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Molecular Basis for Interference of Defective Interfering Particles of Pseudorabies Virus with Replication of Standard Virus

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Serial passage of pseudorabies virus (PrV) at high multiplicity yields defective interfering particles (DIPs), but the sharp cyclical increases and decreases in titer of infectious virus that are observed upon continued passage at high multiplicity of most DIPs of other viruses are not observed with DIPs of PrV (T. Ben-Porat and A. S. Kaplan, Virology 72:471–479). We have studied the dynamics of the interactions of the virions present in a population of DIPs to assess the *cis* functions for which the genomes of the DIPs are enriched. The defective genomes present in one population of DIPs, [PrV(1)42], replicate preferentially over the nondefective genomes present in that virion population at early stages of infection, indicating that the DIP DNA is enriched for sequences that can serve as origins of replication at early stages of infection. This replicative advantage of the DIP DNA is transient and disappears at later stages of infection. The defective DNA does not appear to be encapsidated preferentially over the nondefective DNA present in this virion population, which might indicate that it is not enriched for cleavage-encapsidation sites. However, the nondefective DNA in the DIP virion population has become modified and has acquired reiterations of sequences originating from the end of the unique long (U_L) region of the genome. Furthermore, both the infectious and defective genomes present in the DIP population compete for encapsidation more effectively than do the genomes of standard PrV. These results indicate that the defective genomes in the population of virions studied are enriched not only for an origin of replication but probably also for sequences necessary for efficient cleavage-encapsidation. Furthermore, the nondefective genomes present in this population of DIPs have also been modified and have acquired the ability to compete with the defective genomes for cleavage-encapsidation.

Serial passage at high multiplicity of many viruses, including the herpesviruses, results in the emergence of defective interfering particles (DIPs) which contain altered genome structures. These DIPs, although unable to replicate in the absence of standard helper virus, have a growth advantage over it. The identity of the DIPs is of interest because their emergence has obvious implications concerning the mode of replication and encapsidation of the virus genome.

That DIPs of herpesvirus contain genomes that consist, at least in part, of tandem repeats of restricted regions of the viral genome in head-to-tail alignment has been recognized for some time from their partial denaturation patterns (12, 24). Since populations of DIPs become enriched for virions whose genomes probably can replicate and be encapsidated, common features of these repeated sequences may, in principle, include origins of replication, as well as recognition sites for cleavage and encapsidation. Indeed, it has been established that the DIPs of herpes simplex virus (HSV) contain genomes composed of reiterated segments of DNA that include an origin of replication, as well as the signals required for efficient cleavage-encapsidation (1, 26, 27, 29). Because these DIPs have a clear replicative advantage, cyclical increases and decreases in the level of infectious virus are observed as the populations of DIPs are passaged at high multiplicity.

The genomes present in two independently derived populations of DIPs of pseudorabies virus (PrV) have been partially characterized. The two populations differ in sequence composition (22). PrV DIP DNA contains reiterations of a limited set of sequences of the standard genome (2, 24) which are composed of segments of DNA originating from noncontiguous regions of the standard genome that have become covalently linked (22). One of these populations, [PrV(2)], consists of DIPs with genomes that contain an intact immediate-early gene that is expressed in the absence of helper virus. Coinfection of cells with this population of DIPs and helper virus causes the overproduction of some other viral proteins as well (23). Infection of cells with the other population of DIPs, [PrV(1)], on the other hand, does not detectably affect viral protein synthesis (23). Nevertheless, the biological properties of the two populations of DIPs appear to be quite similar (4). In both, there is a reduction of approximately 90% in the total number of virions produced. Both populations of PrV DIPs behave in a significantly different manner from most DIPs of other herpesviruses (for example, HSV and equine herpesvirus [11, 13, 28]). Thus, the DIPs of PrV accumulate rather slowly; only after 25 passages at high multiplicity can a significant drop in the titer of infectious virus be observed (2, 4). Furthermore, the sharp cyclical increases and decreases in the titers of infectious virus observed upon serial passage of populations of DIPs of HSV and equine herpesvirus (11, 13, 28), for example, do not occur when PrV DIPs are passaged; after an initial drop in the titer of about 10^2 by passage 30, only slight fluctuations in the titer are observed upon continued passage (4). The studies presented in this paper were undertaken to clarify the dynamics of the interactions between the defective and nondefective virions present in the populations of DIPs.

MATERIALS AND METHODS

Virus and cell culture. The preparation of PrV and cultivation of primary rabbit kidney (RK) cells has been described previously (19). Two populations of PrV DIPs [PrV(1) and PrV(2)] were generated by independent serial passage at high multiplicity of the standard PrV(Ka) strain in

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RK cells (4). Populations of DIPs at various levels of passage were used, starting with passage 33 [PrV(1)33] and going to passage 64 [PrV(1)64].

Media and solutions. The following media were used: Eagle synthetic medium (9) plus 5% dialyzed calf serum (EDS); EDS without PO_4 (EDS-PO_4); EDS-PO_4 plus 20 μg of 5-fluorouracil (FU) and 5 μg of thymidine per ml (EDS-PO_4+FU); EDS-PO_4 with 20 μg of FU, 10 μg of bromodeoxyuridine (BUdR), and 5 μg of deoxycytidine (CdR) per ml (BUdR medium); self-digested (nuclease-free) pronase (2 mg/ml) in 0.02 M NaCl–0.02 M Tris (pH 7.3) (pronase solution); and 0.15 M NaCl–0.015 M sodium citrate (pH 7.2) plus 4% sodium lauryl sarkosinate-97 (1× SSC–4% sarkosyl).

Enzymes and chemicals. All restriction enzymes were obtained from Bethesda Research Laboratories, and digestions were performed according to the specifications of the supplier. Inorganic ^{32}P (carrier free) and [α-^{32}P]dCTP were purchased from ICN Pharmaceuticals, Inc., FU and BUdR were obtained from Calbiochem-Behring, and [^3H]thymidine and [^3H]deoxycytidine (^3H-CdR) were obtained from Schwarz/Mann.

Labeling and purification of replicating virus DNA. RK cells were starved of phosphate by incubation in EDS-PO_4+FU for 48 h, a procedure which also inhibits cellular DNA synthesis (18). The cells were then incubated for 24 h in the same medium containing ^{32}P (100 μCi/ml) to label the nucleotide pools. The cultures were infected and further incubated in BUdR medium containing ^{32}P (100 μCi/ml). At the indicated times, the cultures were harvested by scraping the cells into 1× SSC–4% sarkosyl. The samples were heated at 60°C for 15 min, an equal volume of pronase solution was added, and the samples were incubated for 2 h at 37°C. Viral DNA was separated from cellular DNA by isopycnic centrifugation in CsCl, with [^3H]thymidine-containing and [^3H]BUdR-substituted viral DNA as markers, as described previously (6). DNA fractions were collected dropwise (2 drops per tube), the radiolabeled markers were localized, the appropriate fractions were collected and dialyzed, and the DNA was ethanol precipitated.

Plaque purification. Virus was plaque assayed on RK cells grown on 90-mm petri plates. Plaques were picked from plates containing not more than 20 plaques each, and the virus was amplified.

Purification of virions and extraction of viral DNA. Virions were purified and viral DNA was extracted as described previously (2, 5).

Restriction enzyme digestion and gel electrophoresis of DNA fragments. Digestion and agarose gel electrophoresis of virus DNA were carried out as described by Rixon and Ben-Porat (22). Filter strips, to which restriction fragments of PrV were fixed, were prepared by the method of Southern (25).

Nick translation of cloned PrV DNA restriction fragments. PrV DNA restriction fragments cloned in pBR325 as described previously (20) were nick translated by the method of Rigby et al. (21).

RESULTS

Lack of DIP DNA preferential encapsidation. The generation and amplification of DIP DNA may result from a replicative advantage of this DNA, from a selective advantage of this DNA at the level of encapsidation, or from both. To determine whether preferential encapsidation of DIP DNA occurs (i.e., whether this DNA may be enriched for cleavage-encapsidation signals), we compared the restriction patterns of the viral DNA that is packaged into virions with those of the viral DNA that accumulates within the infected cells.

PrV DNA is synthesized in excess, and only part of it becomes encapsidated (3, 17). Furthermore, restriction enzyme digestion of PrV DIP DNA generates aberrantly migrating DNA fragments that are absent from the digests of standard PrV DNA (22). Preferential encapsidation of DIP DNA should therefore be readily detectable, because the presence of these aberrant DNA bands is diagnostic of the presence of DIP DNA. This procedure can be used to study not only the evolution of the DIP upon passage of the virions but also to compare the efficiency of encapsidation of DIP DNA with that of standard DNA.

The restriction pattern of virus DNA that had accumulated in cells infected with populations of PrV(1) at different passage levels was compared with that of DNA that had become encapsidated and was present in preparations of purified virions isolated from these cells (Fig. 1). No significant preferential encapsidation of DIP DNA at any level of passage was detectable; the viral DNA that had accumulated within the infected cells during the infectious cycle had a restriction pattern similar to that of the viral DNA present in the purified virions isolated from these cells. The restriction pattern of the viral DNA changed, however, upon continued passage of the virions. In particular, enrichment for *Bam*HI fragments 10a and 12b (generated from defective DNA) was observed during early passage levels at high multiplicity. By passage 64, two defective DNA fragments, 11a and 12b, made up most of the DNA in the virion population. Thus, although the defective DNA (as exemplified by fragment 12b) did not appear to have a significant selective advantage over nondefective DNA present in the DIP population at the level of encapsidation, it was nevertheless enriched for upon high-multiplicity passage. It appeared possible, therefore, that enrichment of the DIP DNA in the population occurs as a result of preferential replication.

Time course of synthesis of DIP DNA. To determine whether DIP DNA has a replicative advantage either during the first or during later rounds of DNA replication, we followed the time course of its synthesis relative to that of the nondefective DNA present in the population of DIPs.

Phosphate-starved RK cells were pretreated with FU and thymidine (a procedure that completely inhibits cellular DNA synthesis without affecting viral DNA synthesis [18]) and were exposed to ^{32}P to prelabel the nucleotide pools. The cells were then infected with PrV(1)33 and incubated in BUdR medium containing ^{32}P. (BUdR, a density label, was added to the culture to allow the differentiation between viral DNA that had replicated and viral DNA that had become labeled by repair synthesis only.) At various times after infection, some of the cultures were harvested, and the DNA was extracted and banded in CsCl gradients along with [^3H]thymidine-containing and [^3H]BUdR-substituted viral DNA as markers. DNA fractions were collected, and the position in the gradient of ^{32}P-labeled viral DNA was determined. The DNA banding with the [^3H]thymidine-labeled viral DNA and the DNA that had shifted to a position of higher density, i.e., the DNA that had replicated, were collected separately. The DNA preparations were purified, digested with *Bam*HI restriction enzyme, and electrophoresed in agarose gels.

Figure 2 shows that *Bam*HI fragments 10a and 12b [which are the fragments characteristic of PrV(1)33-defective DNA] had replicated preferentially (lane 2) by 3 h 45 min postinfection. Even though these cells had been coinfected with

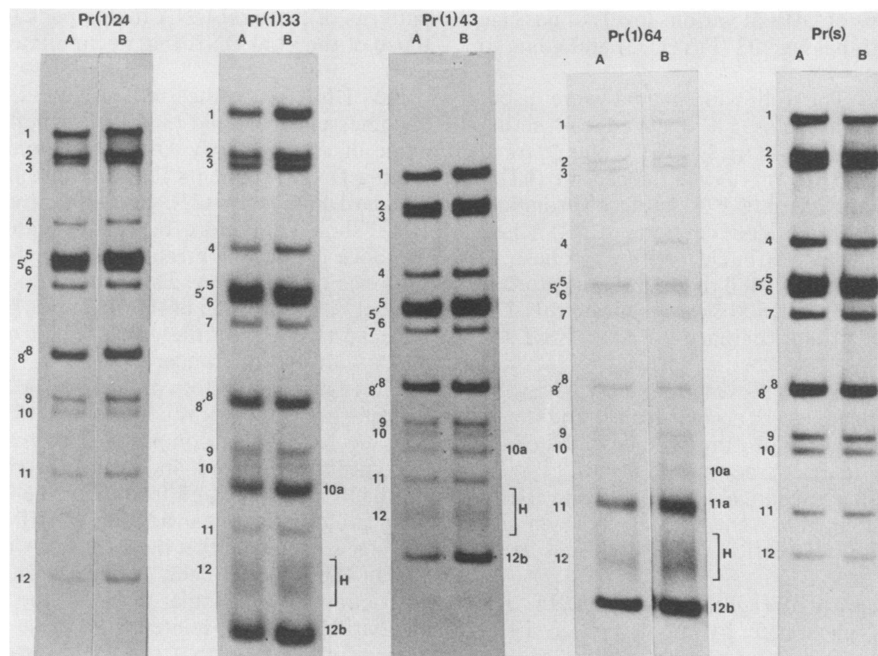

FIG. 1. Comparison of the *Bam*HI restriction patterns of virion DNA with the total intracellular viral DNA. ^{32}P-labeled virion DNA (A) and intracellular viral DNA (B) were purified from cells infected either with PrV(1) at passage levels 24, 33, 43, and 64 or with standard PrV [Pr(s)] as described in Materials and Methods. An equal amount of ^{32}P-labeled DNA obtained from virions and from infected cells was digested with *Bam*HI and electrophoresed in agarose gels, and autoradiograms were prepared. The numbers on the left side of each lane designate the standard PrV *Bam*HI fragments; the numbers on the right side designate the defective DNA fragments, i.e., the fragments that are not generated by digestion of standard PrV DNA. H indicates a region of heterogeneity.

helper virus, fragments representative of the helper PrV genome could barely be detected in the DNA that had replicated (lane 2), i.e., had shifted to a position of higher buoyant density as a result of incorporation of BUdR (normal thymidine-containing DIP DNA has a lower buoyant density than does standard PrV DNA [4]). In this particular experiment a significant amount of repair synthesis had also occurred (lane 1); however, in other experiments much less repair synthesis was observed (data not shown). We do not know the reason for this variation.

Figure 3 shows the microdensitometer tracings of autoradiograms obtained from a similar experiment in which the ^{32}P-labeled viral DNA that had accumulated in the infected cells at different times after infection was analyzed. The results show that the defective DNA, exemplified by fragment 12b, replicated first and, in fact, was the prominent species synthesized up to approximately 6 h postinfection. By 24 h postinfection, however, this band was much less prominent, and the restriction pattern of the ^{32}P-labeled viral DNA that had accumulated in the cells resembled the restriction pattern of the population of virions containing the DIPs that had been used to infect the cells. We conclude that PrV(1)-defective DNA contains origins of replication that are recognized at early times after infection by the DNA-synthesizing machinery of the infected cells. The replicative advantage of the DIP DNA is, however, only transient.

The ^{32}P-labeled DNA accumulating in cells infected with DIPs at various times after infection was also analyzed by the Southern technique (25). The microdensitometer tracings of the hybridization pattern of this DNA to *Kpn*I fragments of standard PrV DNA fixed to nitrocellulose filters are shown in Fig. 4. (See Fig. 6 for map positions of restriction fragments.) Viral DNA synthesized during the first round(s) of replication hybridized exclusively to *Kpn*I fragments B and D (middle panel). (Further exposure did not reveal hybridization to any of the other *Kpn*I bands.) By 24 h postinfection, the DNA synthesized by the cells hybridized to all *Kpn*I fragments (top panel), and the pattern of hybridization mimicked that of the DNA in the population of DIPs used for infection (see also reference 22). These results corroborate the conclusion that the DIP DNA replicates preferentially at early stages of infection in cells infected with a virion population which contains both DIPs and helper virus [PrV(1)33].

Structural modification of genomes of the infectious virions present in the populations of DIPs. The results described above indicate that although defective DNA present in the population of DIPs has a replicative advantage (at early stages of infection), it does not appear to posses an advantage in encapsidation over the genomes of the nondefective virions present in the virion population (Fig. 1). The lack of preferential encapsidation of the defective DNA could be due to a lack of enrichment of this DNA in *cis* functions required for encapsidation. On the other hand, it could be due to the presence in the population of DIPs of infectious virus that has been modified and has acquired the ability to compete effectively with DIP DNA, even though the latter has been enriched for cleavage-encapsidation sites. The emergence in a population of DIPs of infectious virions that, as a result of some modification, have acquired the ability to overcome the selective advantages of the genomes of DIPs has been documented in other systems (10, 14–16).

To determine whether the genomes of the infectious virions present in the population of DIPs of PrV have been modified, we analyzed (as a first step) the structure of the genomes of individual plaque-purified infectious virions obtained from the two independently derived populations of DIPs that we have been studying. Figure 5 illustrates the

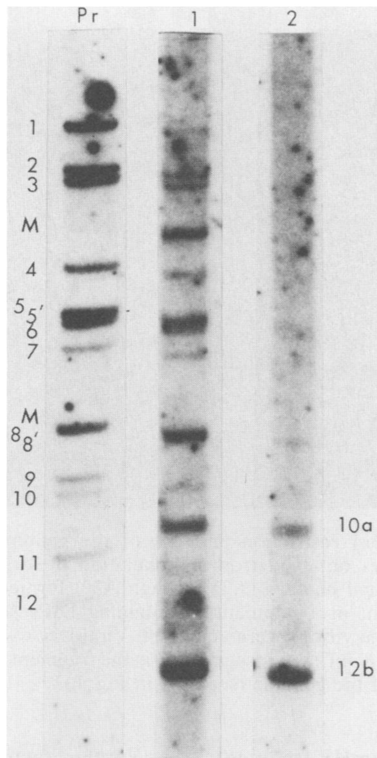

FIG. 2. Defective DNA replicates first in cells infected with a population of PrV(1) DIPs. Cells were incubated in EDS-PO_4+FU (to inhibit the synthesis of cellular DNA) and further incubated in EDS-PO_4+FU containing ^{32}P to equilibrate the nucleotide pools, as described in Materials and Methods. The cells were then infected with standard PrV or PrV(1)33 and were incubated in BUdR medium containing ^{32}P up to 3.75 h postinfection. The cells were harvested, and the DNA was purified by centrifugation in CsCl gradients, as described in Materials and Methods. The purified viral DNA was digested with *Bam*HI and electrophoresed in agarose gels, and an autoradiogram was prepared. Lanes: 1, viral DNA obtained from cells infected with DIPs that had banded in the characteristic position of PrV DNA (6 days of exposure); 2, viral DNA obtained from cells infected with DIPs that banded in a position of higher density (DIP DNA normally has a lower buoyant density than standard PrV DNA), i.e., that had incorporated BUdR (6 days of exposure); Pr, viral DNA obtained from cells infected with standard PrV that banded in a position of higher density, i.e., that had incorporated BUdR (2 days of exposure). M indicates the position of mitochondrial DNA, the synthesis of which is not completely inhibited by preincubation of the cells with FU.

Kpn digest of the DNA in the virions of 10 representative plaque isolates obtained from PrV(1)42. Of interest was the finding that in many cases a ladderlike series of submolar bands was observed; *Kpn*I end fragment D (Fig. 6), which normally is present in molar amounts in standard DNA, was in certain populations of virions either nondetectable or considerably reduced in intensity.

Several restriction digests of the DNA of individual plaque isolates were analyzed by the Southern technique (25) with individual cloned nick-translated restriction fragments encompassing the entire PrV genome as probes, and the genomes of these plaque isolates were mapped. The results showed that although only occasional modifications were observed along various parts of the genome of these plaque isolates, more than 75% had genomes in which sequences from the left end of the genome had become reiterated. The ladderlike arrays of bands derived from the left end of the genome that were observed in the restriction digests were probably the result of unequal (out-of-register) recombination, a process that occurs when sequences are tandemly reiterated.

Examples of the hybridization pattern of the *Bam*HI digests of the DNA of some plaque isolate with some probes of interest are illustrated in Fig. 7. Digestion of the DNA of plaque isolate B7 generated a series of bands which hybridized to cloned *Bam*HI fragment 14′ only (i.e., the left end of the genome; Fig. 6), indicating that sequences from the left end of the genome, which did not include the *Bam*HI cutting site, had become reiterated. On the other hand, digestion of the DNA of plaque isolate A11 generated a multimolar band at approximately the same molecular weight as that of *Bam*HI fragment 14′ that was generated from the standard virus DNA. This fragment hybridized to sequences present in *Bam*HI fragment 14′ only, indicating that the segment of DNA that had become reiterated consisted of the sequences of *Bam*HI fragment 14′ as well as some adjacent sequences

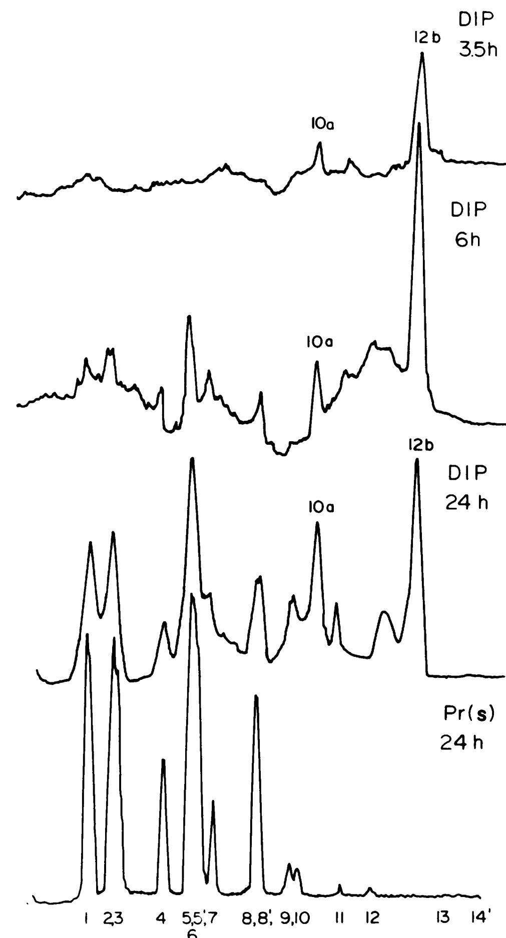

FIG. 3. Preferential replication of defective DNA up to 6 h postinfection. Cells were incubated in EDS-PO_4+FU and further incubated in EDS-PO_4+FU, as described in the legend to Fig. 2. The cells were then infected with PrV(1)33 and further incubated in EDS-PO_4+FU containing ^{32}P. The cells were harvested at the indicated times, and the DNA was purified and digested with *Bam*HI and electrophoresed in agarose gels. Autoradiograms were prepared after an appropriate exposure time and scanned to illustrate the relative abundance of the various restriction fragments.

including the *Bam*HI cleavage site. In other plaque isolates, as, for example, isolate A9, the reiteration not only comprised the extreme left of the genome (i.e., *Bam*HI fragment 14′), but also included significant stretches of sequences derived from the adjacent *Bam*HI 5′ fragment. For isolate A9, the *Bam*HI cleavage sites between the sequences in *Bam*HI fragments 14′ and 5′ were lost. A multimolar fragment hybridizing to both *Bam*HI fragment 14′ and *Bam*HI fragment 5′ was consequently formed. (Isolate A9 also contains inverted repeats of unequal sizes, as indicated by the hybridization pattern of its DNA to *Bam*HI fragment 8′.) Thus, the size of the fragment of DNA originating from the left end of the genome that is reiterated differs in the different virus isolates. This may also be deduced from the increments in the number of nucleotides between the rungs of the ladder observed in the *Kpn*I digest (Fig. 5). Indeed, in some cases, *Kpn*I band D appeared only as a diffuse smear. Analysis of the genomes of more than 100 individual plaque isolates of PrV(1)42 showed that the reiterations at the left end of the genome varied in size from less than 300 to at least 1,500 base pairs (data not shown). The reiterations always included sequences derived from the extreme left end of the genome (*Bam*HI fragment 14′). Similar results were also obtained when plaque isolates obtained from PrV(2)42 were analyzed (8).

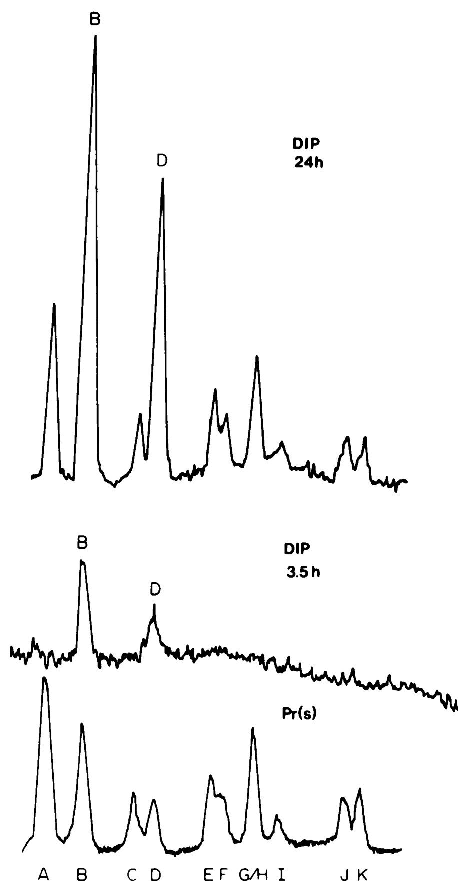

FIG. 4. Microdensitometer tracings of the hybridization pattern of viral DNA synthesized by cells infected with PrV(1)33. Cells were infected with PrV(1)33, as described in the legend to Fig. 3, and harvested at the indicated times. The DNA was purified and hybridized to *Kpn*I restriction fragments of standard PrV DNA that had been fixed to nitrocellulose filter strips. The autoradiograms were scanned after appropriate exposure times. The hybridization pattern of ^{32}P-labeled PrV DNA to the *Kpn*I fragments is also shown [Pr(s)].

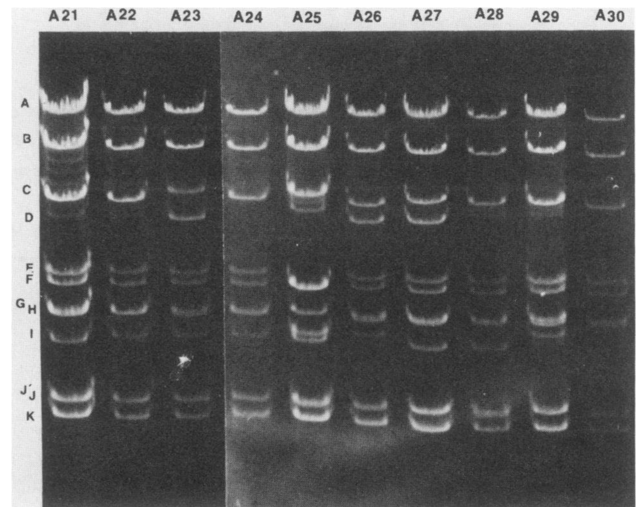

FIG. 5. *Kpn*I restriction patterns of the genomes of different plaque isolates obtained from a population of virions containing DIPs. Individual plaques (A21 through A30) formed by infectious virions present in a population containing DIPs [PrV(1)33] were picked, and the virions were amplified. Viral DNA was purified and digested with *Kpn*I. *Kpn*I fragment D is the fragment generated from the left end of the genome (see map in Fig. 6).

The proportion of infectious virus genomes that had reiterated ends in populations of DIPs was determined at several passage levels. Infectious virions present in PrV(1) at passages 33, 43, and 53 were plaque purified, and 20 plaques were analyzed in each case. No significant differences in the relative number of plaque isolates containing genomes with reiterated ends were found; in all three cases, between 70 and 85% of the plaques tested had genomes with reiterated ends. Thus, the reiterations of the left end of the genome were retained upon passage of the virions in the presence of DIPs. On the other hand, when individual plaque isolates with reiterated end sequences were passaged in cell cultures in the absence of DIPs, the genomes of some of the virions in the progeny acquired nonreiterated ends (Table 1). Thus, it appears that the reiterated sequences at the end of the U_L do not confer on the virions any replicative advantage in the absence of DIPs.

Underproduction of virions with nonreiterated end sequences by cells coinfected with standard virus and a population containing DIPs. Because approximately 75% of the infectious virions present in the population of DIPs have repetitive sequences at the left end of their genomes, these infectious virions can be distinguished from standard infectious virions. It was possible, therefore, to determine whether standard virus would be amplified in unmodified form as efficiently as was the endogenous infectious virus that was present in a population of DIPs when cells were coinfected with both.

Table 2 summarizes the results of this experiment. In the original population of DIPs, PrV(1)42, 25 of 30 plaque isolates (83%) had genomes with reiterated ends. After the population of DIPs was mixed with standard virions, the relative number of plaque isolates with genomes that had reiterated ends decreased, as expected, and only 6 of 30 plaque isolates (20%) were found to have reiterated ends.

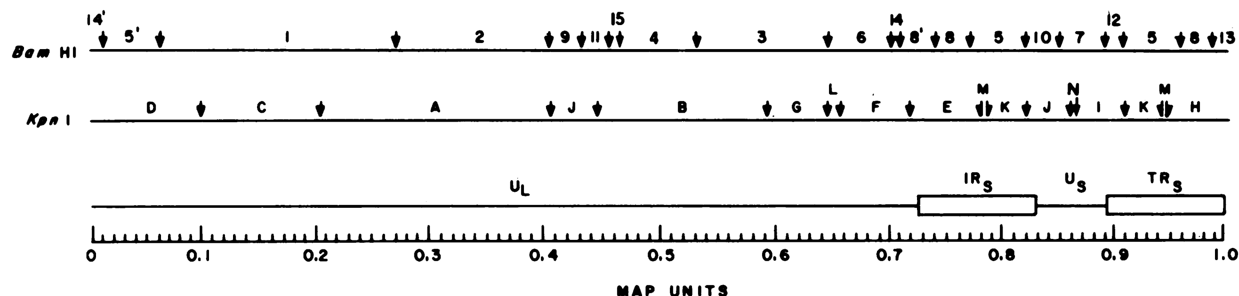

FIG. 6. Restriction maps of the PrV genome. Arrows indicate cleavage sites. Rectangles represent the inverted repeats.

However, after this population of virions was passaged five times at high multiplicity, most (21 of 29, or 72%) of the plaque isolates had reiterated ends, indicating that the standard virus with the nonreiterated ends that had been added to the population of DIPs tended to be lost upon passage. The disappearance of virus with genomes with standard nonreiterated ends observed in this experiment could be the result of a replicative advantage of the virions with reiterated ends that are endogenously present in the population of DIPs over standard virions. On the other hand, the standard viral genomes may have acquired the reiterations at the end of the unique long (U_L) segment as a result of recombination with the virions present in the population of DIPs.

To distinguish between these two possibilities, i.e., to ascertain whether in cells coinfected with DIPs the DNA of standard virus can compete effectively for amplification, we compared (i) the relative amounts of viral DNA synthesized by cells either infected with standard virus alone or coinfected with standard virus and DIPs that became associated with mature virions (Table 3), and (ii) the relative amounts of parental standard PrV DNA that, when added to cells infected with these virion populations, replicated and became associated with mature virions (Table 4).

The amount of viral DNA synthesized by cells infected with standard PrV alone and by cells coinfected with standard and defective virions was approximately the same (Table 3). However, the number of virions produced by the cells coinfected with the DIPs (as determined by the amount of labeled DNA that became associated with preparations of purified virions) was reduced by almost 90%. Thus, in this experiment, 22.6% of the total viral DNA synthesized by cells infected with standard virus alone but only 3.1% of the viral DNA synthesized by cells coinfected with DIPs and standard virus was found in the purified virion preparations. This finding confirms previously published reports (2, 4) which showed that the number of virions produced by the cells coinfected with DIPs of PrV is reduced considerably.

To determine whether standard viral genomes can compete at the level of replication and encapsidation with the viral genomes in the population of DIPs, the fate of

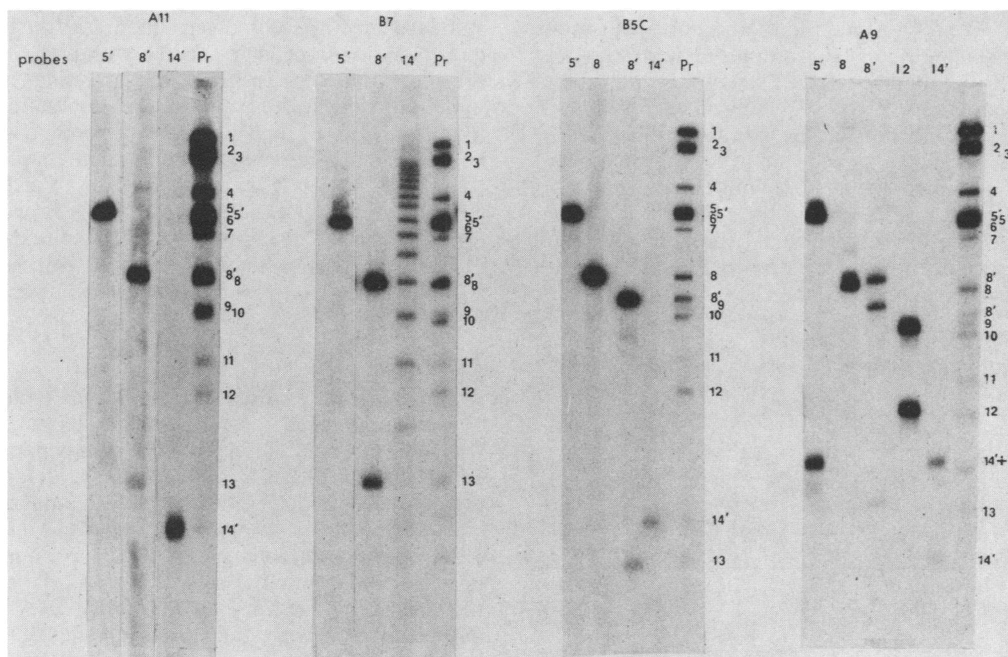

FIG. 7. Hybridization of some nick-translated *Bam*HI restriction fragments of the PrV standard genome to the *Bam*HI restriction digests of the DNA of different plaque isolates. The genomes of the indicated plaque isolates (A11, B7, B5C, and A9) were digested with *Bam*HI and electrophoresed, and the DNA fragments were transferred to nitrocellulose paper. Strips were probed with cloned, nick-translated restriction fragments originating from the fragment encompassing the entire PrV genome; however, the results obtained with some of the probes of interest only (*Bam*HI fragments 5', 8', and 14') are presented. Because of the heterogeneity of some restriction fragments, all of the viral isolates in this experiment were plaque purified again; the results obtained were identical.

TABLE 1. Reversion of virions containing genomes with reiterated ends to virions with nonreiterated end fragments[a]

Plaque isolate	No. of virions with genomes containing standard nonreiterated ends
B1	0/8[b]
B5	6/9
B7	2/9
B12	0/3
A11	9/12

[a] The progeny of some individual plaque isolates obtained from populations of DIPs which contained genomes with reiterated ends (Fig. 7) was passaged five times in RK cells at low multiplicity and then plaque assayed. Individual plaques were picked, the virions were amplified, and their genomes were analyzed with restriction enzymes. The number of plaque isolates which contained DNA with standard nonreiterated ends was determined.

[b] Numerator, number of plaques yielding virions with genomes with standard ends; denominator, number of plaque isolates analyzed.

[^{3}H]thymidine-labeled parental standard viral DNA in cells coinfected with either unlabeled standard virus or unlabeled DIPs was determined (Table 4). This experiment was performed under experimental conditions that were identical to those used in the experiments summarized in Table 3. The results of a representative experiment are summarized in Table 4. This experiment was repeated three times with essentially similar results.

Both in cells infected with standard virus alone and in cells coinfected with DIPs, most of the parental standard viral DNA had replicated by 8 h postinfection. The amount of ^{3}H-labeled parental standard virus DNA that became associated with progeny virions produced by cells coinfected with DIPs was reduced approximately 10-fold compared with that produced by cells infected with standard virus alone (Table 4, column 4). Since the total number of virions produced by these infected cells also differed approximately 10-fold (Table 3), one might conclude that the standard and defective genomes compete equally well for encapsidation. However, a large proportion of the [^{3}H]thymidine-labeled parental virus DNA that was present in the population of progeny virions obtained from cells coinfected with DIPs was DNA that had not replicated in the infected cells (Fig. 8). This DNA was probably derived from input parental virions that had not been uncoated and that copurified with the progeny virions produced by the cells. If one compares

TABLE 2. Loss of virions with genomes with standard ends from a population containing DIPs[a]

Virion population	Before passage		After passage	
	PFU/ml	No. of genomes with reiterated ends	PFU/ml	No. of genomes with reiterated ends
PrV(1) 41	1.3×10^7	25/30	1.3×10^7	24/28
PrV(1) 41 + standard PrV	4.1×10^7	6/30	1.7×10^7	21/29

[a] To a population of DIPs [PrV(1) 41], sufficient standard PrV was added to increase the titer of infectious virus approximately threefold. Part of the virion population was plaque assayed, and part was passaged five times in undiluted form and then plaque assayed. Thirty individual plaques from each virion population were picked; the virions were amplified, and the DNA was extracted and analyzed with restriction enzymes to determine the type of end fragments (standard or reiterated) that were present.

TABLE 3. Synthesis and encapsidation into virions of viral DNA in cells infected with standard PrV alone or in cells coinfected with standard PrV and DIPs[a]

Cells infected with:	^{3}H-CdR in viral DNA (10^3 cpm)	^{3}H-CdR in purified virions (10^3 cpm)	Viral DNA in virion (%)
PrV	97.5	21.9	22.6
PrV + PrV(1) 42	73.2	2.8	3.1

[a] RK cells (4×10^6 per sample) were incubated in EDS-FU for 16 h (to inhibit cellular DNA synthesis) and were then infected with either PrV alone (5 PFU per cell) or a mixture of DIPs (approximately 300 particles; 2 PFU per cell) and standard PrV (3 PFU per cell). Unadsorbed virus was removed after 1 h, and the cells were incubated in BUdR medium containing ^{3}H-CdR (50 μCi/ml) for 48 h. The total amount of ^{3}H-CdR incorporated into viral DNA, as well as the amount of ^{3}H-CdR-labeled DNA that had become encapsidated into mature virions, was determined as described in Materials and Methods.

the amount of ^{3}H-labeled parental standard DNA that had replicated and become part of virions produced by cells coinfected with DIPs with the amount produced by cells infected with standard virus alone, one is led to conclude that the standard DNA was discriminated against at the level of encapsidation in cells coinfected with DIPs. Thus, in cells infected with standard virus alone, the same proportion of the total viral DNA (22.6%) and of the ^{3}H-labeled parental viral DNA that had replicated (24.0%) became encapsidated into virions (see Tables 3 and 4). In cells coinfected with DIPs, 3.1% of the total viral DNA but only 0.9% of the ^{3}H-labeled parental viral DNA that had replicated became encapsidated. These results show that standard viral DNA is discriminated against at the level of encapsidation in cells coinfected with DIPs.

Figure 1 shows that the defective DNA does not appear to be preferentially encapsidated over the nondefective DNA endogenously present in the population of DIPs. On the other hand, Tables 3 and 4 indicate that exogenously added standard PrV DNA is discriminated against at the level of encapsidation in cells coinfected with a mixture of DIPs and the standard virus. Together, these findings indicate that the infectious virions present in the population of DIPs are modified forms of the standard PrV genome in that they have acquired characteristics which allow them to compete effectively with DIPs for encapsidation. Furthermore, these results indicate that the defective DNA is probably enriched for cleavage-encapsidation signals, despite the apparent lack of preferential encapsidation of the defective DNA over the nondefective DNA present in the virion population.

DISCUSSION

The salient features of the results presented in this paper can be summarized as follows. In a population containing DIPs of PrV, the defective DNA has a replicative advantage at early stages of DNA replication. The defective DNA does not, however, appear to have an advantage at the level of cleavage-encapsidation and is not preferentially encapsidated over nondefective viral DNA present in the virion population. Despite the lack of preferential encapsidation of the DIP DNA, it appears that this DNA is nevertheless enriched for cleavage-encapsidation sites. This conclusion is based on the following observations. (i) The genomes of the infectious virions present in the population of DIPs have been altered; notably reiterations of sequences derived from the left end of the genome are present in approximately 75% of the genomes of these virions. (ii) While standard viral genomes are discriminated against at the level of encapsida-

TABLE 4. Replication and encapsidation of [^{3}H] thymidine-labeled standard parental viral DNA in cells coinfected with DIPs[a]

Cells coinfected with:	^{3}H-Pr(s)[b] DNA in cells (10^3 cpm)	^{3}H-Pr(s) DNA that had replicated by 8 h (10^3 cpm)	[^{3}H]Pr(s) DNA in progeny virions (10^3 cpm)	[^{3}H]PrV DNA in progeny virions that had replicated (10^3cpm)	[^{3}H]PrV DNA in progeny virions that had replicated (%)
(^{3}H)PrV(S) + PrV(S)	27.6	19.55	5.49	4.70	24.0
(^{3}H)PrV(S) + PrV(1)42	22.2	14.16	0.59	0.13	0.9

[a] Cells were coinfected with purified [^{3}H]thymidine-labeled virions (3 PFU per cell) and either standard or defective unlabeled virions (2 PFU per cell). After 1 h, unadsorbed virus was removed and the cells were further incubated in BUdR medium. At 8 h postinfection, a portion of the cultures was harvested and the DNA was extracted and centrifuged in a CsCl gradient with ^{32}P-labeled thymidine-containing or BUdR-substituted DNA as markers to determine the amount of parental virus DNA that had replicated (column 3). The remainder of the cultures was incubated up to 48 h postinfection, when the virions produced by the cells were purified. The total amount of parental ^{3}H-labeled PrV DNA (column 4) as well as the amount of DNA that had replicated in the infected cells (column 5; see also Fig. 8) that were transferred to the progeny virions was deterined.

[b] Pr(s), Standard PrV.

[c] P.i., Postinfection.

tion in cells coinfected with DIPs, the nondefective and defective genomes present in these populations of virions compete equally well for encapsidation. It appears, therefore, that both the nondefective and defective genomes in these viral populations have an advantage over standard virus at the level of encapsidation and probably are enriched for cleavage-encapsidation sites.

Our results show that the defective DNA replicates first in the infected cells when the synthesis of the nondefective virus DNA present in the population of DIPs is barely detectable. Therefore, the defective DNA in the population of virions studied is probably enriched for sequences that can serve as origins of replication, sequences that can function at early stages of infection and compete successfully with the origins present in the nondefective DNA. Vlazny and Frenkel (29) showed that the defective DNA present in a population of DIPs of HSV is synthesized mainly at late times after infection, i.e., is enriched for origins of replication that do not function well at early stages

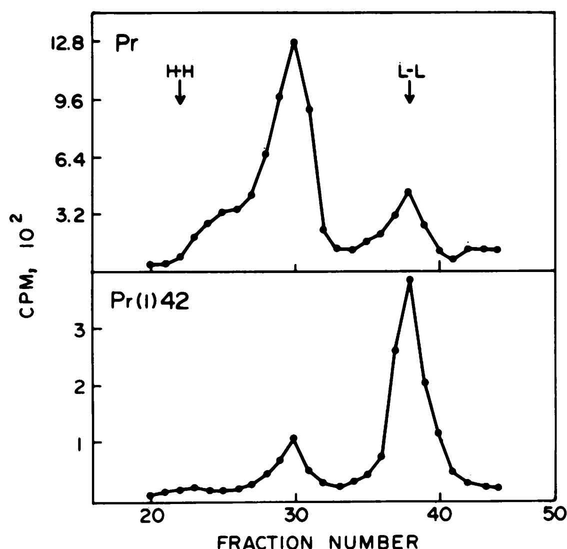

FIG. 8. Analysis of the ^{3}H parental viral DNA strands that were transferred to the virus progeny produced by cells coinfected with DIPs. RK cells were coinfected with either standard PrV or PrV(1)42 and [^{3}H]thymidine-labeled standard PrV, as described in the footnote to Table 4. The DNA of the progeny virions produced by the cells (Table 4, column 4) was centrifuged to equilibrium in CsCl, together with ^{32}P-labeled thymidine-containing DNA and ^{32}P-labeled BUdR-substituted viral DNA as markers. Fractions were collected, and the distribution of label in the gradient was determined. The bottom of the gradient is at the left.

of infection. The differences between our results with PrV DIPs and those of Vlazny and Frenkel (29) with HSV DIPs are probably due to an enrichment of different types of sequences that can serve as origins of replication in the genomes of the two populations of DIPs; it is unlikely that they reflect intrinsic differences between these two herpesviruses.

Normally, during the first round of replication, PrV DNA synthesis is initiated mainly at a site near or within the inverted repeats (7). Southern hybridization analysis of the DNA synthesized during the first round of replication in cells infected with DIPs indicates that the sequences that replicate first do not include sequences located in the inverted repeats (Fig. 4). These results might indicate that an origin other than that in the inverted repeats is present in the DIP DNA and is recognized at early stages of infection. However, because recombinational events occurred during the generation of the DIP DNA, we cannot exclude the possibility that the origin normally present in the inverted repeats has been juxtaposed (by a recombinational event) next to the sequences in the DIP DNA that replicate preferentially at early stages of infection. The presence of a small stretch of sequences derived from the inverted repeats would not have been detected in our experiment. Thus, although out results show that defective DNA is enriched for an origin of replication that can be used at early stages of infection, this origin cannot be conclusively identified in the absence of the appropriate sequencing data. The reason for the transient preferential synthesis only of the DIP DNA is also not clear.

As mentioned above, although our results show that DIP DNA replicates preferentially under certain conditions, it is not, as far as we can tell, preferentially encapsidated. However, the lack of preferential encapsidation of the DIP DNA does not necessarily reflect a lack of enrichment for the cleavage-encapsidation site. It has been established that the dynamics of the interactions between the DIPs and infectious virions present in a population containing DIPs are affected not only by the sequence composition of the DIP DNA but also by mutational changes in the genomes of the endogenous infectious virions (10, 14–16). Indeed, our results show that a selection for infectious virions that have acquired characteristics that promote their ability to compete with the DIP DNA has occurred during the evolution of the population of DIPs. Most of these infectious virions have acquired reiterations of the left end of the genome (Fig. 5 and 7); these reiterations tend to be lost upon passage of the virions in the absence of DIPs (Table 1). Furthermore, infectious virions containing standard PrV DNA with nonreiterated ends are discriminated against in cells coinfected with DIPs (Table 2), and although the DIP DNA and the endogenous nondefective DNA present in the DIP

population compete equally well (Fig. 1), standard PrV genomes compete poorly at the level of encapsidation in cells coinfected with a population of DIPs (Tables 3 and 4). Taken together, these data indicate that both the defective DNA and the genomes of the infectious virions present in the population of DIPs have an advantage in encapsidation over the standard viral genome.

The segments of DNA derived from the left end of the standard viral genome that are reiterated in the genomes of infectious virions present in the population of DIPs contain some of the *cis* functions that are necessary for the efficient cleavage-encapsidation of PrV DNA (30; C. Wu, Ph.D thesis, Vanderbilt-University, Nashville, Tenn., 1986). The possibility that the reiteration of these sequences is linked to the advantage of these genomes in encapsidation is attractive. However, conclusive evidence that these reiterations are the basis for the selective advantage of the modified virions is not available; other, undetected, modifications in the genomes may play a role.

The cyclical changes in the level of infectious virus obtained upon passage of populations of DIPs at high multiplicity that are observed in many other viral systems are not observed upon similar passage of PrV DIP. Furthermore, the accumulation of the DIP DNA in this system is slow (4). The reason for these characteristics is probably related to the fact that the DIPs only have a slight growth advantage over the infectious virus present in the population of DIPs that we have studied. Their genomes have only a transient replicative advantage at early stages of infection (Fig. 2 and 3), and a significant advantage at the level of encapsidation of DIP genomes over the genomes of the infectious virions present in the population of DIPs is also not observed (Fig. 1). As already mentioned, this is probably because modifications of the genomes of the endogenous infectious virions have occurred that allow them to compete for encapsidation.

Despite the fact that the DIP DNA has only a slight replicative advantage, the populations of DIPs do interfere significantly with the growth of standard virus (2). This interference can be ascribed to a preferential encapsidation of the genomes present in the DIP population over that of standard virus. It is also due to an interference with the process of cleavage and encapsidation of viral DNA (standard as well as defective) in DIP-infected cells (Tables 3 and 4). Thus, even though viral protein synthesis (22) and the production of capsids (unpublished results) are not markedly affected, the total number of viral particles produced and the cleavage of concatemeric DNA is reduced by approximately 90% in cells coinfected with DIPs (4). The reduction in cleavage and encapsidation of the virus may be the result of an interference by the DIPs with the orderly expression of the virus genome. On the other hand, it may be due to the direct inhibitory effect of the DIP genomes on the cleavage-encapsidation process as a consequence of the sequence organization of the DIP DNA. This question remains to be clarified.

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