVΔGXTK⁻ (Fig. 1B). The DNA gel in Fig. 2 shows that, as predicted for the desired recombinant, PRVΔGXTK⁻ contained a *Bam*HI fragment (slightly smaller than *Bam*HI-10) that was not present in its parent virus PRVΔGX1.

Since the two recombinant viruses were lacking the DNA coding for the initiation codon and N-terminal amino acids of gX, it was expected that these viruses would not produce gX. By Western blot analysis, neither virus produced gX in the medium of infected cells (Fig. 3) or produced the usual intracellular forms (Bennett et al., in press) of gX. In some experiments with PRVΔGXTK⁻, a small amount of an intracellular protein was observed to react with the gX antiserum in Western blots. This protein did not correspond to one of the intracellular forms of gX seen in the parent virus (Bennett et al., in press) and therefore may represent an internal translation initiation in the coding sequences of the *gX* gene.

The original TK⁻ HR strain of PRV was known to be avirulent in mice (H. E. Renis, personal communication; confirmed in Table 1), as was originally reported by Tatarov (22) for TK⁻ mutants of PRV. HR-B, a derivative of HR in which the *tk*⁻ mutation was rescued by cotransfection with PRV *Bam*HI-11, is fully virulent (Table 1). This indicates that the *tk*⁻ mutation is the only lesion preventing full

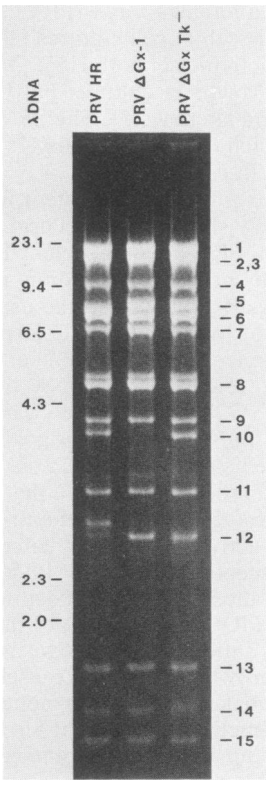

FIG. 2. DNA from gX⁻ PRV. All of the DNAs shown were digested with *Bam*HI. An ethidium bromide-stained agarose gel is shown. The numbers on the left indicate the positions and sizes of the *Hin*dIII fragments of bacteriophage λ on this gel. The numbers on the right indicate the labels assigned to the *Bam*HI fragments and correspond to those on the map in Fig. 1A.

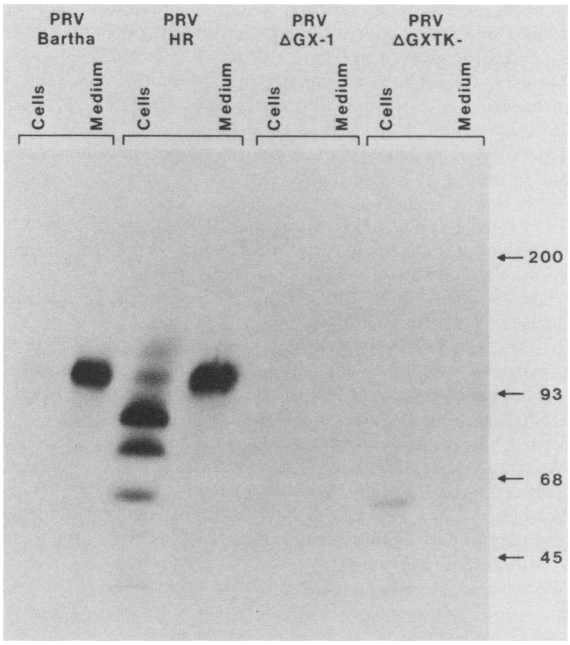

FIG. 3. Analysis of gX synthesis by PRV strains. Samples were run on a 9.25% sodium dodecyl sulfate-polyacrylamide electrophoresis gel (15). A Western blot (25) of samples from four PRV strains was developed with anti-gX antiserum. The antiserum used was raised with the p60-11 gX fusion protein expressed in *Escherichia coli* (19). The lanes labeled cells indicate the total proteins from infected cells, and the lanes labeled medium indicate the medium from the same infected cells.

virulence of HR in mice. Insertion of HSV *tk* into the *gX* gene of HR resulted in a virus as virulent as HR-B, even though PRVΔGX1 produced no gX. When the HSV *tk* gene was removed from PRVΔGX1, the resulting virus, PRVΔGXTK$^-$, showed greatly reduced virulence, as expected for a TK$^-$ virus. The apparently greater virulence of PRVΔGXTK$^-$ over that of HR in the particular experiment summarized in Table 1 was probably due to occasional TK$^+$ revertants.

PRV produces relatively large amounts of gX and excretes it into the medium. Although quantitation has not been attempted, gX is usually observed to be the most abundant of the PRV glycoproteins. From the literature, there appears to be no example of a gX$^-$ PRV arising spontaneously. When field isolates were examined, all were found to make gX (K. F. Bund, D.V.M. thesis, Freie Universität Berlin, Berlin, Federal Republic of Germany). Although *Bam*HI-7 is a region of considerable variability at the DNA level, the variable region has always been traced to the opposite end of the fragment from the *gX* gene (6, 7, 11). Vaccine strains have been shown to produce gX (13). Our results (Fig. 3) contradict the conclusions of Todd and McFerran (24) that the Bartha strain fails to produce gX. Since there appear to be no examples of gX$^-$ PRV arising by any natural process, it is probable that gX serves some function for PRV. What this function may be, however, remains to be determined.

The finding that the gene for a major glycoprotein can be deleted from PRV has interesting implications for PRV vaccine development. A problem with existing PRV vaccines is that vaccinated pigs are not serologically distinguishable from infected pigs. Assays for anti-gX antibodies should be negative for pigs vaccinated with a gX$^-$ PRV strain, but infected pigs should be seropositive when assayed for anti-gX antibodies.

TABLE 1. Virulence of various PRV viruses in mice[a]

PRV strain	Phenotype[b]	LD_{50}[c] (PFU/mouse) in expt:	
		A	B
HR	TK$^-$ gX$^+$	$>1.5 \times 10^7$	$>2.3 \times 10^6$
HR-B	TK$^+$ gX$^+$	1.0×10^0	ND[d]
ΔGX1	TK$^+$ gX$^-$	3.0×10^0	3.4×10^1
ΔGXTK$^-$	TK$^-$ ΔgX	ND	9.4×10^4

[a] CF-1 Mice were inoculated with 50 μl of the respective virus by the footpad route. Groups of 10 mice were injected with at least 5 10-fold dilutions of virus for each determination.
[b] TK, gene encoding TK; gX, gene encoding glycoprotein X.
[c] The 50% lethal dose as calculated by the Spearman Karber method (7) at 14 days postinfection.
[d] ND, Not determined.

We thank Harold Renis for PRV HR and for valuable discussions throughout this work, Jim Timmins for antisera and advice on assays for gX synthesis, and Kathy Hiestand for typing the manuscript.

LITERATURE CITED

1. **Ben-Porat, T., and A. S. Kaplan.** 1970. Synthesis of proteins in cells infected with herpesvirus. V. Viral glycoproteins. Virology **41**:265–273.
2. **Draper, K. G., R. H. Costa, G. T.-Y. Lee, P. G. Spear, and E. K. Wagner.** 1984. Molecular basis of the glycoprotein-C-negative phenotype of herpes simplex virus type 1 macroplaque strain. J. Virol. **51**:578–585.
3. **Dubbs, D. R., and S. Kit.** 1964. Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity. Virology **22**:493–502.
4. **Field, H. J., and P. Wildy.** 1978. The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. J. Hyg. **81**:267–277.
5. **Finney, D. J.** 1978. Statistical method in biological assay, 3rd ed., p. 394–401. Charles Griffin and Co., Ltd., London.
6. **Gielkens, A. L. J., J. T. VanOirschot, and A. J. M. Berns.** 1985. Genome differences among field isolates and vaccine strains of pseudorabies virus. J. Gen. Virol. **66**:69–82.
7. **Hermann, S.-C., B. Heppner, and H. Ludwig.** 1982. Pseudorabies viruses from clinical outbreaks and latent infections grouped into four major genome types, p. 387–401. In G. Wittmann, R. M. Gaskell, and H.-J. Rziha (ed.), Latent herpes virus infections in veterinary medicine. Martinus Nijhoff Publishers, Boston.
8. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208–219.
9. **Johnson, D. C., M. R. McDermott, C. Chrisp, and J. C. Glorioso.** 1986. Pathogenicity in mice of herpes simplex virus type 2 mutants unable to express glycoprotein C. J. Virol. **58**:36–42.
10. **Kit, S., M. Kit, and H. Otsuka.** 1984. Structure of viral thymidine kinase, p. 311–327. *In* F. Rapp (ed.), Herpesviruses. Alan R. Liss, Inc., New York.
11. **Lomniczi, B., M. L. Blankenship, and T. Ben-Porat.** 1984. Deletions in the genomes of pseudorabies virus vaccine strains and existence of four isomers of the genomes. J. Virol. **49**:970–979.
12. **Longnecker, R., and B. Roizman.** 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. J. Virol. **58**:583–591.
13. **Mettenleiter, T. C., N. Lukàcs, and H.-J. Rziha.** 1985. Pseudorabies virus avirulent strains fail to express a major

glycoprotein. J. Virol. **56**:307–311.
14. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22**:243–255.
15. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
16. **Petrovskis, E. A., J. G. Timmins, and L. E. Post.** 1986. Use of λgt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. J. Virol. **60**:185–193.
17. **Post, L. E., A. J. Conley, E. S. Mocarski, and B. Roizman.** 1980. Cloning of reiterated and nonreiterated herpes simplex virus 1 sequences as *Bam*HI fragments. Proc. Natl. Acad. Sci. USA **77**:4201–4205.
18. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25**:227–232.
19. **Rea, T. J., J. G. Timmins, G. W. Long, and L. E. Post.** 1985. Mapping and sequence of the gene for the pseudorabies virus glycoprotein which accumulates in the medium of infected cells. J. Virol. **54**:21–29.
20. **Robbins, A. K., M. E. Whealy, R. J. Watson, and L. W. Enquist.** 1986. Pseudorabies virus gene encoding glycoprotein gIII is not essential for growth in tissue culture. J. Virol. **59**:635–645.
21. **Tatarov, G.** 1968. Apathogenic mutant of the Aujeszky virus induced by 5-iodo-2-deoxyuridine (IUDR). Zentralbl. Veterinaermed. **15**:847–853.
22. **Tatarov, G., and P. Gergov.** 1969. Investigation of harmlessness and immunogenicity of the MK strain of Aujeszky's virus. Vet. Med. Nauki **6**:49–54.
23. **Tenser, R. B., and M. E. Dunstan.** 1979. Herpes simplex virus thymidine kinase expression in infection of the trigeminal ganglion. Virology **99**:417–422.
24. **Todd, D., and J. B. McFerran.** 1985. Control of Aujeszky's disease. Vet. Rec. **117**:647.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
26. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13amp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
27. **Wathen, M. W., and L. M. K. Wathen.** 1986. Characterization and mapping of a nonessential pseudorabies virus glycoprotein. J. Virol. **58**:173–178.