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Genetic Basis of the Neurovirulence of Pseudorabies Virus

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Lomniczi et al. (J. Virol. 49:970–979, 1984) have shown previously that two attenuated vaccine strains of pseudorabies virus have a similar deletion in the short unique (U_S) region of the genome. The region which is deleted normally codes for several translationally competent mRNAs. As expected, these mRNAs are not formed in the cells infected with the vaccine strains. The function specified by these mRNAs is thus not necessary for growth in cell culture. Using intracerebral inoculation of 1-day-old chicks as a test system, we have attempted to determine whether a gene within the region that is missing from the attenuated strains specifies functions that are required for the expression of virulence. An analysis of recombinants between the Bartha vaccine strain and a virulent pseudorabies virus strain (having or lacking a thymidine kinase gene [TK^+ or TK^-]) revealed the following. (i) None of the recombinant plaque isolates that were either TK^- or which had a deletion in the U_S was virulent. (ii) Not all recombinant plaque isolates which were both TK^+ and had an intact U_S were virulent. These results indicate that both thymidine kinase activity and an intact U_S were necessary but not sufficient for the expression of virulence. Marker rescue experiments involving cotransfection of the Bartha strain DNA and a restriction fragment spanning the region of the genome that was missing from the Bartha strain resulted in the isolation of virions to which an intact U_S had been restored. These virions were not virulent but had an improved ability to replicate in the brains of chicks compared with that of the parental nonrescued Bartha strain. Our results show that genes in the U_S region, which are missing from the Bartha strain, were necessary for virulence but that this strain was also defective in other genes required for the expression of virulence. Thus, the virulence of pseudorabies virus, as measured by intracerebral inoculation into chicks, appears to be controlled multigenically.

Pseudorabies virus (PrV) (*Herpesvirus suis*) causes Aujeszky's disease in swine which results in significant economic losses. Cattle are also occasionally infected by virus-carrier pigs, but swine are probably the only natural host, and latently infected swine are the main reservoir of the virus (1). Because of the economic losses caused by PrV, vaccination of swine herds, by using attenuated (nonvirulent) strains of PrV, is practiced in most countries, including the United States.

There are several attenuated strains of PrV currently being used as vaccines. Many of the biological properties of these vaccine strains vary, and the molecular basis for the attenuation of each is unknown. Since most of the strains grow well in cells in culture (for a review, see Baskerville et al. [1]), it is likely that the gene functions essential for the expression of virulence are not essential for growth in vitro. The availability of attenuated vaccine strains provides a means of studying the genetic basis of virulence. We therefore selected two of these strains, Bartha (or K strain) and Norden (a derivative of the BUK strain), to attempt to identify the genes of PrV that are essential for the expression of virulence. Both of these highly passaged, attenuated viruses are avirulent in piglets but provide immunity against superinfection with virulent virus (1).

Lomniczi et al. (12) have reported previously that these two vaccine strains have a similar deletion in the short unique (U_S) region of their genomes. Because these two independently isolated, attenuated strains exhibited a similar deletion, it appeared possible that genes within the regions of the genome that are deleted specify functions required for the expression of virulence. The experiments presented in this paper, in which we have examined this question by using mainly the Bartha strain, show that this is the case. However, although the expression of the genes which have been deleted from the Bartha strain appear to promote the ability of the virus to replicate in chick brains (the model system for virulence used in our studies), the genome of the Bartha strain appears to be defective in at least one other function required for the expression of virulence. Thus, virulence of PrV is polygenically controlled, as is the case for reoviruses (27), myxoviruses (19), and bunyaviruses (20). (A preliminary report of this work was presented at the International Herpesvirus Workshop, 8th, Oxford, England, 1983).

MATERIALS AND METHODS

Virus strains and cell culture. PrV(Ka) is a strain which has been carried in our laboratory for 25 years; its origin is uncertain (9). PrV53 is a recent field isolate. Both strains are virulent in young swine. The Norden and Bartha avirulent vaccine strains were received from P. S. Paul. The origins of these strains have been described previously (13). The region in the U_S sequence of the genome which is deleted from these two strains is shown in Fig. 1. Thymidine kinase-lacking (TK^-) virions were isolated by growth in cells exposed to increasing concentrations of arabinosyl-thymine. In all of the TK^- strains used in this study, the TK lesion is located between ca. 0.43 and 0.45 map units (3; unpublished data). Cells were cultivated in Eagle synthetic medium (6) containing 3% dialyzed bovine serum; virus was titrated by plaque assay on pig kidney (PK) cells (8).

Enzymes and chemicals. Restriction endonucleases were purchased from the Bethesda Research Laboratories, protease K was purchased from Worthington Diagnostics, and nuclease-free pronase was purchased from Calbiochem. [α-^{32}P]dCTP was purchased from ICN Pharmaceuticals Inc., and cycloheximide was purchased from Boehringer-Mannheim Corp.

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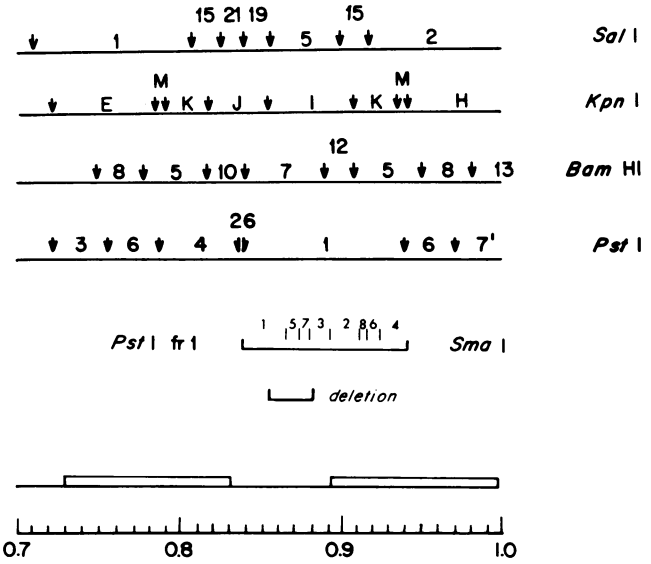

FIG. 1. Restriction maps of the regions of the PrV genome which span the inverted repeats and the U_S. The open rectangles represent the inverted repeats; the line between them represents the U_S. The arrows indicate the cleavage sites on the standard PrV genome of the indicated enzymes; letters or numbers represent fragment designations. The *Sma*I restriction map of *Pst*I fragment 1 obtained from standard virus, as well as the region in that fragment that is deleted in the Bartha and Norden strains, is indicated.

Purification of virions. A continuous line of PK cells (PK_{15}) was infected (multiplicity of infection, 0.5 PFU per cell) and incubated for 48 h in Eagle medium at 37°C. Virions were purified either as described previously (4) or by the following rapid partial purification method. Cells were scraped into the medium, and the sample was sonicated for 1 min and clarified by centrifugation at 5,000 × *g* for 10 min; the resulting supernatant containing the virus was again centrifuged on a 30% sucrose cushion at 20,000 × *g* for 1 h.

Extraction of DNA. DNA extraction was carried out as follows. Sodium sarkosinate (final concentration, 2%) was added to samples which were heated (60°C for 15 min) and digested with nuclease-free pronase (1 mg/ml) for 2 h. The DNA was then extracted four times with phenol-chloroform-isoamyl alcohol (25:24:1) and dialyzed against a buffer (0.1 M Tris, 0.001 M EDTA [pH 7.6]).

Restriction enzyme digestion and gel electrophoresis of DNA fragments. Digestion and agarose gel electrophoresis of virus DNA were carried out as described by Rixon and Ben-Porat (18). Filter strips to which restriction fragments of PrV were fixed were prepared by the method of Southern (21).

Nick-translation of cloned PrV DNA restriction fragments. PrV DNA restriction fragments, cloned in pBR325 as described previously (10), were nick-translated by the method of Rigby et al. (17).

Determination of LD_{50}. Tenfold dilutions of the virus stocks were injected intracerebrally (IC) (0.05 ml) into 1-day-old Hubbard strain chicks (five chicks per dilution) or intraperitoneally (0.1 ml) into 3-week-old BALB/c mice (five mice per dilution). The number of animals which were dead by 2 weeks after inoculation was determined, and the 50% lethal dose (LD_{50}) was calculated.

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 (24).

Selection of mRNA, translation in vitro, and polyacrylamide gel electrophoresis. Selection of mRNA was performed as described by Belle-Isle et al. (2). Translation in vitro was performed in mRNA-dependent rabbit reticulocyte systems (14), and polyacrylamide gel electrophoresis was performed as described previously (10).

Assay of thymidine kinase activity. Thymidine kinase activity was assayed by the method of Zavada et al. (28).

RESULTS

Identification of the mRNA species specified by sequences in the U_S region of the genomes of wild-type, Norden, and Bartha strains. The mRNA species accumulating in PK cells infected with PrV(Ka), Norden, or Bartha strains were identified by Northern blots (24), using nick-translated restriction fragments as probes. The species of mRNAs originating from *Bam*HI fragment 1 were very similar in all three strains (Fig. 2). However, although in RNA preparations from cells infected with wild-type virus at least eight major mRNA species which hybridized to sequences originating from *Bam*HI fragment 7 (which spans the deletion in Bartha and Norden strains) were observed, in similar preparations from cells infected with Norden or Bartha strains, only four species were observed. Thus, the region of the U_S that has been deleted from the two vaccine strains codes for several mRNAs which are normally expressed in PK cells infected with wild-type virus.

Translation of mRNA originating from the U_S specified by wild-type, Bartha, and Norden strains. mRNA present in PK cells infected with the different PrV strains was selected by hybridization with appropriate cloned restriction fragments

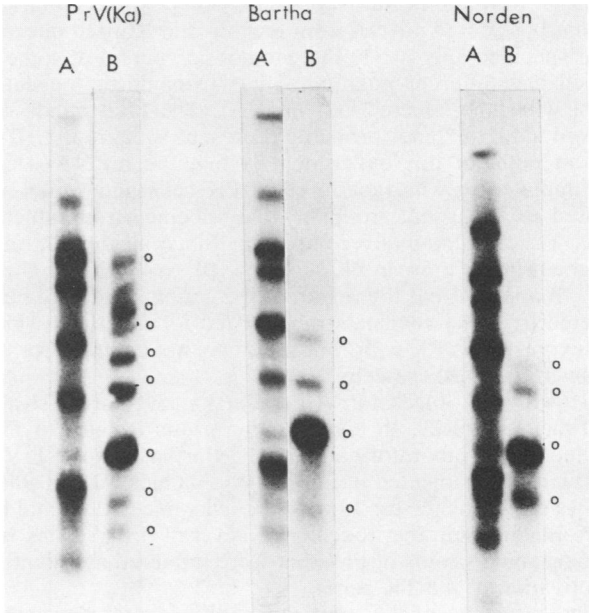

FIG. 2. Autoradiogram of Northern blots of RNA obtained from cells infected with different virus strains. PK cells were infected (multiplicity of infection, 5 PFU per cell) with different PrV strains. At 6 h postinfection, the cells were harvested, the nuclei were separated from the cytoplasmic fractions, and the RNA in the cytoplasmic fractions was purified as described previously (5a). The RNA was denatured, electrophoresed, blotted onto nitrocellulose filters, and probed with nick-translated, cloned PrV DNA *Bam*HI fragments 1 (lanes A) or 7 (lanes B).

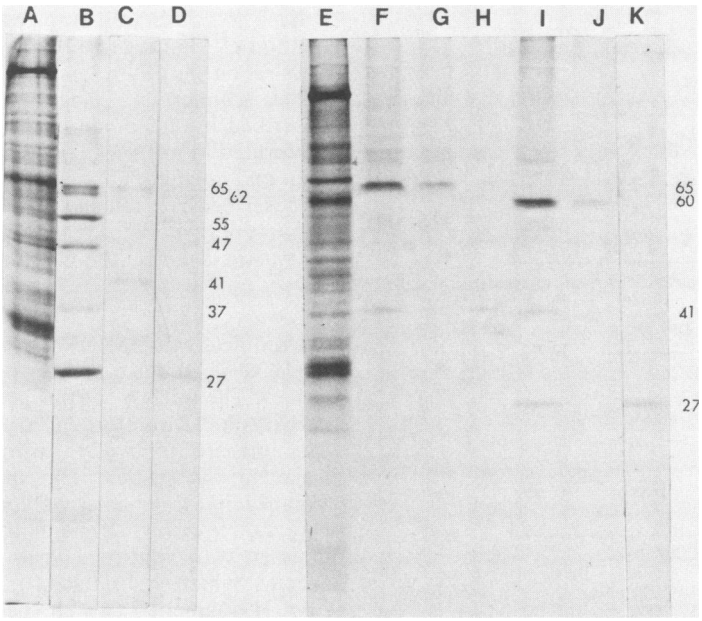

FIG. 3. Autoradiogram of the in vitro translation products obtained with selected mRNA. RNA preparations were obtained as described in the legend to Fig. 2. RNA was selected by hybridization to cloned restriction fragments of PrV DNA, eluted, and translated in a rabbit reticulocyte system. The proteins were analyzed by polyacrylamide gel electrophoresis. The translation product of total cytoplasmic RNA obtained from PrV(Ka)-infected cells is shown in lanes A and E. Translation products of mRNAs from PrV(Ka)-infected cells selected by hybridization to *Bam*HI fragment 7, fragment 10, and fragment 12, are shown in lanes B, C, and D, respectively. Translation products of mRNAs from Norden-infected cells selected by hybridization to *Bam*HI fragment 7, fragment 10, and fragment 12 are shown in lanes F, G, and H, respectively, and those from Bartha-infected cells selected by hybridization to *Bam*HI fragment 7, fragment 10, and fragment 12 are shown in lanes I, J, and K, respectively.

and translated in vitro. The mRNA derived from cells infected with PrV(Ka) and selected for with *Bam*HI fragment 7 translated into six major polypeptides (Fig. 3). Similarly selected mRNA from Bartha- and Norden-infected cells specified only two to three major polypeptides, some of which had different migration rates from those translated from wild-type-infected cell mRNA. (The faint bands observed in all the lanes probably represent background.) The salient point of this experiment is that the mRNA which accumulated in wild-type-infected PK cells and which appeared to be absent from the two vaccine strain-infected cells was translationally competent. Since all three strains reached similar titers in PK cells (2×10^2 to 4×10^2 PFU per cell), it appears that the region of the genome that had been deleted from the vaccine strains coded for functions which are expressed in PK cells, but which are not essential for the virus growth in these cells.

Growth of IC-injected PrV(Ka), Bartha, and Norden strains in 1-day-old chicks. In contrast to various isolates of PrV (including our laboratory strain), the Bartha strain of PrV is avirulent when injected IC into 1-day-old chicks (11). Table 1 shows the virulence for 1-day-old chicks injected IC and for mice injected intraperitoneally of several virus strains (including some strains of PrV that were rendered avirulent by inactivation of the TK gene).

The virulence of TK mutants of the strains was tested because of several reports (7, 16, 22, 23) that TK mutants of herpes simplex virus and PrV are avirulent and do not colonize the ganglia of rabbits or mice. Indeed, none of the PrV TK mutants were virulent when injected IC into the chicks, nor were they virulent when injected intraperitoneally into mice (Table 1). The Bartha strain was also avirulent when injected IC into chicks but exhibited virulence in mice. The Norden strain was virulent for mice and had reduced virulence for chicks. These results corroborate previous reports (see, for example, references 11 and 15) dealing with the virulence of these vaccine strains in various laboratory animals.

Not surprisingly, the ability of the virus to multiply in chick brains correlated with the ability of the virus to kill the animals (Fig. 4). Thus, Bartha and PrV(Ka) TK^- strains were cleared rapidly from the brains, whereas the parental PrV(Ka) strain, as well as the PrV(Ka) TK^- strain which had been rescued with the appropriate restriction fragment to TK^+ (3), grew well in chick brains. The Norden strain replicated to some extent (data not shown) but was cleared thereafter from the brains of surviving chicks (some of the chicks infected with the Norden strain died).

The ability of the different viruses to replicate in chick brains and to kill the chicks appears to mimic the virulence of these viruses for piglets. It has been well established that the two vaccine strains have reduced virulence for piglets

TABLE 1. Susceptibility of mice and chicks to different PrV strains[a]

Virus strain	LD_{50} (PFU) in:	
	Mice (intraperitoneal)	1-day-old chicks (IC)
PrV(Ka)	1×10^4	2×10^2
PrV(Ka) TK^-	$>10^6$	$>10^6$
PrV(Bartha)	5×10^4	$>10^6$
PrV (Bartha) TK^-	$>10^6$	$>10^6$
PrV(Norden)	4×10^4	4×10^3
PrV(Norden) TK^-	$>10^6$	$>10^6$

[a] Animals were inoculated with 10-fold dilutions as described in the text (at least five mice or chicks were used per dilution; the route of inoculation is indicated in parentheses), and the LD_{50}s were calculated.

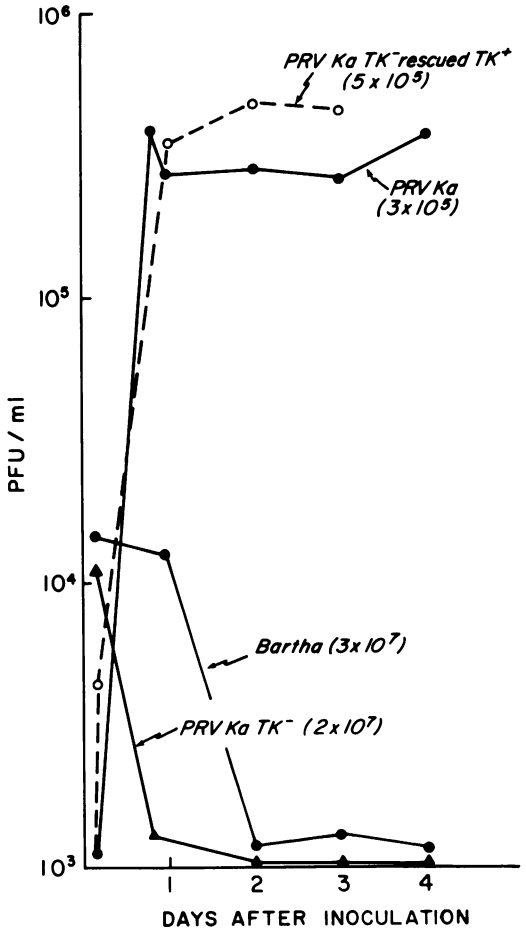

FIG. 4. Replication of different strains of PrV after IC inoculation into 1-day-old chicks. The chicks were inoculated IC as indicated. At daily intervals, some chicks were killed; homogenates of their brains were prepared and plaque assayed as described in the text. Numbers in parentheses indicate the doses, in PFU per chick.

(1). The TK⁻ strains that we have used are also avirulent for pigs (unpublished data). IC inoculation into chicks thus appears to be a good test model system for PrV virulence, and we chose it to try to localize the regions of the genome which are defective in the avirulent Bartha strain. Eventually, the ability of various virulence mutants to grow in chick brains (and to kill these animals) will be correlated with their ability to kill piglets.

Do the genes in the U_S which are deleted from the vaccine strains play a role in virulence? Because two vaccine strains which were derived independently have the same deletion, it seemed possible that the deleted region codes for functions involved in virulence. In the experiments described below, we attempted to ascertain whether the sequences that are deleted from Bartha and Norden vaccine strains are necessary for the expression of virulence, i.e., whether the genomes of all virulent virions contain an intact U_S, and also whether an intact U_S region (as well as a functional TK gene) is sufficient for virulence, i.e., whether all virions that have both the intact U_S region and the functional TK gene are virulent.

Two approaches were used to identify the region of the PrV genome which contains genes the expression of which is required for virulence. The first approach involved an analysis of recombinants between two avirulent strains or between a virulent and an avirulent strain; the second involved marker rescue by using cotransfection of intact DNA from avirulent virus with various restriction fragments of the DNA from a virulent virus.

TABLE 2. Ability of recombinants between PrV(Ka) TK⁻ and Bartha strain virions to kill chicks[a]

Virus strain	No. of dead chicks at following PFU inoculation:			
	10^2	10^3	10^4	10^5
Bartha	0	0	0	0
PrV(Ka) TK⁻	0	0	0	0
PrV(Ka) TK⁻ × Bartha	ND[b]	6	6	8

[a] For each test performed, eight 1-day-old chicks were injected IC and kept for 2 weeks.
[b] ND, Not done.

(i) Analysis of recombinants between the Bartha and PrV(Ka) TK⁻ strains. In these experiments, cells were coinfected with two avirulent viruses, a PrV(Ka) TK⁻ mutant and the Bartha vaccine strain (Table 1; Fig. 4), and the virulence of the progeny recombinant population was tested. Although, as expected, the Bartha strain and the PrV(Ka) TK⁻ mutant grown individually were completely avirulent when inoculated IC into 1-day-old chicks, the progeny from cells coinfected with these two virus strains was virulent (Table 2).

With the expectation that only virulent viruses would replicate in chick brains (which would provide a method for selecting virulent recombinants), we analyzed the virions present in the brains of moribund chicks which had been inoculated with 2×10^5 PFU of recombinant virus. A 20% suspension of these brains was made and plaque assayed. The titer of virus obtained from brains of chicks inoculated with the population of recombinants was, as expected, higher than that obtained with each of the parental strains which were rapidly cleared from the brains (Table 3). Individual plaques of virus obtained from the brains of chicks infected with recombinant virus were picked; the virions in the plaques were amplified and analyzed with restriction enzymes (to detect the characteristic Bartha-like deletion in the U_S region) and for thymidine kinase activity.

A total of 35 virion populations obtained from randomly selected individual plaques isolated from the brains of seven moribund chicks were analyzed for thymidine kinase activity; 3 of these populations were TK⁻. Of the virion popula-

TABLE 3. Recovery of virions from chick brains 3 days after inoculation with PrV(Bartha), PrV(Ka) TK⁻, and a population of virions containing recombinants between the two[a]

Virus inoculated	PFU per brain	TK⁻ virions (No. TK⁻/ no. tested)	Virions with deleted U_S (No. with deletions/ no. tested)
Bartha × PrV(Ka) TK⁻	5.0×10^5	3/35	8/112
Bartha	1.1×10^2	ND[b]	10/10
PrV(Ka) TK⁻	9.5×10^1	ND	ND

[a] Chicks were inoculated IC with 2×10^5 PFU. At 3 days postinfection, the chicks were killed, and the amount of virus in their brains was assayed. Virions in individual plaques chosen at random were assayed for thymidine kinase activity, and the DNA was analyzed by restriction enzymes to ascertain the presence of an intact U_S.
[b] ND, Not done.

tions obtained from 112 randomly selected individual plaques that were analyzed with restriction endonucleases, 8 had a Bartha-like deletion in the U_S region (Table 3). The finding that a relatively large proportion of the viruses isolated from the brains of the chicks were avirulent (ca. 16% were either TK^- or had a deleted U_S) indicates that although the selection procedure enriches for virulent virus, the virulent virus present in the population of recombinants may provide helper functions to the avirulent viruses, allowing the viruses to replicate in the brains.

Figure 5 illustrates typical cleavage patterns obtained with some recombinants with either an intact or deleted U_S. Virion populations were also analyzed for virulence by IC injection into 1-day-old chicks (Table 4). The salient finding from these experiments was that none of the virions which were TK^- or which had a deletion in the U_S were virulent. Furthermore, 3 of 16 plaques analyzed which contained an intact U_S and which were also TK^+ were completely avirulent; the others had different LD_{50}s.

To be certain that the avirulent recombinant virions with an intact U_S had acquired a complete PrV(Ka)-like U_S, we analyzed this region of the genome in greater detail (Fig. 6). The *Pst*I fragment I derived from the recombinant virion genomes was excised from gels and redigested with *Sma*I (see Fig. 1 for restriction maps). This enzyme cleaves the U_S of wild-type PrV into several fragments. The Bartha strain lacked two of these fragments and had shortened versions of two other fragments (Fig. 6, lane B). Several of the virulent, as well as avirulent, recombinants which appeared to have an intact U_S were also tested; all had an apparent PrV(Ka)-like U_S (Fig. 6, lanes C to G). Thus, it appears that the presence of an intact U_S, as well as the expression of thymidine kinase activity, is probably not sufficient for the expression of virulence of the virus for chicks.

In the experiment summarized in Tables 3 and 4, only

TABLE 4. Characteristics of virions isolated from brains of chicks injected with PrV(Ka) TK^- × Bartha recombinants[a]

Recombinant plaque	Thymidine kinase activity	Wild-type U_S	Lethality to chicks (no. of dead chicks)[b]	LD_{50} in chicks
L8	−	−	0	$>10^6$
M2	−	+	0	$>10^6$
P5	−	+	0	$>10^6$
K4	+	−	0	$>10^6$
K5	+	−	0	$>10^6$
K15	+	−	0	$>10^6$
L2	+	−	0	$>10^6$
L5	+	−	0	$>10^6$
P1	+	−	0	$>10^6$
F2	+	−	0	$>10^6$
M1	+	+	0	$>10^6$
M2	+	+	0	$>10^6$
K1	+	+	0	$>10^6$
K20	+	+	1	
L1	+	+	1	1×10^5
L3	+	+	2	2×10^3
K13	+	+	1	
K9	+	+	2	
M3	+	+	2	
K3	+	+	3	
L7	+	+	3	3×10^2
K2	+	+	5	4×10^2
P2	+	+	5	9×10^2
P4	+	+	5	1×10^2
P6	+	+	5	1×10^2
P3	+	+	5	1×10^3

[a] Individual plaques obtained from brains of moribund chicks injected IC with Bartha × PrV(Ka) TK^- recombinants (see Table 3) were analyzed for thymidine kinase activity, the presence of intact U_S, and virulence as determined by IC inoculation into five 1-day-old chicks. The virus was reinoculated (10^5 PFU per chick) into five chicks for each plaque, and the number that died was determined. The LD_{50} of some of the virus populations was subsequently determined as described in the text.

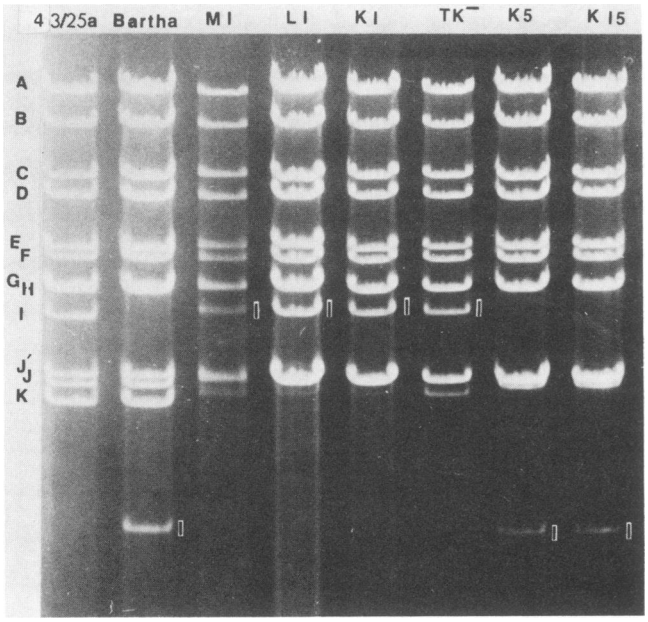

FIG. 5. Digestion with *Kpn*I of the genomes of different plaque isolates from the brains of chicks inoculated IC with Bartha × PrV(Ka) TK^- recombinants. Parental strains (lanes M1, L1, K1, K5, and K15), PrV(Ka) TK^- (lane TK^-), and Bartha. Lane 43/25a is a plaque isolate obtained after cotransfection of Bartha DNA and *Pst*I fragment I of PrV(Ka) DNA (see Fig. 7).

eight plaque isolates with a Bartha-like deletion (seven of which were TK^+) were obtained. Three of these isolates showed no evidence of recombination between PrV(Ka) TK^- and the Bartha strain; i.e., these virion populations were Bartha-like in their restriction patterns. To obtain a larger sample of virion populations with a deletion in the U_S to help determine whether genes in the U_S are really necessary for virulence, we also used a series of plaque-purified virions which were obtained during unrelated studies. In this experiment, a field isolate of PrV (PrV53) and the Bartha vaccine strain were allowed to recombine, and individual plaques formed by the progeny population were analyzed with restriction enzymes (*Kpn*I, *Bam*HI, *Sal*I, and *Pst*I). We selected plaques of recombinant virions, which had restriction patterns indicating that they had acquired sequences from both parental strains and determined their virulence. Of these plaque isolates, 10 had deletions in the U_S, and 9 had an intact U_S. The 10 recombinants which had a deletion in the U_S were avirulent, whereas 4 of the 9 recombinants which had an intact U_S were virulent (all 19 plaque isolates were TK^+) (Table 5). These results confirm those obtained from the previous experiment which showed that recombinant plaques with a deletion in the U_S are not virulent. We conclude therefore that genes located in the U_S play a role in the expression of virulence. However, because not all of the virions with an intact U_S were virulent in both experiments (see Tables 4 and 5) and, furthermore, because

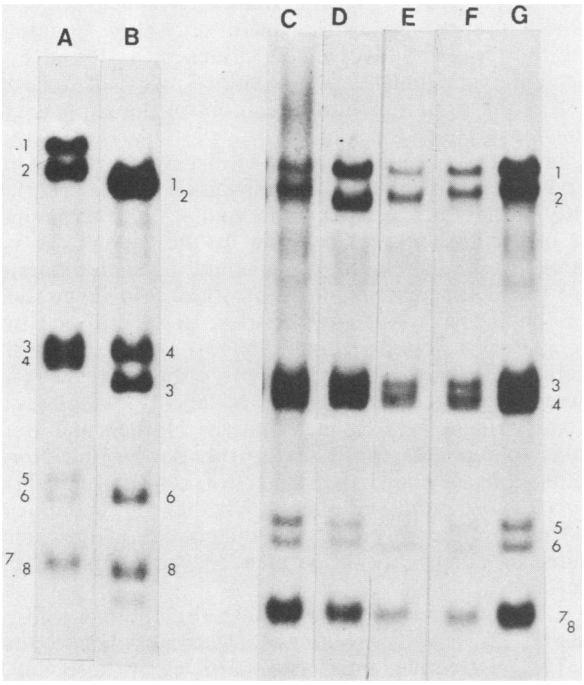

FIG. 6. Digestion of *Pst*I fragment 1 from the genomes of different plaque isolates with restriction enzyme *Sma*I. ^{32}P-labeled DNA from purified virion preparations of different plaque isolates was digested with *Pst*I, and the fragments were separated by electrophoresis. *Pst*I fragment 1 was excised from the gel, and the DNA was digested with *Sma*I. The fragments were separated by electrophoresis and autoradiographed. Lane A, PrV(Ka); lane B, PrV-Bartha; lane C, plaque isolate K1; lane D, plaque isolate K2; lane E, plaque isolate M1; lane F, plaque isolate L3; lane G, PrV(Ka).

their LD_{50}s varied, we conclude that, in addition to an intact U_S and thymidine kinase activity, one or more factors are necessary for the expression of full virulence as determined by IC injection into 1-day-old chicks. These factors are missing from the Bartha strain. These conclusions are reinforced by the experiments described below.

(ii) Marker rescue. We determined whether virulence would be restored to the Bartha strain by rescue with restriction fragments of DNA derived from wild-type virus which span the deleted region. Figure 7 summarizes the protocol used in this experiment, as well as some of the results.

PK cells were cotransfected with intact Bartha DNA and *Pst*I fragment 1 of wild-type PrV DNA (which spans the sequences which have been deleted from the Bartha strain [Fig. 1]). The progeny virions were injected IC into 1-day-old chicks with the expectation that the virus containing DNA which had recombined with the wild-type *Pst*I fragment, i.e., which had been rescued, would be able to replicate, whereas the nonrescued Bartha strain would not. At daily intervals, the chicks were killed, and the virus in their brains was plaque assayed. Whereas the Bartha strain is normally cleared rapidly from the brains, the virion population that was derived after transfection with Bartha DNA and *Pst*I fragment 1 replicated somewhat better or was cleared less rapidly than the Bartha strain (data not shown), indicating that some modification of the virion population had occurred as a result of cotransfection with *Pst*I fragment 1. Individual virus plaques obtained from the brains of chicks inoculated with the rescued Bartha strain were picked and analyzed to determine whether an intact U_S was indeed present in these virions. Virions in only 3 of 18 plaques that were analyzed contained genomes with a PrV(Ka)-like U_S region; the remainder were Bartha-like. (The restriction pattern of one recombinant strain [43/25a] is illustrated in Fig. 5.) The virions with the intact U_S did not kill the chicks when injected IC, although, as shown in Fig. 8, they replicated in the brains to a greater extent than did the Bartha strain. Thus, although restoration of an intact U_S region to the Bartha strain did not restore virulence, it did provide the virus with a limited ability to replicate in chick brains.

TABLE 5. Analysis of virulence of 19 recombinants between strains PrV53 (virulent) and PrV-Bartha (avirulent)[a]

No. of plaques	Characteristic	
	Intact U_S	Virulence
0	−	+
10	−	−
4	+	+
5	+	−

[a] PK cells were coinfected with PrV53 and PrV-Bartha (multiplicity of infection, 5 PFU of each per cell). After adsorption for 1 h, the cells were washed and overlaid with Eagle medium. At 48 h postinfection, the virus was harvested and plaque assayed. Plaques were picked at random, the virus was amplified, and the virus DNA was purified and digested with restriction enzymes *Pst*I, *Bam*HI, and *Kpn*I as described in the text. Of 32 individual plaques analyzed, the genomes of 19 had restriction patterns that indicated clearly that they were recombinants between the parental strains. Ten of these had a Bartha-like deletion in the U_S (−); the other 9 had an intact U_S (+). These recombinant virion populations were analyzed for virulence, i.e., their ability to kill chicks after IC inoculation was determined.

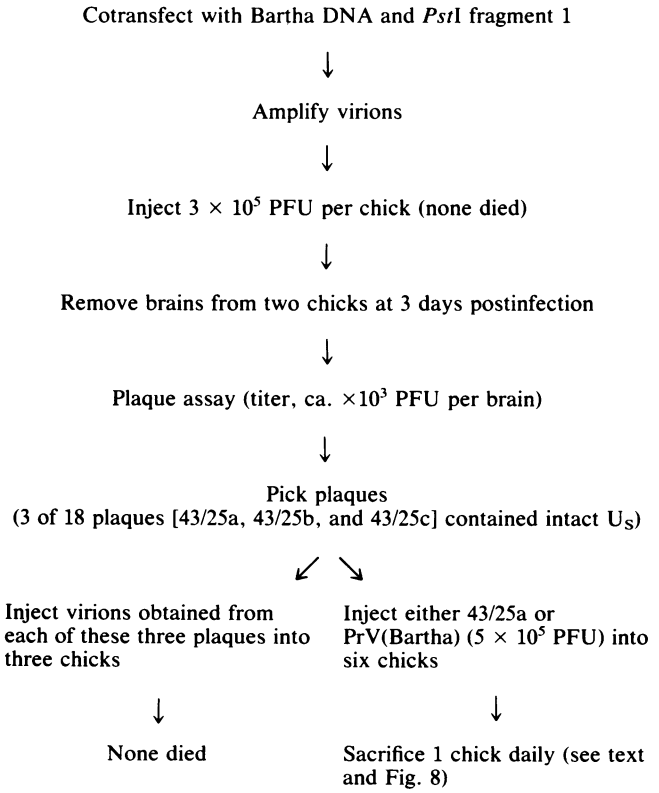

FIG. 7. Rescue of Bartha by cotransfection with *Pst*I fragment 1 DNA of wild-type PrV.

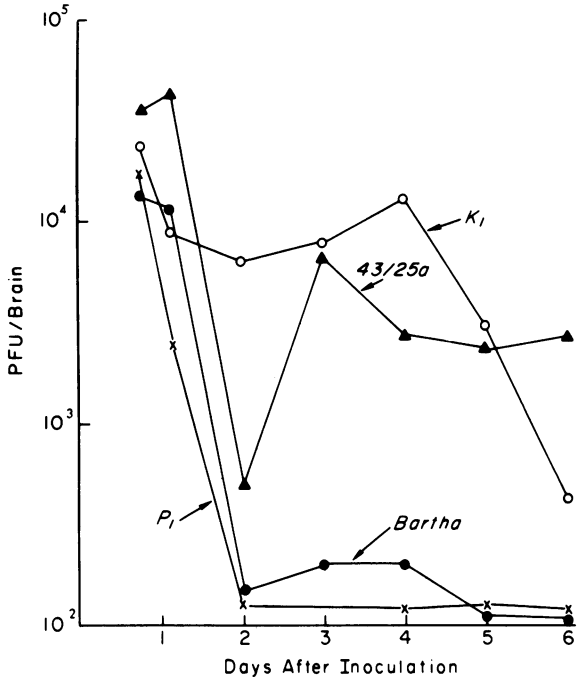

FIG. 8. Replication of virions from different plaque isolates after IC inoculation into 1-day-old chicks. The chicks were inoculated IC with 10^6 PFU of each plaque isolate. At daily intervals, some of the chicks were sacrificed, and homogenates of their brains were prepared and plaque assayed as described in the text.

Indeed, some of the avirulent plaque isolates obtained from the recombination experiment which had acquired both thymidine kinase activity and an intact U_S (as for instance K1, [see Table 4]) also appeared to replicate somewhat in (or be cleared less rapidly from) the chick brains.

DISCUSSION

The experiments described in this paper are part of a study designed to ascertain the functions of the genome of PrV, which are necessary for the expression of virulence. Our strategy has been to use attenuated vaccine strains of PrV and to attempt to render them virulent either by marker rescue or by recombination, identify the regions of the genome that might be implicated in virulence, and eventually try to ascertain the functions involved. In essence, our experimental approach is similar, though not identical, to that recently used by Centifanto-Fitzgerald et al. (5), Thompson and Stevens (25), and Thompson et al. (26), who have attempted to localize the genes of herpes simplex virus which are responsible for the expression of virulence of that virus.

We have used the ability of the virus to kill 1-day-old chicks after IC injection as a model system to test virulence. Virulent PrV (i.e., virus that causes disease in piglets) replicates well in chick brains and kills the chicks; the avirulent virus, such as the vaccine strains, replicates poorly or not at all in this system. This correlation may be only coincidental, and the ability of the virus to kill the chicks and its ability to cause disease in its natural host may eventually be found to be controlled by different functions. However, most laboratory animals are highly susceptible to wild-type strains as well as to the vaccine strains of PrV, and since it is impractical to use piglets as experimental animals (too expensive), the use of chicks as a model system appears to be the best approach at this stage of our study. Eventually, once the functions involved in virulence, as tested by IC inoculation into chicks, are identified, we will determine whether the same functions also control the expression of virulence in piglets.

Many factors could affect the virulence of a virus strain. As pointed out recently by Thompson and Stevens (25), the ability of the virus to replicate in vivo at the body temperature of the host may play a role. In the case of the virus strains that we have used in this study, no such differences were found (manuscript in preparation). However, differences in the growth characteristics, in several cell lines, among Bartha, Norden, and PrV(Ka) strains, as well as several field isolates, do exist. Thus, although there is little difference between the ability of the laboratory isolates and several virulent field isolates and the Norden and Bartha strains to replicate in PK cells, the Bartha and Norden strains replicate poorly in primary RK cells and remain cell associated (unpublished data). Whether any relationship between the altered growth characteristics of the vaccine strains and their lack of virulence exists remains to be ascertained.

TK^- mutants are known to be less virulent than wild-type virus (7, 22); however, both vaccine strains that we tested are TK^+. Defective, interfering particles are also not the basis for the lack of virulence of these virus strains because the virions used in these studies were twice plaque purified and amplified at low multiplicity only once thereafter.

We have focused on the possibility that the deletion present both in PrV-Norden and PrV-Bartha plays a role in virulence. The relatively large deletion (ca. 4.0 kilobase pairs) in both Bartha and Norden vaccine strains does not affect the ability of the virus to grow in a continuous line of PK cells, despite the lack of synthesis of several virus mRNAs (and several proteins). Thus, these genes appear to be nonessential for growth in PK cells. As shown by the results described in this paper, however, they appear to be necessary but not sufficient for unrestricted growth of the virus in chick brains.

Two sets of observations lead us to conclude that the genes which have been deleted from the vaccine strains play a role in virulence. (i) Of more than 100 virion populations originating from individual plaques recovered from chick brains injected with a population of virions containing recombinants between Bartha and Pr(Ka) TK^- virus, 8 had retained the Bartha-like deletion in the U_S; none of these was virulent. Furthermore, although 10 recombinants between Bartha and PrV53 with a deletion in the U_S were also avirulent, approximately one-half of those with an intact U_S were virulent. However, some of the avirulent recombinants with an intact U_S had an improved ability to replicate in chick brains.

(ii) Cotransfection of PrV-Bartha DNA with *PstI* fragment 1 of wild-type PrV DNA led to the isolation of rescued PrV-Bartha (with an intact U_S) which, though avirulent (i.e., did not kill the animals), could replicate somewhat in chick brains, whereas the original Bartha strain was rapidly cleared from the brains.

Thus, our results show that functions specified by the U_S, although nonessential for virus replication in PK cells in vitro, are essential for replication of the virus in chick brains. Furthermore, preliminary results showed that the rescued PrV-Bartha 43/25a with an intact U_S but not PrV-Bartha could be rendered virulent, i.e., the strain could kill the chicks when it, in turn, is rescued with wild-type restriction

fragments which span sequences occupying approximately the middle of the repeat (manuscript in preparation). These results indicate that although functions in the U_S are essential for the expression of virulence of PrV when injected IC into chicks, the Bartha strain is deficient in at least one other function involved in virulence. Thus, our results show that the expression of virulence of PrV (as determined by IC injection into chicks) is multigenically controlled.

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