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Role of Glycoprotein gIII of Pseudorabies Virus in Virulence

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Deletion mutants of pseudorabies virus unable to express glycoprotein gIII, gI, or gp63 or double and triple mutants defective in these glycoproteins were constructed, and their virulence for day-old chickens inoculated intracerebrally was determined. Mutants of wild-type pseudorabies virus defective in glycoprotein gIII, gI, or gp63 were only slightly less virulent (at most, fivefold) for chickens than was the wild-type virus. However, mutants defective in both gIII and gI or gIII and gp63 were avirulent for chickens, despite their ability to grow in cell culture in vitro to about the same extent as mutants defective in gIII alone (which were virulent). These results show that gIII plays a role in virulence and does so in conjunction with gI or gp63. The effect of gIII on virulence was also shown when the resident gIII gene of variants of the Bartha vaccine strain (which codes for $gIII^{B}$) was replaced with a gIII gene derived from a virulent wild-type strain (which codes for $gIII^{Ka}$); $gIII^{Ka}$ significantly enhanced the virulence of a variant of the Bartha strain to which partial virulence had been previously restored by marker rescue. Our results show that viral functions that play a role in the virulence of the virus (as measured by intracerebral inoculation of chickens) may act synergistically to affect the expression of virulence and that the ability of the virus to grow in cell culture is not necessarily correlated with virulence.

Pseudorabies virus (PrV), a herpesvirus of pigs, causes latent, as well as acute, often fatal, infection of the nervous system in pigs and acute infection in other domestic and wild animals. Not much is known about the viral functions that affect the virulence of either human herpesviruses, such as herpes simplex virus and varicella-zoster virus, or PrV. Because of their complexity, information concerning the genetic basis for the virulence of these viruses is just beginning to emerge (1, 4, 5, 9, 11, 13–16, 19, 20, 23, 27, 31–34, 36).

To determine the functions necessary for the expression of virulence of PrV, we have undertaken a series of studies, using two complementary approaches. (i) We have restored partial virulence to avirulent strains by marker rescue, thereby attempting to identify the functions that are defective in these strains, and (ii) we have introduced mutations into wild-type PrV(Ka) genes encoding functions nonessential for growth in vitro and ascertained how these mutations affect virus growth in cell culture and how they affect virulence.

Most of our studies to date have involved the analyses of the defects in vaccine strains. The main conclusions that could be drawn from these analyses are that virulence is controlled multigenically and that defects in several different loci of the PrV genome may affect virulence without necessarily affecting virus growth in cell culture. Furthermore, most vaccine strains have multiple defects contributing to their lack of virulence (14–16).

By deleting parts of specific genes, we have also shown that whereas inactivation of the gene encoding glycoprotein gI in wild-type virus strains has only a slight effect on virus virulence, inactivation of this gene in a partially attenuated virus variant affects virus virulence much more drastically (19), indicating that inactivation of several viral functions that play a role in virulence may be necessary before an effect on virulence can be observed.

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The purpose of the studies described in this paper was to show that three nonessential glycoproteins, gI, gp63, and gIII, play a role in the virulence of PrV. However, whereas defects in the gene coding for gI, gp63, or gIII alone affected virulence only slightly, defects in gIII and gI or in gIII and gp63 affected virulence drastically. Thus, in addition to being determined multigenically, the virulence of PrV (as measured by intracerebral [i.c.] inoculation of day-old chickens) is affected synergistically by some viral functions. The synergistic effect of defects in these functions on the virulence of the virus was not reflected by a similar effect on the growth of the virus in several different cell types cultured in vitro.

MATERIALS AND METHODS

Virus strains and cell cultures. Rabbit kidney (RK), pig kidney (PK), and Madin-Darby bovine kidney (MDBK) cells and chicken embryo fibroblasts (CEF) were cultivated in Eagle synthetic medium containing 3% dialyzed bovine serum; virus titers were determined by plaque assay in RK, PK, or MDBK cells. The titers obtained with the different cell types were in all cases similar.

PrV(Ka) is a strain which has been carried in our laboratory for more than 25 years; its origin is uncertain (10). The Bartha avirulent vaccine strain was received from P. S. Paul. The origin of this strain has been described earlier (22). The Bartha strain has a deletion in the S component (13) which includes the genes encoding gp63 and gI (17, 21). Bartha 43/25a is a Bartha variant to which an intact S component has been restored by marker rescue with the appropriate restriction fragment of PrV(Ka) and which expresses gI and gp63; it is not virulent for day-old chickens (15). Bartha 43/25aB4 is derived from Bartha 43/25a and contains at least a part of the *Bam*HI 4 fragment (which encodes four capsid proteins) of PrV(Ka) (Fig. 1) in place of its own homologous sequences. This variant has acquired a low level of virulence for chickens.

Determination of LD_{50}. Tenfold dilutions of virus stocks were injected i.c. (0.03 ml) into day-old chickens (Hubbard strain; six animals were used per dilution). The number of

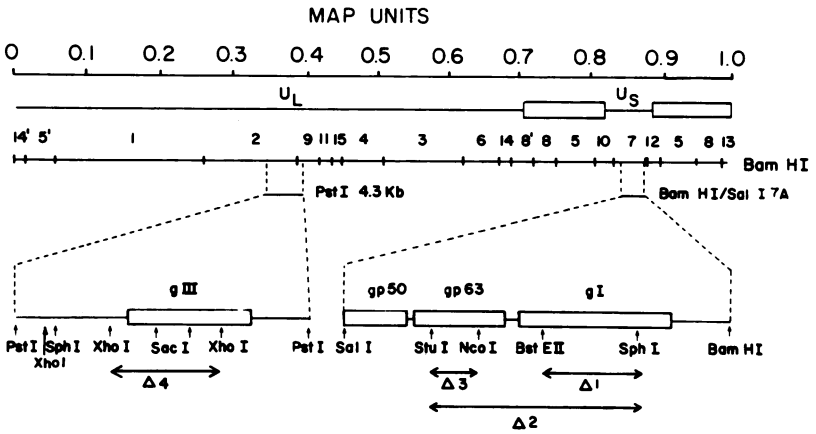

FIG. 1. Restriction fragment map of the PrV(Ka) genome indicating the regions coding for glycoproteins gIII, gp50, gp63, and gI. The map positions of the regions coding for the glycoproteins have been determined by Petrovskis et al. (21) and Robbins et al. (25). The deletions that were introduced into the virions to render them gI$^-$ (Δ1), gp63$^-$ (Δ3), gI$^-$ gp63$^-$ (Δ2), and gIII$^-$ (Δ4) are indicated.

animals that had died by 2 weeks after inoculation was determined and, the 50% lethal dose (LD_{50}) was calculated by using the Reed-Muench method (24).

Immunoprecipitation. Immunoprecipitation was performed as described previously (7, 12). The monoclonal antibodies against glycoproteins gI, gII, and gIII have been described previously (3, 7). The monoclonal antibody against gp63 (3M8) was the generous gift of P. Desmettre, Rhone-Merieux, France.

RESULTS

Isolation of gIII$^-$, gIII$^-$ gI$^-$, gIII$^-$ gp63$^-$, and gIII$^-$ gI$^-$ gp63$^-$ mutants of PrV(Ka). Glycoprotein gIII is nonessential for the growth of PrV in cell culture (3, 26, 35). Mutants defective in the expression of gIII were constructed essentially as described by Robbins et al. (25). The 4.3-kilobase-pair (kb) *Pst*I fragment, which includes the whole gIII gene (Fig. 1), was cloned into the *Pst*I site of pBR322. The *Pst*I-*Sph*I fragment was then subcloned into pUC18 (to remove the *Xho*I site to the left of the *Sph*I site). The plasmid was digested with *Xho*I, thereby removing an approximately 1.45-kb fragment that includes most of the regulatory and coding sequences of the gIII gene (25). The linear plasmid was electrophoresed on an agarose gel, eluted, and ligated. Ampicillin-resistant colonies were isolated after transfection of *Escherichia coli* HB101 with this DNA. The plasmids were characterized by restriction analysis and by Southern hybridization (30) to ensure that they had the appropriate structure. One of these plasmids (4.3-kb gIII$^-$A) was used to isolate mutants of PrV defective in the gIII gene.

Deletions in the gIII gene were introduced into wild-type PrV(Ka), PrV(Ka)Δ1 (which has a deletion in the gene encoding glycoprotein gI), PrV(Ka)Δ3 (which has a deletion in the gene encoding glycoprotein gp63), or PrV(Ka)Δ2 (which has a deletion in both genes [Fig. 1]). The methods used to obtain PrV(Ka)Δ1 and PrV(Ka)Δ2 and their structures have been described previously (18). PrV(Ka)Δ3 was obtained by using similar methods (unpublished results).

RK cells were cotransfected with plasmid 4.3-kb gIII$^-$A and with viral DNA obtained from the various virus mutants. Progeny virions were plaque assayed, plaques were picked, and those with a deletion in the gIII gene were identified by probing with the nick-translated *Sac*I fragment of the 4.3-kb *Pst*I fragment (Fig. 1). Figure 2 shows the results of Southern analysis of representative gIII$^-$, gIII$^-$ gI$^-$, gIII$^-$ gp63$^-$, and gIII$^-$ gI$^-$ gp63$^-$ mutants. It is clear that all mutants were gIII$^-$. All the mutants lacked the 1.45-kb *Xho* fragment which had been excised from the gIII gene (Fig. 2A). The gIII$^-$ mutants had a deletion in the 4.3-kb *Pst*I fragment of the expected size (Fig. 2B). All the mutants also had the

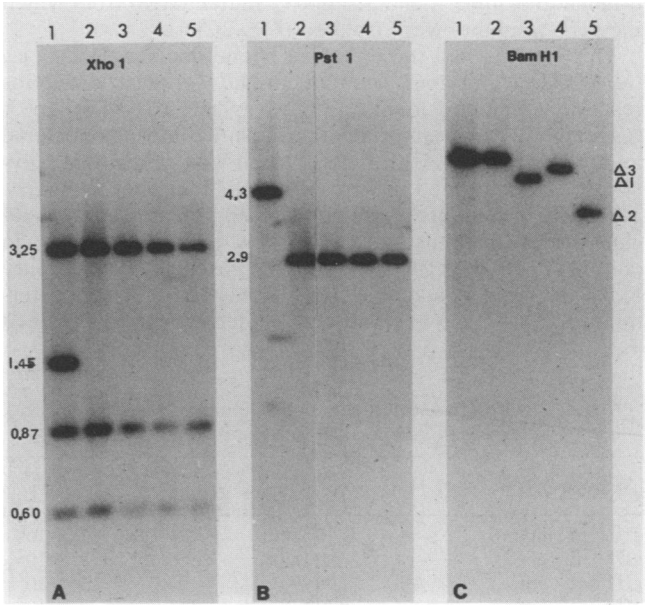

FIG. 2. Southern blot analysis of the genomes of virus deletion mutants. Wild-type PrV(Ka) (lanes 1), PrV(Ka) gIII$^-$ (lanes 2), PrV(Ka) gIII$^-$ gI$^-$ (lanes 3), PrV(Ka) gIII$^-$ gp63$^-$ (lanes 4), and PrV(Ka) gIII$^-$ gI$^-$ gp63$^-$ (lanes 5) were cleaved with *Xho*I and probed with the 4.3-kb *Pst*I fragment (A), cleaved with *Pst*I and probed with the 4.3-kb *Pst*I fragment (B), or cleaved with *Bam*HI and probed with the *Bam*HI 7 fragment (C). The approximate sizes of the fragments in kilobases are indicated on the left side of panels A and B. In panel C, the positions of the *Bam*HI 7 fragments carrying the different types of deletions (Δ1, Δ2, and Δ3; described in the legend to Fig. 1) are indicated. Details concerning the structure of the mutants carrying deletions in the genes encoding gI, gp63, or both have been reported (18).

TABLE 1. Immunoprecipitation of glycoproteins of various mutants and variants of PrV(Ka) and Bartha strains[a]

Virus strain	Amt of radioactivity (cpm [10^2])[b] immunoprecipitated					
	gII with M2	gI with		gp63 with 3M8	gIII with	
		M133	M156		M1	M16
PrV(Ka) mutants						
Wild type	225	123	146	79	298	306
gIII$^-$	275	113	173	59	0	0
gIII$^-$ gI$^-$	295	0	0	49	0	0
gIII$^-$ gp63$^-$	195	89	97	0	0	0
gIII$^-$ gI$^-$ gp63$^-$	215	0	0	0	0	0
Bartha variants[c]						
Parental Bartha	257	0	0	0	110	0
gIII^{Ka}	217	0	0	0	259	257
43/25a	283	93	113	37	167	0
43/25a gIII^{Ka}	219	81	83	42	315	301
43/25aB4$^+$	256	74	82	52	195	0
43/25aB4$^+$ gIII^{Ka}	199	69	102	37	346	316

[a] [^{35}S]methionine-labeled cell extracts obtained from RK cultures infected with the virus mutants or variants were immunoprecipitated with the indicated monoclonal antibodies. The amount of radioactivity immunoprecipitated was determined. The identity of the immunoprecipitated glycoprotein was determined by polyacrylamide gel electrophoresis (data not shown).

[b] After subtraction of background counts per minute ($<10^3$).

[c] The variants that have been marker rescued with the *Bam*HI 4 fragment are designated by B4$^+$.

deletions in the *Bam*HI 7 fragments that were present in the original gI$^-$, gp63$^-$, and gI$^-$ gp63$^-$ mutants (18) from which the gIII$^-$ mutants were isolated (Fig. 2C).

The mutants had the expected phenotype (Table 1); i.e., the gIII$^-$, gI$^-$, or gp63$^-$ mutants did not synthesize proteins reacting with monoclonal antibodies against gIII, gI, or gp63, respectively. Immunoprecipitation with other monoclonal antibodies reactive with different epitopes of the same glycoproteins confirmed these conclusions (data not shown).

Growth of mutants in cell culture and virulence for day-old chickens. The ability of the various mutants to grow in cultures of RK and PK cells and CEF, as well as their ability to kill day-old chickens when inoculated i.c., was determined. Table 2 shows the titers of infectious virus obtained from RK cells. Similar results were obtained with PK cells and CEF (data not shown). As reported previously (18, 19), mutants of PrV defective in either gI (gIII$^+$ gI$^-$ gp63$^+$) or gp63 (gIII$^+$ gI$^+$ gp63$^-$) or in both gI and gp63 (gIII$^+$ gI$^-$ gp63$^-$) grew as well as the wild type in cell culture and, at most, had only a fivefold increase in LD_{50}. Cultures infected with a mutant defective in gIII (gIII$^-$ gI$^+$ gp63$^+$) produced a virus population that generated only approximately 25% as many PFU as did cultures infected with the wild-type virus. The reduction in titers of infectious virus obtained from gIII$^-$-infected cultures is due, in large part, to a less effective and slower rate of adsorption of the gIII$^-$ mutants to their host cells, thereby reducing the number of PFU that are scored (28). The reduction in titers was not due to a smaller number of viral particles produced by cells infected with gIII$^-$ mutants compared with those produced by cells infected with PrV(Ka) (28). Despite the lower yield of PFU by cells infected with the gIII$^-$ mutant, the LD_{50} of this mutant for chickens was at most five times higher than that of the PrV(Ka) virus (Table 2).

TABLE 2. Growth and virulence of deletion mutants of PrV(Ka) defective in synthesis of different glycoproteins

Virus phenotype	Virus yield (PFU/cell)[a]	Virulence (LD_{50} [PFU])[b]
gIII$^+$ gI$^+$ gp63$^+$	220 (100)	$<1 \times 10^2$
gIII$^+$ gI$^-$ gp63$^+$	190 (87)	2×10^2
gIII$^+$ gI$^+$ gp63$^-$	200 (90)	3×10^2
gIII$^+$ gI$^-$ gp63$^-$	170 (78)	2×10^2
gIII$^-$ gI$^+$ gp63$^+$	54 (25)	2×10^2
gIII$^-$ gI$^-$ gp63$^+$	23 (10)	$>10^5$
gIII$^-$ gI$^+$ gp63$^-$	28 (12)	$>10^5$
gIII$^-$ gI$^-$ gp63$^-$	33 (15)	$>10^5$

[a] RK cells were infected with 5 PFU per cell of the virus mutant and incubated for 48 h at 37°C in Eagle medium containing 5% dialyzed calf serum. The cells were scraped into the culture fluid, the samples were sonicated, cellular debris was removed by centrifugation, and the virus yield was assayed in MDBK cells. Numbers in parentheses indicate the percentage of wild-type virus (gIII$^+$ gI$^+$ gp63$^+$) produced by cells infected with each mutant.

[b] Day-old chickens (six per dilution) were inoculated i.c., and the number of PFU required for LD_{50} was calculated as described in Materials and Methods.

Cells infected with the double mutants gIII$^-$ gI$^-$ (gIII$^-$ gI$^-$ gp63$^+$) and gIII$^-$ gp63$^-$ (gIII$^-$ gI$^+$ gp63$^-$) or the triple mutant gIII$^-$ gI$^-$ gp63$^-$ yielded only slightly lower titers of infectious virus (about two- to threefold lower) than did cells infected with mutants defective in gIII alone. However, in contrast to the gIII$^-$ virions, which were almost as virulent as the wild-type virus, the double or triple mutants were completely avirulent for chickens after i.c. inoculation.

Thus, whereas inactivation of gI, gp63, or gIII alone affected virulence only slightly, inactivation of either gI and gIII or gp63 and gIII completely inhibited the expression of virus virulence. The lack of virulence of the double mutants gIII$^-$ gI$^-$ and gIII$^-$ gp63$^-$ cannot be ascribed to their general inability to replicate; there were only modest differences between the virus yields from cultures of RK cells (as well as PK cells and CEF; data not shown) infected with the gIII$^-$ mutant, which is virulent, and those from cultures infected with the gIII$^-$ gI$^-$ or gIII$^-$ gp63$^-$ mutant, each of which is avirulent (Table 2). It is, of course, possible that the loss of virulence of these double mutants is related to the loss of their ability to replicate in certain other cell types.

Isolation of variants of Bartha strain in which resident gIII gene was replaced by PrV(Ka) gIII gene. The Bartha vaccine strain and its derivatives encode a gIII glycoprotein (gIII^B) that differs from the wild-type PrV(Ka) gIII (gIII^{Ka}) in the following two respects: at most, (i) only 50% as much glycoprotein gIII accumulates in cells infected with the Bartha strain as in cells infected with wild-type PrV(Ka), and (ii) only 10% as much gIII is present in purified Bartha virion preparations as in PrV(Ka) virion preparations (2, 3; F. Zuckermann and T. Ben-Porat, manuscript in preparation).

Because the results described above (Table 2) indicated that glycoprotein gIII, in conjunction with glycoprotein gI or gp63, plays a role in virulence, it seemed of interest to ascertain whether the defects in the gIII gene of the Bartha strain contribute to its avirulence. We therefore replaced the resident Bartha strain gIII gene with the gIII gene of PrV(Ka) in various Bartha strain derivatives and determined how this change would affect their virulence.

We have previously restored partial virulence to the Bartha vaccine strain by two cycles of marker rescue (16). The original Bartha vaccine strain is gI$^-$ gp63$^-$ as a result of a deletion in the U_S component which spans the sequences encoding gI and gp63 (12, 17, 21). An intact U_S component was introduced into Bartha 43/25a by marker rescue with the appropriate PrV(Ka) restriction fragment (15), thereby re-

storing the ability of the virus to synthesize gp63 and gI (Table 1). Bartha 43/25a is not virulent (15), but after further marker rescue with another sequence of PrV(Ka) DNA (the *Bam*HI 4 fragment), a sequence that encodes four capsid proteins, the variant Bartha 43/25aB4 that was obtained was partially virulent (16).

The gIII gene of PrV(Ka) (coding for $gIII^{Ka}$) was inserted by marker rescue into the Bartha parental strain and its variants as follows. Cells were cotransfected with the 4.3-kb *Pst*I fragment of PrV(Ka) (which includes the regulatory and coding sequences of gIII [24]) and with either parental Bartha DNA, Bartha 43/25a DNA (a Bartha variant with an intact U_S whose ability to express gI and gp63 had been restored), or Bartha 43/25aB4 DNA (the doubly rescued, partially virulent Bartha variant). The virus progeny was plaque assayed, and the plaques were stained by the immunoperoxidase, black plaque assay (8) with a monoclonal antibody against gIII (M16) that reacts with the PrV(Ka) glycoprotein gIII ($gIII^{Ka}$) but not with the Bartha glycoprotein gIII ($gIII^{B}$) (3). Plaques that reacted with monoclonal antibody M16 were picked, purified again by plaque assay, and restained with M16 to confirm their $gIII^{Ka}$ phenotype. Their identity as Bartha variants was established by their characteristic restriction enzyme profiles (data not shown).

The reactivities of the proteins synthesized by cells infected with the various Bartha derivatives with monoclonal antibodies against glycoprotein gI, gII, gp63, or gIII are summarized in Table 1. Cells infected with the parental Bartha strain, as expected, did not synthesize gI or gp63. These infected cells synthesized at most only 50% as much glycoprotein gIII as did cells infected with the wild-type virus (as indicated by reactivity with monoclonal antibodies M1 [Table 1] and M7 [data not shown]) and did not synthesize glycoproteins that reacted with M16. The variants Bartha 43/25a and Bartha 43/25aB4 expressed gI and gp63 and a gIII glycoprotein similar to that expressed by the parental Bartha strain. After having acquired the gene coding for $gIII^{Ka}$, all three Bartha derivatives behaved as expected with respect to the expression of glycoprotein gIII; there was an increase in the amount of gIII immunoprecipitated by M1 (and M7; data not shown), and gIII became reactive with M16. (The identity of the glycoproteins immunoprecipitated by the monoclonal antibodies was confirmed by polyacrylamide gel electrophoresis.)

Growth in cell culture and virulence for chickens of Bartha variants which acquired gIII gene of PrV(Ka). Table 3 shows the titers of infectious virus produced by PK cells infected with the different Bartha variants, as well as their LD_{50}s for chickens. Whereas there was no significant difference between the titers of infectious virus produced by PK cells (and by CEF; data not shown) infected with the different Bartha variants, considerable differences in the LD_{50}s of these variants were observed. As expected from earlier studies, the parental Bartha strain and variant Bartha 43/25a were avirulent and the doubly rescued variant Bartha 43/25aB4 had a low level of virulence. After acquisition of the gene coding for $gIII^{Ka}$, the parental Bartha strain and Bartha 43/25a remained avirulent but Bartha 43/25aB4 $gIII^{Ka}$ became 20-fold more virulent than Bartha 43/25aB4 $gIII^{B}$. These results show that the resident $gIII^{B}$ gene in the Bartha strain was defective and that this defect contributed to the low level of virulence of Bartha 43/25aB4. These findings corroborate the results, obtained with the deletion mutants of PrV(Ka) described in the first section of this paper, that showed that glycoprotein gIII plays a role in virulence but that it does so in conjunction with other viral functions.

TABLE 3. Virulence of different marker-rescued Bartha variants compared with that of wild-type strains

Virus strain	Phenotype[a]	Virus yield (PFU/cell)[b]	Virulence (LD_{50} [PFU])[c]
Bartha variants			
Parental Bartha	gI^- $gp63^-$ $B4^-$ $gIII^B$	210	$>10^6$
$gIII^{Ka}$	gI^- $gp63^-$ $B4^-$ $gIII^{Ka}$	250	$>10^6$
43/25a	gI^+ $gp63^+$ $B4^-$ $gIII^B$	220	$>10^6$
43/25a $gIII^{Ka}$	gI^+ $gp63^+$ $B4^-$ $gIII^{Ka}$	230	$>10^6$
$43/25aB4^+$	gI^+ $gp63^+$ $B4^+$ $gIII^B$	250	2×10^4
$43/25aB4^+$ $gIII^{Ka}$	gI^+ $gp63^+$ $B4^+$ $gIII^{Ka}$	240	9×10^2
Wild-type strains			
PrV(Ka)		270	5×10^1
PrV(90)		290	3×10^1

[a] The Bartha variants that were marker rescued with the *Bam*HI 4 fragment are indicated by $B4^+$; those that were not marker rescued are indicated by $B4^-$.

[b] PK cells were infected (5 PFU/cell) and incubated for 48 h in Eagle medium containing 5% dialyzed calf serum. The virus yield was determined as described in Materials and Methods.

[c] LD_{50} was determined as described in Materials and Methods.

DISCUSSION

Several conclusions can be drawn from the results described in this report. (i) Mutants of PrV(Ka) defective in the expression of glycoprotein gIII are at most five times less virulent for chickens after i.c. inoculation than is wild-type PrV(Ka). Similar results were also obtained with $gIII^-$ mutants of another PrV strain, the Becker strain (L. Enquist and T. Ben-Porat, unpublished results). (ii) Mutants of PrV(Ka) defective in the expression of glycoproteins gI, gp63, or both are also only slightly less virulent for chickens than is wild-type PrV(Ka). This latter observation corroborates our previously reported finding (19). (iii) Whereas mutants defective in the expression of gI, gp63, or gIII are virulent, mutants defective in gI or gp63 in combination with gIII are avirulent for chickens, despite the fact that they grow in vitro to only slightly lower titers of infectious virus than do mutants defective in gIII alone (which are virulent). (iv) Replacement of the resident gIII gene of the Bartha strain (coding for $gIII^{B}$) by a gIII gene derived from the virulent PrV(Ka) strain (coding for $gIII^{Ka}$) significantly enhances the virulence of a variant of the Bartha vaccine strain to which partial virulence had been previously restored by marker rescue.

The results obtained with the deletion mutants of PrV(Ka) show that glycoproteins gp63 or gI and gIII play a role in virulence but that the deletion of one of these glycoproteins has a significant effect only when it occurs in conjunction with the deletion of the other. Thus, glycoprotein gI or gp63 appears to act synergistically with gIII in the expression of virulence. A similar synergistic effect on viral growth in cell culture was not observed in several different cell types.

The conclusions drawn from the results obtained with the deletion mutants of PrV(Ka) defective in the synthesis of gIII are consistent with those obtained with the marker-rescued variants of the Bartha vaccine strain. In both cases, gI, gp63, or both, in combination with gIII, were shown to play a role in virulence and to affect virulence only by acting in conjunction with each other. Interestingly, expression of

$gIII^{Ka}$ by Bartha 43/25a [a marker-rescued strain which expressed both PrV(Ka) glycoproteins gI^{Ka} and $gp63^{Ka}$] did not restore its virulence. Thus, Bartha 43/25a $gIII^{Ka}$, which as a result of marker rescue expressed three PrV(Ka) glycoproteins (gI^{Ka}, $gp63^{Ka}$, and $gIII^{Ka}$), remained avirulent, indicating that the expression of functional glycoproteins gI, gp63, and gIII is not sufficient to confer virulence on this vaccine strain. On the other hand, expression of $gIII^{Ka}$ by Bartha 43/25aB4 considerably enhanced its virulence. A defect in one of the capsid proteins has been marker rescued by another DNA segment of PrV(Ka), the *Bam*HI 4 fragment, in Bartha 43/25aB4 but not in Bartha 43/25a. This defect thus appears to play a pivotal role in the virulence of the Bartha strain. Rescue of this function did not restore virulence to the parental Bartha strain and restored only a low degree of virulence to the Bartha 43/25a variant that was marker rescued and that expressed functional gI and gp63 glycoproteins (16); it did, however, increase significantly the virulence of a Bartha variant virus that had acquired genes coding for gI or gp63 and gIII from a virulent PrV(Ka) strain (Table 3).

It is interesting that while the virulence of PrV for chickens inoculated i.c. was not affected significantly by the inactivation of gIII alone, inactivation of glycoprotein gC of herpes simplex virus, which is the homolog of gIII of PrV (25), reduces considerably the virulence of herpes simplex virus for mice (11). Whether the different effects of inactivation of gIII of PrV and gC of herpes simplex virus are due to differences in the pathogenesis of these viruses in the systems used to test virulence or to intrinsic differences between the functions of these two homologous glycoproteins remains to be ascertained.

The fact that PrV defective in the expression of gIII has only a slightly reduced level of virulence is interesting, because glycoprotein gIII is important in mediating the attachment of the virus to the host cells. In the absence of gIII, adsorption is considerably slower and is inefficient (28). Because the attachment of a virus to its host cell is a major determinant in pathogenesis and determines the tropism of the virus (6, 29), the finding that despite the role of gIII in adsorption its inactivation affects the virulence of the virus for chickens inoculated i.c. only slightly is unexpected. However, cell-to-cell spread does not appear to be affected by gIII (28), and it is possible that $gIII^{-}$ mutants retain their virulence after i.c. infection of chickens, because in this system, the virus spreads mainly cell to cell.

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LITERATURE CITED

1. **Becker, Y., J. Hadar, E. Tabor, T. Ben-Hur, I. Raibstein, A. Rosen, and G. Darai.** 1986. A sequence in HpaI-P fragment of herpes simplex virus-1 DNA determines intraperitoneal virulence in mice. Virology **149**:255–259.
2. **Ben-Porat, T., J. DeMarchi, J. Pendrys, R. A. Veach, and A. S. Kaplan.** 1986. Proteins specified by the short unique region of the genome of pseudorabies virus play a role in the release of virions from certain cells. J. Virol. **57**:191–196.
3. **Ben-Porat, T., J. M. Demarchi, B. Lomniczi, and A. S. Kaplan.** 1986. Role of glycoproteins of pseudorabies virus in eliciting neutralizing antibodies. Virology **154**:325–334.
4. **Berns, A., A. van den Ouweland, W. Quint, J. van Oirschot, and A. Gielkens.** 1985. Presence of markers for virulence in the unique short region or repeat region or both of pseudorabies hybrid viruses. J. Virol. **53**:89–93.
5. **Centifanto-Fitzgerald, Y. M., T. Yamaguchi, H. E. Kaufman, M. Tognon, and B. Roizman.** 1982. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. J. Exp. Med. **155**:475–489.
6. **Fields, B. N., and M. I. Greene.** 1982. Genetic and molecular mechanisms of viral pathogenesis; implications for prevention and treatment. Nature (London) **300**:19–24.
7. **Hampl, H., T. Ben-Porat, L. Ehrlicher, K.-O. Habermehl, and A. S. Kaplan.** 1984. Characterization of the envelope proteins of pseudorabies virus. J. Virol. **52**:583–590.
8. **Holland, T. C., R. M. Sandri-Goldin, L. E. Holland, S. D. Marlin, M. Levine, and J. C. Glorioso.** 1983. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. J. Virol. **46**:649–652.
9. **Javier, R. T., R. L. Thompson, and J. G. Stevens.** 1987. Genetic and biological analyses of a herpes simplex virus intertypic recombinant reduced specifically for neurovirulence. J. Virol. **61**:1978–1984.
10. **Kaplan, A. S., and A. E. Vatter.** 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7**:394–407.
11. **Kümel, G., H. C. Kaerner, M. Levine, C. H. Schröder, and J. C. Glorioso.** 1985. Passive immune protection by herpes simplex virus-specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenicity. J. Virol. **56**:930–937.
12. **Ladin, B. F., S. Ihara, H. Hampl, and T. Ben-Porat.** 1982. Pathway of assembly of herpesvirus capsids: an analysis using DNA^{+} temperature-sensitive mutants of pseudorabies virus. Virology **116**:544–561.
13. **Lomniczi, B., M. L. Blankenship, and T. Ben-Porat.** 1984. Deletions in the genomes of pseudorabies vaccine strains and existence of four isomers of the genomes. J. Virol. **49**:970–979.
14. **Lomniczi, B., A. S. Kaplan, and T. Ben-Porat.** 1987. Multiple defects in the genome of pseudorabies virus can affect virulence without detectably affecting replication in cell culture. Virology **161**:181–189.
15. **Lomniczi, B., S. Watanabe, T. Ben-Porat, and A. S. Kaplan.** 1984. Genetic basis of the neurovirulence of pseudorabies virus. J. Virol. **52**:198–205.
16. **Lomniczi, B., S. Watanabe, T. Ben-Porat, and A. S. Kaplan.** 1987. Genome location and identification of functions defective in the Bartha vaccine strain of pseudorabies virus. J. Virol. **61**:796–801.
17. **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.
18. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, and T. Ben-Porat.** 1987. Role of pseudorabies virus glycoprotein gI in virus release from infected cells. J. Virol. **61**:2764–2769.
19. **Mettenleiter, T. C., L. Zsak, A. S. Kaplan, T. Ben-Porat, and B. Lomniczi.** 1987. Role of a structural glycoprotein of pseudorabies in virus virulence. J. Virol. **61**:4030–4032.
20. **Oakes, J. E., W. L. Gray, and R. N. Lausch.** 1986. Herpes simplex virus type 1 DNA sequences which direct spread of virus from cornea to central nervous system. Virology **150**: 513–517.
21. **Petrovskis, E. A., J. G. Timmins, T. M. Gierman, and L. E. Post.** 1986. Deletions in vaccine strains of pseudorabies virus and their effect on synthesis of glycoprotein gp63. J. Virol. **60**: 1166–1169.
22. **Platt, K. B., C. J. Mare, and P. N. Hinz.** 1979. Differentiation of vaccine strains and field isolates of pseudorabies (Aujeszky's disease) virus: thermal sensitivity and rabbit virulence markers. Arch. Virol. **60**:13–23.
23. **Quint, W., A. Gielkens, J. van Oirschot, A. Berns, and H. T. Cuypers.** 1987. Construction and characterization of deletion mutants of pseudorabies virus: a new generation of "live" vaccines. J. Gen. Virol. **68**:523–534.
24. **Reed, L. J., and M. Muench.** 1938. A simple method of

estimating fifty percent end points. Am. J. Hyg. **27**:493–497.
25. **Robbins, A. K., R. J. Watson, M. E. Whealy, W. W. Hays, and L. W. Enquist.** 1986. Characterization of a pseudorabies virus glycoprotein gene with homology to herpes simplex virus type 1 and type 2 glycoprotein C. J. Virol. **58**:339–347.
26. **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.
27. **Rösen, A., H. Gelderblom, and G. Darai.** 1985. Transduction of virulence in herpes simplex virus type 1 from a pathogenic to an apathogenic strain by a cloned viral DNA fragment. Med. Microbiol. Immunol. **173**:257–278.
28. **Schreurs, C., T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat.** 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. J. Virol. **62**:2251–2257.
29. **Sharpe, A. H., and B. N. Fields.** 1985. Pathogenesis of viral infections. Basic concepts derived from the reovirus model. N. Engl. J. Med. **312**:486–497.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
31. **Thompson, R. L., M. L. Cook, G. B. Devi-Rao, E. K. Wagner, and J. G. Stevens.** 1986. Functional and molecular analyses of the avirulent wild-type herpes simplex virus type 1 strain KOS. J. Virol. **58**:203–211.
32. **Thompson, R. L., G. V. Devi-Rao, J. G. Stevens, and E. K. Wagner.** 1985. Rescue of a herpes simplex virus type 1 neurovirulence function with a cloned DNA fragment. J. Virol. **55**:504–508.
33. **Thompson, R. L., and J. G. Stevens.** 1983. Biological characterization of a herpes simplex virus intertypic recombinant which is completely and specifically non-neurovirulent. Virology **131**: 171–179.
34. **Thompson, R. L., E. K. Wagner, and J. G. Stevens.** 1983. Physical location of a herpes simplex virus type 1 gene function(s) specifically associated with a 10-million-fold increase in HSV neurovirulence. Virology **131**:180–192.
35. **Wathen, M. W., and L. M. K. Wathen.** 1986. Characterization and mapping of a nonessential pseudorabies virus glycoprotein. J. Virol. **58**:173–178.
36. **Weise, K., H. C. Kaerner, J. Glorioso, and C. H. Schröder.** 1987. Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes of plaque morphology and neuropathogenicity. J. Gen. Virol. **68**:1909–1919.