JOURNAL OF VIROLOGY, Mar. 1987, p. 796–801
0022-538X/87/030796-06$02.00/0

Vol. 61, No. 3

Genome Location and Identification of Functions Defective in the Bartha Vaccine Strain of Pseudorabies Virus

BELA LOMNICZI,† SHOZO WATANABE,‡ TAMAR BEN-PORAT, AND ALBERT S. KAPLAN*
Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received 14 April 1986/Accepted 7 November 1986

We have shown previously (Lomniczi et al., J. Virol. 52:198–205, 1984) that the Bartha vaccine strain of pseudorabies virus has a deletion in the short unique (U_S) region of its genome—a deletion that is related to the absence of virus virulence. This strain is, however, also defective in other genes involved in virulence. We show here that virulence can be restored by marker rescue of the Bartha strain to which an intact U_S has been restored (but not to the parental Bartha strain) by sequences derived from approximate map units 0.460 and 0.505 of the wild-type virus genome. No difference in the ability to grow in cell culture was observed between parental Bartha, Bartha 43/25a (Bartha to which an intact U_S has been restored), or the doubly rescued Bartha strains. However, only the doubly rescued Bartha strain was virulent for both chickens and pigs and replicated to high titers when inoculated directly into the brains of chickens. The sequences that could restore virulence to the Bartha 43/25a strain encode four genes, all of which are involved in processes leading to the assembly of nucleocapsids. Since these sequences rescue virulence, it appears that a function that plays a role in nucleocapsid assembly is defective in the Bartha strain and that this defect contributes to the lack of virulence of this virus.

Pseudorabies virus (PrV) (herpesvirus suis) causes Aujeszky's disease in swine, which results in severe economic losses. Vaccination with attenuated strains of the virus is practiced in most countries, including the United States. Several attenuated strains have been isolated by repeated passage in embryonated eggs or in cell culture. In general, these attenuated strains do not cause disease in swine and provide varied degrees of immunity against superinfection with virulent strains (8).

We have undertaken a series of studies designed to elucidate the genetic basis for the lack of virulence of some of the vaccine strains of PrV. The aims of these studies are to identify the functions of the genes responsible for the expression of virulence. We have shown previously that one vaccine strain, Bartha, has a deletion in the short unique region (U_S) of its genome (approximate map units 0.855 to 0.882) that is related to its lack of virulence (16). A similar observation has also been made by Berns et al. (4). However, the deletion in the U_S in the Bartha strain is not the only lesion that is responsible for its lack of virulence (16); restoration of the deleted sequences to the virus genome restores to it a limited ability to grow in chicken brains but does not restore its ability to kill day-old chickens when inoculated intracerebrally (16).

The experiments described in this paper were designed to identify the other region(s) of the Bartha genome that has been modified, resulting in defective functions and contributing to the avirulence of this strain.

We show here that the Bartha vaccine strain, in addition to being defective in functions encoded by a sequence in the U_S (which is deleted), is also defective in a gene that appears to be involved in nucleocapsid assembly.

* Corresponding author.
† Present address: Veterinary Medical Research Institute, H-1581 Budapest, Hungary.
‡ Present address: Third Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514, Japan.

MATERIALS AND METHODS

Virus strains and cell culture. PrV(Ka) is a strain which has been carried in our laboratory for more than 25 years; its origin is uncertain (11). The Bartha avirulent vaccine strain was received from P. S. Paul.

Rabbit kidney (RK) and pig kidney (PK) cells and chicken embryo fibroblasts (CEF) were cultivated in Eagle synthetic medium (7) containing 3% dialyzed bovine serum; virus was titrated by plaque assay in RK or PK cells. The number of plaques obtained on the different cell monolayers was approximately the same.

Chemicals and radiochemicals. [^{3}H]leucine (specific activity, 45 Ci/mmol) was purchased from Schwarz/Mann. [α-^{32}P]dCTP was purchased from New England Nuclear Corp. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc.

Marker rescue. Marker rescue was performed as described previously (10).

Immunoprecipitation. Immunoprecipitation was performed by the method of Kessler (12) as described previously (9). Antibodies against capsids were obtained by intramuscular injection of purified empty capsids in rabbits (14).

Determination of LD_{50}. Tenfold dilutions of the virus stocks were injected intracerebrally (0.05 ml) into day-old Hubbard strain chickens (five per dilution). The number of animals dead by 2 weeks after inoculation was determined, and the 50% lethal dose (LD_{50}) was calculated by the Reed and Muench method (16a).

Electrophoresis of RNA for Northern transfers. RNA was denatured, glyoxylated, and electrophoresed in 1% agarose gels. The RNA was transferred to nitrocellulose by the method of Thomas (17).

Purification of cytoplasmic and polysomal RNA. Cytoplasmic and polysomal RNAs were purified as described previously (6).

Selection of mRNA translation in vitro, and polyacrylamide gel electrophoresis. Selection of mRNA was carried out

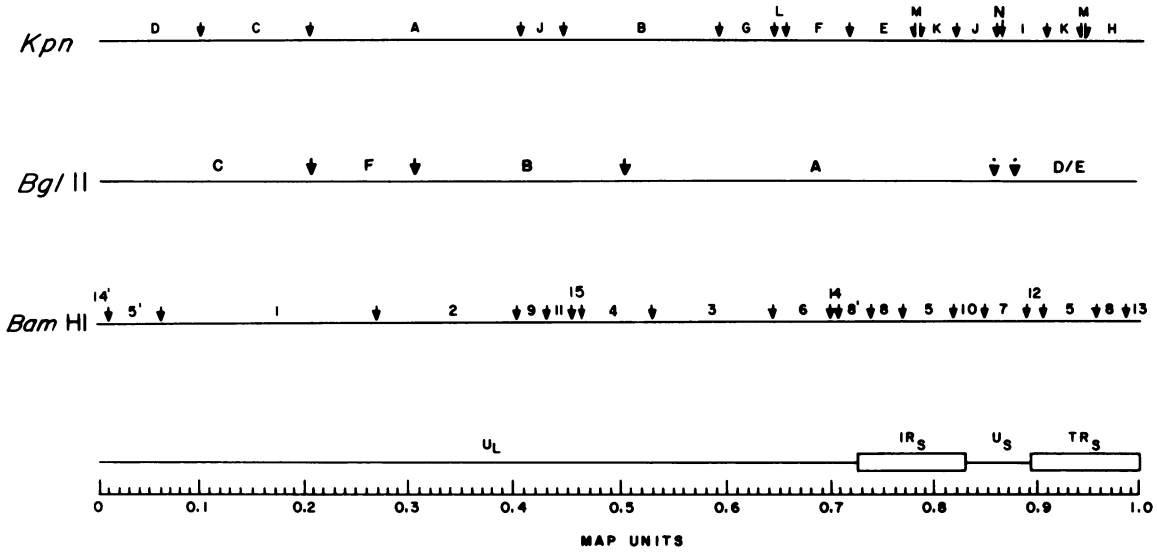

FIG. 1. Restriction fragment map of the genome of PrV. The open rectangles indicate the inverted repeats. The arrows indicate cleavage sites.

essentially as described by Belle Isle et al. (1). Translation in vitro was performed in mRNA-dependent rabbit reticulocyte systems as described previously (16). Urea and sodium dodecyl sulfate (final concentrations, 1 M and 2%, respectively) were added to the protein samples, which were boiled for 2 min and then reduced by incubation with dithioerythritol (final concentration, 0.01 M) at 37°C for 30 min. The proteins were then electrophoresed in polyacrylamide gels as described previously (14).

RESULTS

Restoration of virulence to the Bartha vaccine strain by marker rescue. We have previously shown that the Bartha strain has a deletion in the U_S spanning approximate map units 0.855 to 0.882 and that these sequences are necessary for the expression of virulence of this strain (15, 16). However, this virus strain is also defective in other functions required for the expression of virulence. Thus, the Bartha strain to which an intact U_S had been restored by marker rescue (Bartha 43/25a) is avirulent, yet, in contrast to the parental Bartha strain, it has acquired a limited ability to replicate in the brains of day-old chickens (16).

To locate the functions that are defective in Bartha 43/25a, we cotransfected cells with DNA from either Bartha or Bartha 43/25a virions and individual *Bgl*II restriction fragments of wild-type PrV DNA. (Digestion of PrV DNA yields only six *Bgl*II restriction fragments [Fig. 1].) On the assumption that passage of the virions in chicken brains will select for recombinants that have acquired an enhanced ability to grow in the chicken brains, the progeny virions obtained from the cotransfected cells were injected i.c. into day-old chickens. Four days after inoculation, the brains of the chickens were collected, homogenized, sonicated, and centrifuged to remove cellular debris; the virus in the supernatant fluids was amplified in PK cells. The virions produced by these cells were then reinoculated into day-old chickens, and the animals were observed for signs of disease or death.

Six out of seven chickens injected i.c. with the virus population obtained after cotransfection of cells with Bartha 43/25a DNA (but not with parental Bartha DNA) and *Bgl*II fragment B died (Table 1). These results indicate that virulence can be restored to the Bartha 43/25a strain but not to the parental Bartha strain by cotransfection with *Bgl*II fragment B. Some animals injected with virions produced by cells cotransfected with Bartha 43/25a and *Bgl*II fragments other than B also died. The virus recovered from these dead or moribund animals was, however, not virulent, and only the virions obtained from chickens inoculated with Bartha 43/25a DNA and *Bgl*II fragment B were virulent (Table 1). The death of the animals inoculated with Bartha 43/25a and other *Bgl*II fragments was therefore probably unrelated to the formation of virulent recombinant virus. Occasionally, a

TABLE 1. Marker rescue of Bartha 43/25a with *Bgl*II fragments of PrV(Ka) DNA[a]

*Bgl*II fragment	Approximate map units	Cells cotransfected with:			
		Bartha DNA		Bartha 43/25a DNA	
		Virulence[b]	LD_{50} (PFU)	Virulence	LD_{50} (PFU)
A	0.505–0.850	0/6	ND[c]	0/6	ND
B	0.297–0.505	0/6	ND	6/7	8×10^3
C	0.000–0.205	0/6	ND	1/8	$>10^6$
D	0.850–1.000	0/6	ND	0/8	ND
E	0.866–1.000	0/6	ND	1/8	$>10^6$
F	0.205–0.291	0/6	ND	1/12	$>10^6$

[a] PK cells were cotransfected with either Bartha or Bartha 43/25a DNA and the indicated *Bgl*II fragment of PrV(Ka) DNA as described in Materials and Methods. After complete virus-induced degeneration of the monolayers, the progeny virus was collected, and 10^5 PFU were inoculated i.c. into day-old chickens. None of the chickens showed signs of disease or died. Four days after inoculation, the chickens were killed, and their brains were homogenized. The homogenates of each group of nine chickens were pooled, and a sample was inoculated into PK cells. After complete virus degeneration of the cell monolayers, the virus was harvested, and 2×10^5 PFU were injected i.c. into day-old chickens, and the chickens were observed for signs of disease or death. The brains of dead or moribund chickens were homogenized, the virus in the brains was amplified in PK cells, and the LD_{50}s were determined as described in Materials and Methods.

[b] Number of chickens that became moribund or died/number of chickens injected.

[c] ND, not determined.

small percentage of chickens inoculated i.c. with either Bartha or Bartha 43/25a will die. However, even multiple serial passages in chicken brains of the virus recovered from these chickens will not restore virulence to the virus.

To identify further the region of the genome in which the defect in the Bartha strain is localized, the same experiment was performed with smaller *Bam*HI restriction fragments of the wild-type PrV genome that span the sequences within *Bgl*II fragment B (approximate map units 0.298 to 0.505). Virulence was restored to Bartha 43/25a by *Bam*HI fragment 4 (Table 2), indicating that a defect involved in virulence is present in this strain within the sequences of *Bam*HI fragment 4. Analysis with restriction enzymes of the *Bam*HI fragment 4 derived from the DNA of the Bartha and PrV(Ka) strains did not reveal differences in restriction patterns that might indicate that a deletion was present in the genome of the Bartha strain (data not shown).

Individual plaques were picked from the population of virions isolated from the brains of chickens inoculated with the Bartha 43/25a strain that had been rescued with *Bam*HI fragment 4, and the LD_{50} of these plaque-purified virions (Bartha 43/25a *Bam*HI-4/7, -4/17, and -4/18) was determined (Table 3). Although these plaque isolates were virulent, their LD_{50} was approximately 50-fold higher than that of the virulent PrV(Ka) laboratory strain or two other primary virus isolates (PrV53 and PrV90) (Table 3).

Virulence of the marker-rescued Bartha strains for pigs. Although it was unrealistic because of the expense to test virulence in pigs during the initial phase of these studies, once we had obtained marker-rescued virus isolates that were virulent for chickens, we proceeded to test them for virulence in pigs—the natural host. Whereas the Bartha and Bartha 43/25a (Bartha with an intact U_S) strains were nonvirulent, the doubly rescued Bartha strain (Bartha 43/25a Bam HI-4/7) was virulent for piglets (Table 4). Thus, the defect in the Bartha genome that is rescued by sequences present in *Bam*HI fragment 4 is necessary for the expression of virulence in both chickens and the natural host.

Growth in vitro of the marker-rescued Bartha strains in various cell types. The ultimate aim of the experiments described in this paper is to identify the functions of PrV that affect virulence without affecting the ability of the virus to grow in cell culture. Therefore we compared the ability of the avirulent and virulent rescued Bartha strains to grow in different cell types in cell culture. We also determined their ability to grow in the brains of chickens after i.c. inoculation.

TABLE 2. Marker rescue of Bartha 43/25a by various *Bam*HI fragments[a]

*Bam*HI fragment	Map units	Virulence[b]
2	0.271–0.406	0/8
9	0.406–0.435	0/8
11	0.435–0.455	0/8
15	0.455–0.460	0/8
4	0.460–0.524	4/8
None		0/8

[a] RK cells were cotransfected with Bartha 43/25a DNA and the indicated cloned *Bam*HI fragment. After complete degeneration of the monolayers (4 days), 10^5 PFU of the virus produced by the cells were injected i.c. into day-old chickens. Three days later the chickens were killed, the brains were homogenized, and a portion was inoculated into PK culture cells; the cultures were incubated until complete virus-induced degeneration of the monolayers had occurred. The recovered virus (10^5 PFU) was reinoculated into day-old chickens.

[b] Number of chickens that died/number of chickens injected.

TABLE 3. Virulence of different doubly rescued Bartha virions

Virus strain or mutant	Virulence (LD_{50})[a]
PrV(Ka)	$>10^2$
PrV53	$>10^2$
PrV90	$>10^2$
Bartha	$<10^6$
Bartha 43/25a	$<10^6$
Bartha 43/25a *Bam*HI-4/7	8×10^3
Bartha 43/25a *Bam*HI-4/17	2×10^3
Bartha 43/25a *Bam*HI-4/18	5×10^3

[a] The LD_{50} (PFU) of the different virus strains was determined as described in Materials and Methods.

We have previously shown that the Bartha strain grows as well as PrV in both RK and PK cells, but that it is not released from RK cells (2). Bartha, Bartha 43/25a (Bartha with an intact U_S), and Bartha 43/25a Bam HI-4/7 (doubly rescued Bartha) strains grow equally well, as does PrV(Ka), in all three cell types, including CEF (Table 5). However, there is a significant difference in their ability to grow in brains of day-old chickens when inoculated i.c. Bartha does not appear to replicate at all after i.c. inoculation of chickens, Bartha 43/25a can replicate to a limited extent, and the doubly rescued virulent Bartha 43/25a Bam HI-4/7 replicates almost as well as virulent PrV(Ka). Thus, whereas no difference in the ability of virulent and avirulent variants of the Bartha strain to replicate in CEF was observed, a correlation between their virulence and their ability to replicate in chicken brains was found.

Functions encoded by the sequences in *Bam*HI fragment 4. The experiments summarized in Tables 2 and 3 show that virulence can be restored to the Bartha 43/25a strain by cotransfection with *Bam*HI fragment 4. The experiments described below were performed to identify the functions encoded in *Bam*HI fragment 4. The aim of these experiments was to identify the functions that may be defective in the Bartha strain that contribute to its lack of virulence.

We have shown previously that mutants belonging to four different complementation groups, 11, 12, 13, and 14, can be rescued by cotransfection with *Bam*HI fragment 4 (3, 10). The mutants in these four complementation groups complement one another well and recombine with one another at relatively high frequency, indicating that complementation is not intragenic (3, 10). Mutants in three out of these four complementation groups are defective in the assembly of capsids; one mutant encodes a temperature-sensitive capsid protein (142K) (14). The mutants in all four complementation groups are DNA positive and all are defective in the cleavage

TABLE 4. Virulence of marker-rescued Bartha for pigs[a]

Virus strain	Virulence[b]	
	Death	Signs of Disease
Bartha	0/10	0/10
Bartha 43/25a	0/12	0/12
Bartha 43/25a *Bam*HI-4/7	4/8	2/8
PrV(Ka)	7/11	4/11

[a] Four-week old pigs were inoculated with approximately 10^8 PFU intranasally as well as 10^8 PFU subcutaneously and observed for signs of disease and death.

[b] Number of pigs that died or showed signs of disease (loss of appetite, respiratory signs, fever, loss of weight)/number of pigs inoculated.

TABLE 5. Virus replication in various cell types in culture and in the brains of day-old chickens[a]

Virus strain	Virus yield (PFU/culture)			Virus yield (PFU/ml) from chicken brains[b]
	CEF	PK	RK	
Bartha	9.8×10^8	8.9×10^{10}	8.6×10^9	$<10^2$
Bartha 43/25a	4.1×10^8	9.6×10^9	8.0×10^9	9.0×10^2
Bartha 43/25a *Bam*HI-4/7	6.2×10^8	9.8×10^9	1.1×10^{10}	1.2×10^5
PrV(Ka)	3.1×10^8	3.5×10^{10}	2.4×10^{10}	1.0×10^6

[a] Cells in culture (2×10^6) were infected with the indicated virus strains at a multiplicity of 3 PFU per cell and incubated for 48 h in Eagle medium at 37°C. The cells were scraped into the culture fluid, the samples were sonicated, and cellular debris was removed by centrifugation. The virus yield was determined by plaque assay in PK cells.

[b] Three chickens were injected i.c. with 10^4 PFU of each strain. Three days later the chickens were killed, the brains were collected, and the virus was titrated as described in Materials and Methods. The results are expressed as PFU per milliliter of 20% brain suspension of the three brains obtained from each group.

and encapsidation of concatemeric DNA (13, 14). Thus, mutants belonging to all four complementation groups that are rescued by *Bam*HI fragment 4 are defective in the assembly of nucleocapsids.

*Bam*HI fragment 4 appears to encode only four mRNAs (Fig. 2). Thus, at both early (2 h) and late (5 h) stages of infection, the same four RNA species can be detected in the cytoplasm of the infected cells. Furthermore, the same RNA species are polysome associated at both early and late stages of infection. Overexposure of autoradiograms prepared with several different RNA preparations did not reveal the consistent presence of any other RNA bands.

The mRNA species originating from *Bam*HI fragment 4 were selected by hybridization and were translated in vitro. Figure 3 shows the polyacrylamide gel electrophoresis pattern of the proteins synthesized by reticulocyte lysates primed with total cytoplasmic RNA from infected cells as well as those primed with the same RNA that had first been selected by hybridization to *Bam*HI fragment 4. The polyacrylamide gel electrophoresis pattern of the proteins synthesized in vitro with two different RNA preparations obtained from the cytoplasm of infected cells varied somewhat, probably because of differences in the degree of virus-induced degradation of cellular mRNAs between the two experiments. Translation of the RNA that had been selected with *Bam*HI fragment 4 yielded three protein bands, approximately 142, 62, and 32 kilodaltons in size; these are also the approximate molecular sizes of the major virus capsid proteins (14). (Although some other minor bands can also be seen, these were not reproducibly obtained and were seen also in some cases when RNA selected with other restriction fragments of PrV were translated in vitro.) All three major proteins synthesized in vitro by RNA selected with *Bam*HI

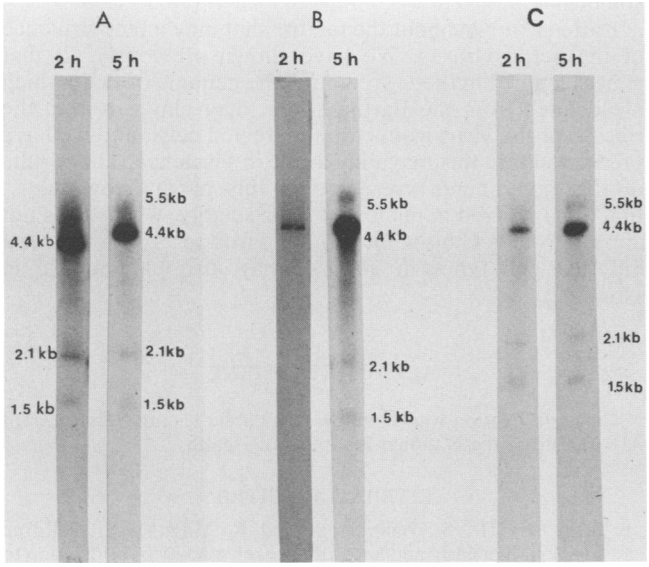

FIG. 2. Autoradiogram of Northern blots of RNA obtained from infected cells probed with *Bam*HI fragment 4. PK cells were infected (10 PFU/cell) and at the indicated times after infection (2 or 5 h), the cells were harvested, and the nuclei were separated from the cytoplasm. In some cases the polysomes were isolated from the cytoplasmic fractions. The RNA was purified as described previously (6). (A and B) Results obtained in two different experiments with cytoplasmic RNA. (C) Results obtained with polysomal RNA isolated from the infected cells. kb, Kilobases.

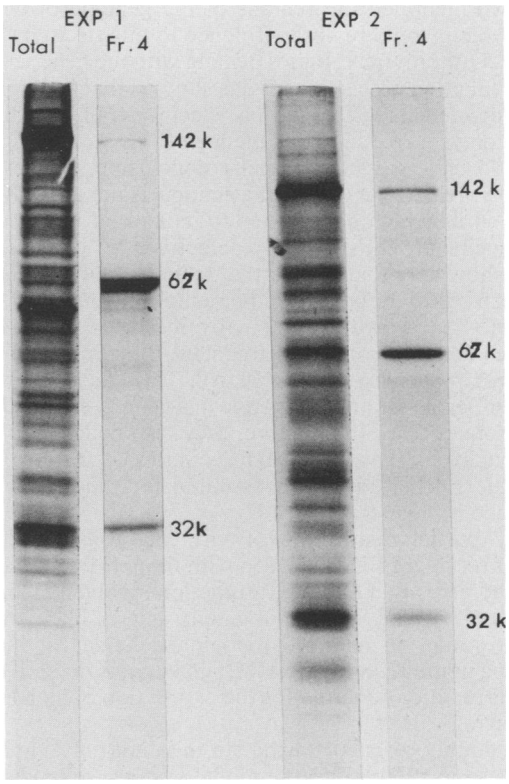

FIG. 3. Autoradiograms of the in vitro translation products obtained with RNA selected with *Bam*HI fragment 4. Cytoplasmic RNA preparations were obtained 5 h after infection as described in the legend to Fig. 2 and were hybridized to cloned *Bam*HI fragment 4. After elution from the DNA, the RNA was translated in vitro as described in Materials and Methods. The proteins were analyzed by polyacrylamide gel electrophoresis. Lanes: Total, translation product of total unselected RNA; Fr. 4, translation product of RNA selected by hybridization to *Bam*HI fragment 4. k, Kilodaltons.

fragment 4 were immunoprecipitated by rabbit sera prepared against purified capsids of PrV (data not shown). These results indicate that the sequences in *Bam*HI fragment 4 encode three major capsid proteins. Because mutants in at least four different complementation groups, as well as four different mRNAs, originate from that region of the genome, another protein (in addition to the three capsid proteins) must also be specified by this region of the genome. Although we do not know the identity of this protein, the evidence obtained from the analysis of temperature-sensitive mutants (see above) indicates that this protein is also involved either in capsid assembly or in cleavage of concatemeric DNA (i.e., nucleocapsid assembly).

The results described above show that *Bam*HI fragment 4, which restores virulence to Bartha 43/25a, appears to encode only four genes, all of which are involved in nucleocapsid assembly. These results suggest that the function deficient in the Bartha strain that is required for the expression of virulence is involved in nucleocapsid assembly. It appears therefore that in certain cases modification of a protein involved in nucleocapsid assembly may play a role in the expression of virulence.

DISCUSSION

The experiments presented in this paper deal with the identification of the lesions contributing to the lack of virulence of the Bartha vaccine strains of PrV. As a first step, we identified the regions of the genome of wild-type virus which would restore virulence to this strain by marker rescue. Our previous studies dealing with the genetic basis of the attenuation of vaccine strains showed that a region in the U_S is deleted from the Bartha vaccine strain and that this region plays a role in the virulence of this strain (16). Berns et al. (4) have reached a similar conclusion. However, the deletion in the U_S in the Bartha genome is not the only defect in this virus which contributes to its lack of virulence (16); this vaccine strain is multiply defective.

We show here that the Bartha strain to which an intact U_S has been restored, but not the parental Bartha strain, can be converted to a virulent strain by marker rescue with sequences derived from *Bgl*II fragment B or *Bam*HI fragment 4 (approximate map units 0.460 to 0.505). These results show that the Bartha strain is defective in a gene(s) encoded within these sequences. Furthermore, they also confirm our previous conclusion that the sequences in the U_S that are deleted from the Bartha strain are essential for the expression of virulence in this strain.

Only partial restoration of virulence was obtained by marker rescue of Bartha 43/25a with *Bam*HI fragment 4. It is clear therefore that although a function involved in virulence is located within *Bam*HI fragment 4 it is likely that the Bartha strain, in addition to being deficient in functions mapping in the U_S and *Bam*HI fragment 4, is defective in yet another function required for the expression of high levels of virulence.

The doubly rescued Bartha strain is not only virulent for chickens inoculated i.c. (the model system we have used to test virulence) but has also acquired virulence for pigs (the natural host). Whereas the parental Bartha and the Bartha with an intact U_S were both avirulent for chickens and pigs, the doubly rescued Bartha was virulent for both. Thus, good correlation was obtained in this case between virulence for chickens and pigs.

No difference in the ability of all three variants to grow in cell culture was observed. However, whereas all three variants replicated well in CEF in culture, only the virulent doubly rescued Bartha replicated to high titers when inoculated directly into the brains of chickens. Thus, whereas the functions defective in Bartha do not affect its ability to grow in chicken cells, the virus cannot replicate in chickens in vivo.

*Bam*HI fragment 4 appears to specify four proteins only, all of which are involved in nucleocapsid assembly. This can be deduced from the following observations. (i) Mutants belonging to four different complementation groups are marker rescued by sequences within *Bam*HI fragment 4 (10), and mutants in all four of the complementation groups are defective in the assembly of nucleocapsids (13, 14). (ii) Only four mRNAs originate from that region of the genome (Fig. 2). These mRNAs are translated into three major capsid proteins of PrV (Fig 3); all three proteins are immunoprecipitated by antisera against purified PrV capsids.

Because *Bam*HI fragment 4 appears to contain only four genes, all of which are involved in nucleocapsid assembly, and because a defect which contributes to the lack of virulence of the Bartha strain maps in *Bam*HI fragment 4, it is likely that in this strain a protein that plays a role in nucleocapsid assembly is modified and contributes to the lack of virulence. The kind of role that modification in a protein involved in nucleocapsid assembly may play in virulence is uncertain. Since nucleocapsid assembly appears to occur in association with the cellular matrix (5), it is possible that interactions between virus and cell proteins during the assembly of the nucleocapsids are necessary. Whereas the Bartha strain grows well in cell culture (Table 5) and nucleocapsids are assembled efficiently in vitro in cells infected with this virus (unpublished results), this process could nevertheless be defective in certain specific target cells in vivo. Alternatively, the uncoating of the parental nucleocapsids, a process which also is mediated by cellular functions, may not occur readily in certain target cells infected with a virus whose capsid proteins have been modified somewhat.

Little is known about the factors that may affect virulence of the herpesviruses. We have shown previously (2) that genes encoded in the U_S region of the genome of PrV, which are deleted from the Bartha strain, may play a role in the release of the virus from certain infected cells, and we have postulated that this may play a role in virulence. The results of the experiments presented in this paper show that a function involved in nucleocapsid assembly, which does not appear to affect the ability of the virus to grow in several different cell types in culture, may also play a role in virulence.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-10947 from the National Institutes of Health.

LITERATURE CITED

1. **Belle Isle, H., S. Venkatesan, and B. Moss.** 1981. Cell-free translation of early and late mRNAs selected by hybridization to cloned DNA fragments derived from the left 14 million to 72 million daltons of the vaccine virus genome. Virology **112**:306–317.
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., P. Hoffmann, L. Brown, L. Feldman, and M. L. Blankenship.** 1982. Partial characterization of temperature-

sensitive mutants of pseudorabies virus. Virology **122**:251–267.
4. **Berns, A., A. Van der 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. **Bibor-Hardy, V., M. Pouchelet, E. Pierre, M. Herzberg, and R. Simard.** 1982. The nuclear matrix is involved in herpes simplex virogenesis. Virology **121**:296–306.
6. **Deatly, A. M., and T. Ben-Porat.** 1985. Relation between the levels of mRNA abundance and kinetics of protein synthesis in pseudorabies virus infected cells. Virology **143**:558–568.
7. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
8. **Gustafson, D. P.** 1975. Pseudorabies, p. 391–410. *In* H. W. Dunne and A. D. Leman (ed.) Diseases of swine. The Iowa State University press, Ames.
9. **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 **42**:583–590.
10. **Ihara, S., B. F. Ladin, and T. Ben-Porat.** 1982. Comparison of the physical and genetic maps of pseudorabies virus shows that the genetic map is circular. Virology **122**:268–278.
11. **Kaplan, A. S., and A. E. Vatter.** 1959. A comparison of herpes simplex and pseudorabies virus. Virology **7**:394–407.
12. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **119**:1617–1624.
13. **Ladin, B. F., L. M. Blankenship, and T. Ben-Porat.** 1980. Replication of herpesvirus DNA. V. The maturation of concatemeric DNA of pseudorabies virus to genome length is related to capsid formation. J. Virol. **33**:1151–1164.
14. **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.
15. **Lomniczi, B., M. L. Blankenship, and T. Ben-Porat.** 1984. Deletions in the genome of pseudorabies virus vaccine strains and existence of four isomers of the genome. J. Virol. **49**:970–979.
16. **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.

16a. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**:493–497.

17. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments to nitrocellulose filters. Proc. Natl. Acad. Sci. USA **77**:5201–5205.