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Evolution of Pseudorabies Virions Containing Genomes with an Invertible Long Component after Repeated Passage in Chicken Embryo Fibroblasts

B. LOMNICZI,[1]† A. GIELKENS,[2] I. CSOBAI,[1]† AND T. BEN-PORAT[1]*

Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232,[1] and Department of Virology, Central Veterinary Institute, 8221 RA Lelystad, The Netherlands[2]

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The genome of pseudorabies virus consists of two components, short (S) and long (L). Only the S component is bracketed by inverted repeats, and only the S component inverts itself relative to the L component, giving rise to two isomeric forms of the genome. An attenuated vaccine strain of pseudorabies virus (Norden), however, has a genome which is found in four isomeric forms (B. Lomniczi, M. L. Blankenship, and T. Ben-Porat, J. Virol. 49:970–979, 1984). To determine the basis for the atypical structure of the genome of the Norden strain, we examined more than 40 field isolates of pseudorabies virus; all contained genomes in which the L component was fixed in only one orientation relative to the S component. Several independently generated vaccine strains which have been passaged extensively in chicken embryos and chicken embryo fibroblast (CEF) cell cultures were also analyzed; they possessed an invertible L component. Furthermore, emergence of pseudorabies virus variants with an invertible L component was observed after passage of the virus in CEF, but not in rabbit kidney or pig kidney, cells. The invertibility of the L component was associated consistently with a translocation of sequences from the left end of the genome to a position next to the inverted repeat sequence of the S component. Three observations indicate that genomes with an invertible L component (and the translocation) have a selective growth advantage over standard pseudorabies virus when grown in CEF. (i) The proportion of virions with such genomes does not increase linearly as would be expected if the translocation events occurred repeatedly, most genomes eventually experiencing the translocation. Instead, after a lag, the proportion of such virions in the population increases relatively rapidly. (ii) The genome structures that are generated upon independent passage in CEF of each virion population were relatively homogeneous. Some heterogeneity was observed at relatively early stages of the emergence of the genomes carrying the translocation; at later stages, virions with genomes with a specific size translocation predominated in the virus population. (iii) Parallel passages in CEF of the same pseudorabies virus strain resulted in the emergence of populations of virions with genomes with different size translocations. However, in each of the passaged populations of virions the majority of virions had genomes with the same size translocation. The most likely interpretation of these results is that virions with genomes carrying the translocations that emerge upon passage of the virus in CEF have a selective advantage when grown in these cells.

The genomes of 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 genome of herpes simplex virus, for example, both the L and S components are bracketed by inverted repeat sequences, and both components invert themselves relative to each other, giving rise to four isomeric forms of the genome. Genomes with this structure have been designated class 3 DNA molecules (6). In the genome of pseudorabies virus (PrV), only the S component is bracketed by inverted repeats, and only the S component inverts itself relative to the L component, giving rise to just two isomeric forms. Genomes with this structure have been designated class 2 DNA molecules (6).

The functions of the inverted repeats in the genomes of the herpesviruses, the functional significance of the inversions they promote, and the processes which lead to the evolution of class 2 to class 3 DNA molecules (or vice versa) in the different herpesviruses are not known. No effect of the inversions of the genomes on the growth of the viruses has been documented to date; both isomers of PrV have been found to be infectious for rabbit kidney (RK) cells growing in vitro (4). Similarly, inversions of the S or the L component are not essential for growth in vitro of herpes simplex virus; virus mutants that are frozen in one or two of the isomeric forms of the DNA (because of the acquisition of unequal inverted repeats or because of loss of inverted repeats) are able to grow in cell culture (9, 14).

We have identified previously an attenuated vaccine strain of PrV (the Norden strain) in which the genome is found in four isomeric forms rather than the two found in wild-type PrV (8). In the Norden strain, a sequence of nucleotides that is normally present only at the left end of the PrV genome has been translocated in inverted form next to the internal repeat (IR) sequence at the junction between the L and the S components of the genome. As a consequence, the L component inverts and is found in two orientations relative to the S component. Restoration of a normal junction fragment to the Norden genome by marker rescue converts it to a typical class 2 molecule in which the L component is present in only one orientation (T. Ben-Porat, unpublished data), indicating that the invertibility of the L component is linked to the translocation.

* Corresponding author.
† Present address: Veterinary Medical Research Institute, Hungarian Academy of Sciences, H-1581 Budapest, Hungary.

The atypical structure of the genome of the Norden strain could be a reflection of the presence in nature of a minority of PrV strains containing genomes with an invertible L component, the Norden strain being derived from such an atypical field isolate. On the other hand, the genome of the Norden strain may have become modified in vitro as a result of the procedures used during its attenuation (i.e., passage in embryonated eggs and in chicken embryo fibroblasts [CEF]).

The experiments described in this paper were performed to distinguish between these two possibilities. We report here that whereas all newly isolated field isolates of PrV that we examined contained genomes in which the L component is fixed in only one orientation relative to the S component, several vaccine strains (which were passaged extensively in cell culture) acquired an invertible L component. Furthermore, we show that PrV strains with an invertible L component emerge upon passage of the virus in CEF but not in RK or pig kidney (PK) cells. The invertibility of the L component is consistently associated with a translocation of sequences derived from the left end of the genome to a position next to the IR sequence. Furthermore, the kinetics of emergence of virions with genomes with an invertible L component upon passage in CEF, as well as the fact that each virion population with an invertible L component that emerges is relatively homogeneous with respect to the size of the translocation, indicates that virions with genomes carrying this translocation may have a selective growth advantage in CEF.

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. PrV(7), PrV(53), and PrV(90) are recent virulent field isolates. The Bartha and Norden avirulent vaccine strains were received from P. S. Paul. The Tatarov (MK-25) strain (13) is a vaccine strain produced by Pharmachim, Sofia, Bulgaria.

RK, PK, and CEF cells were cultivated in Eagle synthetic medium (5) containing 5% bovine serum. Virus was titrated by plaque assay, usually in RK cells but sometimes in PK cells.

Chemicals and radiochemicals. [α-^{32}P]dCTP was purchased from New England Nuclear Corp. (Boston, Mass.). Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Purification of virions. A continuous line of PK (PK_{15}) cells were infected (multiplicity of infection, 0.5 PFU per cell) and incubated for 48 h in Eagle medium at 37°C. Virions were purified as described previously (1).

Extraction of DNA. Sodium sarkosinate (final concentration, 2%) was added to the 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 (50:48:2, vol/vol/vol) and either dialyzed against buffer (0.01 M Tris, 0.001 M EDTA, pH 7.6) or alcohol precipitated.

Restriction enzyme digestion and gel electrophoresis of DNA fragments. Digestion and agarose gel electrophoresis of viral DNA was done as described previously (2). Filter strips to which restriction fragments of PrV DNA were fixed were prepared by the method of Southern (12).

Electron microscopy of DNA. Electron microscopy of DNA was performed as described previously (3).

Nick translation of cloned PrV DNA restriction fragments. PrV DNA restriction fragments cloned in pBR325 as described previously (7, 15) were nick translated by the method of Rigby et al. (10).

RESULTS

Analysis of genomes of primary virus isolates obtained from different geographic areas. In contrast to other PrV strains that have been analyzed, the Norden vaccine strain of PrV has a genome with an invertible L component (8). To ascertain whether some primary isolates of PrV have genome structures similar to that of the genome of the Norden strain, more than 40 primary virus isolates obtained from infected animals in the United States or Europe (Hungary, Roumania, Bulgaria, or Czechoslovakia) were mapped by the Southern technique (12), using cloned nick-translated restriction fragments of PrV(Ka) as probes. In all cases, the genomes exhibited the characteristic class 2 structure, i.e., the S component consisted of a unique sequence (U_S) which was bracketed by inverted repeats and was present in two orientations relative to the L component; the L component was present in only one orientation and was not bracketed by inverted repeats (data not shown). Thus, most PrV field isolates contained typical class 2 genomes. It is probable therefore that the genome of the Norden strain had been modified and had acquired a class 3-like genome during the large number of passages in cells cultivated in vitro that had been carried out in the attempt to attenuate this strain.

Analysis of PrV genomes present in populations of virions after they were passaged extensively in cell culture. The genomes of more than 200 plaque isolates obtained from a population of virions [PrV(Ka)] that were passaged in RK cells 80 times at high multiplicity (and contained a high proportion of defective, interfering particles) or that were passaged 40 times at low multiplicity were analyzed. All the plaque isolates had a genome organization similar to that of the parental strain, i.e., consisted of characteristic class 2 DNA molecules.

The genomes of several independently derived vaccine strains, all of which were extensively passaged in cell culture, were also analyzed. Of these, the MK-25 (Tatarov) strain and all the derivatives of the Buk family of strains (of which the Norden strain is a member) had an invertible L component. Figure 1 shows an example of the hybridization of the genome of one of these vaccine strains, MK-25, to selected restriction fragments of PrV(Ka) DNA. The sequences in *Bam*HI fragment 14′ (see Fig. 2 for restriction maps) hybridize normally to a single restriction fragment generated by digestion of standard viral DNA with either *Bam*HI or *Kpn*I. However, digestion of the MK-25 genome with *Kpn*I yielded four fragments (fragments D/E and F/E comigrate), and digestion with *Bam*HI yielded two fragments that hybridized to *Bam*HI fragment 14′. Further analysis of the DNA of this strain with other restriction enzymes as well as with other probes revealed that sequences derived from the left end of the genome had been translocated to a site next to the IR. As a result of this translocation, two restriction fragments now hybridized to sequences of *Bam*HI fragment 14′. Furthermore, as a result of the translocation, the L component had become invertible; the sequences of *Bam*HI fragment 14′ therefore hybridized to four 0.5 M fragments in the *Kpn*I digest. Because the sequences that had been translocated in the MK-25 genome included, in addition to sequences derived from *Bam*HI 14′, adjacent sequences from *Bam*HI fragment 5′ and included the *Bam*HI cleavage site (i.e., the repeat generated by the translocation was cleaved by *Bam*HI), the sequences of *Bam*HI fragment 14′ (as well as those of *Bam*HI fragment 5′) hybridized to only two fragments in the *Bam*HI digests (Fig. 1). (Despite recent plaque purification. *Bam*HI fragment 12a,

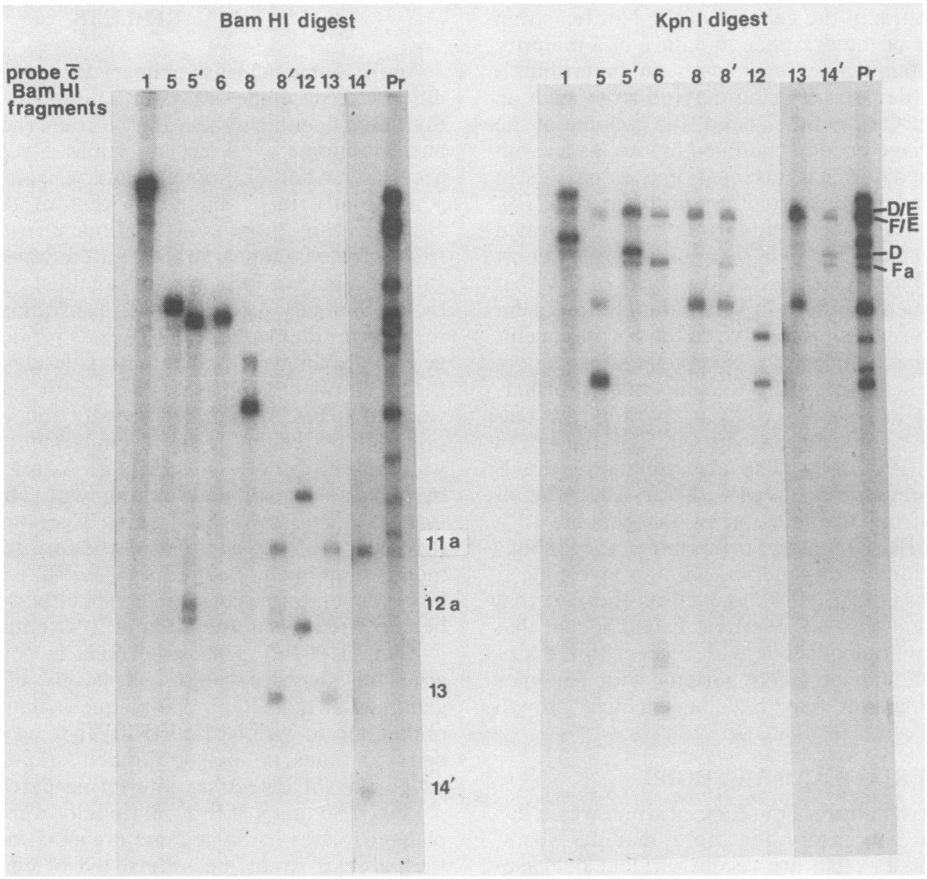

FIG. 1. Southern analysis of the DNA of the MK-25 strain of PrV. The DNA digests of the MK-25 strain were fixed to nitrocellulose filters by the method of Southern (12) and were hybridized to selected nick-translated cloned *Bam*HI fragments of PrV(Ka) (see Fig. 2 for map positions). The fragments that behave in an atypical manner are indicated at the right of each panel. The *Bam*HI fragments used as probes are indicated at the top of the figure. The whole PrV genome was also used as probe (Pr).

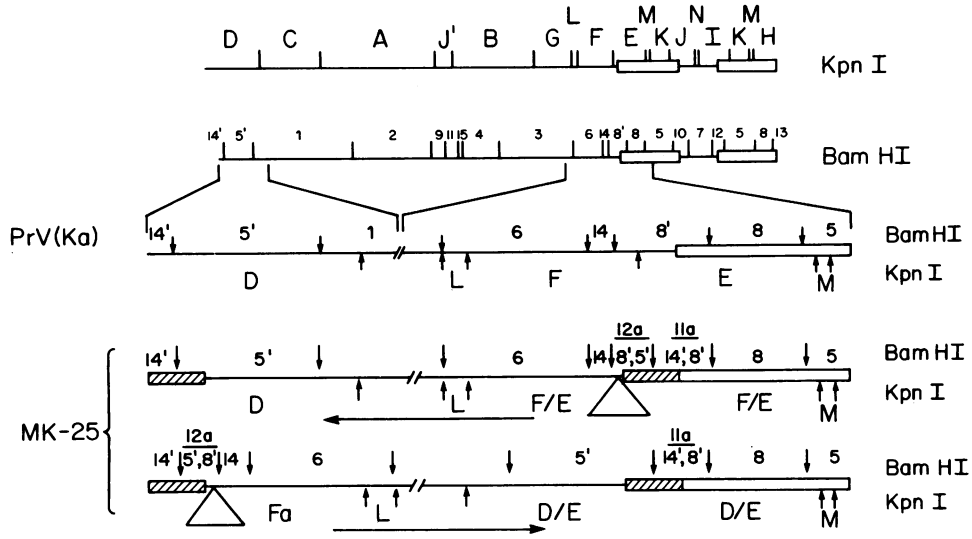

FIG. 2. Restriction maps of the MK-25 strain genome, illustrating the inversion of the L component. The map of the MK-25 genome was deduced from the hybridization patterns in Fig. 1, as well as others (not shown; see text). The *Kpn*I and *Bam*HI restriction maps of standard PrV(Ka) DNA are illustrated at the top of the figure. The regions of interest of the restriction map of the two isomeric forms of the MK-25 strain genome (prototype L [top] and inverted L [bottom]) are shown below. Open rectangles represent the inverted repeats bracketing the U_S; striped rectangles represent the inverted repeats bracketing the U_L. The size of the deletion in fragment 8′ is also indicated.

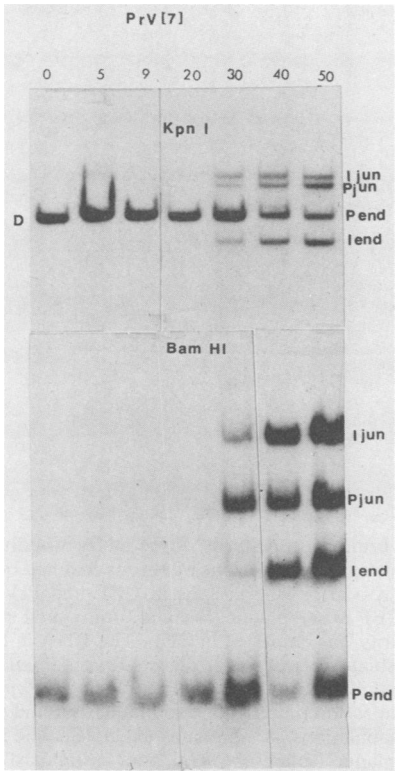

FIG. 3. Hybridization to *Bam*HI fragment 14′ of *Kpn*I and *Bam*HI restriction digests of DNA obtained from PrV(7) after different numbers of passages in CEF. PrV(7) was passaged repeatedly at low multiplicity (0.1 PFU per cell) in CEF. After the indicated number of passages (top of lanes), virions were purified, and the DNA was extracted and digested with either *Kpn*I or *Bam*HI. The digests were electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated *Bam*HI fragment 14′ of PrV(Ka). I jun, Inverted junction, the junction fragment between the prototype left end of the genome and the S component that is obtained after inversion of the L component; P jun, prototype junction between the L and S component, *Bam*HI fragment 8′ or *Kpn*I fragment E into which sequences from the left end of the genome have been translocated; I end, inverted end, the end fragment of the L component generated after its inversion; P end, prototype left end of the genome, *Kpn*I fragment D or *Bam*HI fragment 14′ (see map in fig. 2).

which hybridizes to *Bam*HI fragments 5′ and 8′, was heterogeneous in size.) The sizes of the fragments spanning the junction between the L and S component indicated that a deletion of sequences from the right end of the U_L (adjacent to the translocation) had also occurred in the genome of the MK-25 strain.

The *Bam*HI and *Kpn*I maps of the genome of the MK-25 strain, as deduced from the hybridization of *Bam*HI and *Kpn*I digests to all *Bam*HI restriction fragments of PrV(Ka) (and corroborated by analysis of *Pst*I and *Sal*I digests), are illustrated in Fig. 2.

Evolution of genomes of PrV with an invertible L component after passage of CEF. The attenuated vaccine strains that have acquired an invertible L component have a history of being passaged in embryonated eggs or in CEF. On the other hand, PrV(Ka) that had been passaged extensively in RK cells had retained a noninvertible L component (see above). We therefore considered the possibility that passage of PrV in embryonated eggs or CEF (i.e., cells of avian origin) might promote the emergence of or the selection or both of virions with genomes with an invertible L component.

To test this possibility, we passaged different strains of PrV repeatedly at low multiplicity (0.1 PFU per cell) in CEF, RK, or PK cells. The DNA of partially purified virion populations produced by these cells at different passage levels was digested with restriction enzymes and analyzed by the Southern technique (12) to determine whether the virions had undergone the characteristic translocation seen in some of the vaccine strains and whether the L component of their genome had become invertible. *Bam*HI fragment 14′, which originates from the left end of the viral genome, or *Bam*HI fragment 8′, which includes sequences that are derived from the junction of the L and S components (see restriction map in Fig. 2), were used as the probe.

Figure 3 shows the hybridization patterns to *Bam*HI fragment 14′ of *Bam*HI and *Kpn*I digests of DNA extracted from a population of PrV(7) virions (a field isolate from the United States) after different numbers of passages in CEF. As expected, only one fragment in each of the DNA digests of standard PrV(7) virions (before passage in CEF) hybridized to *Bam*HI fragment 14′. After passage of the virus in CEF, *Bam*HI fragment 14′ hybridized to four *Kpn*I fragments and to four *Bam*HI fragments (Fig. 3).

The hybridization of the DNA of the same virion populations to *Bam*HI fragment 8′ after passage in CEF or PK cells is illustrated in Fig. 4. Normally, these sequences hybridized to *Bam*HI fragments 8′ and 13, both of which include part of the sequences within the inverted repeats (Fig. 2). Passage of the virus in PK cells did not change the hybridization pattern of the viral genomes to *Bam*HI fragment 8′, but after passage of the virus in CEF this probe hybridized to four instead of two *Bam*HI restriction fragments. Concomitant with the appearance of additional bands hybridizing to *Bam*HI fragment 8′, a deletion of sequences from *Bam*HI fragment 8′ was observed.

To determine the reproducibility of these phenomena, we

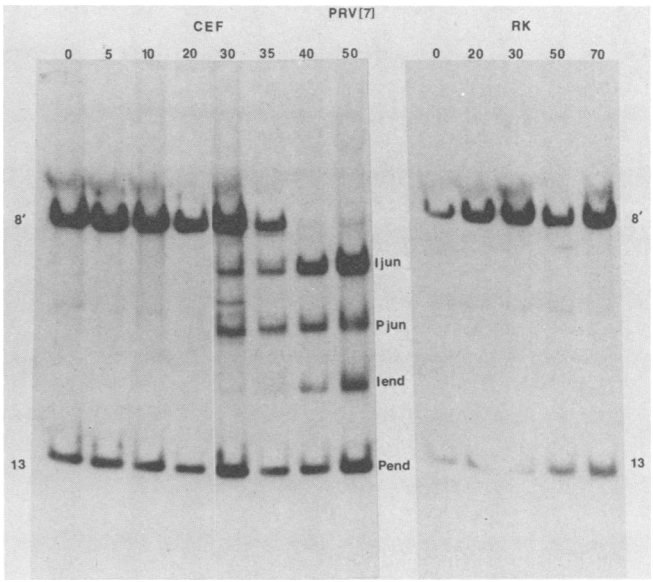

FIG. 4. Hybridization to *Bam*HI fragment 8′ of the *Bam*HI digests of PrV(7) DNA after passage of the virions in CEF and RK cells. The experiment was performed as described in the legend to Fig. 3.

analyzed the hybridization patterns obtained after passage of eight other PrV strains in CEF, RK, or PK cells. In all cases, modifications of the genomes similar to the ones observed with PrV(7) were observed after passage of the virions in CEF but not in RK or PK cells. One example, PrV strain PrV(Ka) passaged in CEF, is illustrated in Fig. 5. The sizes of the translocation and the sizes of the deletion varied in the different passaged strains.

The structures of the genomes of individual plaque isolates obtained from populations of virions passaged in CEF were analyzed. Plaques were picked, virus was amplified, and viral DNA was extracted and analyzed by the Southern technique. Figure 6 shows an example of hybridization of the genomes of two individual plaque isolates obtained from two different virion populations to a probe consisting of *Bam*HI fragment 14′. Although small differences in the sizes of the small end fragments of individual plaques obtained from the same virion population could occasionally be observed (Fig. 6), the restriction digests of the genomes of plaque-purified virions obtained from the same population were similar. This finding is not surprising because after extensive passage in CEF, each population of virions appeared to be relatively homogeneous with respect to the aberrant restriction fragments that were generated (Fig. 3 to 5). The results also show that the restriction patterns of the genomes of plaque isolates obtained from different independently passaged virion populations were quite different (Fig. 6; compare also Fig. 3 to 5).

The modifications in the restriction digests of the genomes of the different PrV strains that had been passaged in CEF were analyzed with several restriction enzymes, and the genomes were mapped. Examples of the results obtained with two virion populations are summarized in Table 1. In both PrV(7) and PrV(53) a translocation of sequences de-

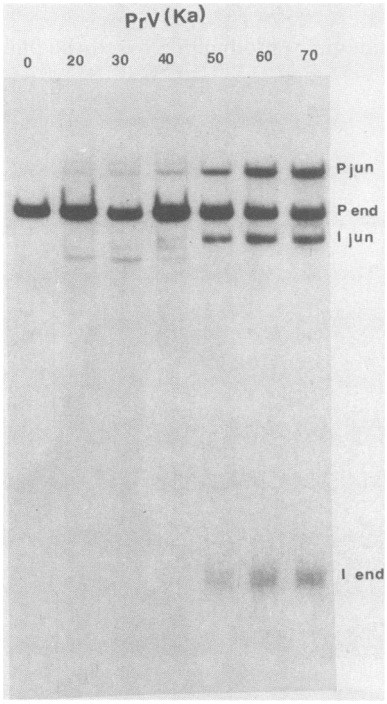

FIG. 5. Hybridization to *Bam*HI fragment 14′ of *Kpn*I digests of PrV(Ka) after passage of the virions in CEF. The experiment was performed as described in the legend to Fig. 3.

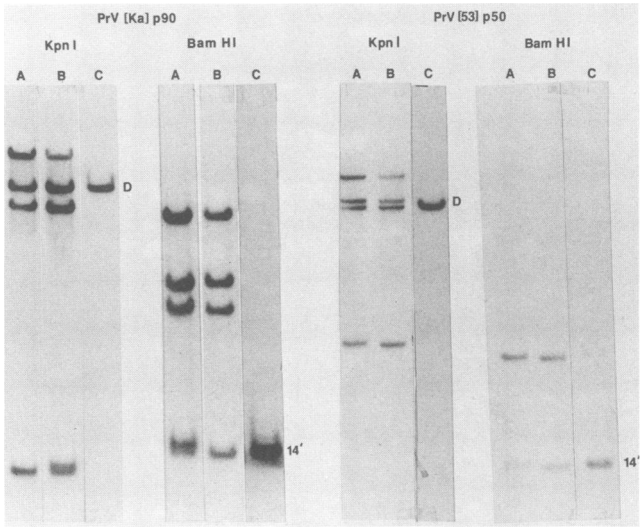

FIG. 6. Hybridization patterns of the DNA of individual plaque isolates obtained from populations of virions that had been passaged in CEF. PrV(Ka) after 90 passages in CEF and PrV(53) after 50 passages in CEF were plaque assayed. Individual plaques were picked, and virus was amplified in CEF. The DNA in the virions of each plaque isolate was purified, digested with *Bam*HI and *Kpn*I, electrophoresed, and hybridized to nick-translated *Bam*HI fragment 14′. The hybridization patterns of two plaques (A and B) from each of the virion populations are shown as examples. The hybridization patterns of a plaque isolate obtained from a standard PrV(90) (not passaged in CEF) are also shown (C).

rived from the left end of the standard genome next to the IR occurred after passage of the virus in CEF, and the L component of the genomes became invertible. The size of the translocation varied in the two virion populations. In PrV(53), the fragment of DNA that was translocated was larger than in PrV(7) and included the *Bam*HI cleavage site located between *Bam*HI fragments 14′ and 5′. Consequently, *Bam*HI cleaved within the newly formed repeat, and therefore *Bam*HI fragment 14′ hybridized to only two *Bam*HI restriction fragments. A deletion of sequences from *Bam*HI fragment 8′ similar to that observed in the MK-25 strain was observed in PrV(7) but not in PrV(53).

A similar analysis of the genomes of other virus strains that had been passaged in CEF showed that the size of the DNA fragment derived from the left end of the genome that had been translocated next to the inverted repeat varied from between 500 and 4,500 base pairs (data not shown). The sizes of the deletions in *Bam*HI fragment 8′ that often accompanied the translocation also varied from undetectable (<200 base pairs) to 1,700 base pairs. These modifications were observed only after the virus had been passaged in CEF; passage of the virus in PK cells or RK cells did not result in similar modifications of the genomes of the virion populations.

Parallel passage of the same strain of PrV in CEF leads to the emergence of virions containing genomes with different size translocations. To determine whether the sizes of the DNA segments that were translocated in the genomes of PrV after passage in CEF depend on the virus strain or whether parallel passages of the same virus strain would lead to the emergence of virions with genomes in which segments of DNA of different sizes had been translocated, we did independent serial passages of PrV(53) in CEF and in PK cells. At passage 30, the virion populations were purified, and the

TABLE 1. Analysis of hybridization of restriction digests of the DNA of PrV(7) and PrV(53) passaged in CEF to *Bam*HI fragment 14'[a]

PrV fragment	*Kpn*I digest[b]	Fragment size (10^6 daltons)	*Bam*HI digest[b]	Fragment size (10^6 daltons)
PrV(7)p50				
Inverted junction	Fr[b] D (8.3) + Fr H (5.3)	13.6	Fr 13 (1.1) + Fr 14' (0.75)	1.85
Prototype junction	Fr E + F (6.3 + 6.1) − deletion (1.1) + translocation (0.7)	12.0	Fr 8' (2.9) − deletion (1.1) + translocation (0.7)	2.5
Inverted end	Prototype junction (12.0) − Fr H (5.3)	6.7	Prototype junction (2.5) − Fr 13 (1.1)	1.4
Prototype end	Fr D (8.3)	8.3	Fr 14 ' (0.75)	0.75
PrV(53)p50C				
Inverted junction	Fr D (8.3) + Fr H (5.3)	13.6	Fr 13 (1.1) + Fr 14' (0.75)[c]	1.85
Prototype junction	Fr E (6.3) + translocation (2.9)	9.2		
Inverted end	Prototype junction (9.2) − Fr H (5.3)	3.9	Fr 14 ' (0.75)[c]	0.75
Prototype end	Fr D (8.3)	8.3		

[a] *Kpn*I or *Bam*HI digests of the DNA of PrV(7) passaged 50 times in CEF or PrV(53) passaged 50 times in CEF were hybridized to nick-translated sequences from BamHI fragment 14' of standard PrV DNA, and the number and sizes of the fragments that hybridized were determined. The sizes of the sequences that were translocated and deleted were deduced from the analysis of these results, as well as those results obtained by analyzing the DNA preparation after digestion with other restriction enzymes and hybridizing to the same and other probes.

[b] Fr, fragment; sizes of fragments (in kilodaltons) are shown in parentheses.

[c] (*Bam*HI cleavage site present in repeat bracketing L component. Consequently, only two fragments hybridize to *Bam*HI fragment 14'.)

DNA was extracted and analyzed. Figure 7 shows the following. (i) As expected, passage of the virus in PK cells did not alter significantly the hybridization pattern of the viral DNA; *Bam*HI fragment 14' hybridized to *Kpn*I fragment D only. (ii) In all three parallel passages of the virus in CEF cells, viral genomes emerged in which the characteristic translocation had occurred and in which the L component had become invertible. (iii) The three populations of virions that had been passaged independently in CEF cells differed with respect to the sizes of their end and junction fragments, indicating that fragments of different sizes had been translocated. (iv) Although not completely homogeneous, each population of virions appeared to be enriched considerably for genomes with a translocation of a specific size. (This was corroborated by analysis of plaque-purified virus isolated from these virion populations.)

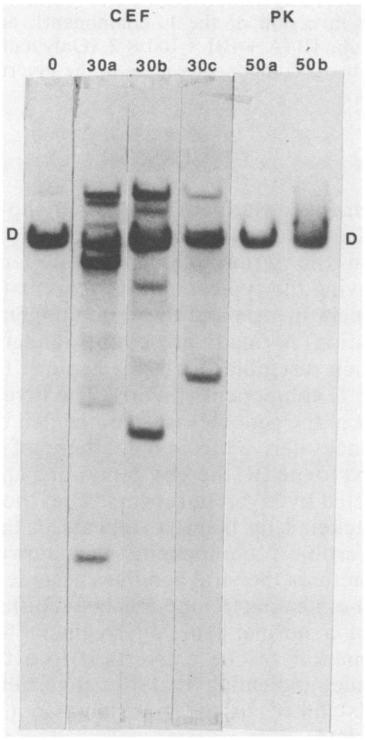

FIG. 7. Hybridization patterns of the genomes present in three populations of PrV(53) that were independently passaged in CEF. Three independent passages of PrV(53) (a, b, and c) were done in parallel in CEF, and two (a and b) were done in PK cells. The DNA in the virion population after 30 passages in CEF and after 50 passages in PK cells was purified, digested with *Kpn*I, and hybridized to nick-translated *Bam*HI fragment 14'.

The findings that the sizes of the segments of DNA that had been translocated varied after parallel passages in CEF of the same virus strain and that each population of virions was enriched for genomes with a translocation of a specific size suggests that virions with genomes that carry the translocation have a growth advantage in CEF cells.

Kinetics of appearance of PrV with genomes that had acquired an invertible U_L upon passage in CEF. Inspection of the hybridization patterns illustrated in Fig. 3 to 5 reveals that the accumulation of virions with an invertible L component is not linear. Thus, during early passages in CEF (the number of which varies in different parallel passages) the viral genomes remained noninvertible; thereafter, the entire population was converted relatively rapidly to virions with genomes with an invertible L component.

Scans of autoradiograms obtained after hybridization of *Bam*HI fragment 14' to digests of DNA from virions after different passage levels showed that indeed the transition from virions with genomes with a noninvertible L component to virions with genomes with an invertible L component was rapid, once it had been initiated (Fig. 8). After 16 passages in CEF, *Bam*HI fragment 14' hybridized mainly to the end fragment generated from the left (prototype) end of the genome (*Kpn*I fragment D); less than 5% of the molecules were invertible as ascertained by the relative amounts of hybridization of the probe to the prototype end fragment and to the junction fragment that is generated after the L component has inverted. However, by passage 28, the amount of hybridization of *Bam*HI fragment 14' to the prototype end and inverted junction fragments was almost equal. Quantitation of the amount of probe hybridized to these two bands showed that by passage 28, approximately 90% of the virus genomes had an invertible U_L.

We analyzed similarly several other populations of virions and found that, in all cases, upon passage in CEF the transition from virions containing a noninvertible L compo-

nent to virions containing an invertible L component was relatively rapid, once genomes with invertible L components became detectable in the population (Fig. 9). In one case [PrV(90)], however, while genomes with a translocation and an invertible L component emerged upon initial passages in CEF, upon further passages the population reverted to virions which had lost the translocation and contained genomes with a noninverting L component. Of a total of 14 independent passages of nine different strains of PrV in CEF, this was the only case in which such a reversion occurred. In all other cases, the virions in the population retained an invertible L component once they had acquired it, whether they were passaged further in CEF, RK, or PK cells.

The finding that independently passaged virion populations differed with respect to the size of the translocation in their genomes but that each population of virions was enriched in virions with genomes with the same size translocation indicates that the translocation event leading to the invertibility of the L component is relatively rare. The kinetics of accumulation in the virion populations of genomes that acquired the translocation indicate that virions containing such genomes possess a selective advantage when grown in CEF. In the one case [PrV(90)] in which the population reverted upon further passage in CEF cells to virions containing genomes which had lost the translocation and which had reacquired a noninverting L component, a compensating mutation had most likely occurred.

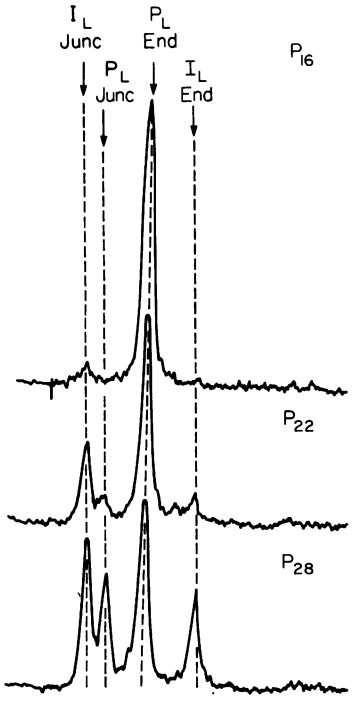

FIG. 8. Scans of the hybridization to nick-translated *Bam*HI fragment 14′ of *Kpn*I-digested DNA obtained from PrV(53) virions after various numbers of passages in CEF. The experiment was performed as described in the legend to Fig. 3. After exposure to X-ray films for the appropriate times (to ensure linearity of exposure), films were scanned with a Joyce-Loebl microdensitometer, and the relative amounts of DNA hybridized were determined with a planimeter. For abbreviations, see the legend to Fig. 3.

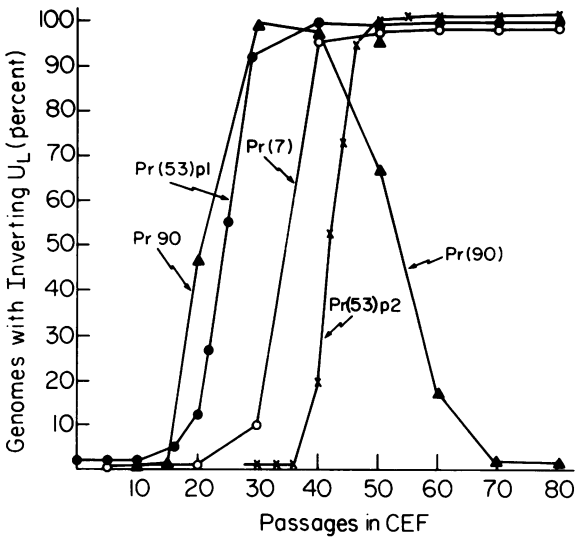

FIG. 9. Accumulation of genomes with an invertible L component within the virion population after different numbers of passages in CEF. Several PrV strains were passaged in CEF. After the indicated number of passages, the DNA in the virion populations was extracted and digested with restriction enzymes. The digests were electrophoresed, fixed to nitrocellulose filters, and hybridized to nick-translated *Bam*HI fragment 14′. After exposure of the filter strips to X-ray film for the appropriate times, the films were scanned. The percentage of genomes in the virion population that had an invertible L component was determined from the relative amounts of the probe that hybridized to the prototype end fragment (A) and to the inverted junction fragment (B) (i.e., the prototype end fragment after inversion of the L component), according to the following formula: $[B/(A + B)] \times 100 \times 2$. (Only half of the genomes with an invertible L component will be in the inverted orientation).

DISCUSSION

The experiments described in this paper show that passage of PrV in CEF, but not in RK or PK, cells results in the emergence in the virion population of genomes which, instead of having the typical class 2 genome (in which only the S component inverts and the L component is found in a single orientation) normally present in standard PrV, have a structure which resembles a class 3 genome (in which both the S and the L components invert). The invertibility of the L component of the genomes is accompanied by a translocation of sequences derived from the left end of the genome to a position next to the IR, thereby generating an L component that is bracketed by inverted repeats. That the L component is indeed bracketed by inverted repeats in the genomes of PrV with invertible L components was shown by the characteristic structures the viral genomes assume after denaturation and self-annealing (8; unpublished data). Furthermore, restoration of a normal junction fragment to the Norden genome by marker rescue converts it to a typical class 2 DNA molecule, indicating that the invertibility of the L component is linked to the translocation (T. Ben-Porat, unpublished data).

The finding that translocations that generated genomes in which the L component had become bracketed by inverted repeats had occurred was interesting though not particularly surprising. Thus, after passage of PrV(Ka) at high multiplicity in RK cells, infectious virus can occasionally be isolated whose genomes carry various translocations (T. Ben-Porat,

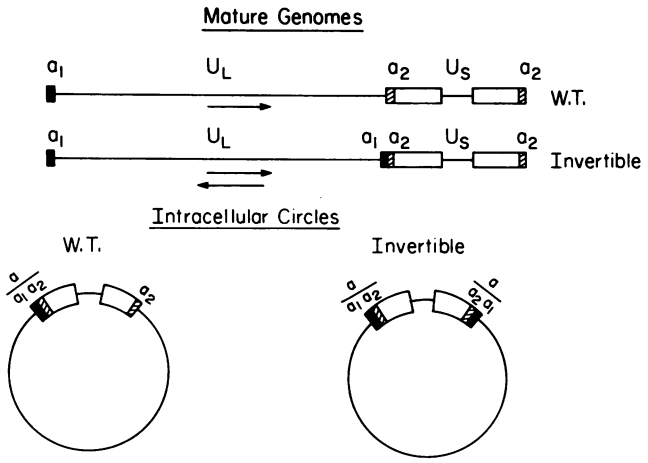

FIG. 10. Schematic diagram illustrating the structure of the linear and circular forms of the genome of standard PrV and of the genome of a PrV variant that has an invertible L component and carries a translocation of sequences derived from the left end of the genome next to the IR. W.T., Wild type.

unpublished data). Indeed, defective PrV DNA is composed of segments of DNA originating from different regions of the standard genome that have become covalently linked (11, 15). The translocation, in inverted form, of sequences derived from the left end of the genome next to the IR and the consequent formation of genomes with an invertible L component could thus, in principle, be the result of commonly occurring random translocation events. Alternatively, it could be linked to specific structures the viral DNA assumes during replication.

Whatever the basis for the observed translocations, the salient finding described in this paper is that the accumulation in the virion population of genomes with an invertible L component does not appear to reflect merely a high frequency of translocation events. The results indicate that at least some genomes that have acquired a translocation of sequences derived from the left end of the genome next to the IR have a selective advantage over standard PrV when grown in CEF. This conclusion can be derived from the following three observations. (i) The proportion of virions with genomes with an invertible L component in the virion population does not increase linearly, as would be expected if the translocation events occurred repeatedly, most of the genomes eventually experiencing the translocation. Instead, after a lag, the proportion of such virions in the population increases rapidly upon continued passage in CEF. (ii) While some heterogeneity in the size of the translocation (Fig. 4 and 7, for example) in the genomes of a given virion population passaged in CEF is observed at relatively early passage levels, at later passage levels most of the genomes in the virion population have the same size translocation. (iii) Three parallel passages in CEF of the same PrV strain resulted in the emergence of three populations of virions with genomes carrying different size translocations; however, the majority of the virions in each of these populations had genomes with a similar size translocation.

The emergence of virions with genomes with invertible L components which have a selective growth advantage in CEF may not be a single-step event. Thus, during early passages in CEF the population of virions was not homogeneous with respect to the size of the translocation the virions carried (Fig. 5 and 7). Furthermore, the results in Fig. 3 indicate that translocation of sequences derived from the right end of the genome next to the IR may not always result in inversion. Thus, while at passage 30 sequences derived from the end of the U_L (*Bam*HI fragment 14') hybridized abundantly to both the prototype end and junction fragments, hybridization to either the inverted end or the inverted junction fragments was slight, indicating that the L component of only a small proportion of the genomes with the translocation had inverted. At later passage levels hybridization to the fragment derived from the prototype and inverted forms of the genome was of equal intensity. Thus, it appears that the acquisition of an invertible L component may, at least in some cases, occur in more than one step.

The molecular basis for the growth advantage that PrV strains with genomes with an invertible L component appear to have in CEF is unknown but is probably related to the juxtaposition in these genomes of sequences normally present at the two ends of the mature virus genome. Figure 10 shows the structure of the linear mature form and of the circular form (the form the viral genome assumes upon entering the cell [4]) of the genome of standard PrV and of a variant with an invertible L component. In the standard genome the sequences found at the two ends of the mature linear genome become juxtaposed after the formation of circles (or concatemers). In the variants carrying the translocation these sequences are juxtaposed even in the linear form of the genome, and after circle (or concatemer) formation these juxtaposed sequences are present twice in each genome equivalent.

Circle formation occurs as efficiently in CEF, PK, or RK cells infected with either standard virus or virus with genomes with an invertible L component (T. Ben-Porat, unpublished data), and it is unlikely therefore that a lack of juxtaposition of the two ends of the genome plays a role in the poorer growth in CEF of standard PrV than of PrV with an invertible U_L. However, the double dosage of the juxtaposed sequences present in the genomes carrying the translocation could somehow affect transcription, DNA replication, or cleavage-encapsidation. Indeed, efficient cleavage-encapsidation is dependent on signals that are present at both ends of the genome (15). We are currently investigating these possibilities and are determining the organization of the juxtaposed end sequences in the genomes of the variants at the nucleotide level in an attempt to gain some understanding of the possible mechanism by which the translocation may affect virus growth.

The basis for the evolution of the genomes of the herpesviruses from class 2 to class 3 DNA molecules (or vice versa) is obscure. To date, no effect of the inversions of the genome on the growth characteristics of the virus has been documented. Thus, for example, both isomers of PrV are infectious for cells grown in vitro (4), and inversion of the S or the L components is not essential for growth in vitro of HSV (9, 14). We show here that PrV genomes with characteristics similar to those of a type 3 herpesvirus DNA molecule appear to have a selective advantage when grown in avian cells, i.e., cells that are distantly related to the normal host of PrV (swine). This finding has implications concerning the evolutionary pressures that may have led to the emergence of the different types of herpesvirus genomes and indicates that the characteristics of herpesvirus genomes that function in the generation of the different isomeric forms of the genomes may also, under certain conditions, affect the growth of the virus.

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