JOURNAL OF VIROLOGY, Nov. 1991, p. 5839–5847
0022-538X/91/115839-09$02.00/0

Vol. 65, No. 11

The Ability of Pseudorabies Virus To Grow in Different Hosts Is Affected by the Duplication and Translocation of Sequences from the Left End of the Genome to the U_L-U_S Junction

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Received 2 May 1991/Accepted 22 July 1991

Pseudorabies virus is a herpesvirus which has a class 2 genome. However, under certain growth conditions it acquires a genome with class 3-like characteristics. In these variants, the leftmost sequences of the long (L) component of the viral genome have been duplicated and translocated to the right of the L component next to the short (S) component, resulting in an L component that is bracketed by inverted repeats. Consequently, the L component can invert and is found in two orientations relative to the S component. The translocation is accompanied invariably by a deletion of sequences that are normally present in the wild-type genome at the right end of the L component. The virion variants with an invertible L component have a growth advantage over wild-type virus in chicken embryo fibroblasts and chickens; they also have a growth disadvantage in mice or rabbit kidney cells. The changed growth characteristics of the variants reside entirely in the changed structure of the junction between the S and L components. Replacement of that region of the DNA with wild-type sequences restores the wild-type phenotype. To determine whether the modified growth characteristics of the variants are related to the translocation or to the deletion, mutants that have a deletion or that have a deletion as well as a translocation similar to those observed in the variants were constructed, and the growth characteristics of these mutants were determined. We show that the modified growth characteristics of the mutants with an invertible L component can be attributed to the translocation of the leftmost terminal sequences of the genome next to the inverted repeat; they are not related to the deletion of the sequences normally present at the right end of the L component. The translocation of the leftmost 325 bp of the genome is sufficient to confer upon the virus the modified cell-type-specific growth characteristics. Furthermore, the modified growth characteristics are contingent upon the presence of 68 bp spanning the internal junction between the L and S components.

The genomes of the herpesviruses consist of large, linear, double-stranded, noncircularly permuted molecules. Some herpesviruses contain genomes that are best described as being composed of two covalently linked components, the long (L) and short (S) components. In the genomes of herpes simplex virus, for example, both the L and S components are bracketed by inverted repeat sequences (19); both components invert themselves relative to each other, giving rise to four equimolar isomeric forms of the genome (17, 20, 23, 24). Genomes with this structure have been designated class 3 DNA or class E molecules (7, 18). The genomes of pseudorabies virus (PrV) also consist of an L and S component, but only the S component is bracketed by perfect inverted repeats; thus, only the S component is found in two equimolar isomeric forms (2, 22). Genomes with this structure have been designated class 2 or class D DNA molecules (7, 18).

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The functions of the inverted repeats in the genomes of the herpesviruses and the functional significance of the inversions they promote are not known. Virions containing either isomeric form of the genome of PrV are functionally equivalent (3). Furthermore, inversions of the S or L component are not essential for growth in vitro of herpes simplex virus; virus mutants that are frozen in one isomeric form are able to grow in cell culture (14).

The pressures that have led to the evolution of herpesviruses with different class genomes are obscure. The observation that PrV, which has a class 2 genome, may acquire a genome with class 3 characteristics (10, 11) under certain growth conditions was therefore of interest. Furthermore, the finding that these virion variants have a growth advantage over wild-type virus in cells of avian origin (11) may provide an initial insight into the biological and evolutionary significance of the invertible elements present in the genomes of different herpesviruses.

The nucleotide sequence spanning the junctions of the S and L components in the genomes of PrV variants that had acquired an invertible L component has been determined (12). In these variants the leftmost sequences of the viral genome have been duplicated and translocated in inverted form to the right of the L component next to the S component, resulting in an L component bracketed by inverted repeats. In three independently isolated variants with an invertible L component, which have been analyzed in detail, the sizes of the translocated fragments were similar—approximately 1.4 kbp (12). The translocated segment of DNA

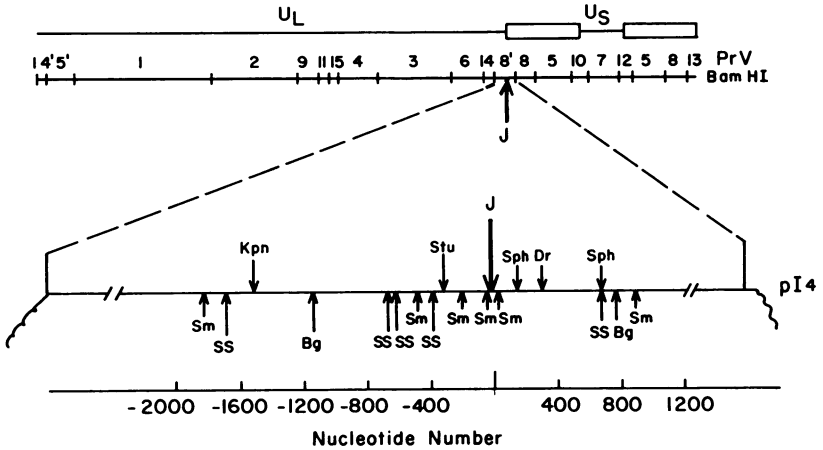


FIG. 1. Restriction map of the region of the junction between the L and S component of mature PrV DNA. Lines 1 and 2, structure and the *Bam*HI restriction map of the PrV genome. The open rectangles indicate the inverted repeats bracketing the U_S; Line 3, restriction map of the region spanning the junction between the L and S components; *Bam*HI fragment 8′ in pI4. Starting with the first nucleotide at the junction, nucleotides of the S component are numbered 1, etc. Starting with the first nucleotide of the L component, nucleotides of the L component are numbered −1 etc. Abbreviations: Sm, *Sma*I; SS, *Sst*II; Kpn, *Kpn*I; Bg, *Bgl*II; Stu, *Stu*I; Sph, *Sph*I; Dr, *Dra*I.

had not undergone sequence modifications and was, in all cases, derived solely from sequences normally present at the left end of the L component (the left end of the genome). In all three variants, the translocation was accompanied by a deletion of sequences that are normally present in the wild-type genome at the right end of the L component, adjacent to the internal repeat (or S component). The sizes of the deleted segments of DNA differed significantly in the different isolates (between 0.8 and 2.3 kbp). The nucleotide sequences of the DNA adjacent to the translocated sequences remained unchanged (12).

The class 3-like PrV variants thus have two modifications: (i) a duplication and translocation of the leftmost sequences of the genome to the internal junction region between the S and L components, and (ii) a deletion of sequences normally present at the right end of the L component (adjacent to the S component). The studies described in this paper are part of an attempt to clarify the molecular basis for the cell-specific growth advantage of PrV variants that have acquired an invertible L component. We show here that the growth characteristics of the invertible mutants can be attributed to the translocation of the leftmost terminal sequences of the genome next to the inverted repeat and are not related to the deletion of the sequences normally present at the right end of the L component.

MATERIALS AND METHODS

Virus strains and cell culture. PrV(Ka) is a strain that has been carried in our laboratory for more than 30 years. The isolation of PrV variants PrV(Ka)B8 and PrV(USA)A4 has been described previously; they are two class 3-like variants that have been selected after extensive passage in avian cells (11). The construction and characterization of vLD68 is detailed elsewhere (8). The construction of the other mutants is described below. The viruses were grown in PK or RK cells which were cultivated in Eagle's synthetic medium containing 5% bovine serum. Chicken embryo fibroblasts (CEF) were made from 10- to 12-day-old embryos.

Enzymes and chemicals. Restriction enzymes, DNA polymerase I, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. T4 polynucleotide kinase was purchased from U.S. Biochemical Corp. [α-^{32}P]dCTP was purchased from New England Nuclear Corp.

Purification of virions and of viral DNA. Virions were purified as described previously (1).

Cloning of restriction fragments. The procedures described previously (4, 6, 12) were used.

Southern and sequence analyses. Southern analysis, nick translation of DNA probes, and sequencing were performed as described previously (4, 12, 16, 21).

Construction of deletion plasmids. (i) pE10. The parent plasmid I4 (which contains *Bam*HI fragment 8′ of the PrV genome cloned in the *Bam*HI site of pBR325) was digested with *Kpn*I and *Stu*I (Fig. 1) and religated, thereby deleting approximately 1.2 kbp from the unique L component (U_L; from nucleotides −1509 to −322).

(ii) pTN155. A *Bgl*I subclone of plasmid I4 which spans the junction between the S and L components (Fig. 1) was subcloned into the *Sma*I site of pTM. (pTM was obtained by digesting pBR322 with *Bam*HI and *Sal*I and blunt-end ligating and subsequently introducing the multiple cloning cassette of M13 between its *Eco*RI and *Hin*dIII site.) To remove the *Sst*II and *Sph*I sites in the *Bgl*I insert (around bp 690) (Fig. 1), the plasmid was cleaved at the *Bam*HI site present in the M13 sequences at the right side of the insert and approximately 150 bp was removed by *Bal* 31 digestion. This procedure removed the region of the PrV DNA insert up to approximately bp 600; it also removed most of the M13 sequences that had been present in pTM. The plasmid was religated. The resulting plasmid (p118) was digested with *Sst*II, causing the removal of 287 bp (from bp −677 to −390), and a *Hae*III fragment of M13 containing the multiple cloning cassette was introduced at that position in both orientations. In one of the resulting plasmids, pTN155a, the multiple cloning cassette of the M13 DNA is close to the *Stu*I site. In the other, pTN155b, the M13 insert is in the opposite orientation.

The M13 sequences were introduced for two reasons. (i) Since PrV and M13 do not cross-hybridize, the mutated plasmids as well as the viral mutants to be constructed using these plasmids would be marked by the M13 sequence. (ii) The multiple cloning site of M13 is helpful in the construc-

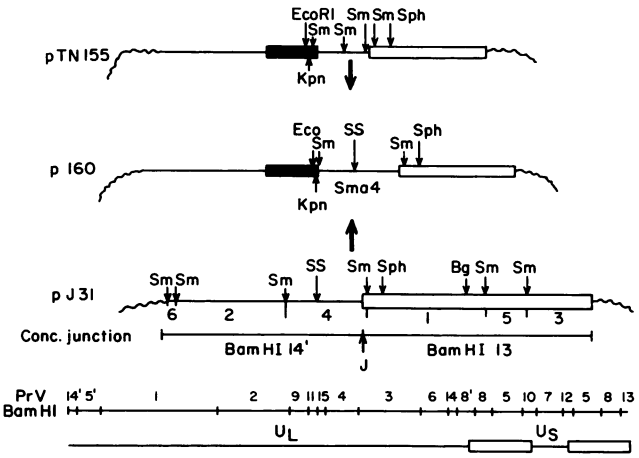


FIG. 2. Construction of p160. Open rectangles denote segments of viral DNA derived from the inverted repeat of the S component. Filled rectangles denote an M13 segment of DNA that includes the multiple cloning site. The straight line denotes segments of DNA derived from the L component of the genome. Line 1, structure of pTN155a (see text for detail); line 2, structure of p160 derived by inserting *Sma*I fragment 4 derived from J31 (see line 3) into *Sma*I-digested pTN155; lines 3 and 4, restriction map of the concatemeric junction of PrV DNA which consists of the joined *Bam*HI fragments 13 and 14'; lines 5 and 6, structure and *Bam*HI restriction map of the PrV genome.

tion of deletion and translocation mutants near the junction between the L and S component (see below).

(iii) pYa. To derive pYa, p118 (see above) was digested with *Sma*I, resulting in a deletion of 503 bp, ranging from positions −487 to +16 (Fig. 1). The plasmid was religated with a *Hae*III fragment of M13 DNA (bp 5940 to 6040). Thus, pYa has a 16-bp deletion at the end of the internal inverted repeat and a deletion of 487 bp at the right terminus of the U_L; it also has an insertion of 100 bp of M13 DNA (see Fig. 3 for a summary of the structure of the deletion mutants).

Construction of deletion-translocation plasmids. (i) p160. pJ31 contains the *Bam*HI concatemeric junction fragment of the fused end fragments of PrV (*Bam*HI 14' and *Bam*HI 13 [Fig. 2]). *Sma*I fragment 4, derived from clone J31 contains 586 bp of *Bam*HI 14' and an additional 16 bp of the inverted repeat (Fig. 2). To construct p160, pTN155a (in which the cloning cassette of M13 is proximal to the inverted repeat sequences [see above]) was digested with *Sma*I, thereby deleting approximately 400 nucleotides between the *Sma*I site in the MCS of the M13 sequences and the *Sma*I site present 16 bp into the internal inverted repeat (IR). The truncated plasmid was ligated to *Sma*I fragment 4, obtained by digestion of pJ31 (Fig. 2). In the correct orientation, this restores an intact IR (i.e., the 16 first nucleotides of the IR are replaced) and also inserts adjacent to the IR 586 nucleotides derived from the left end of the genome (left end of the U_L).

(ii) p173/3 and p6DG3. Plasmids p173/3 and p6DG3 were constructed from p160. *Kpn*I cleaves within the multiple cloning site to the left of the *Sma*I fragment 4 insert (Fig. 2). *Sma*I fragment 2, also obtained from pJ31 (Fig. 2), was ligated in both orientations next to the *Sma*I fragment 4 insert of p160. p173/3 has *Sma*I fragment 2 in the standard orientation relative to *Sma*I fragment 4; in p6DG3 *Sma*I fragment 2 is in the inverted orientation relative to *Sma*I fragment 4 (see Fig. 4).

(iii) p160/2.5X. Plasmid p160/2.5X is similar to p160 but contains the terminal 325 nucleotides of *Sma*I fragment 4 only. p160/2.5X was obtained by digesting p160 with *Sst*II and *Eco*RI (Fig. 2) and religating.

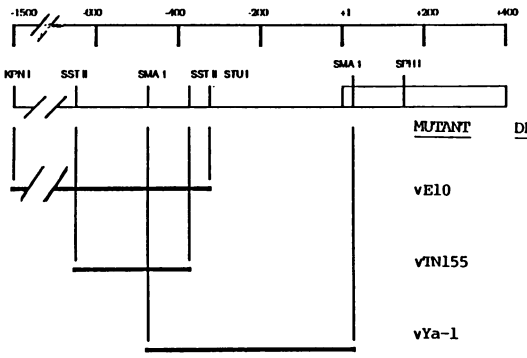
Characterization of plasmids. All constructs were analyzed by restriction endonuclease digestion for the presence or absence of the appropriate restriction sites. In most cases, they were also sequenced.

Isolation of viral mutants. To isolate virions with genomes that harbor the specific deletions and translocations, plasmids containing viral inserts with the appropriate mutations were cotransfected with wild-type PrV DNA into RK cells by the calcium chloride precipitation method (5). Recombination between the sequences flanking the mutated regions in the inserts of the plasmid and the PrV genome will occur, giving rise to virus mutants with modifications near or at the junction between the S and L components. Following virus-induced cell degeneration, virions were plaque assayed. Individual plaques were picked into 96-well plates in which RK cells had been grown. A portion of the infected cells was dot blotted onto nitrocellulose filters and probed with a nick-translated fragment that distinguishes wild-type PrV DNA from the mutant viral DNA (in most cases an M13 probe was used; in the case of E10, the *Kpn*I-*Sph*I fragment that had been deleted from E10 was used). Once mutant plaques were identified, they were replaque purified twice and characterized.

Characterization of viral mutants. All putative mutant DNAs were digested with restriction endonucleases with sites within the area of interest and hybridized to appropriate restriction enzyme fragments as probes. The internal junction fragments (the equivalents of *Bam*HI fragment 8' of the wild-type genome [Fig. 1]), into which the mutation had been introduced, were cloned and analyzed for the presence of restriction sites diagnostic of the desired deletion or deletion plus translocation. In some cases (vE10, v160, and vYa) the regions spanning the junction bordering the deletions or the insertions were sequenced. The data all showed that the virus mutants had indeed the desired structure. Because these experiments were performed only to ensure the identity of the mutants and are not otherwise informative, they are not presented. Figures 3 and 4 summarize the structures of the virus mutant genomes at the junctions between the L and S components.

Marker rescue of natural translocation mutant PrV(Ka)B8. PrV(Ka)B8 viral DNA was cotransfected with pI4 [a plasmid carrying the *Bam*HI fragment 8' of wild-type PrV(Ka) (Fig. 1)] into RK cells. Progeny plaques were picked and amplified, and DNA was extracted and analyzed for the presence or absence of an invertible U_L. A high proportion (3 of 12) of marker-rescued virions had a noninvertible U_L. This can be attributed to the fact that variants with an invertible U_L have a growth disadvantage in RK cells.

Passage of virus in vitro. Primary RK cells or primary CEF monolayers growing in 90-mm petri plates were infected at low multiplicity (0.01 PFU per cell) with the virus mixtures. Following detection of cytopathic effects, 10 μl of the media obtained from these infected cultures was transferred onto fresh RK or CEF monolayers. This process was repeated five times. The viral particles present in the original mixture, as well as after the fifth passage, were purified, and viral DNA was isolated. The DNA was digested with the appropriate restriction enzyme, electrophoresed, transferred to nitrocellulose, and hybridized with the appropriate nick-translated probe, and the ratio of the two types of virions was determined.



MUTANT	DELETION SIZE (in bp)	DELETION RANGE (nucleotide number)	
vE10	1183	-1509	-326
vIN155	287	- 677	-390
vYa-1	503	- 487	+ 16

FIG. 3. Summary of structure of deletion mutants. Line 2, open rectangles denote the inverted repeat of the S component. Restriction sites of interest are indicated. Lines 3 to 5, heavy lines indicate the segments of DNA that have been deleted.

Passage of virus in vivo. One-day-old chickens or 12-week-old BALB/c mice were injected in the wing or in the hind-leg muscle, respectively, with the viral mixtures. The brains of moribund animals were removed aseptically and homogenized. Virus was purified, and the viral DNA was subjected to the same analysis as described above. The original reason for using animals was to compare the pathogenesis of the mutants and of the wild type. However, the results obtained also illustrated the host (species)-specific advantage or disadvantage of the mutants in various cell types (or tissues) of the two species.

Quantitation of viral DNA bands. Several exposures of Southern blots were scanned by using the Gel Scan function of the Beckman D4-70 spectrophotometer or a Joyce Loebl microdensitometer, and the bands were quantitated.

RESULTS

Natural translocation variants have a growth advantage in CEF and a growth disadvantage in RK cells compared with wild-type virus. All primary isolates of PrV that have been analyzed to date (approximately 40) have a genome with a class 2 structure, i.e., only the U_S is bracketed by perfect inverted repeats and inverts relative to the fixed U_L. Thus, only two isomeric forms of the genome are found. Passage of wild-type virions in CEF but not in RK, PK, or MDBK cells invariably results in the accumulation of virions with genomes that have a translocation of sequences from the end of the U_L next to the internal repeat and in which the U_L inverts, so that four isomeric forms of the genome exist (11). The kinetics of accumulation of these genomes as well as the relative homogeneity of the structure of the genomes in each independently derived population of virions passaged in CEF indicated that the translocation occurs rarely but that it confers upon the virus a selective growth advantage in CEF (11).

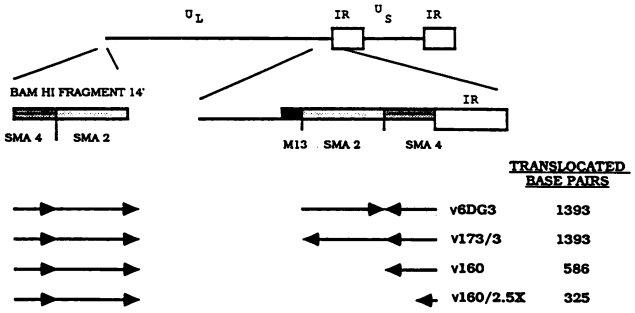


FIG. 4. Summary of the structure of deletion-translocation mutants. All mutants have a deletion of nucleotides −1 to −677 from the right end of the U_L. The sequences from the extreme left of the genomes (*Sma*I fragments 4 and 2) have been inserted directly adjacent to the inverted repeat. Mutant 160/2.5X has an insertion of the leftmost 325 bp of the genome; mutant 160 has an insertion of the leftmost 586 bp of the viral genome. Mutant 6DG3 and 173/3 both have insertions of 1,393 bp, but *Sma*I fragment 2 and *Sma*I fragment 4, derived from the terminal region of the genome, are present in different orientations, one relative to the other.

Figure 5 shows the results of experiments in which two natural translocation variants of PrV (variants that had arisen after passage of two different isolates of wild-type PrV in CEF [11]) were mixed with wild-type PrV(Ka) and either passaged in CEF or in RK cells or injected into mice or chickens. The progeny virions obtained after five passages in cell culture or isolated from the brains of the infected animals were purified, and their restriction patterns were analyzed.

*Kpn*I digests of wild-type PrV contain a single fragment that hybridizes to a probe derived from the leftmost 1,400 bp of the genome (*Bam*HI 14'); *Kpn*I digests of the translocation mutants contain four such fragments, two end fragments, and two junction fragments (see Fig. 6 for restriction maps). The sizes of fragments C and D may vary in different translocation mutants, depending on the sizes of the fragments that have been deleted from the right terminus of the U_L. Before passage in CEF or RK cells, approximately one-third of the genomes in the mixture were wild type, as determined by the relative amount of wild-type terminal fragment (band B) present in the digest (Fig. 5). After passage in CEF, the prototype end fragment (band B) was not as abundantly represented as it was in the initial mixture, confirming our previous conclusion (11) that wild-type genomes have a growth disadvantage in CEF compared with the translocation mutants. Interestingly, after passage in RK cells, the virion population consisted mainly of wild-type virus; the relative amount of invertible genomes was greatly decreased and mostly the prototype end (band B) was found. Thus, the invertible mutants have a distinct growth disadvantage compared with that of the wild type in RK cells.

Similar results were obtained when the virus was injected into mice and chickens. In mice, mostly the wild type replicated while in chickens the growth of the translocation mutants was favored over that of wild-type virus. Table 1 summarizes the results of several experiments. The growth advantage of the variants in CEF and in chickens was

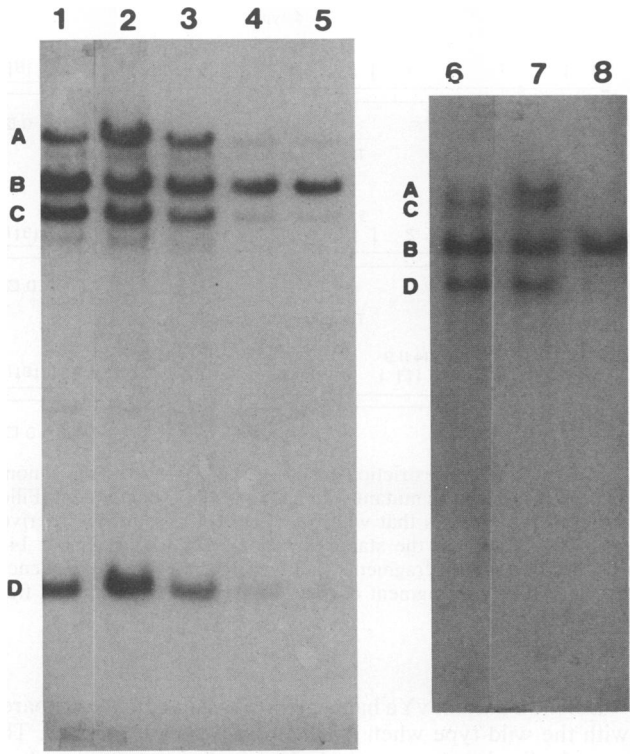


FIG. 5. *Kpn*I digests of a mixture of standard PrV and natural translocation mutants before and after passage in cell culture or in animals. A mixture of standard PrV and PrV(Ka)B8 virions (original mixture, lane 1) was injected into the wings of chickens (lanes 2 and 3) or the hind legs of mice (lanes 4 and 5). The brains of moribund animals were collected, and the virions were purified. A mixture of standard PrV and PrV(USA7)A4 virions (original mixture, lane 6) was passaged five times, as described in Materials and Methods, either in CEF (lane 7) or in RK cells (lane 8), and the virions were purified. The DNA was extracted, digested with *Kpn*I, electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated *Bam*HI fragment 14 (see Fig. 6 and 8 for restriction maps).

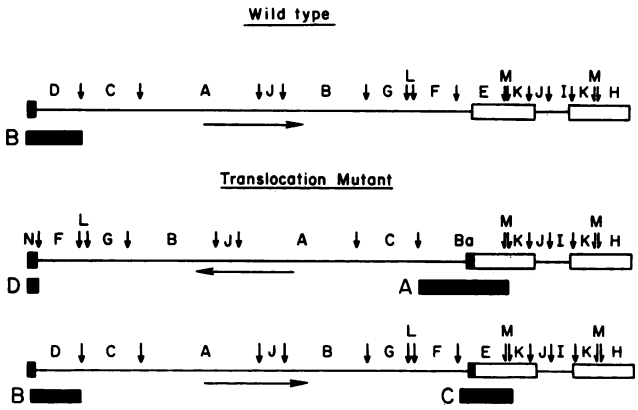


FIG. 6. *Kpn*I restriction map of the wild-type PrV genome and of a translocation mutant with an invertible L component. Filled rectangles, fragments that will hybridize to sequences derived from the left end of the standard viral genome (*Bam*HI fragment 14 [Fig. 8]).

relatively small. However, in each case, the amount of wild-type virus was reduced at least twofold, as ascertained by scanning appropriately exposed autoradiograms. The growth disadvantage of the variants in mice or in RK cells was much more pronounced. In most cases, the presence of the variants was barely detectable after passage of the virus mixtures in RK cells or mice.

The modified growth characteristics of the mutants are the result of the genome modifications at the internal junction between the L and S components. Marker rescue of the variant PrV(Ka)B8 with *Bam*HI fragment 8′ of wild-type PrV(Ka) generated a virus that had regained wild-type growth characteristics, i.e., did not have a growth disadvantage in RK cells or a growth advantage in CEF over the wild-type virus (Table 1).

As mentioned above, the natural translocation mutants all have experienced two modifications: (i) a duplication and translocation of a segment of approximately 1.4 kb of DNA derived from the left end of the L component to a position directly adjacent to the internal inverted repeat, and (ii) a deletion of sequences normally present at the right end of the L component (12). The experiments described below were performed to ascertain whether the growth characteristics of the natural translocation mutants could be attributed to one or the other of these modifications.

Growth characteristics of deletion mutants. In principle, deletions of specific regions of the virus genome can confer upon the virus a growth advantage under certain growth conditions. For example, the expression of PrV glycoprotein gI is deleterious to the release of the virus from CEF but not RK cells (13); gI-null mutants have a growth advantage in CEF over the wild-type virus. It seemed possible, therefore, that the deletion of sequences at the right end of the U_L, which is observed in all natural translocation mutants, could be responsible for the modified growth characteristics of these mutants.

Three viral mutants that harbor deletions adjacent to the internal repeat encompassing sequences deleted from the natural translocation mutants were constructed (Fig. 3). Each of these deletion mutants was mixed with an equal amount of wild-type PrV, and the growth advantage of one over the other was determined with the aim of ascertaining whether any of these mutants behaved like the class 3-like

TABLE 1. Growth advantage summary for translocation mutants selected by passage in CEF[a]

Virus mixture	RK cells	CEF	Mice	Chickens
PrV(Ka) + PrV(USA7) A4				
A4	0	++	0	+++
Wild type	+++	0	+++++	0
Neither	0	0	0	+
PrV(Ka) + PrV(Ka)B8				
A4	0	++	0	+++++
Wild type	+++	0	+++++	0
Neither	0	+	0	0
PrV(Ka) + marker-rescued PrV(Ka)B8 [B8(12)]				
B8(12)	0	0		
Wild type	0	0		
Neither	+++	+++		

[a] Each + denotes an individual animal or series of passage in cell culture in which a minimum of a twofold advantage for the indicated virus variant was observed.

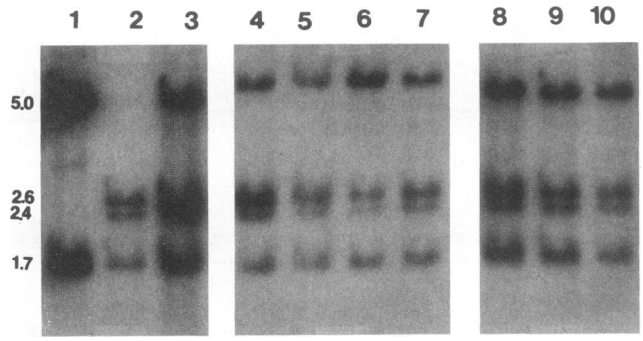


FIG. 7. Comparison of the ability of vTN155 and PrV to replicate in chickens and mice. A mixture of wild-type PrV and vTN155 was injected intramuscularly into either mice (10^3 PFU) or chickens (10^5 PFU). The brains of moribund animals were collected, and virus was isolated. Viral DNA was extracted, digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated *Bam*HI fragment 8′. Because of the presence of the multiple cloning cassette of M13, digestion of vTN155 with *Bam*HI will yield two fragments instead of *Bam*HI fragment 8′. The 2.4-kb fragment includes the sequences in the inverted repeat (1.7 kb), the first 390 bp of the right side of the L component, and approximately 250 bp of M13. The other band consists of 2.6 kb of fragment 8′, starting at bp −677. Lane 1, wild-type DNA; lane 2, vTN155 DNA; lane 3, original mixture; lanes 4 to 7, virus DNA obtained from infected mice; lanes 8 to 10, virus DNA obtained from infected chickens.

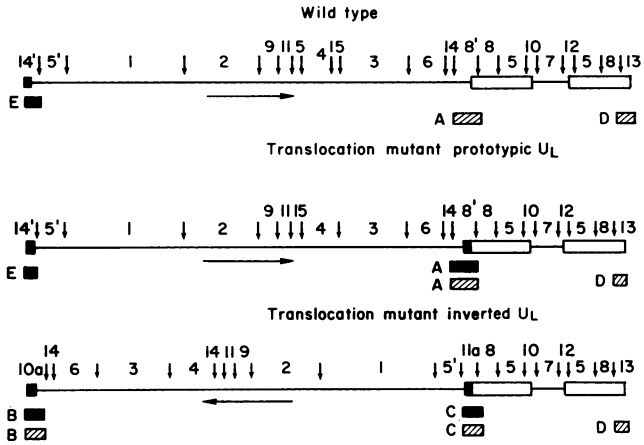


FIG. 8. *Bam*HI restriction maps of the wild-type PrV genome and of a translocation mutant with an invertible L component. Filled rectangles, fragments that will hybridize to the sequences derived from the left end of the standard genome (*Bam*HI fragment 14′). Hatched rectangles, fragments that will hybridize to the sequences from the junction fragment of the wild-type genome, *Bam*HI fragment 8′.

natural translocation mutants, i.e., had a growth advantage in CEF and a growth disadvantage in RK cells relative to that of wild-type virus. The mutant–wild-type virus mixtures were passaged in CEF and RK cells; they were also injected intramuscularly into both mice and chickens, and the virus was isolated from the brains of moribund animals.

Figure 7 is an example of a representative experiment in which a mixture of PrV and vTN155 was passaged in chickens and mice. The first lane shows the restriction pattern of *Bam*HI-digested wild-type PrV probed with *Bam*HI fragment 8′. Two bands, corresponding to fragment 8′ (5.0 kb) and fragment 13 (1.7 kb), hybridize (see Fig. 8 for restriction map). Lane 2 shows the pattern of the vTN155 mutant similarly analyzed. The introduction of the M13 multiple cloning cassette into *Bam*HI fragment 8′ results in the presence of an additional *Bam*HI restriction site, causing fragment 8′ of vTN155 to be digested into two smaller fragments of approximately 2.6 and 2.4 kb in size. Lane 3 represents the pattern of the DNA obtained from the original virus mixture of PrV and vTN155 prior to passaging. Lanes 4 through 7 show the pattern of the DNA in the viral populations isolated from the brains of four infected mice, and lanes 8 through 10 are those isolated from the brains of three infected chickens.

The ratio of PrV-specific bands to vTN155-specific bands is similar in the original virus mixture and after it has been passaged in either chickens or mice. Thus, vTN155 replicates as well as wild-type virus in both species. The same results were obtained when the vTN155-wild type mixture was passaged in cell culture (Table 2).

Similar experiments also were performed with deletion mutant vE10 (Table 2). In this case also, the ability of the mutant to grow in chickens or mice could not be distinguished from that of wild-type virus. Thus, both deletion mutants vE10 and vTN155 behave like wild-type virus with respect to their growth characteristics.

Deletion mutant vYa had a growth disadvantage compared with the wild type when analyzed in a similar manner. The growth disadvantage was observed upon passage in both chickens and mice, as well as in CEF and RK cells (Table 2). Thus, while vYa did not grow as well as wild-type virus, it did not have the cell-specific growth characteristics observed with the naturally occurring class 3-like mutants. The reason for the growth disadvantage of vYa will be dealt with elsewhere.

The results obtained with the deletion mutants showed that none had acquired the growth characteristics of the natural variants with class 3-like genomes that harbor deletions in the same regions of the genome.

Growth characteristics of deletion-translocation mutants. We next determined whether the duplication and translocation (in inverted form) of sequences derived from the left end of the genome to a position adjacent to the internal repeat could confer upon the virus the modified growth character-

TABLE 2. Growth advantage summary for deletion mutants[a]

Virus mixture	RK cells	Mice	Chickens
PrV(Ka) + vE10			
Wild type		+	+
vE10		0	+
Neither		++++++++++	++
PrV(Ka) + vTN155			
Wild type	0	0	0
vTN155	0	0	0
Neither	+++	++++	+++++++
PrV(Ka) + vYa-1			
Wild type	+++	+++++	++++++
vYa-1	0	0	0
Neither	0	+	0

[a] Each + denotes an individual animal or series of passages in cell culture in which a minimum of a twofold advantage for the indicated virus was observed.

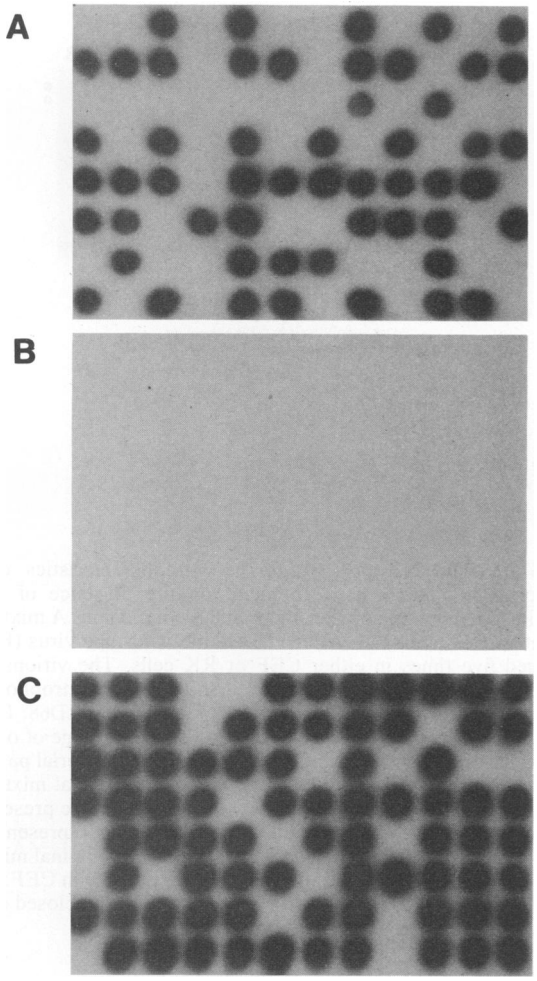


FIG. 9. Translocation mutant v6DG3 has a growth advantage in CEF and a growth disadvantage in RK cells compared with wild-type PrV: infectious virus assay. A mixture of wild-type PrV and of translocation mutant v6DG3 was passaged five times either in RK or in CEF monolayers, as described in Materials and Methods. The virions in the original virus mixture and after passage in RK cells or CEF were plaque assayed. Individual plaques were picked and inoculated in 96-well plates in which RK cells had been grown. Parts of the infected-cell supernatants were blotted onto nitrocellulose filters and hybridized to nick-translated M13 DNA (the M13 probe will hybridize to v6DG3 but not with wild-type PrV DNA). To ensure that viral DNA was present in all the wells, the filters were subsequently hybridized to nick-translated PrV DNA; all wells were positive. (A) Original mixture—45% wild type, 55% v6DG3; (B) After passage in RK cells—100% wild type, 0% v6DG3; (C) After passage in CEF—20% wild type, 80% v6DG3.

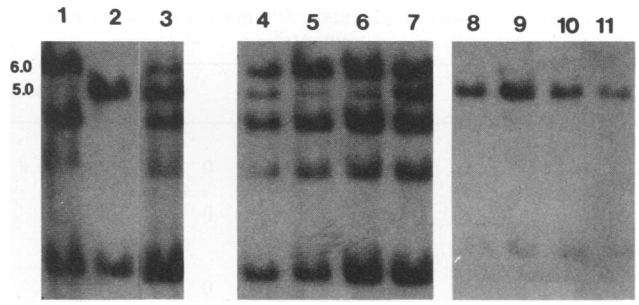


FIG. 10. Translocation mutant v6DG3 has a growth advantage in chickens and a growth disadvantage in mice compared with wild-type PrV: Southern blot analysis of virus DNA. A mixture of v6DG3 and wild-type PrV was injected into the wings of chickens (10^5 PFU per animal) or the hind legs of mice (10^3 PFU per animal). The brains of moribund animals were collected, the virus was isolated, and the DNA was digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and hybridized to *Bam*HI fragment 8′. Lane 1, v6DG3 DNA; lane 2, wild-type DNA; lane 3, DNA isolated from the original virus mixture used for inoculation of the animals; lanes 4 to 7, virus DNA isolated from brains of chickens; lanes 8 to 11, virus DNA isolated from brains of mice.

istics of the natural translocation variants. Four viral mutants in which various size segments of DNA derived from the extreme left end of the genome were translocated adjacent to the internal repeat sequences were constructed (Fig. 4).

Each of the mutants was mixed with wild-type PrV and grown either in RK or CEF monolayers or injected into mice or chickens. Figure 9 shows an example of the results of an experiment in which a mixture of mutant v6DG3 and wild-type PrV was passaged in cell culture in RK and in CEF. Following completion of the passaging regimen, the virions were plaque assayed and randomly chosen plaques were picked into 96-well plates containing RK cells. After cell degeneration, the relative number of mutant and wild-type plaques was determined by dot blot assay with M13 as a probe (M13 will hybridize with v6DG3 but not with wild-type virus DNA). The results showed that v6DG3 had a significant growth advantage in CEF over the wild type but that the wild type had a distinct growth advantage over v6DG3 in RK cells (Fig. 9).

Southern hybridization of virus DNA obtained from the brains of chickens and mice that had been inoculated with the a mixture of v6DG3 and wild-type virus are illustrated in Fig. 10. Lane 1 represents the DNA of v6DG3 translocation mutant. The presence of four bands in the *Bam*HI digest hybridizing to fragment 8′ reflects the ability of the L component, now bracketed by inverted repeats, to invert (Fig. 8). Lane 2 represents wild-type PrV DNA, and lane 3 represents the DNA of the original virus mixture used to inoculate the animals. Viral DNA isolated from chicken brains is shown in lanes 4 through 7, and that from mice brains is shown in lanes 8 through 10. The results parallel those seen in the plaque dot blot assay; v6DG3 had a significant growth advantage over wild-type PrV upon passage in chickens and a distinct growth disadvantage in mice.

Identical results (data not shown) were also obtained with mutant v160 as well as with mutant v173/3 (see Fig. 4 for structures of mutants). Thus, neither the presence nor the orientation of *Sma*I fragment 2 sequences appeared to affect the outcome. This was further confirmed by a complete growth neutrality of the mutants v160, v173/3, v6DG3, and PrV(Ka)B8, one relative to the other. The results of several such experiments are summarized in Table 3.

Mutant v160/2.5X, in which only the terminal 325 nucleotides of the genome have been translocated next to the IR, also behaved as did the other deletion-translocation mutants. It was neutral relative to v160 after passage in CEF or RK cells or after passage in chickens and mice. It did have a growth advantage in CEF and chickens and a growth disadvantage in RK cells and in mice compared with wild-type virus (Table 3). Thus, it can be concluded that the translocation of the terminal 325 nucleotides of the genome adjacent to the internal inverted repeat is sufficient to duplicate the

TABLE 3. Growth advantage summary for translocation mutants[a]

Virus mixture	RK cells	CEF	Mice	Chickens
PrV(Ka) + v160				
v160	0	+++	0	++++
PrV(Ka)	+++	0	+++++	0
Neither	0	0	0	+
v173/3 + v160				
v173/3	0	0	0	0
v160	0	0	0	0
Neither	+++	+++	+++++	+++++
PrV(Ka) + v160/2.5X				
v160/2.5X	0	++	0	+++
PrV(Ka)	+++	0	+++++	0
Neither	0	+	0	0
v160 + v160/2.5X				
v160	0	0	0	0
v160/2.5X	0	0	0	0
Neither	++	++	+++	+++
PrV(Ka)B8 + v160				
B8	0	0		
v160	0	0		
Neither	+++	+++		

[a] Each + denotes an individual animal or series of passage in cell culture in which a minimum of a twofold advantage for the indicated virus variant was observed.

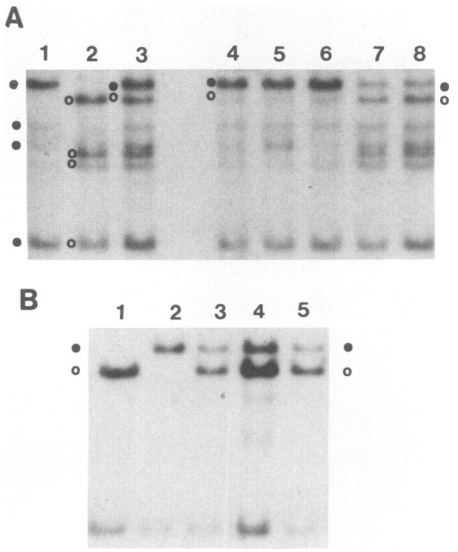


FIG. 11. The cell-type-specific growth characteristics of the translocation mutants are dependent on the presence of 68 bp spanning the junction between the L and S component. A mixture of mutant vLD68 and v160 (A) or vLD68 and wild-type virus (B) was passaged five times in either CEF or RK cells. The virions were isolated, and the DNA was digested with *Bam*HI, electrophoresed, and probed with *Bam*HI fragment 8′. (A) Lane 1, vLD68; lane 2, v160; lane 3, original mixture; lanes 4 to 6, after passage of original mixture in RK cells (the results of three independent serial passages are presented); lanes 7 and 8, after passages of original mixture in CEF (the results of two independent serial passages are presented). Closed circles represent vLD68 DNA; open circles represent v160 DNA. (B) Lane 1: wild type; lane 2, LD68; lane 3, original mixture; lane 4, after passage in RK cells; lane 5, after passage in CEF. Open circles represent a band diagnostic of wild-type DNA; closed circles represent a band diagnostic of vLD68 DNA.

growth advantage characteristics of the natural translocation mutants.

The sequences spanning the junction between the S and L components affect the cell-specific growth characteristics of the deletion-translocation mutants. Mutant vLD68 is a translocation mutant which is similar to v173/3 or variant PrV(Ka)B8 but in which 68 bp of the internal junction (64 bp from the teminus of the L component and 4 bp from the terminus of the S component) have been deleted. A detailed description of the construction and characterization of this mutant is presented elsewhere (8). Pertinent to the problem discussed here is the fact that vLD68 has growth characteristics similar to those of wild-type virus; it is neutral relative to the wild-type when grown both in RK cells or CEF and has a distinct growth advantage in RK cells and a disadvantage in CEF relative to v160 (Fig. 11). Thus, the 68 bp spanning the internal junction between the L and S components in translocation mutants are essential for the expression of the host-cell-specific growth characteristics of the deletion-translocation mutants.

DISCUSSION

The experiments described in this paper were designed to clarify the basis for the evolution of wild-type PrV with a class 2 genome into variants with class 3-like genomes after passage in CEFs (11). The naturally arising class 3-like PrV variants have altered host cell growth characteristics. They have a distinct growth disadvantage in RK cells (or in mice) but have a significant growth advantage in CEF (or in chickens) compared with wild-type virus. The changed growth characteristics of the natural translocation mutants reside entirely in the changed structure of the junction between the S and L component. Replacement of that segment of DNA with wild-type sequences restores the wild-type phenotype. These class 3-like virus genomes possess two modifications: (i) an inverted translocation of sequences derived from the left terminus of the genome to a position adjacent to the right terminus of the U_L, and (ii) a deletion of sequences from the right terminus of the U_L (11, 12).

To analyze the factors that contribute to the emergence of the variants as well as to elucidate the molecular basis for the modified growth characteristics of the variants, mutants that harbor only one (the deletion) or both (the deletion and translocation) of the modifications that had been observed in the natural class 3-like variants were constructed. (For technical reasons, we were unable to obtain variants that harbor only the translocation.) The following results were obtained: (i) no viral mutant harboring only a deletion of the sequences at the right end of the U_L component of the genome had a cell-type-specific growth advantage over wild-type virus, and (ii) viral variants harboring a deletion next to the internal junction between the L and S component, as well as a translocation of the leftmost terminal sequences of the genome, had acquired the altered cell-specific growth characteristics of the variants. The translocation of the 325 terminal nucleotides of the genome in inverted form adjacent to the internal repeat was sufficient to duplicate the CEF-specific growth advantage and the RK-specific growth disadvantage seen in the naturally occurring variants. Since the deletion alone did not result in a cell-type-specific change in

the virus growth characteristics but the translocation in addition to the deletion did, the conclusion that it is the translocation that affords the virus with the altered growth characteristics appears warranted.

Although the size of the translocations seen in the naturally arising class 3-like mutants is in all cases approximately 1.4 kbp, the translocation of most of these sequences to confer the modified growth characteristics upon the virus is unnecessary. The translocation of the terminal leftmost 325 nucleotides of the genome appears to be sufficient. However, the deletion of 68 bp spanning the junction of the L and S components ablates the host-cell-specific growth characteristics of these mutants, as indicated by the results obtained with the deletion-translocation mutant vLD68.

The fact that most natural translocation mutants that emerge upon passage in CEF have a translocation approximately 1,400 bp in size while 325 bp are sufficient to confer upon the virus a growth advantage in CEF may be a reflection of the mode of genesis of these mutants. We have postulated previously that these mutants are generated by a double recombinational event between two concatemeric molecules in different orientations (12). It is possible that the second recombinational event occurs preferentially at a certain distance (approximately 1,400 bp rather than 325 bp) from the end of the inverted repeat (in which the first recombinational event has occurred).

The 325 terminal bp, the translocation of which is sufficient to confer the host-cell-specific growth characteristics, can function as an origin of replication (9, 25). They also contain a portion of the sequence necessary for cleavage of concatemeric viral DNA into unit-length genomes (25). The juxtaposition of the translocated segment of DNA and the internal inverted repeat that has occurred in the class 3-like mutants generates an internal alternative cleavage signal. Our results show that it is the creation of this alternative cleavage site that may be responsible, at least in part, for the altered growth characteristics of the deletion-translocation mutants (15).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10947 from the National Institutes of Health. Glenn F. Rall and Linda M. Reilly were supported by training grant 5T32 CA09385 from the National Cancer Institute.

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