Functions of the Sequences at the Ends of the Inverted Repeats of Pseudorabies Virus

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Two mutants were constructed to explore the functions of the sequences at the end of the S terminus of pseudorabies virus (PrV). In mutant vYa, 17 bp from the internal inverted repeat, as well as adjacent sequences from the L component, were deleted. In mutant v135/9, 143 bp from the internal inverted repeat (including sequences with homology to the pac-1 site of herpes simplex virus), as well as adjacent sequences from the L component, were deleted. Our aim in constructing these mutants was to ascertain whether equalization of the terminal regions of the S component would occur, whether genome termini that lack either the terminal 17 or 143 bp would be generated as a result of equalization of the repeats (thereby identifying the terminal nucleotides that may include cleavage signals), and whether inversion of the S component would occur (thereby ascertaining the importance of the deleted sequences in this process). The results obtained show the following. (i) The removal of the terminal 17 or 143 bp of the internal S component, including the sequences with homology to the pac-1 site, does not affect the inversion of the U_S. (ii) The equalization of both the vYa and the v135/9 inverted repeats occurs at high frequency, the terminal repeats being converted and becoming similar to the mutated internal inverted repeat. (iii) Mutants in which the 17 terminal base pairs (vYa) have been replaced by unrelated sequences are viable. However, the 143 terminal base pairs appear to be essential to virus survival; concatemeric v135/9 DNA with equalized, mutant-type, inverted repeats accumulates, but mature virions with such equalized repeats are not generated at high frequency. Since concatemeric DNA missing the 143 bp at both ends of the S component is not cleaved, the terminal 143 bp that include the sequences with homology to the pac-1 site are necessary for efficient cleavage. (iv) v135/9 intracellular DNA is composed mainly of arrays in which one S component (with two equalized inverted repeats both having the deletion) is bracketed by two L components in opposite orientations and in which two L components are in head-to-head alignment. Because (i) no homology exists in the circularized (or concatemeric) genomes of these mutants between the sequences adjacent to the two ends of the S component which would allow equalization of the repeats by recombination, and (ii) the alternate product of recombination, i.e., DNA with wild-type sequences at the ends of both inverted repeats, does not accumulate, we postulate that equalization of the termini of the repeats occurs by a copying mechanism. Since it is the terminal repeat that is converted consistently and becomes identical to the internal repeat, we propose that the terminal repeat invades the internal repeat, which acts as a template for its conversion. To account for the accumulation in v135/9-infected cells of concatemeric DNA in which the L components are in head-to-head alignment, the model we propose postulates that the sequences with homology to the pac-1 site (present at the ends of the inverted repeats in wild-type but missing from v135/9 intracellular DNA) are somehow responsible for preventing strand elongation beyond the ends of inverted repeats. In the absence of these sequences, strand elongation would continue after the internal end has been copied, and concatemeric molecules in which L components are in head-to-head alignment would be formed by a process akin to that postulated for poxvirus DNA replication.

The genomes of the herpesviruses are large double-stranded molecules which have been grouped into three or five classes (13, 29). Class 2 and 3 genomes (or D and E) are composed of two components, the L and the S, which may be bracketed by large inverted repeats and may invert their orientation relative to each other (6). While homologous recombination via the inverted repeat flanking the S and L components could be responsible for their inversion (30, 32, 33), it has been suggested that the "a" sequence of herpes simplex virus (HSV) (the terminal 300 to 500 bp of the

inverted repeats bracketing both the L and S components) includes *cis*-acting elements that mediate site-specific inversion (7, 20, 21). However, mutants of HSV, in which the internal "a" sequence has been deleted, retain a reduced ability to invert their L component (17), indicating that the "a" component is not essential for inversion. Indeed, several reports have appeared (24, 35, 36) that indicate that the inversion process may lack sequence specificity and that the sequences at the ends of the inverted repeats may be inherently recombinogenic, i.e., recombination may occur in the absence of specific herpesvirus-encoded proteins (36).

The "a" sequence of HSV also includes signals required for cleavage and encapsidation of concatemeric DNA. Two elements (pac-1 and pac-2, as defined in reference 8) that are highly conserved among herpesviruses include signals for site-specific cleavage (8, 23, 31).

Another attribute of the inverted repeats bracketing the

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unique components of the herpesvirus genomes is their propensity to equalize. Equalization could be mediated by a copying mechanism in which one repeat acts as a template for the other, as initially proposed by Roizman (28). Alternatively, it could occur by intermolecular recombination followed by segregation. Evidence that intermolecular recombination occurs at a frequency that is sufficient to account for equalization of the repeats (33) and that equalization of the repeats can indeed occur by intermolecular recombination (2) has been obtained.

Pseudorabies virus has a class 2 or D genome (1). Only the S component is bracketed by inverted repeats and inverts relative to the L component. The sequences at the L and S termini of the genome of PrV differ (12). A sequence with homology to the pac-1 element of the "a" sequence of HSV is present at the ends of the inverted repeat bracketing the S component. A sequence with homology to the pac-2 element of the "a" sequence of HSV is present at the left terminus of the genome (11). Thus, both ends of the genome include elements with homologies to the "a" sequences, and upon entering the cell nucleus, a sequence that bears similarities to the "a" sequence of HSV is generated after the genome of PrV circularizes (5, 14).

We constructed two mutants with small deletions encompassing the terminal nucleotides of the internal inverted repeat. The mutants thus created should, in principle, have genomes that are heterozygous with respect to the end sequences of the inverted repeats. These mutants were constructed because we hoped they might clarify some aspects of the processes by which the repeats equalize. Because no sequence homology exists in these mutants between the two termini of the L component, i.e., between the sequences adjacent to the two inverted repeats in concatemeric DNA, equalization of the end sequences of the repeats, were it to occur, could not be attributed to homologous recombination. The equalization of the ends of the inverted repeat (or lack thereof), i.e., the acquisition at the end of the genome of the deletion that had been introduced at the end of the internal repeat, should also help delimit the terminal nucleotides of the genome essential for cleavage and encapsidation. Finally, we hoped to ascertain the role of the terminal sequences of the inverted repeat bracketing the S component, sequences with homology to the pac-1 sequence of HSV, in inversion of that 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 construction of the mutants used in this study is described below. The viruses were grown in pig kidney (PK) or rabbit kidney (RK) cells which were cultivated in Eagle's synthetic medium containing 5% bovine serum.

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 and dATP were purchased from Dupont, NEN Research Products.

Purification of virions and of viral DNA and cloning of restriction fragments. The procedures used to purify virions and viral DNA were described previously (3). Cloning was performed essentially by the method of Maniatis et al. (19). Briefly, to clone the end fragments of the PrV genome, DNA preparations obtained from mature virions were digested to completion with either *Bgl*III or *Hind*III and run on a 0.5%

FIG. 1. Restriction map of the region of the junction between the L and S components of wild-type (W.T.), vYa, and v135/9 mature genomes. Lines 1 and 2, structure and *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 of the wild-type genome (*Bam*HI fragment 8'). 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. Lines 5 and 7, deletions introduced into vYa and v135/9 are indicated by thin lines. Lines 6 and 8, translocations of M13 sequences are indicated by black rectangles. Line 9, restriction map of the region spanning the junction between the L and S components of vYa. Line 10, restriction map of the region spanning the junction between the L and S components of v135/9. Sm, *Sma*I; SS, *Sst*II; Kpn, *Kpn*I; Bg, *Bgl*II; Stu, *Stu*I; Sph, *Sph*I; Dr, *Dra*I; Eco, *Eco*RI; Bam, *Bam*HI.

agarose gel, and the terminal fragments (*Bgl*III fragment D or E or *Hind*III fragment C) were excised from the gels and purified. The DNA was blunt ended, *Eco*RI linkers were attached, and the fragments were cloned into the *Eco*RI site of pBR325. To clone internal *Bam*HI fragments, the DNA was digested with *Bam*HI, the desired fragment was isolated from gels, purified, and cloned into the *Bam*HI site of pBR325.

Southern and sequence analyses. Southern analysis, nick translation of DNA probes, and sequencing were performed as described previously (9, 18).

Construction of deletion plasmids. (i) p135/9. The parent plasmid was pTN155, the construction of which has been described previously (27). Briefly, pTN155 includes the sequences spanning the internal junction between the S and L components of PrV approximately between bp -1142 and +741 (Fig. 1). However, the sequences between the *Sst*II sites at nucleotides -677 and -390 have been deleted and replaced with a 285-bp M13mp19 *Hae*III fragment that includes the multiple cloning site (MCS). This plasmid was constructed by T. Mettenleiter.

To construct p135/9, we digested pTN155 with *Sph*I, which cleaves 143 bp within the inverted repeat as well as in the MCS of the M13 sequences. The linearized plasmid was purified from agarose gels and religated. Thus, in p135/9 the junction between the S and L components has a deletion

FIG. 3. Restriction maps of wild-type (W.T.) and v135/9 mature virion DNA. The black rectangles on the maps of the v135/9 genome represent the M13 sequences; the arrows above these rectangles indicate the restriction sites in the MCS of the M13 sequences.

The *Bgl*II fragments D and E (the two S-terminal fragments of the PrV genome derived from the two orientations of the S component; see Fig. 3 and 6) were cloned as described in Materials and Methods, and the sequence at their termini was determined. The results (Fig. 4) showed that the terminal nucleotides of vYa, starting at the *Sma*I site, i.e., 15 bp from the ends of the genome (in the mature PrV genome there is a 2-bp 3' overhang at the S terminus [12]) were different from those of wild-type DNA. Instead of the terminal sequences normally found in wild-type DNA, the M13 sequences that had been inserted next to the internal inverted repeat were present. Thus, the M13 sequences that had been inserted next to the internal inverted repeat had become part of the terminal inverted repeat, and equalization of the repeats had occurred. Both *Bgl*II fragments D and E had acquired the same M13 sequence at their S terminus, i.e., the same conversion had occurred at the ends of both isomeric forms of the DNA (data not shown).

The sequence at the S terminus of five additional vYa mutants that had been independently isolated from four different transfections was also ascertained to determine whether replacement of the terminal repeat sequences with sequences that had been introduced at the internal repeat had occurred consistently. *Bam*HI fragment 13, which includes the terminal sequences of the S component (Fig. 1), obtained from the genomes of these mutants, was excised from the gels and sequenced directly (without cloning), as described previously (12). The termini of all those mutants were identical; the terminal 15 bp normally present at the S terminus of the wild-type genome had been replaced by the M13 sequence that had been inserted in the vYa mutants adjacent to the deleted internal inverted repeat.

(ii) v135/9. The sequence of the S terminus of v135/9 was ascertained as follows. The unique *Hind*III end fragment derived from the right terminus of the genome (*Hind*III fragment C; Fig. 3) was isolated from v135/9 DNA and either

FIG. 4. Nucleotide sequence of the termini of vYa and v135/9. The sequences were obtained as described in the text. The vertical arrow indicates the internal junction between the S and L components in the wild-type (WT) PrV and vYa genomes. The M13 sequence that has been inserted at the junction of vYa is also shown. The homologous sequences are indicated. The sequence at the internal junction and the right terminus of wild-type PrV has been previously reported (9, 12).

sequenced directly or cloned and then sequenced. The sequence at the S terminus of v135/9 that was obtained was identical to that of the wild-type genome (Fig. 4). Thus, the genome of mature v135/9 has unequal repeats; the internal inverted repeat lacks the last 143 bp of the S component, while the terminal inverted repeat does not (Fig. 2 and 4).

The inequality of the termini of the two inverted repeats of v135/9 should be reflected in the restriction digestion pattern of mature v135/9 DNA. The internal *Bam*HI junction fragment normally generated from wild-type DNA (*Bam*HI fragment 8') is cleaved in v135/9 because of the presence of a *Bam*HI site in the M13 MCS at the junction between the S and L components, giving rise to two fragments, *Bam*HI fragment Δ 13 and *Bam*HI fragment 12a. The terminal *Bam*HI fragment 13 normally generated from the S terminus of the wild-type genome (Fig. 1) will also be generated from v135/9 DNA. *Bam*HI fragment 13 is 1.7 kbp in size, but the fragment derived from the internal inverted repeat of v135/9, *Bam*HI Δ 13, should be approximately 120 bp smaller. (The internal repeat has a 143-bp deletion at its terminus, but the internal *Bam*HI fragment that is generated will include 24 bp of M13 DNA.) The result of a Southern blot analysis of a *Bam*HI digest of mature v135/9 genomes (Fig. 5E) shows that indeed two fragments that hybridize to *Bam*HI fragment 13 are present in equimolar amounts: *Bam*HI fragment 13 (1.7 kbp) and a fragment approximately 120 bp smaller, *Bam*HI fragment Δ 13. (In wild-type genomes, only one *Bam*HI fragment 13 is present.)

The restriction patterns of v135/9 DNA digested with *Kpn*I, *Bgl*II, *Hind*III, and *Sal*I were similar to those of wild-type DNA digests with the exception that the internal junction fragments generated from wild-type DNA were cleaved in *Kpn*I and *Sal*I digests of v135/9 (as they were in *Bam*HI digests) because of the presence of the M13 MCS. The 120-bp difference in size between the end fragments of the S component derived from the internal and terminal inverted repeats was, however, not clear in these digests because the fragments that were generated were too large. Differences between the sizes of the two end fragments of the inverted repeats generated from v135/9 were, however, observed when the viral DNA was doubly digested with an enzyme that cleaves within the M13 MCS (such as *Kpn*I) and

FIG. 5. Southern blot analysis of mature and intracellular v135/9 DNA. RK cells were infected (5 PFU per cell) with either v135/9 or wild-type virions and harvested 12 h thereafter (intracellular DNA). Virions were purified as described in Materials and Methods. The DNA was extracted and digested with either *Kpn*I or *Bam*HI and transferred to nitrocellulose filters. Strips were hybridized with nick-translated PrV DNA (lanes 1), *Bam*HI fragment 8' (lanes 2), *Bam*HI fragment 13 (lanes 3), or *Bam*HI fragment 14' (lanes 4). (A) *Kpn*I digests of intracellular wild-type DNA. (B) *Kpn*I digests of intracellular v135/9 DNA. (C) *Bam*HI digests of intracellular wild-type DNA. (D) *Bam*HI digests of intracellular v135/9 DNA. (E) *Bam*HI digest of mature v135/9 virion DNA. Numbers and letters to side of gels designate restriction fragments.

enzymes that cleave near the ends of the inverted repeats (such as, for example, *Xho*I, which cleaves approximately 1.5 kbp from the S terminus, or *Bgl*I, which cleaves 741 bp from the S terminus). In these cases, two fragments were generated from the ends of the inverted repeats of mature v135/9 genomes that were present in equimolar amounts and differed by approximately 120 bp (data not shown), confirming the conclusion that the majority of the genomes in populations of v135/9 virions are heterozygous with respect to the terminal sequences of the inverted repeats.

Inversion of the S component. PrV has a class 2 genome, i.e., its S component is bracketed by inverted repeats and inverts. It was of interest to ascertain whether the S component of v135/9 from which the last 143 bp of the internal inverted repeat are missing would invert at high frequency.

*Bgl*II cleaves asymmetrically within the U_S of PrV, generating two equimolar end fragments that are derived from the two isomeric forms of the genome, fragments D and E. Virions isolated from individual small plaques of either v135/9 or vYa mutants were amplified in 4×10^6 cells, and their DNA was analyzed by digestion with the restriction enzyme *Bgl*II to ascertain whether their S components were invertible. The restriction patterns of wild-type PrV(Ka), of the vYa mutants, and of v135/9 were the same; *Bgl*II fragments D and E were present in equimolar amounts (Fig. 6), indicating efficient inversion. The presence at both ends of the inverted repeats of the terminal 143 bp, which include the sequences homologous to the pac-1 site of HSV, is thus not essential for high-frequency inversion.

Growth characteristics of the mutants. Table 1 shows the virus titer obtained from RK cells infected with various mutants with deletions near or at the internal junction between the S and L components. As previously reported (27), the deletion of sequences from the right end of the L component and the insertion of M13 sequences (as, for example, in mutant vTN155) does not detectably affect the growth of the virus. Mutant vYa, in which, in addition to the deletion of sequences at the right end of the L component

FIG. 6. S components of vYa and v135/9 invert. Virus populations present in individual plaques were amplified in 4×10^6 cells. (A) DNA obtained from purified wild-type PrV virions (lane 1), v135/9 virions (lane 2), vYa1 virions (lane 3), and vYa4 virions (lane 4) was digested with *Bgl*II, and the bands were separated. (B) DNA was transferred to nitrocellulose filters and probed with nick-translated *Bam*HI fragment 13 (see Fig. 3 for map).

and the insertion of M13 sequences, the terminal 17 bp of both inverted repeats have been replaced by M13 sequences (see above), also yielded virus titers that were similar to those of wild-type virus. However, mutant v135/9, which is similar to vYa but has a deletion of the terminal 143 bp of the internal inverted repeat and also has unequal repeats, produced significantly lower virus titers.

To further document the growth disadvantages of v135/9, we infected cells at high multiplicity with a mixture of wild-type and v135/9 virus, and the ratio between virions that included the M13 sequences (i.e., the mutants) and those that did not (i.e., wild type) used to infect the cells and in the virus progeny produced by the cells was ascertained. Figure 7 shows the results of a representative experiment.

In cells doubly infected with a mixture of v135/9 and wild-type virus, the difference between the degree of replication of genomes that included the M13 sequences and those that did not was significant even after a single cycle of growth. Thus, genomes with the v135/9 mutation (M13 sequences) had a distinct growth disadvantage (Fig. 7). When mixtures of wild type and vYa mutants were similarly passaged, no growth disadvantage was observed for vYa after a single cycle of growth (Table 2). However, after

TABLE 1. Titer of infectious virus produced by RK cells infected with PrV deletion mutants^a

Virus mutant	Titer (PFU/cell)		Deletion (bp) ^b
	Expt 1	Expt 2	
Wild type	270	520	None
vE10	310	390	-1506 to -322
vTN155	190	410	-677 to -390
vYa	230	450	-487 to +17
v135/9	49	73	-677 to +143

^a RK cell monolayers were infected with 2 PFU per cell. After a 1-h adsorption period, the monolayers were washed and incubated for 48 h, by which time cytopathic effect was complete. Virus was assayed on MDBK cell monolayers, and plaques were counted 4 days later.

^b See Fig. 1 for map.

FIG. 7. Wild-type virus has a growth advantage relative to v135/9. RK cells were infected with a mixture of wild-type PrV and v135/9 virions (approximately 10 PFU per cell each). The virions in the original virus mixture and after being amplified in the cells were plaque assayed. Individual plaques were picked and inoculated in 96 wells in which RK cells had been grown. Part of the infected cell supernatants were dot blotted onto nitrocellulose filters and hybridized to nick-translated M13 DNA (the M13 probe will hybridize to v135/9 but not to wild-type PrV DNA). To ensure that the wells had been infected, i.e., that viral DNA was present, the filters were subsequently hybridized to nick-translated PrV DNA. (A) Original mixture; (B) after being grown in RK cells.

several cycles of growth, a disadvantage of vYa relative to wild type was observed. Thus, vYa has only a slight growth disadvantage relative to wild-type virus, as expected from the fact that vYa- and wild type-infected cells yielded similar virus titers (Table 1). Since vYa and v135/9 have a similar deletion of sequences within the U_L and a similar insertion of M13 sequences (neither of which affects virus growth [27]), it is either the absence of the terminal 143 bp of the internal inverted repeat or the lack of identity of the inverted repeats in v135/9 that significantly affects its growth.

Viral DNA synthesis in mutant-infected cells. The results described above (Fig. 7 and Table 2) show that even under

TABLE 2. Growth disadvantage of mutants relative to wild-type virus^a

Mixture	No. of mutants/no. of total virions ^b		
	Original mix	Passaged once at 20 PFU/cell	Passaged 5 times at 0.01 PFU/cell
WT + vYa	29/92 (31)	28/96 (30)	9/93 (10)
WT + v135/9	40/94 (42)	9/89 (10)	0/96 (0)
WT + vTN155	50/95 (53)	ND ^c	47/95 (50)

^a Mixtures of either wild-type (WT) virus and vYa or wild-type virus and v135/9 were used to infect RK cell monolayers at a multiplicity of 20 or 0.01 PFU per cell. After a 1-h adsorption period, the monolayers were washed and incubated until complete cytopathic effect was seen. The virus produced by the cultures infected at low multiplicity was used to infect a new set of cultures at low multiplicity, a procedure that was repeated five times. The cultures infected at high multiplicity were harvested after only one cycle of virus growth. The virus produced by the cultures was plaque assayed. Plaques were picked and used to infect 96-well plates in which RK cells had been grown. After cytopathic effect had developed, an aliquot from each well was blotted onto nitrocellulose filters and probed first with nick-translated M13 DNA (to identify the mutants) and then with nick-translated PrV DNA (to identify all the virus-positive wells).

^b The numbers in parentheses represent the percentage of the virions in the population that are mutants.

^c ND, not determined.

FIG. 8. Synthesis of viral DNA and of virions in wild type (W.T.), *vYa*-, and *v135/9*-infected cells. RK cell monolayers were infected with wild-type PrV(Ka), with *vYa*, or with *v135/9* virions (5 PFU per cell). At the indicated times after infection, the cells from some cultures were harvested. Mature virions produced by the cells were purified from other cultures. The DNA was extracted, and the amount of viral DNA in the samples was estimated by dot blotting twofold dilutions of denatured DNA and hybridizing it to nick-translated PrV DNA. ×, total cellular viral DNA synthesized. ●, viral DNA associated with mature purified virions produced by the culture.

conditions in which the cells were coinfecting with *v135/9* and wild-type virus and in which wild type could, in principle, complement *v135/9*, genomes that included the *v135/9* junction (M13 sequences) had a significant growth disadvantage. It therefore appeared probable that the growth disadvantage is determined by a defect in a *cis* function, most likely one that affects either the synthesis or the processing of *v135/9* DNA.

Figure 8 shows the relative amounts of viral DNA that accumulated at different times after infection in cells infected with wild type, *vYa*, or *v135/9*, as well as the relative amounts of viral DNA that became part of the mature virion populations produced by these infected cells. The results obtained with wild type and *vYa* were indistinguishable. However, while approximately the same amount of viral DNA accumulated in *v135/9*-infected cells as in wild type-infected cells, considerably less *v135/9* DNA became part of mature virus particles, indicating that the *v135/9* DNA that accumulated in the infected cells was not a good substrate for cleavage and encapsidation.

Structure of intracellular *v135/9* DNA. The decreased virion formation in *v135/9*-infected cells could be due to a low level of accumulation of viral structural (or other) proteins, to poor cleavage of concatemeric DNA, or possibly to other modifications in the intracellular structure of *v135/9* DNA. Because the time of initiation and the level of accumulation of the viral proteins were similar in *v135/9*- and wild type-infected cells (data not shown), we surmised that differences in the structures of intracellular *v135/9* DNA and wild type might exist.

We first ascertained whether *v135/9* intracellular concatemeric DNA is cleaved poorly, i.e., remained in the form of large, rapidly sedimenting molecules. The DNA present in *v135/9*- or in wild type-infected cells was centrifuged in a sucrose gradient, and the distribution of the viral DNA in the gradient was ascertained by dot-blot analysis. By 12 h postinfection, most (95%) of the wild-type viral DNA was found in the peak of mature virus genomes, i.e., the DNA had been cleaved (wild-type concatemeric DNA is cleaved

FIG. 9. Sucrose gradient analysis of DNA obtained from cells infected with *v135/9* virus. RK cells were infected (5 PFU per cell) with *v135/9* virions. At 12 h postinfection, the cells were harvested, lysed with sarcosyl, heated at 60°C for 15 min, pronase treated, and sedimented on a neutral sucrose gradient as described in Materials and Methods. Fractions (1 ml) were collected, dialyzed, digested with *Bam*HI, electrophoresed, and hybridized to a *Bam*HI fragment 13 probe. The top of the gradient is fraction 1. Mature DNA sedimented to fraction 18 (DNA from purified virion DNA sedimented to that position in a parallel gradient). Concatemeric DNA sedimented to the bottom of the tube (pellet; fraction 30). The pellet was resuspended in 2 ml of buffer before being extracted. The open circles represent distinct bands.

efficiently in infected RK cells [26]). In *v135/9*-infected cells, however, most of the viral DNA sedimented to the bottom of the tube. Even by 16 h postinfection, approximately 75% of the total viral DNA in the cells was in the form of rapidly sedimenting molecules (data not shown).

Southern blot analysis of the DNA in the different fractions of a gradient in which *v135/9* DNA had been centrifuged (Fig. 9) showed that the two unequal *Bam*HI fragments derived from the termini of the inverted repeats of mature *v135/9* genomes were, as expected, present in equimolar amounts in the peak of mature DNA (fractions 18 to 20). However, *Bam*HI fragment 13, derived from the S terminus of the genome, was missing from DNA that sedimented to the bottom of the tube, and only *Bam*HI-Δ13 was present. This per se is not surprising because in concatemeric DNA, *Bam*HI fragment 13 (which is derived from the S terminus of the genome) should be linked to *Bam*HI fragment 14' (the left-end fragment of the mature genome) and a 3.1-kb junction fragment composed of the two covalently linked end fragments should appear. While the expected 3.1-kb concatemeric junction fragment was present in the rapidly sedimenting DNA, it was not present in sufficient abundance to account for the absence of *Bam*HI fragment 13 (Fig. 9). A similar analysis of the DNA present in wild type-infected cells showed that, as expected, only one *Bam*HI fragment 13 was generated from the DNA in the peak of mature DNA and that the concatemeric DNA yielded mainly the 3.1-kb junction fragment (data not shown). Thus, while a large proportion of intracellular *v135/9* DNA is present in the form of rapidly sedimenting molecules, this DNA does not appear to be in the form of conventional head-to-tail concatemers.

To try to elucidate the structure of the rapidly sedimenting viral DNA present in *v135/9*-infected cells, we analyzed DNA obtained from *v135/9*-infected cells by Southern blots after digestion with various enzymes. Figure 5 shows the results obtained with *Bam*HI and *Kpn*I digests; Fig. 10 shows the results obtained with *Bgl*III and *Hind*III digests. The restriction maps of wild-type and *v135/9* mature genomes are shown in Fig. 3.

FIG. 10. Southern blot analysis of *Hind*III and *Bgl*II digests of intracellular wild-type and v135/9 DNA. The DNA was obtained as described in the legend to Fig. 5. It was digested, electrophoresed, and transferred to nitrocellulose filters. Strips were hybridized to nick-translated *Bam*HI fragment 5' (lane 1), *Bam*HI fragment 7 (lane 2), *Bam*HI fragment 8 (lane 3), *Bam*HI fragment 8' (lane 4), *Bam*HI fragment 13 (lane 5), and *Bam*HI fragment 14' (lane 6). Wild-type intracellular DNA was digested with *Hind*III (A) and with *Bgl*II (D). v135/9 intracellular DNA was digested with *Hind*III (B) and with *Bgl*II (E); v135/9 intracellular DNA was digested with *Hind*III and *Eco*RI (C) and with *Bgl*II and *Eco*RI (F).

The results obtained with the *Bam*HI digests of total intracellular DNA confirmed those obtained from the analysis of the sucrose gradient fractions. *Bam*HI fragment 13 (the fragment derived from the S terminus of the genome) was barely detectable in intracellular (concatemeric) v135/9 DNA (Fig. 5D, lane 3). The fragment derived from the L terminus, *Bam*HI fragment 14', was, however, present (Fig. 5D, lane 4). In addition, a fragment approximately twice the size of *Bam*HI fragment 14' that hybridized to the *Bam*HI fragment 14' probe only (i.e., hybridized exclusively to sequences derived from the left terminus of the genome [Fig. 5D, lane 4, asterisk]) was also present. The underrepresentation of the S-terminal fragment in intracellular v135/9 DNA is impossible to ascertain in *Kpn*I digests because the fragments derived from the two ends of the inverted repeats comigrate. However, a fragment approximately twice the size of the L-terminal fragment (*Kpn*I fragment D) that hybridized to the *Bam*HI fragment 14' sequences (Fig. 5B, lane 4, asterisk) was also observed. No evidence for the presence of large amounts of head-to-tail concatemeric arrays in which either *Bam*HI fragment 13 plus fragment 14' or *Kpn*I fragment D plus *Kpn*I fragment H are covalently linked and that hybridize to the sequences of both *Bam*HI fragments 13 and 14' was obtained in either digest. Thus, clearly, no significant amounts of conventional head-to-tail concatemers were present.

In principle, the intracellular concatemers of v135/9 could be in one of several forms. Three of these forms are depicted in Fig. 11. Form 1 is the form wild-type concatemeric DNA assumes; this form has been excluded by the results described above; head-to-tail concatemers (form 1) were present in low abundance only. In form 2 concatemers, the

FIG. 11. Graphic representation of the structures of three different forms of concatemeric DNA. Open rectangles, inverted repeat. Black rectangle, 143-bp deletion and M13 insertion. Open circles, sequences at the left end of the genome. Vertical arrows, complete cleavage signal resulting from the juxtaposition of the sequences at both ends of the mature genome. Horizontal arrow, orientation of the L component.

TABLE 3. Approximate sizes of the end fragments derived from the L terminus of the PrV genome and the sizes of the starred L-L fragments^a

Digest	L end fragment (kbp)	Starred fragment (L-L) (kbp)
<i>Bam</i> HI	1.4	2.8
<i>Kpn</i> I	12.5	26.0
<i>Sal</i> I	2.7	5.5
<i>Hind</i> III	30.0	>50.0
<i>Bgl</i> II	27.0	>50.0
<i>Pst</i> I	2.6	5.0
<i>Nco</i> I	1.2	2.4
<i>Sma</i> I	0.6	1.2

^a The L-L fragments were identified by probing transfers of gels on which various restriction digests of v135/9 intracellular DNA had been run with nick-translated *Bam*HI fragment 14' (the 1.4 terminal kilobase pairs of the L terminus of the genome). The sizes of the fragments were estimated by comparing their migration rates with those of size markers or of PrV restriction fragments of known size.

possibility is invoked that the MCS, which had been introduced at the internal inverted repeat, has been transferred in intracellular concatemeric DNA to the S terminus, i.e., that the inverted repeats in concatemeric DNA have equalized, both repeats now being of the mutant type. The concatemeric array in form 2 is in head-to-tail alignment as it is in form 1, but the MCS of M13 is present at both junctions between the S and L components. Digestion with enzymes present in the MCS such as *Bam*HI or *Kpn*I would cleave within the concatemeric junction, which would therefore not be detectable. In *Bam*HI digests, for example, only *Bam*HI fragment Δ 13 and *Bam*HI fragment 14' would be observed instead of the covalently linked junction fragment of the two. However, digestion of the DNA with enzymes such as *Bgl*II and *Hind*III that do not cleave either in the M13 MCS present in v135/9 (the *Hind*III cleavage site of the MCS has been removed during construction of the mutant by removal of the sequences up to the *Sph*I site) or close to either the S or L terminus of the genome should leave the concatemeric junction intact. Southern blots of *Hind*III and *Bgl*II digests are illustrated in Fig. 10 (see Fig. 3 for restriction maps).

The digests were probed with sequences derived from the end of either the L component (*Bam*HI fragments 14' and 5') or the S component (*Bam*HI fragments 13, 8, 8', and 7). The hybridization patterns obtained from v135/9 intracellular DNA (Fig. 10B and E) showed the following. (i) The fragments characteristic of the S terminus of the genome (*Bgl*II fragments D and E or *Hind*III fragment C) were barely detectable, but the fragments characteristic of the L terminus (*Bgl*II fragment C and *Hind*III fragment B) were present. (ii) In addition to the L-terminal fragments, *Bgl*II fragment C and *Hind*III fragment B, a larger fragment (indicated by an asterisk) (starred band) hybridizing to the sequences derived from the L terminus of the genome was also present in abundance in v135/9 intracellular DNA. These larger starred bands hybridized to the *Bam*HI fragment 14' and 5' probes with an intensity that was almost equal to that of the band generated from the L terminus of the genome. The presence of starred bands approximately twice the size of the band obtained from mature genomes that hybridize to the sequences derived from the L terminus of the genome was also observed in the *Bam*HI and *Kpn*I digests (Fig. 5). Such bands were also present in other restriction enzyme digests of v135/9 intracellular DNA. The sizes of these bands are summarized in Table 3.

Digestion of PrV with both *Bgl*II and *Hind*III generates relatively large fragments. The large starred band that hybridizes to the terminal sequences of the L component comigrated with bands hybridizing to the terminal sequences of the S component; i.e., they could represent fragments derived from the junctions of head-to-tail concatemeric DNA. The structure of the DNA could thus be as depicted in form 2 (Fig. 11). However, digestion of the *Bgl*II or *Hind*III digests with *Eco*RI (which has no restriction site in wild-type DNA but has a site in the MCS introduced into v135/9 adjacent to the internal inverted repeat) cleaves, as expected, within the internal junction fragment (bands A) but does not cleave the starred fragment, which now hybridizes only to the sequences derived from the end of the L component of the genome (Fig. 10C and F). Thus, the starred fragments are not derived from concatemeric junctions.

As mentioned above, the starred bands that hybridized with the end sequences of the L terminus were in all cases approximately twice the size of the terminal fragment generated from the L component of the mature genomes (Table 3). Although the starred fragments have not been cloned and sequenced (we have been unsuccessful in our attempts to clone these fragments), the fact that after digestion with several different restriction enzymes they are always twice the size of the fragments generated from the mature L terminus (Table 3) indicates that these fragments must be generated from head-to-head alignments of L components, i.e., that they represent the junctions between two L components in different orientations.

Few free S-component termini were detectable in *Hind*III and *Bgl*II digests of intracellular v135/9 DNA; the probes derived from the end of the S component hybridized mainly to junction fragments. After *Hind*III-*Eco*RI or *Bgl*II-*Eco*RI digestion, the junction fragments were digested to completion and fragments with the approximate size of the free ends appeared. Thus, it is clear that the sequences adjacent to the termini of the S components include the *Eco*RI site, i.e., that the great majority of the inverted repeats of v135/9 DNA have equalized. This could also be deduced from the fact that only *Bam*HI fragment Δ 13 was present in *Bam*HI digests and that *Bam*HI fragment 13, as well as concatemeric junction fragments, was present in low abundance only.

Analysis of v135/9 intracellular DNA thus revealed the following. (i) In all digests, the restriction fragments derived from the S terminus of the genome were considerably underrepresented (they were not detectable in some cases), but those derived from the L terminus were present. (ii) In addition to the normal L-terminus fragment, another fragment hybridizing exclusively to probes derived from the sequences of the L terminus of the genome was generated. This starred fragment was in all cases approximately twice the size of the normal L-terminal fragment. (iii) No fragment appeared that was consistent with the presence of concatemers in which genomes are in head-to-tail arrays. (iv) The restriction pattern of the intracellular DNA showed that the inverted repeats had equalized. The fragments generated showed that in most of the intracellular DNA, both inverted repeats had the modifications that had been introduced at the internal inverted repeat of the virus.

The above data are consistent with intracellular v135/9 DNA being in concatemeric form 3A and form 3B (Fig. 11). These concatemers are defective because the required cleavage signals, i.e., the sequences normally present at both termini of the mature genome (37), are not juxtaposed in the junctions of concatemeric v135/9 DNA.

FIG. 12. Ratio of L to S components in intracellular v135/9 DNA. The DNA preparations were obtained as described in the legend to Fig. 5. The DNA was digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and hybridized to a mixture of nick-translated *Bam*HI fragment 4 and fragment 7. Appropriately exposed films were scanned.

The relative intensity of hybridization of the L terminus and the L-L junction fragment obtained after analysis of different v135/9-infected cell preparations varied somewhat. While in some cases the intensities of the mature L end fragment and the L-L fragment were almost equal, suggesting that the DNA is mostly in concatemeric form 3A, in other experiments the intensity of hybridization to the L end fragment was much greater than that of the L-L fragment. The free S-terminal fragment was, however, always only barely detectable. We therefore conclude that the relative amounts of form 3B (of various lengths) and of form 3A concatemers present in different preparations of intracellular v135/9 DNA differ.

Ratio of L to S components in v135/9 intracellular DNA. The schematic representation of form 3 (A or B) DNA in Fig. 11 shows arrays in which two L components are present for every S component. The ratio of hybridization of intracellular v135/9 DNA to a mixture of two probes, one derived from sequences in the L component (*Bam*HI fragment 4) and one derived from sequences in the S component (*Bam*HI fragment 7), should therefore be twice that of mature v135/9 or wild-type DNA. Figure 12 shows scans of Southern blots of *Bam*HI digests of mature v135/9 DNA, of v135/9 intracellular DNA, and of wild-type intracellular DNA hybridized to a probe composed of a mixture of *Bam*HI fragment 4 and *Bam*HI fragment 7. The amount of *Bam*HI fragment 7 relative to that of *Bam*HI fragment 4 that was present in intracellular v135/9 DNA was considerably lower than in either mature v135/9 DNA or intracellular wild-type DNA. Quantitation of the bands showed that while the ratio of *Bam*HI fragment 4 to fragment 7 was 2.1 and 2.3 for mature v135/9 and intracellular wild-type DNA, respectively, it was 3.5 for intracellular v135/9 DNA. Thus, the L component was overrepresented by 60% relative to the S component in intracellular v135/9 DNA. This finding supports our conclusion that a large part of v135/9 intracellular DNA is present as form 3 concatemers.

Time of appearance of head-to-head concatemers in mutant- and wild type-infected cells. Wild-type PrV DNA replicates in the form of head-to-tail concatemers (4). However, in the experiments presented above (Fig. 5 and 10), small amounts of L-L fragments were detected in intracellular DNA obtained from wild type-infected cells. To ascertain whether L-L fragments would be present in larger proportion at other times after infection, we analyzed the relative amounts of restriction fragments diagnostic of head-to-tail concatemeric DNA, mature S or L termini, and of the L-L fragment present at various times after infection in cells infected with wild-type, vYa, and v135/9 virus.

In the DNA obtained from wild type-infected cells at any time of the infectious cycle, a small amount of L-L junction appeared; a maximum of 10% of the total intracellular DNA was in the form of concatemers in which the L component was in head-to-head alignment. As expected, conventional concatemeric DNA molecules (S-L junctions) appeared early, but their relative amounts decreased at later times after infection when mature S and L terminal fragments both accumulated abundantly. The results obtained with vYa-infected cells were very similar to those obtained with wild type-infected cells. In v135/9-infected cells, however, while molecules with the concatemeric S-L junction fragment were also present, they were never as abundant as in wild type-infected cells. However, molecules with the L-L junction fragment accumulated abundantly throughout the infectious process, and most of the total intracellular viral DNA was in a form in which the L component was in head-to-head alignment. Mature L ends also accumulated abundantly in v135/9-infected cells, but the S ends were always considerably underrepresented (data not shown).

These results indicate that in wild type-infected cells, a small amount of form 3 concatemers is formed at any time during the infectious process but that most of the DNA that accumulates in the cells throughout infection in v135/9-infected cells is in this form. Since only a small proportion of v135/9 DNA becomes part of virions (Fig. 8), i.e., is withdrawn from the intracellular pool of viral DNA, we conclude that most of the v135/9 DNA that is synthesized is in form 3 concatemers.

DISCUSSION

The mutants discussed in this report were constructed to explore the functions of sequences at the end of the S terminus. Two mutants were constructed. In vYa, 17 bp from the internal inverted repeat (as well as some adjacent sequences from the L component) were deleted; in v135/9, 143 bp from the internal inverted repeat (as well as some adjacent sequences in the L component) were deleted. Our aim in constructing these mutants was to ascertain whether (i) equalization of the terminal regions of the S component would occur; (ii) genome termini which lack either the 17 or 143 bp would be generated as a result of equalization of the repeats, thereby identifying the terminal nucleotides that may include cleavage signals; and (iii) inversion of the S component would occur, thereby ascertaining the importance of the terminal sequences of the S component in the processes leading to isomerization of this component.

Isomerization. The mechanism by which the unique components of the genomes of herpesviruses invert is controversial despite being the subject of intense interest. Two schools of thought exist. (i) Inversion occurs by general recombination, hot spots mediating recombination being present near the ends of the inverted repeats. (ii) Inversion occurs by a site-specific mechanism mediated by specific proteins that bind to sequences present near or at the ends of the repeats.

Our results show that the terminal 143 bp bracketing the S component of the genome (that are missing from the internal inverted repeat of v135/9) are not essential for isomerization; high-frequency isomerization occurred in the absence of these sequences at one end of the inverted repeat. By the time a small plaque was amplified (we estimate that a maximum of seven cycles of replication have taken place), equimolar amounts of genomes with either of the two isomeric forms of the S component were present in the virion population. Thus, site-specific recombination within the 143

terminal base pairs bracketing the inverted repeats (which include the sequences with homology to the *pac*-1 element of HSV) are not essential for efficient isomerization.

An extensive dissection of the elements within the "a" sequence that may affect inversion has recently been done by Smiley et al. (31). On the basis of their results, they have proposed that the processes leading to the isomerization events are stimulated by site-specific breaks. (In particular, they have reported that deletion of the *pac* sites drastically reduces the frequency of isomerization.) Within the limits of sensitivity of the experiments presented here, it appears that the presence of sequences with homology to the *pac*-1 site at both termini of the inverted repeat of the S component of PrV DNA is not essential for high-frequency inversion of the S component.

Signal recognition for cleavage. In vYa, a mutation had been introduced which deleted 17 bp from the end of the internal repeat and replaced them with M13 sequences. These 17 bp were also absent from the S terminus of the genome of vYa and had been replaced by the same M13 sequences that had been introduced adjacent to the internal inverted repeat. The vYa mutants grew well; the deletion of the terminal 17 bp at the ends of both inverted repeats and their replacement with M13 sequences (as well as the deletion of the rightmost sequences of the L component) only conferred upon the virus a small growth disadvantage. The terminal 17 bp therefore do not include specific sequences that are essential for cleavage of concatemeric precursor DNA to mature genomes. Cleavage of PrV concatemeric precursors thus appears to occur at a defined distance from a recognition site distal to the ends. The finding that the terminal 17 nucleotides can be replaced by an unrelated sequence confirms previously reported results of Varmuza and Smiley (34), who showed that cleavage can occur within non-"a" sequences at an analogous distance from the *pac* sites.

In mutant v135/9, 143 bp, including the sequence with homology to the *pac*-1 sequence, were deleted from the internal inverted repeat. v135/9 mature genomes retain unequal repeats; the terminal repeats have the wild-type structure, while the internal repeat has the 143-bp deletion. These mutants grew poorly; viral DNA was synthesized, but the formation of virions was impaired. Analysis of the intracellular viral DNA synthesized by v135/9-infected cells showed that most of it had acquired equal, mutant-type, inverted repeats, i.e., that the terminal 143 bp of the genome were absent from both ends of the inverted repeats. This DNA remained concatemeric, i.e., was not cleaved to form mature genomes and virions.

Cleavage of herpesvirus concatemeric DNA appears to depend on the highly conserved sequences *pac*-1 and *pac*-2 (8, 11, 31). In PrV, the *pac*-1 homologous sequences are present near the end of the inverted repeats (within the 143 bp deleted from v135/9), and the *pac*-2 homologous sequences are near the L terminus of the genome. The fact that the DNA that remained concatemeric had S components from which the terminal 143 bp were missing, while the DNA that found its way into virions had retained these sequences, indicates that the 143 bp which include the sequences with homology to the *pac*-1 site are indeed essential for the cleavage-encapsidation process.

Equalization of the inverted repeats bracketing the U_S . It is well established that sequences within the inverted repeat tend to equalize rapidly. In principle, the equalization of the repeats can occur either by a specialized mechanism that ensures their identity (such as one repeat acting as a tem-

plate for the synthesis or repair of the other) or from intermolecular recombination and then segregation. Recombination provides a simple mechanism by which equalization can occur, and this notion is appealing; evidence that recombination plays a role in equalization of the repeats has been presented previously (2, 33). No direct support for the existence of a copying mechanism by which one repeat acts as a template for the repair of the other has to our knowledge been obtained to date.

Equalization of the inverted repeats was observed in both the vYa and the v135/9 mutants. In six independently isolated vYa mutants that we analyzed, the deletion of 17 bp at the end of the internal repeat and their replacement with M13 sequences was accompanied by the replacement of the sequences at the S terminus of the genome with the same M13 sequence that had been introduced at the internal repeat. The equalization of the repeats of vYa genomes that had occurred was in all cases unidirectional; the terminal repeat had become modified and had acquired the sequences that had been introduced at the internal repeat. It is unlikely that the fact that all six mutants were similar in this respect is related to the method we used to identify the mutants, i.e., hybridization to an M13 probe. Most of the M13 sequences in vYa DNA are part of the L component, and these sequences should be retained by the genomes even if the ends of the inverted repeats had reverted to wild-type sequences. Indeed, a mutant, vLD68, which also has a deletion at the junction between the L and S components and an insertion of M13 sequences and which reacquires wild-type sequences at high frequency, does not lose the M13 sequences at the junction (16).

The fact that conversion of the repeats is indeed unidirectional is also clearly indicated by the results of analyses of intracellular v135/9 DNA. While the genome of mature v135/9 mutants retains unequal repeats (the internal repeat of mature genomes had a deletion of 143 bp but the terminal repeat retained its wild-type structure), the majority of the intracellular viral DNA consists of DNA in which both inverted repeats have acquired the modifications that had been introduced at the internal repeat, modifications that were present in mature DNA at the internal repeat only. The massive conversion of the repeats in v135/9-infected cells which resulted in both repeats acquiring the mutant structure is especially significant because it occurred even though this DNA is defective, i.e., could not be cleaved and encapsidated, thereby conferring upon the virus a significant growth disadvantage.

Accumulation of concatemeric DNA in which the L components are in head-to-head alignment. Analysis of intracellular wild-type, vYa, and v135/9 DNA revealed an accumulation in cells infected with the latter of large amounts of form 3 concatemers in which two L components were in head-to-head alignment and shared a single S component. In wild type- and vYa-infected cells, such molecules were also present, but they accounted for only a small proportion of the viral DNA synthesized. In v135/9-infected cells, however, we estimate that they accounted for approximately 80% of the total viral DNA.

The differences in the properties of vYa and of v135/9 mutants can only be ascribed to differences in the sizes of the deletion at the internal inverted repeat. Both mutants have similar deletions of sequences at the right end of the L component and a similar insertion of M13 sequences. However, while in v135/9 143 bp have been deleted from the end of the internal inverted repeat, in vYa only 17 bp have been deleted. The absence in v135/9 of the 143 bp at the end of the

FIG. 13. Model for the equalization of the terminal sequences of the inverted repeat (IR) and for the generation of form 3 concatemers. Open rectangles, inverted repeats. Black rectangles, pac-1 sequences. Small circle, pac-2 sequences. Large circle, origin of replication (the presence of the origin of replication near or at the end of the L component [15] could be important and is indicated even though it has not been assigned a specific role in the sequence of events depicted in the proposed model). Curved arrow, single-stranded nicks. (A) Wild-type DNA. The sequences at the end of the internal and terminal inverted repeats interact, resulting in the copying of the end sequences of the internal repeat by the terminal repeat (TR). Two concatemeric molecules, A and B, in inverse orientation are depicted. Step 1, single-stranded gap is introduced at the junction that will become the S terminus. Step 2, 3' single-stranded end of molecule B invades the internal repeat of molecule

internal inverted repeat that include the sequences with homology to the pac-1 site thus appears to cause the accumulation of form 3 concatemeric molecules. We therefore postulate that the deletion of sequences that include those with homology to the pac-1 site and the accumulation of form 3 concatemers are related.

Equalization of the ends of the inverted repeats occurs by a copying mechanism. The sequences adjacent to the terminal nucleotides of the internal and the terminal inverted repeats differ in circular or in head-to-tail concatemeric DNA of both vYa and v135/9. In these mutants, the sequences normally present at the U_L adjacent to the internal S-L junction have been replaced by M13 sequences, i.e., sequences that bear no homology to the sequences at the S-L junction that is formed when the left end of the genome becomes linked to the S terminus in circular or concatemeric DNA. Equalization of the terminal sequences of the inverted repeats can therefore not occur by homologous recombination within sequences adjacent to the ends of internal and terminal inverted repeats. Furthermore, the alternate products of such recombinational events, i.e., molecules with wild-type sequences at the ends of both inverted repeats, were not observed. Therefore, to account for the equalization of the ends of the inverted repeats, we postulate a copying mechanism in which the terminal repeat invades and copies the internal repeat.

The model. Figure 13 illustrates the basic features of the model we propose to account for our results. The main elements of these results that have led to the formulation of this model are that (i) the sequences of the terminal repeat are converted to those of the internal repeat at high frequency, i.e., conversion is unidirectional, and that (ii) the absence of the 143 bp of the internal inverted repeats (which include the sequences with homology to the pac-1 site) leads to the accumulation of form 3 concatemers.

The model we propose postulates that copying of the internal repeat by the terminal repeat occurs within a cleavage-encapsidation complex and that this is an intrinsic aspect of the replication strategy of PrV. In this complex, the inverted repeats of two concatemeric molecules in different orientations (or the inverted repeats of the same molecule) align (Fig. 13A, step 1). During a preliminary stage of the process that will ultimately lead to cleavage of the concatemeric DNA, a single-stranded nick is introduced at the junction that will become the S terminus. After being acted on by an exonuclease, the single-stranded end invades and copies the internal repeat (Fig. 13A, step 2). The pac-1 sequence that is present at the internal repeat is somehow responsible (because of its structure or because of the proteins it binds) for preventing the continued elongation of

A and the 5' strand of molecule A is copied by the 3' strand of molecule B. The pac-1 sequences are responsible for preventing elongation beyond the sequences at the end of the inverted repeat. Steps 3 and 4 show how both strands of molecule B might become identical to those of molecule A. (B) v135/9 DNA. In the mature genome of v135/9, the pac-1 site at the internal junction is missing. Steps 1 and 2 are similar to those in the wild type, but because of the absence of the pac-1 site at the end of the internal inverted repeat in molecule A, elongation of the invading strand continues, as shown in step 3. (C) Formation of L-L junctions. An 82-bp inverted repeat (heavy lines) is present at the ends of the L component of PrV (9). The strand that has been elongated up to the end of the L component can fold back and replication can continue. Form 3 concatemers (either 3a or 3b) will be generated.

the invading strand once it reaches the end of the repeat sequences. The invading strand that had copied the sequences of the internal repeat can, in turn, now serve as a template for the conversion of its complementary strand by a process that involves the introduction of a single-stranded nick and exonuclease digestion of the complementary strand (Fig. 13A, steps 3 and 4). Cleavage of the concatemeric DNA would then occur. The sequence of events culminating in the copying of the internal strand by an invading terminal strand that is specified in the model is only one of several possible scenarios. The main point of the model is that copying of the internal repeat by the terminal repeat occurs and that the pac-1 sequences at the internal inverted repeat somehow act as a stop signal that prevents the elongation of the invading strand beyond the end of the inverted repeat.

Steps 1 and 2 for v135/9 DNA (Fig. 13B) are the same as those diagrammed in Fig. 13A for wild-type DNA. However, v135/9 DNA lacks the pac-1 sequences at the internal inverted repeat, which, according to the model, prevent continued synthesis beyond the end of the inverted repeat. Elongation of the invading strand will therefore continue (Fig. 13B, step 3).

An 82-bp inverted repeat is present at the end of the L component of the PrV genome (9, 18). As the newly synthesized strand reaches the end of the L component, this inverted repeat could form a hairpin and replication could proceed, first generating form 3B and then form 3A concatemeric molecules (Fig. 13C) by a mechanism akin to that proposed for poxvirus DNA replication (22).

The model in Fig. 13 accounts for all the observations described in this report. It should be mentioned, however, that approximately 20% of the genomes of v135/9 are able to replicate without equalizing their repeats. The processes leading to repeat equalization can therefore not be absolutely essential to the process of cleavage of the PrV genome.

The minority of the head-to-head (L-L) structures that are observed in wild type-infected cells may result from leakage and elongation in a small minority of the molecules beyond the stop signal specified by the pac-1 sequences at the internal inverted repeat. It is interesting that L-L junction fragments accumulate in considerably greater abundance in wild-type PrV-infected chicken embryo fibroblasts in which cleavage of concatemeric DNA is poor (and in which only a low level of structural viral proteins accumulate) than in wild type-infected RK cells in which cleavage of concatemeric DNA is efficient (26). This observation is consistent with the model we propose.

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