JOURNAL OF VIROLOGY, Sept. 1987, p. 2764–2769
0022-538X/87/092764-06$02.00/0

Vol. 61, No. 9

Role of Pseudorabies Virus Glycoprotein gI in Virus Release from Infected Cells

THOMAS C. METTENLEITER, CHRISTA SCHREURS, FEDERICO ZUCKERMANN, AND TAMAR BEN-PORAT*

Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received 13 April 1987/Accepted 22 May 1987

The Bartha vaccine strain of pseudorabies virus has a deletion in the short unique (U_S) region of its genome which includes the genes that code for glycoproteins gI and gp63 (E. Petrovskis, J. G. Timmins, T. M. Gierman, and L. E. Post, J. Virol. 60:1166–1169, 1986). Restoration of an intact U_S to the Bartha strain enhances its ability to be released from infected rabbit kidney cells and increases the size of the plaques formed on these cells (T. Ben-Porat, J. M. DeMarchi, J. Pendrys, R. A. Veach, and A. S. Kaplan, J. Virol. 57:191–196, 1986). To determine which gene function plays a role in virus release from rabbit kidney cells, deletions were introduced into the genomes of both wild-type virus and the "rescued" Bartha strain (Bartha strain to which an intact U_S had been restored) that abolish the expression of either the gI gene alone or both gI and gp63 genes. The effect of these deletions on the phenotype of the viruses was studied. Deletion mutants of wild-type virus defective in either gI or gI and gp63 behave like wild-type virus with respect to virus release and plaque size on rabbit kidney cells. Deletion of gI from the rescued Bartha strain, however, strongly affects virus release and causes a decrease in plaque size. We conclude that gI affects virus release but that at least one other viral function also affects this process. This function is defective in the Bartha strain but not in wild-type virus; in its absence gI is essential to efficient release of the virus from rabbit kidney cells.

Pseudorabies virus (PrV), a herpesvirus, causes latent and acute, often fatal, infection of the nervous system in swine, as well as acute infection of other domestic and wild animals (1). Because PrV causes severe economic losses, vaccination of pigs with attenuated or killed vaccines is practiced in many countries with varying degrees of success (7).

The attenuated vaccine strains of PrV that have been isolated grow well in vitro, even though they do not cause disease in swine. Some information concerning the genomic organization and the genetic basis for the avirulence of the vaccine strains is available (6, 12). In some of these strains, for example, Bartha or Norden, a region in the short unique (U_S) part of the genome is deleted, a deletion related to the avirulence of the Bartha strain (4, 13, 14). Furthermore, although the Bartha strain grows well in rabbit kidney (RK) cells, it is not released readily from these cells. Restoration of the region in the U_S that had been deleted from the Bartha strain restores partially its ability to be released from RK cells (2). Thus, the region in the U_S of the genome that is deleted from the Bartha strain plays a role in virulence and in the release of the virus from certain cell types but does not appear to be otherwise esssential for virus growth in vitro.

The region in the U_S that is deleted from the Bartha strain codes for glycoproteins gI and gp63 (15–18). It may also encode several other viral functions, since several additional mRNAs transcribed from that region are present in the cytoplasm of cells infected with wild-type virus and are absent from the cytoplasm of Bartha strain-infected cells (13). To determine which of the genes in this segment of DNA are involved in virus release from RK cells, deletions were introduced into the genomes of both wild-type PrV and the "rescued" Bartha strain (Bartha strain to which an intact U_S had been restored) that abolish the expression of either gI alone or both gI and gp63. The effect of these deletions on the phenotype of the viruses was studied.

* Corresponding author.

MATERIALS AND METHODS

Virus strains. PrV(Ka) is a strain which has been carried in our laboratory for more than 25 years; its origin is uncertain (10). The Bartha strain was obtained from P. S. Paul; the origin of this strain has been described before (19). Bartha 43/25aB4 is a Bartha strain to which an intact U_S has been restored by marker rescue (13) and which has also been rescued by *Bam*HI fragment 4 of the wild-type virus, a procedure that restores partial virulence to this strain (14).

Media and solutions. EDS is Eagle synthetic medium (5) plus 3% dialyzed bovine serum, and EDS-FU is EDS plus 5-fluorouracil (20 μg/ml) and thymidine (5 μg/ml). TBSA consists of 0.136 M NaCl, 2.6 mM KCl, 0.01 M Tris hydrochloride, 20 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.0, plus 1% crystalline bovine albumin.

Radiochemicals. [^{3}H]glucosamine (specific activity, 32 Ci/mol) and [^{3}H]thymidine (specific activity, 50 Ci/mmol) were purchased from New England Nuclear Corp.

Monoclonal antibodies. The monoclonal antibodies used in this study were isolated and characterized as described previously (8).

Immunoprecipitation. Immunoprecipitation was performed essentially by the method of Kessler (11), as described previously (8).

Selection of deletion mutants. Originally, we used the method described by Villareal and Berg (21) to identify deletion mutants. Cells were cotransfected with virion DNA and plasmid pTM7AΔ1 or pTM7AΔ2, both of which carry PrV *Bam*HI/*Sal*I fragment 7A with the desired deletion (see Fig. 1). The progeny was plaque assayed, and plaque isolates that did not hybridize to the segments of DNA deleted from the plasmids were identified. Using this technique, we found that there was a degree of uncertainty in the identification of the negative plaques. Consequently, repeated cycles of plaque purification were required before pure preparations of deletion mutants were obtained. The procedure was therefore modified as follows.

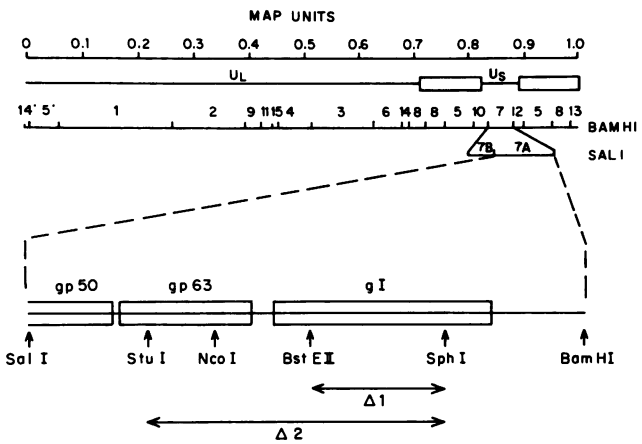

FIG. 1. Restriction fragment map of the genome of PrV(Ka) indicating the location of the glycoprotein genes in *Bam*HI/*Sal*I fragment 7A. The map positions of the glycoproteins have been previously determined by Petrovskis et al. (18). Δ1 and Δ2 denote the deletions introduced into plasmid pTM-7A (pBR325 into which *Bam*HI/*Sal*I fragment 7A has been inserted).

Progeny virus obtained from cells cotransfected with virus DNA and the appropriate plasmids were exposed to monoclonal antibodies against gI and complement to enrich for mutants that do not express gI (16a). Surviving virus was plaque assayed. Well-separated plaques were picked by aspiration of the agar plug over the plaques into an Eppendorf pipette. Wells containing RK cells (in 96-well plates) were inoculated with each of the plaque isolates. After virus-induced degeneration of the cells was observed, 100 μl of culture fluids was dot blotted onto nitrocellulose filters. The filter sheets were placed for 5 min sequentially on filter paper saturated with the following: (i) 0.5 N NaOH; (ii) 0.6 M NaCl, 1.0 M Tris hydrochloride, pH 7.4; (iii) 1.5 M NaCl, 0.5 M Tris hydrochloride, pH 7.4; and (iv) 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filters were dried, baked, and hybridized with the appropriate probes to reveal which of the plaque isolates had a deletion in either gI or both gI and gp63. This method allows for rapid and reliable identification of the deletion mutants. To ensure purity, the deletion mutants that had been identified were repurified by two cycles of plaque purification. However, most of the plaque isolates appeared to be pure after the original plaque purification.

Labeling and purification of virions. Primary RK cells were treated with EDS-FU for 24 h (to inhibit cellular DNA synthesis [9]). The cells were infected (multiplicity, 5 PFU per cell) and incubated for 24 h in EDS containing [^{3}H]thymidine (20 μCi/ml). To obtain extracellular virus, the culture medium was clarified and the virus in the supernatant was purified, as described previously (3). To obtain intracellular virus, the cells were sedimented by centrifugation at 1,000 × *g* for 5 min, suspended in TBSA, and sonicated. After removal of cellular debris by centrifugation at 5,000 × *g* for 10 min, the virus was purified.

RESULTS

Construction of deletion mutants defective in gI or gI and gp63. The region of the genome of PrV encoding glycoproteins gI and gp63 has been sequenced by Petrovskis et al. (18). The availability of this information enabled us to construct mutants with deletions in specific regions of each of these genes.

Figure 1 illustrates the physical map of the genome of wild-type PrV with an expansion of *Bam*HI/*Sal*I fragment 7A that includes the region encoding gI and gp63. To construct the deletion mutants, *Bam*HI/*Sal*I fragment 7A from wild-type virus DNA was cloned into pBR325. The resulting plasmid, pTM-7A, was cleaved with either *Bst*EII and *Sph*I, thereby deleting a large part of gI (Δ1), or *Stu*I and *Sph*I, thereby deleting most of gI and gp63 (Δ2; Fig. 1). The DNA was blunt ended and religated, and the plasmids were amplified. Figure 2 illustrates the patterns of Southern hybridization of plasmids pTM7AΔ1 and pTM7AΔ2 to the *Bst*EII/*Sph*I fragment (which was deleted from the plasmid with Δ1) and to the *Stu*I/*Nco*I fragment (which was deleted from the plasmid with Δ2). The sizes of the fragments and the hybridization patterns to the probes indicated that the plasmids with the desired deletions had been obtained.

To isolate mutants of PrV or Bartha 43/25aB4 with deletions in gI or gI and gp63, Bartha 43/25aB4 DNA or wild-type PrV(Ka) DNA was cotransfected with plasmid pTM7AΔ1 or pTM7AΔ2. The progeny virions obtained from the cells were exposed to monoclonal antibodies against gI and complement (to enrich for virions which do not express gI [16a]), and the surviving virions were plaque assayed. Plaques were picked and virus was grown in 96-well plates. Portions of medium from each well were used to identify by dot blot hybridization those virion populations carrying the desired deletion. Figure 3 shows the results of a representative experiment in which wild-type PrV(Ka) was cotransfected with pTM7AΔ1. Several of the virion populations, each derived from a single plaque, did not hybridize with the *Bst*EII/*Sph*I probe but did hybridize to total PrV(Ka) DNA, indicating the presence of the desired deletion. The progeny

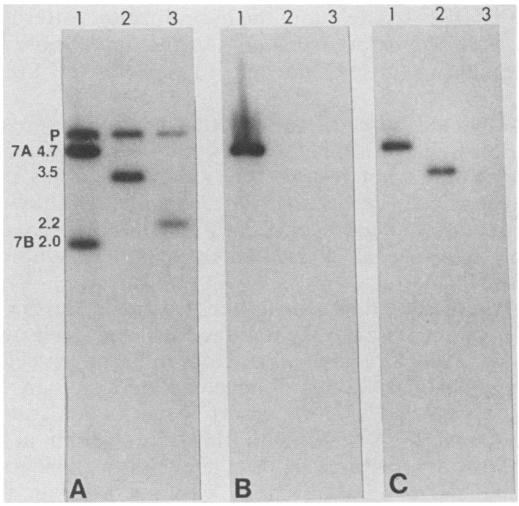

FIG. 2. Southern blot analysis of plasmid pTM-7AΔ1 and pTM-7AΔ2. Plasmids pBR325-*Bam*HI fragment 7 (lanes 1), pTM-7AΔ1 (lanes 2), and pTM-7AΔ2 (lanes 3) were cleaved with *Bam*HI and *Sal*I to release the inserts; the resulting fragments were electrophoresed in a 1% agarose gel. After transfer onto nitrocellulose (20), the filters were hybridized with nick-translated (A) pBR325-*Bam*HI fragment 7; (B) fragment *Bst*EII/*Sph*I representing Δ1 (Fig. 1); or (C) fragment *Stu*I/*Nco*I diagnostic of Δ2 (Fig. 1). Clone pBR325-*Bam*HI fragment 7 hybridizes to plasmid DNA (P) as well as to fragments 7A (4.7 kilobases [kb]), 7B (2.0 kb), 7AΔ1 (3.5 kb), and 7AΔ2 (2.2 kb). The *Bst*EII/*Sph*I fragment hybridizes only to intact *Bam*HI fragment 7A; these sequences have been deleted from both *Bam*HI fragments 7AΔ1 and 7AΔ2. The *Stu*I/*Nco*I fragment hybridizes to *Bam*HI fragments 7A and 7AΔ1, but not to 7AΔ2 (Fig. 1).

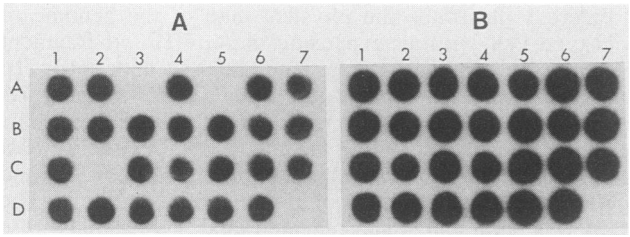

FIG. 3. Screening of single plaque isolates by dot blot assay. Cells were cotransfected with PrV(Ka) DNA and pTM-7AΔ1. The progeny was plaque assayed and plaques were picked. RK cells grown in 96-well plates were infected with these plaque isolates. After virus-induced degeneration of the cell cultures, a portion of the medium in each well was spotted onto nitrocellulose filters. The DNA was denatured and immobilized, as described in Materials and Methods. (A) Autoradiogram after hybridization of the filter with nick-translated fragment *Bst*EII/*Sph*I (Δ1; Fig. 1). The absence of a signal in A3, A5, and C2 denotes that these virus populations carry deletion Δ1. (B) Same filter after hybridization with nick-translated PrV(Ka) DNA. D7 represents medium obtained from a well with uninfected cells.

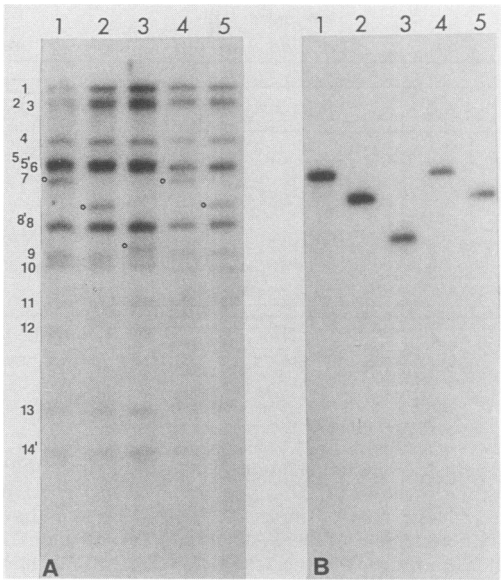

FIG. 4. Southern hybridization analysis of the genomes of the virus deletion mutants: evidence for a deletion in *Bam*HI fragment 7. DNA obtained from wild-type PrV(Ka) (lanes 1), PrV(Ka)Δ1 (lanes 2) and PrV(Ka)Δ2 (lanes 3) as well as from Bartha 43/25aB4 (lanes 4) and Bartha 43/25aB4Δ1 (lanes 5) was cleaved with *Bam*HI, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose. (A) The filters were hybridized to nick-translated PrV DNA: the positions of all viral DNA fragments remain unchanged except that of *Bam*HI fragment 7 (○). (B) The filters were hybridized to *Bam*HI fragment 7: deletions in that fragment were detected in the mutants.

of cells cotransfected with Bartha 43/25aB4 and pTM7AΔ1 or pTM7AΔ2, as well as that of cells cotransfected with PrV(Ka) DNA and pTM7AΔ2, were similarly analyzed with either the *Bst*EII/*Sph*I (diagnostic for a deletion in gI) or the *Stu*I/*Nco*I (diagnostic for a deletion in gI and gp63) fragment as probe (data not shown).

The virion populations that had been identified by dot blot hybridization as carrying the desired deletions were subjected to two further cycles of plaque purification and were analyzed by Southern hybridization (Fig. 4 and 5). The hybridization patterns showed that the virions did indeed carry the expected deletion; the hybridization patterns of the genomes of the deletion mutants to the appropriate probes were similar to those of the deleted plasmids (cf. Fig. 2 and 5).

As expected, none of the deletion mutants expressed gI (Table 1). Because we do not have antibody specific against gp63, we could not test for the synthesis of this protein. However, since the Southern analysis clearly showed that most of the sequences encoding gp63 were deleted from mutants carrying Δ2, it is unlikely that they synthesize a functional gp63.

Effects of deletion of gI from the "rescued" Bartha strain. We have shown previously that the Bartha strain grows well in pig kidney, RK, and chicken embryo fibroblast cells. The virus yield from these cells is approximately the same as that of wild-type PrV(Ka) virus (2). However, when cultures of RK cells were infected with this virus strain at a low multiplicity of infection (0.1 PFU per cell), the appearance of virus-induced cytopathic effect was much slower than in cells infected with wild-type virus, reflecting the poor release of strain Bartha from RK cells. Restoration of an intact U_S to the Bartha strain restored its ability to be released from RK cells, indicating that a function encoded in the region of the U_S deleted from the Bartha strain is responsible (at least in part) for the poor release of the virions from RK cells (2).

To determine which of the viral functions encoded in the region of the genome that had been deleted from the Bartha strain affects the release of virions from RK cells, we compared the release of the parental Bartha strain with that of rescued Bartha 43/25aB4 (14), as well as with that of Bartha 43/25aB4 into which Δ1 had been introduced (i.e., which does not express gI). To this end, RK cells were

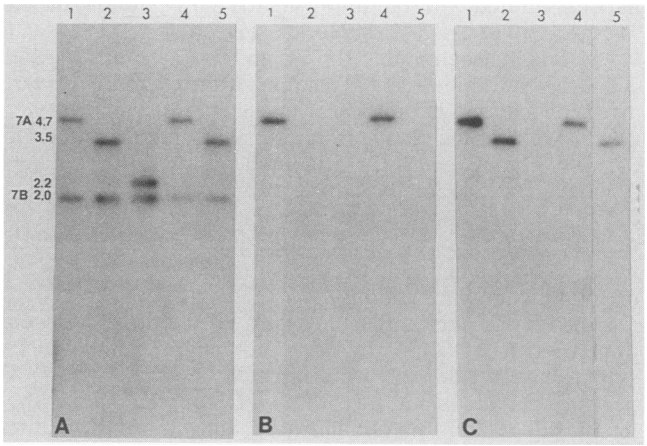

FIG. 5. Further characterization of deletions in the mutant viruses. DNA isolated from PrV(Ka) (lanes 1), PrV(Ka)Δ1 (lanes 2), PrV(Ka)Δ2 (lanes 3), Bartha 43/25aB4 (lanes 4), and Bartha 43/25aB4Δ1 (lanes 5) was cleaved with the *Bam*HI and *Sal*I restriction enzymes and electrophoresed in a 1% agarose gel. The DNA was transferred to nitrocellulose filters and hybridized to nick-translated fragment *Bam*HI 7 (A), fragment *Bst*EII/*Sph*I (diagnostic of Δ1; Fig. 1) (B), or fragment *Stu*I/*Nco*I (diagnostic of Δ2; Fig. 1) (C). Fragment *Bam*HI 7 hybridized as expected to *Bam*HI/*Sal*I fragments 7A and 7B. While fragment 7B remained unchanged in all strains, part of fragment 7A was deleted from the mutant viruses. The *Bst*EII/*Sph*I probe hybridized only to wild-type PrV(Ka) and Bartha 43/25aB4. The *Stu*I/*Nco*I probe hybridized to the mutants with deletion Δ1 but not to those with deletion Δ2. The sizes of the fragments are indicated in kilobases.

TABLE 1. Lack of synthesis of gI by deletion mutants[a]

Virus mutant	Immunoprecipitation (cpm, 10^2)	
	gI	gII
Bartha	10	2,327
Bartha 43/25aB4	352	2,717
Bartha 43/25aB4Δ1	11	2,234
PrV(Ka)	679	1,476
PrV(Ka)Δ1	30	1,217
PrV(Ka)Δ2	22	2,078

[a] RK cells were infected with the various virus mutants at a multiplicity of 5 PFU per cell. The cells were labeled with [^{3}H]glucosamine (20 μCi/ml) at 3 to 20 h postinfection. The cells were harvested, and the membrane fraction was isolated and immunoprecipitated with monoclonal antibodies against gI or gII, as described in Materials and Methods.

infected with the virus variants and labeled with [^{3}H]thymidine and the amount of extracellular or intracellular virus present in the culture at 24 h postinfection was determined. Figure 6 shows the results obtained when the extracellular virions were centrifuged in sucrose gradients. As expected, little of the parental Bartha strain was released from the cells, whereas a significant amount of Bartha 43/25aB4 (Bartha strain with an intact U_S) was released. However, Bartha 43/25aB4Δ1 (which does not express gI) behaved like the parental Bartha strain and was not released well from the cells.

Table 2 summarizes the distribution of ^{3}H-labeled virus in the extracellular and intracellular fractions obtained from another, similar experiment. Deletion of gI from Bartha 43/25aB4 resulted in poor release of the virus from RK cells, as seen in two independently obtained isolates. These deletion mutants behaved like the parental Bartha strain. Bartha 43/25aB4Δ2, which is gI$^-$ gp63$^-$, also behaved like the parental Bartha strain (data not shown). We conclude from this experiment that gI plays a role in the release of Bartha 43/25aB4 from RK cells.

We have shown previously that restoration of an intact U_S to the Bartha strain increases the size of the plaques the virus forms on RK cells (2). Figure 7 shows that the plaques formed by the Bartha 43/25aB4 variant (B) (which has an intact U_S) were larger than those of the parental Bartha strain (A) or those of Bartha 43/25aB4Δ1 (C), i.e., Bartha 43/25aB4 which has a deletion in gI. Thus, in strain Bartha gI affects the size of the plaques on RK cells as well as virus release from these cells (Table 2; Fig. 5).

Effect of deletion of gI or gI and gp63 from wild-type PrV(Ka) virus. Experiments similar to the ones performed with the rescued Bartha strain described above were also performed with the wild-type virus PrV(Ka). Figure 6 shows the distribution in sucrose gradients of virions released by RK cells infected with either PrV(Ka) or deletion mutants of this virus, PrV(Ka)7AΔ1 or PrV(Ka)7AΔ2. The results of another, similar experiment are summarized in Table 2. No difference in the release of the virus was found between the wild type and either one of the deletion mutants of PrV(Ka). Thus, while deletion of gI from Bartha 43/25aB4 affects the release of the virus from RK cells, the deletion of gI from wild type did not affect virus release. Deletion of both gI and gp63 from PrV(Ka) also did not affect virus release. Furthermore, in contrast to the decrease in plaque size observed after deletion of gI from Bartha 43/25aB4, no effect on the sizes of the plaques was detectable after deletion of either gI or gI and gp63 from PrV(Ka) (data not shown).

Thus, in contrast to the rescued Bartha strain, in which gI plays an important role in the release of virus from RK cells,

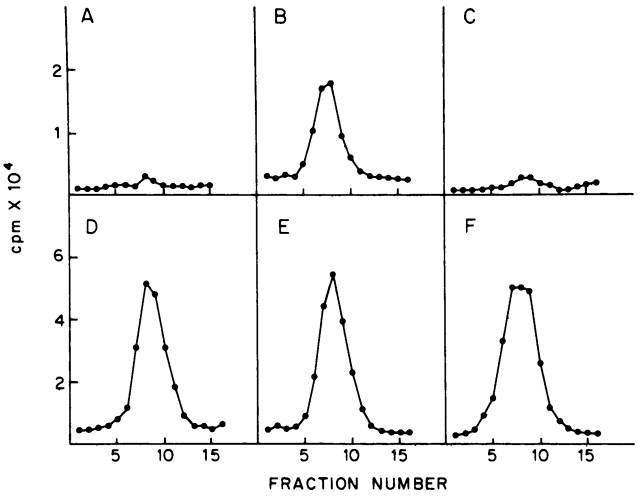

FIG. 6. Sedimentation in sucrose gradients of virus released from RK cells. Cells were pretreated with ELS-FU to inhibit cellular DNA synthesis (9) and were then infected with 5 PFU per cell and incubated in EDS containing [^{3}H]thymidine (20 μCi/ml). At 24 h postinfection, the medium over the cells was harvested and clarified (to remove cellular debris), and the virus was concentrated on a sucrose cushion and centrifuged in sucrose gradients, as described in Materials and Methods. (A) Strain Bartha; (B) Bartha 43/25aB4; (C) Bartha 43/25aB4Δ1; (D) PrV(Ka); (E) PrV(Ka)Δ1; (F) PrV(Ka)Δ2.

deletion of that gene from PrV(Ka) does not affect virus release. These findings lead us to conclude that gI plays a role in virus release but that another factor(s) also can affect virus release. In contrast to wild-type PrV(Ka), the Bartha strain is defective in this factor.

DISCUSSION

As part of a study designed to identify the genes responsible for the virulence of PrV, we have examined several aspects of the biology and molecular biology of avirulent vaccine strains. We have shown previously that the Bartha vaccine strain has a deletion in the U_S. This deletion is related to the lack of virulence of this virus strain. Berns et al. (4) arrived at a similar conclusion. The deletion in strain Bartha includes the sequences encoding glycoprotein gI (15, 16), as well as glycoprotein gp63 (17), and may also affect the

TABLE 2. Release of deletion mutants of PrV(Ka) and Bartha 43/25aB4 from RK cells[a]

Virus mutant	cpm (10^3) per sample		Released (%)
	Extracellular	Intracellular	
PrV(Ka)	152	122	55
PrV(Ka)Δ1	163	142	54
PrV(Ka)Δ2	169	153	52
B.43/25aB4	69	259	21
B.43/25aB4Δ1 (isolate 1)[b]	2	328	<1
B.43/25aB4Δ1 (isolate 2)[b]	2	295	<1

[a] RK cells (4×10^6 per sample) were pretreated with 5-fluorouracil to inhibit cellular DNA synthesis (9). The cells were then infected (multiplicity, 5 PFU per cell) and incubated in EDS containing [^{3}H]thymidine (20 μCi/ml). At 24 h after infection, the extracellular virus in the medium and the intracellular virus obtained after sonication of the cells were collected separately and the virions were purified by centrifugation in sucrose gradients. The amount of radioactivity associated with the virus peaks was determined.

[b] Independently obtained isolates with Δ1.

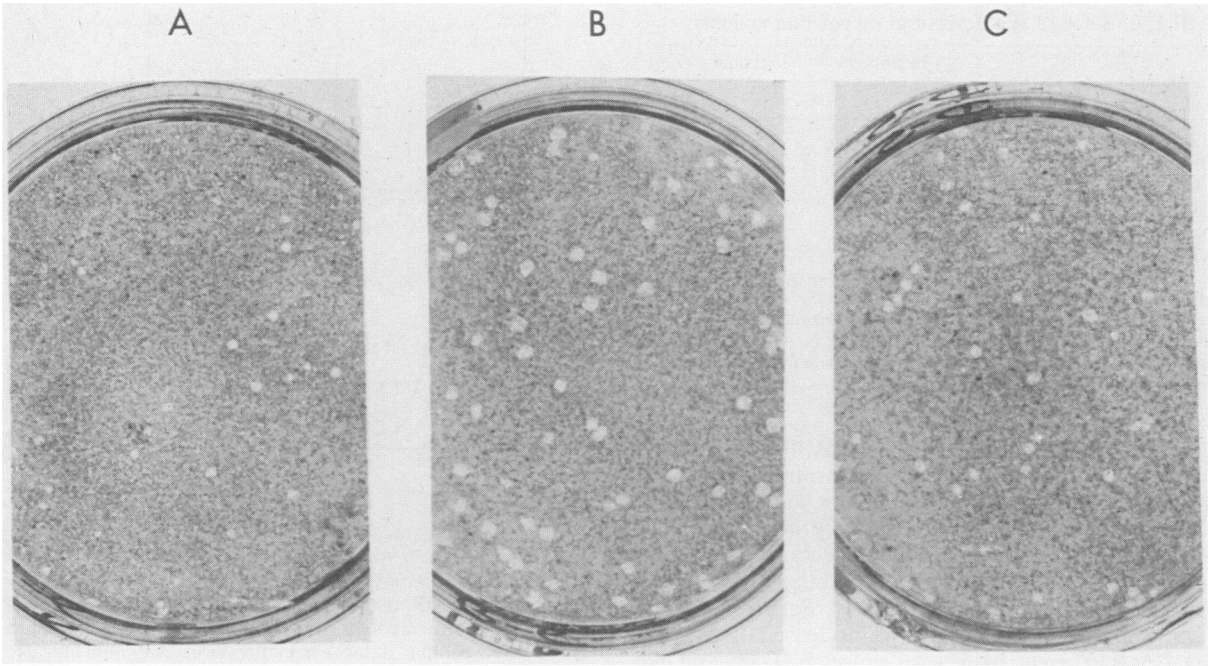

FIG. 7. Sizes of plaques formed by different variants of the Bartha strain: (A) Bartha; (B) Bartha 43/25aB4; (C) Bartha 43/25aB4Δ1.

expression of other gene products. (Several mRNAs are encoded in that region of the genome [13]). Restoration of an intact U_S to strain Bartha restores to it a limited ability to replicate in the brains of day-old chickens (13). Furthermore, while the parental Bartha strain is released poorly from RK cells and forms small plaques on these cells, the Bartha strain to which an intact U_S has been restored is released more efficiently and forms larger plaques on RK cells. This virus, however, is not released as efficiently as PrV(Ka) (2).

To ascertain which of the genes in the U_S that were deleted from the Bartha strain affect virus release, we deleted from the Bartha 43/25aB4 (a virulent, doubly marker rescued Bartha strain to which an intact U_S has been restored [14]) parts of the coding sequences of either gI or both gI and gp63. These sequences were also deleted from PrV(Ka) virus. The salient findings of these experiments may be summarized as follows. (i) Deletion of gI from Bartha 43/25aB4 affects the release of the virus from RK cells (Fig. 6; Table 2); it also affects the size of the plaques the virus produces in these cells (Fig. 7). Thus, it was the restoration of gI to Bartha 43/25a (Bartha with an intact U_S) which caused the improved release and the larger plaque formation of this strain (2). (ii) Deletion of gI from wild-type virus does not affect the release of the virus from RK cells nor does it affect the size of the plaques the virus forms on these cells. Furthermore, deletion of both gI and gp63 also does not detectably affect the behavior of the virus in this respect.

That deletion of gI affects both virus release and plaque size of the rescued Bartha virus, but does not affect PrV(Ka) in the same way, indicates that the effect of gI on virus release depends on the genetic background of the virus strain. Virus release thus appears to be affected by at least two gene products. One of those is glycoprotein gI; the other gene product is defective in the Bartha strain but not in PrV(Ka). In the absence of this function, gI is essential for efficient release of PrV from RK cells. This conclusion is based on the following considerations. (i) A defect in gI or gI and gp63 [PrV(Ka)Δ1 or PrV(Ka)Δ2] does not affect virus release from RK cells. (ii) A defect in an unknown other function(s) affects release partially. Bartha 43/25aB4 which has an intact U_S and expresses gI is not released as efficiently as is PrV(Ka) from RK cells; Bartha is presumably defective in this function(s). (iii) A defect in both gI and the other function(s) (strain Bartha or Bartha 43/25aB4Δ1) results in poor virus release.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10947 from the National Institutes of Health. Thomas C. Mettenleiter was supported by research fellowship Me 854/1-1 from the Deutsche Forschungsgemeinschaft. Federico Zuckermann was supported by Public Health Service training grant 5T32-CA09385 from the National Cancer Institute.

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