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cis Functions Involved in Replication and Cleavage-Encapsidation of Pseudorabies Virus

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Serial passage at high multiplicity of pseudorabies virus generates defective interfering particles (DIPs) whose genomes consist at least in part of reiterations of segments of DNA in which sequences originating from different regions of the genome have become covalently linked (F. J. Rixon and T. Ben-Porat, Virology 97:151–163). To determine whether some *cis* functions present in these reiterated DNA sequences may be responsible for the amplification of DIP DNA, *Bam*HI restriction fragments of this DNA were cloned. These fragments were analyzed and tested for their ability to promote the amplification of covalently linked pBR325 DNA when cotransfected into cells with helper pseudorabies virus DNA. The cloned DIP *Bam*HI DNA fragments consisted of various combinations of sequences originating from either one or both ends as well as sequences from the middle of the unique long (U_L) segment of the genome. Only plasmids with inserts consisting of segments of defective DNA originating from the middle of the U_L, as well as from both ends of the genome, were able to replicate and be encapsidated autonomously. This finding indicated that signals present at both ends of the genome may be necessary for efficient cleavage-encapsidation. To confirm this observation, we constructed plasmids in which DNA segments containing an origin of replication and sequences from either one or both ends of the virus genome were linked. These experiments showed that efficient cleavage-encapsidation requires the presence of sequences derived from both ends of the genome. Two origins of replication, one at the end of the U_L segment and one in the middle of the U_L segment, were also identified.

The genome of pseudorabies virus (PrV) is composed of a linear, double-stranded DNA molecule that can be divided into a unique long (U_L) component and a unique short (U_S) component, the U_S component being bracketed by inverted repeats (6, 33). Soon after entering the cell nucleus, the viral DNA forms circles (7, 14). Because the two ends of the linear DNA molecules are unique and do not share sequence homology (L. Harper and T. Ben-Porat, manuscript in preparation), circle formation (a process that does not require the expression of virus functions [14]) must occur by direct ligation of the ends. During the early rounds of viral DNA replication, replicating DNA is associated with circular structures; thereafter it is associated with concatemers in head-to-tail alignment (5, 7). Cleavage of this concatemeric DNA requires the expression of at least nine viral gene functions and is intimately linked to capsid assembly (18).

Several regions of the genome of PrV containing *cis* functions that can serve as origins of replication have been identified (7). The signals necessary for efficient cleavage and encapsidation of PrV DNA are unknown, however. These signals may reside on only one or on both of the unique ends of the virus genome.

We have shown that the DNA of defective interfering particles (DIPs) is enriched for sequences that can serve as origins of replication and probably also for sequences that are necessary for cleavage-encapsidation (42). The experiments described in this paper were designed to further identify these *cis* functions. To this end, we cloned restriction fragments of the DIP DNA and used these clones in a manner similar to that previously used to define the amplicons of herpes simplex virus (HSV) (31, 32, 35). This method tests the ability of restriction fragments of either standard or defective DNA to promote the amplification of covalently linked pBR325 sequences.

A number of reports relating to the localization on the HSV genome of the *cis* functions necessary for replication and replication-encapsidation have appeared (15, 31, 32, 34, 35, 37–39, 41). These studies have localized the cleavage-encapsidation signals of HSV within the "a" sequence of HSV, specifically to a 250-base-pair fragment within the "a" sequence (37). The "a" sequence is present at both ends of the HSV genome (and is also present in an inverted orientation at the junction between the U_L and the U_S regions of the HSV genome). Since the linear genome of HSV is terminally redundant for the "a" sequence, the question of whether cleavage occurs as a result of recognition of signals present on both sides of the cleavage site or only on one has not as yet been resolved. Because the two ends of the genome of PrV do not share sequence homology, it is possible with this system to ascertain whether signals required for efficient cleavage-encapsidation are present in this herpesvirus at both ends of the genome. The results presented in this paper show that this is indeed the case.

MATERIALS AND METHODS

Virus and cell culture. The preparation of PrV and cultivation of primary rabbit kidney (RK) cells have been described previously (16). DIPs of PrV were generated by serial, undiluted passage of the standard virus in RK cells (3).

Media and solutions. The following media were used: Eagle synthetic medium (9) plus 5% dialyzed calf serum (EDS); 0.9 M NaCl–0.09 M sodium citrate (pH 7.2) (6× SSC); and HEPES- (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffered saline (8.0 g of NaCl, 0.37 g of KCl, 0.125 g of Na_2HPO_4, 1.0 g of dextrose, 5.0 g of HEPES per liter [pH 7.5]) (HBS).

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Enzymes and radiochemicals. Restriction endonucleases were obtained from Bethesda Research Laboratories; digestions were carried out as specified by the supplier. [α-^{32}P]dCTP was purchased from ICN Pharmaceuticals, Inc.

Cloning of defective PrV DNA fragments. RK cells were infected with PrV(1)33 (multiplicity, 5 PFU per cell). Twenty-four hours later the cells were harvested, and the DNA was extracted (8). Viral DNA was separated from cellular DNA by equilibrium sedimentation in CsCl and purified as described previously (4). The viral DNA was digested with *Bam*HI and cloned into the *Bam*HI site of pBR325 essentially as described previously (18).

Cloning of standard PrV end fragments and junction fragments. Standard PrV end fragments and junction fragments were cloned by the method described by Maniatis et al. (19). *Bam*HI end fragments 13 and 14′ were excised from an agarose gel and purified on hydroxyapatite columns. The DNA was blunt-ended by treatment with DNA polymerase I large fragment (Klenow), and *Bam*HI linkers were added. The DNA fragments were then cloned into the *Bam*HI site of pBR325, as described previously (18). The *Bam*HI junction fragment was obtained from the DNA of cells infected with PrV *ts*N, a temperature-sensitive mutant; concatemeric DNA accumulates in RK cells infected with this mutant at the nonpermissive temperature (17). Viral DNA was separated from cellular DNA as described above; it was digested with *Bam*HI and electrophoresed in agarose gels. The band containing a junction fragment was excised from the gels and cloned into the *Bam*HI site of pBR325.

Construction of plasmid C109. Partial digests of plasmid C14 (*Bam*HI fragment 15 cloned into pBR325) with *Bam*HI were electrophoresed in low-melting-temperature agarose gel (0.8%). The linearized plasmid C14 was visualized by ethidium bromide staining and cut from the gel. The agarose containing the band was melted by heating to 68°C and diluted in 4 volumes of H_2O. A fivefold excess of *Bam*HI fragment 13 was ligated to the linearized C14 plasmid. The orientation of the two inserts was determined by restriction enzyme mapping.

Nick translation. Nick translation of cloned fragments was performed by the method of Rigby et al. (24).

Transfection. The calcium phosphate precipitation method of Graham and Van der Eb (12) was used. In brief, the appropriate DNA preparations were mixed in 1 ml of 1× HBS. $CaCl_2$ was added (final concentration, 125 mM), and the mixture was incubated at room temperature for 30 min and added to RK monolayers in 90-mm petri dishes. After incubation of the mixture for 45 min at room temperature, EDS was added, and the cells were further incubated at 37°C for 4 h. The medium was then replaced with fresh EDS, and incubation at 37°C was continued up to 3 days postinfection.

Preparation of DNA samples for analysis of the amplification of plasmid sequences. Amplification of plasmid sequences was determined in (i) total intracellular DNA of transfected cells and (ii) purified virions produced by these cells. (i) For preparation of intracellular DNA, cells were scraped into the medium 3 days posttransfection and centrifuged at 5,000 × *g* for 15 min. The cellular pellet was resuspended, and the DNA was extracted as described previously (8). (ii) For purification of DNA from virions, the supernatant from the transfected cells 3 days after transfection was inoculated into fresh RK cultures. Twenty-four hours after infection, virions were purified and viral DNA was extracted as described previously (6).

Restriction enzyme digestion, electrophoresis, and Southern transfer. The DNA was digested with the indicated restriction enzyme, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose filters by the method of Southern (30), as described previously (5).

Dot-blotting of DNA. The DNA was denatured by boiling in 0.2 M NaOH for 5 min and then neutralized with an equal volume of 1.33 M Tris (pH 4.6). Twofold dilutions of the DNA were made in 6× SSC. The samples were blotted onto nitrocellulose paper and hybridized with the appropriate ^{32}P-labeled nick-translated probe.

RESULTS

Cloning of *Bam*HI restriction fragments of defective PrV DNA. Serial passage of PrV at high multiplicity results in the generation of DIPs (2). The DNA from one population of DIPs was found to be enriched for genomes of lower sequence complexity than that of the standard virus genome (2, 3); it was composed, at least in part, of tandem reiterations (26) of sequences originating from noncontiguous regions of the standard PrV DNA (25).

Since the reiterations in the defective DNA may include *cis* functions necessary for the propagation of defective DNA, such as an origin of replication and cleavage-encapsidation recognition sites, we used this DNA as a potential source of DNA fragments containing both of these *cis* functions.

Digestion of defective DNA with *Bam*HI generates several fragments not found in digests of standard viral DNA (25). To determine whether some of the aberrantly migrating fragments generated from defective DNA possess the *cis* functions required for their amplification, a *Bam*HI digest of viral DNA obtained from cells infected with a population of DIPs [PrV(1)33] was cloned into the *Bam*HI site of pBR325. Forty plasmids containing inserts of viral DNA were analyzed with respect to their size, as well as their homology to different regions of the standard viral DNA (as determined by their pattern of hybridization to strips to which *Bam*HI, *Kpn*I, *Sal*I, and *Pst*I-digested standard PrV DNA had been fixed). A summary of the results obtained is given in Fig. 1.

While similarly cloned *Bam*HI-digested standard PrV DNA yielded, as expected, plasmids with inserts consisting of standard *Bam*HI restriction fragments only, of the 40 plasmids obtained after cloning defective DNA, only 2 had inserts consisting of standard PrV *Bam*HI fragments; 38 had inserts which were derived from defective DNA. Of these 38 plasmids, 13 contained as an insert *Bam*HI fragment 12b (molecular weight, 1.25×10^6), which is the most abundantly represented fragment observed in *Bam*HI digests of defective DNA. The other plasmids had *Bam*HI fragment inserts that varied in size.

The viral DNA sequences in the cloned defective *Bam*HI DNA fragments originated from various regions of the viral genome, some consisting of sequences that are normally noncontiguous on the standard genome. On the basis of their homology with sequences originating from different regions of the standard PrV genome, the plasmids could be grouped into the following four classes: (i) class I, containing inserts with sequences derived from the middle of the U_L region, as well as from both ends of the standard PrV genome; (ii) class II, containing inserts with sequences arising from the middle of the U_L region and from the left end of the genome; (iii) class III, containing inserts with sequences originating from the middle of the U_L region only; and (iv) class IV, containing inserts with sequences derived from the left end of the genome only. Although this classification indicates the re-

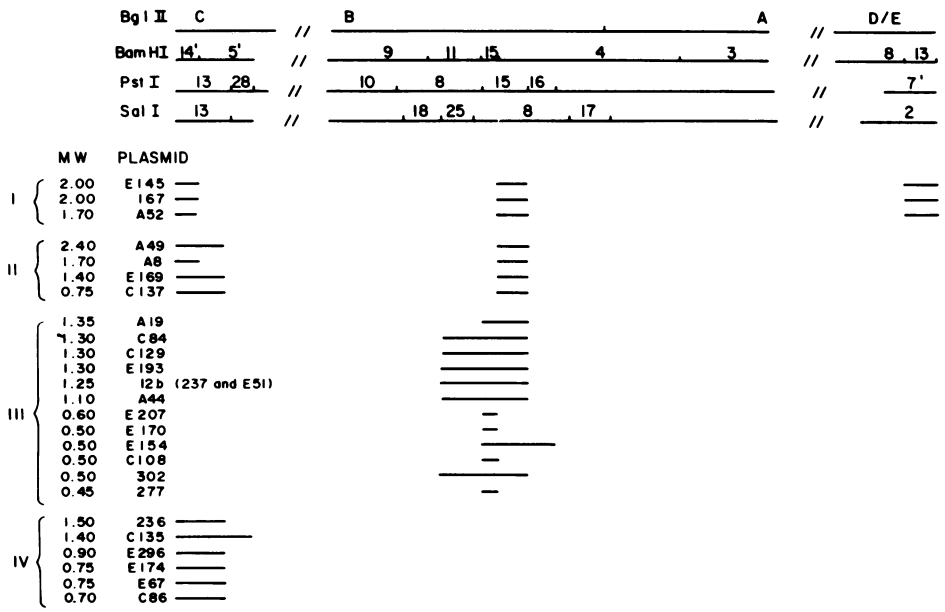

FIG. 1. Analysis of cloned *Bam*HI-defective DNA fragments. *Bam*HI-digested defective PrV(1) DNA was cloned into the *Bam*HI site of pBR325, as described in Materials and Methods. The sizes of the inserts, their designation, and their homology to regions of the PrV genome, as determined by hybridization of the ^{32}P-labeled plasmid DNA preparations to filter strips to which *Kpn*I, *Bam*HI, *Pst*I, and *Sal*I restriction fragments of standard PrV DNA had been fixed, are indicated by lines. These lines indicate the limits of map positions of the restriction fragments of standard PrV DNA to which the plasmids hybridize. The whole region indicated by the line is not necessarily included in the cloned DNA fragment, as indicated by the molecular weight of the inserts in the plasmids. Based on their homology with regions of the standard genome, these plasmids can be grouped into the indicated four classes. Regions of interest of the restriction enzyme maps of the PrV genome are also indicated.

gions of the standard genome from which most of the sequences in the defective *Bam*HI fragments originate, the entire regions (indicated as lines in Fig. 1) are not necessarily included in the cloned defective DNA fragments. Furthermore, these fragments may also contain some sequences derived from other regions of the PrV genome that were not detected under the hybridization conditions that we used.

The results of the analysis of the cloned defective DNA fragments (Fig. 1) indicate that defective PrV(1)33 DNA is composed of heterogeneous DNA segments and that, as indicated by previous studies (25), recombination between sequences that are noncontiguous on the standard PrV genome has occurred.

Amplification of pBR325 sequences in cells cotransfected with plasmids containing PrV DNA inserts and helper PrV DNA. The plasmids containing inserts derived from defective DNA were screened for their ability to be amplified when cotransfected into cells with helper PrV DNA by determination of whether amplification of the covalently linked pBR325 sequences occurs. The virions produced by the transfected cells were purified, and the DNA was extracted, dot-blotted onto nitrocellulose paper, and probed with nick-translated pBR325 DNA. The dot-blots were also probed with nick-translated PrV DNA to determine whether similar amounts of DNA in the different preparations had been blotted onto the filters.

In cells transfected with PrV DNA alone, (Fig. 2, lane -0-) or in cells transfected with helper PrV DNA and pBR325 DNA without an insert of PrV DNA (lane pBR325), hybridization with pBR325 sequences was not observed. In cells cotransfected with PrV DNA and most of the plasmids containing an insert of defective PrV DNA, pBR325 sequences were amplified, although to different degrees, depending on the type of inserts the plasmids carried. The best amplification of pBR325 sequences was observed with plasmids A52, E145, and 167, all of which are class I plasmids (see above). Various degrees of amplification of pBR325 sequences were also observed with plasmids with type II inserts. Some of the other plasmids containing type III and IV inserts also promoted pBR325 sequence amplification, but to a much lesser extent.

It is interesting that plasmids containing *Bam*HI fragment 12b (for example, plasmids E51 and 237), the most frequently cloned defective DNA fragment (see above) and the most abundant *Bam*HI fragment generated from defective DNA (25), are amplified only to a minimal extent. Thus, *Bam*HI fragment 12b does not appear to include the *cis* functions necessary for its amplification. However, we have found (42) that the sequences included in fragment 12b replicate first in cells infected with a population containing DIPs. It is probable that *Bam*HI fragment 12b is part of a somewhat larger repeat unit that contains a functional origin of replication but also includes at least one *Bam*HI cleavage site. The fragments produced after *Bam*HI digestion of the putative repeat unit would therefore include fragment 12b and one (or several) other relatively small fragments that include an origin of replication and that may have run off the gel under the conditions of electrophoresis that we used.

Distinction between autonomous replication, autonomous replication and encapsidation, and amplification of plasmid sequences as a result of recombination into helper PrV DNA. Figure 2 showed that pBR325 sequences linked to PrV defective DNA sequences were in most cases amplified when cotransfected into cells with helper PrV DNA. Some amplification of pBR325 sequences was also observed when standard PrV fragments cloned into pBR325 were similarly transfected into cells (data not shown). However, the degree

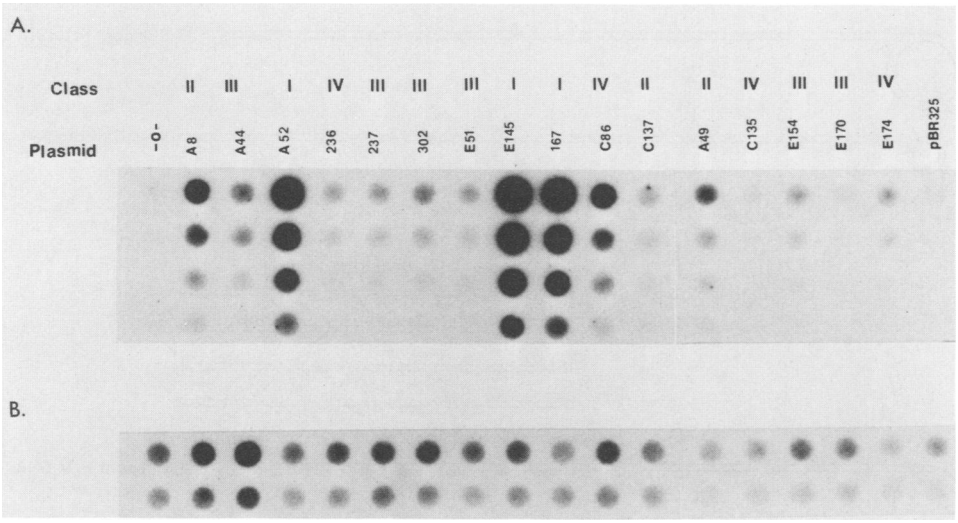

FIG. 2. Amplification and encapsidation of pBR325 sequences in cells cotransfected with plasmids containing defective DNA inserts and PrV DNA. Cells were cotransfected with the indicated plasmids and helper PrV DNA. Virions produced by the cells were purified, their DNA was extracted and denatured, and twofold dilutions were blotted onto nitrocellulose paper. These dot-blots were probed with (A) nick-translated pBR325 DNA (exposure time, 7 days) or (B) nick-translated PrV DNA (exposure time, 6 h). The class to which each plasmid belongs (Fig. 1) is indicated at the top of the figure.

of amplification of the pBR325 sequences varied greatly, depending upon the PrV DNA sequences in the plasmid. This indicates that the mechanism leading to amplification of the pBR325 sequences may vary for the different plasmids, depending upon the *cis* functions present in the PrV DNA sequences.

One of the following three mechanisms might be responsible for the amplification and encapsidation of the pBR325 sequences. (i) The PrV DNA sequences may include an origin of replication as well as cleavage-encapsidation sites, thereby promoting the autonomous replication and encapsidation of the covalently linked pBR325 sequences. (ii) PrV DNA sequences may include an origin of replication, resulting in the autonomous replication of the pBR325 sequences. These replicated sequences would, however, have no means of becoming encapsidated into virions unless they recombined into helper PrV DNA (presumably via the PrV DNA sequences in the insert) before encapsidation. (iii) The PrV DNA sequences may include neither an origin of replication nor encapsidation recognition signals, and thus amplification of pBR325 sequences would occur only after recombination of the plasmid into helper DNA; the differences in the degree of amplification of pBR325 sequences observed with different plasmids could be due to "hot spots" of recombination.

We attempted to distinguish between these possibilities by restriction enzyme analysis of the virion DNA by using the rationale illustrated in Fig. 3. If the plasmid contains sequences that include an origin of replication, as well as the signals necessary for cleavage-encapsidation, virion-size genomes composed entirely of plasmid sequences can be generated by replication of the plasmid via a rolling-circle mode of replication (1, 31) and cleavage of concatemeric DNA. After the virion DNA produced by cells cotransfected with this plasmid and helper PrV DNA is digested with a restriction enzyme that does not cleave the plasmid but cleaves the PrV genome, pBR325 sequences should remain associated with genome-size DNA (Fig. 3A). pBR325 sequences could also be associated with molecules of smaller than genome size if the plasmid either has replicated and then recombined into the helper PrV or has recombined into the helper PrV DNA prior to its replication.

On the other hand, if a plasmid contains an origin of replication only but no cleavage-encapsidation signals, the plasmid sequences would replicate but could be encapsidated into virions only after they had recombined with the helper virus genome. After the DNA present in virions produced by these cells is digested with an enzyme that does not cleave the plasmid but does cleave PrV DNA, pBR325 sequences should not remain associated with genome-size molecules. However, if the plasmid has replicated autonomously prior to its recombination into helper DNA, pBR325 sequences should be associated with fragments of DNA larger than the sum of the sizes of the largest fragment of standard DNA and the input plasmid. Furthermore, the autonomous replication of the plasmid (presumably by a rolling-circle mechanism) followed by its recombination into standard DNA should generate, after digestion of the virion DNA with an enzyme that cleaves the PrV genome but not the plasmid, a ladderlike array of bands containing pBR325 sequences (Fig. 3B).

Finally, if the plasmids contain neither an origin nor cleavage-encapsidation signals, the plasmid sequences can replicate and become encapsidated only after their recombination into the helper PrV genome. Digestion of the virion DNA with an enzyme that cleaves PrV DNA but does not cleave the plasmids should generate fragments which include the pBR325 sequences that are not larger than the sum of the sizes of the largest PrV DNA fragment and the linear plasmid (Fig. 3C).

Figure 4 shows the results of a representative experiment of the type discussed above. RK cells were cotransfected with the indicated plasmids and helper PrV DNA. The DNA in the virions produced by the transfected cells was purified and digested with *Xho*I (a restriction enzyme that does not cleave any of the plasmids used but does cleave the PrV genome into at least 30 fragments). The DNA digests (Fig. 5), as well as undigested virion DNA (Fig. 4), were electrophoresed in the same gel (to establish the position of genome size DNA) and transferred onto nitrocellulose filters by the

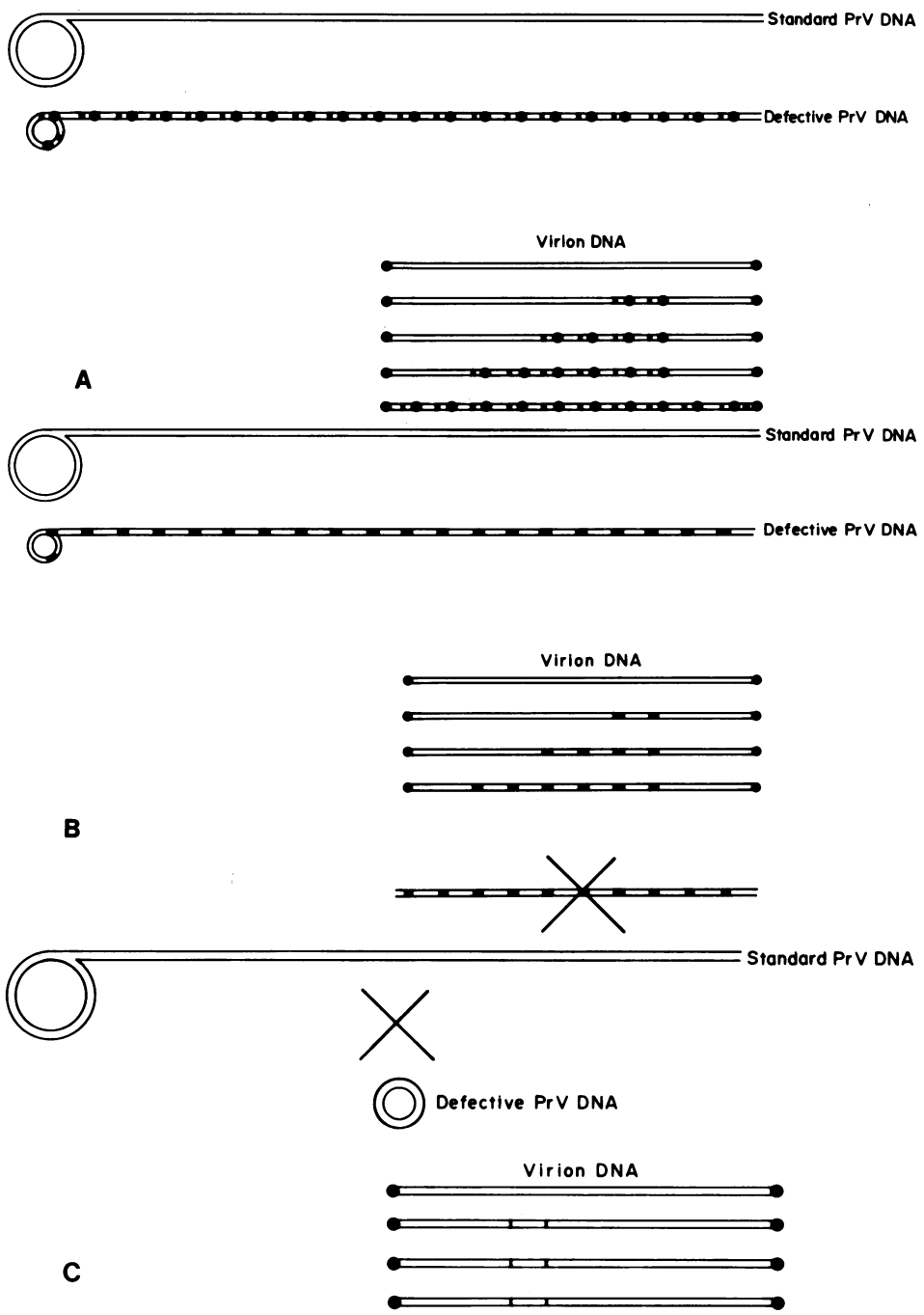

FIG. 3. Scheme used to differentiate between autonomous replication-encapsidation and autonomous replication only, or amplification of the pBR325 sequences after recombination into the standard PrV DNA. (A) Plasmid with inserts of viral DNA that contain an origin of replication (solid bars) and cleavage-encapsidation signals (solid circles) will be able to replicate autonomously (presumably by a rolling-circle mechanism) and be encapsidated autonomously. The virions produced will contain some DNA molecules composed entirely of plasmid sequences. Upon digestion of the virion DNA with an enzyme that cleaves PrV helper DNA several times but fails to cleave the input plasmid, pBR325 DNA sequences associated with PrV genome-size molecules will be obtained. (B) Plasmids with inserts of viral DNA that contain only an origin of replication (solid bars) will be able to replicate autonomously. Encapsidation into virus particles will, however, only occur after recombination of the plasmid sequences into the helper PrV DNA, and therefore the virions produced will not contain DNA molecules composed entirely of plasmid sequences. After digestion of the virion DNA with an enzyme that cleaves PrV DNA but fails to cleave the input plasmid, no pBR325 sequences associated with PrV genome-size DNA will be obtained (indicated by the cross). Instead, if replication has occurred via a rolling-circle mechanism, the pBR325 sequences that have replicated autonomously will be associated with restriction fragments of DNA larger than the sum of the largest PrV restriction fragment and the linear input plasmid. A ladderlike array of bands containing pBR325 sequences will be found as a consequence of recombination of the replicated plasmid sequences into the PrV genome. (C) If the plasmid does not contain an origin, amplification of the plasmid sequences will occur only after its recombination (indicated by the cross) into the helper virus DNA.

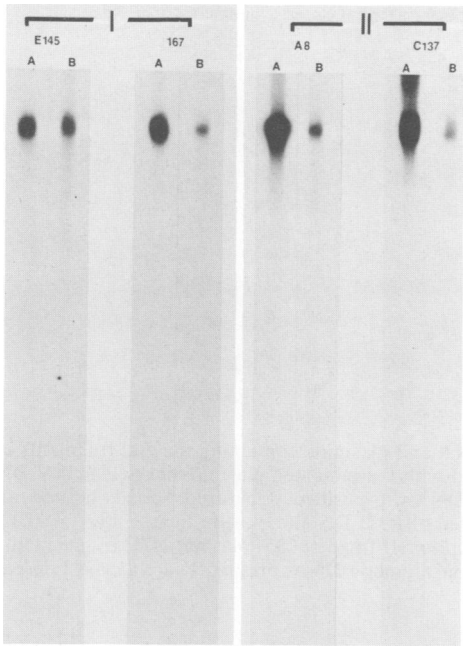

FIG. 4. pBR325 DNA sequences present in virion preparations produced by cells cotransfected with plasmids containing viral inserts and PrV helper DNA are associated with genome-size molecules. RK cells were cotransfected with PrV DNA and the indicated plasmid. The virions produced by the cells were purified, and the DNA was extracted and electrophoresed into agarose cells. The DNA was transferred to nitrocellulose paper by the method of Southern (30), and strips were probed with nick-translated PrV DNA (A) or pBR325 DNA (B). Lanes B were exposed eight times longer than lanes A.

method of Southern (30), and filter strips were probed with nick-translated pBR325 DNA or PrV DNA.

In all cases, the pBR325 sequences present in undigested virion DNA were found in the gels in a position expected of genome-size DNA (approximately 90×10^6 daltons) (Fig. 4). After the DNA obtained from virions produced by cells cotransfected with helper PrV DNA and plasmids E145 or 167 (two class I plasmids) was digested with *Xho*I, pBR325 sequences associated with genome-size molecules were observed (Fig. 5). This indicates that these plasmids had both replicated and become encapsidated autonomously. However, relatively few viral DNA sequences remained associated with genome-size molecules, indicating that while the plasmid had replicated and become encapsidated autonomously, it only accounted for a small proportion of the DNA in the virion population. In addition, pBR325 sequences were also associated with molecules smaller than virion genome size, indicating that some plasmid sequences had recombined into helper DNA before encapsidation. Some fragments smaller than the original plasmid were also observed. Deletions of plasmid sequences must therefore have occurred. Indeed, deletions of plasmid sequences have been found to occur randomly along the plasmid molecule in the transfected cells (C. A. Wu, Ph.D. thesis, Vanderbilt University, Nashville, Tenn., 1986).

*Xho*I digestion of DNA obtained from virions produced by cells cotransfected with plasmid A8 (a class II plasmid) and helper PrV DNA generated a ladderlike array of fragments containing pBR325 sequences (Fig. 5). Some of these fragments were larger than the sum of the sizes of the largest *Xho*I PrV fragment and the linear plasmid A8 (12×10^6 daltons). Thus, plasmid A8 probably can replicate autonomously (see above). However, pBR325 sequences associated with genome-size molecules were not found, and we conclude that autonomous cleavage-encapsidation of plasmid A8 had not occurred and that the plasmid sequences had recombined with helper PrV DNA before encapsidation.

In the case of plasmid C137 (another class II plasmid), no evidence for autonomous replication (or autonomous encapsidation) was obtained. pBR325 sequences were associated with fragments whose size did not exceed the sum of the sizes of the largest *Xho*I PrV fragment and linear plasmid C137 DNA (11×10^6 daltons). It is possible, therefore, that this plasmid was amplified only after its recombination into the helper viral genome. Analysis of other class II plasmids was not possible because we could not identify restriction enzymes that did not cleave the plasmid but cleaved the DNA of PrV frequently.

A similar analysis of the DNA present in populations of virions obtained from cells cotransfected with helper PrV DNA and some class III or class IV plasmids did not produce evidence for autonomous replication and encapsidation or autonomous replication of the plasmids.

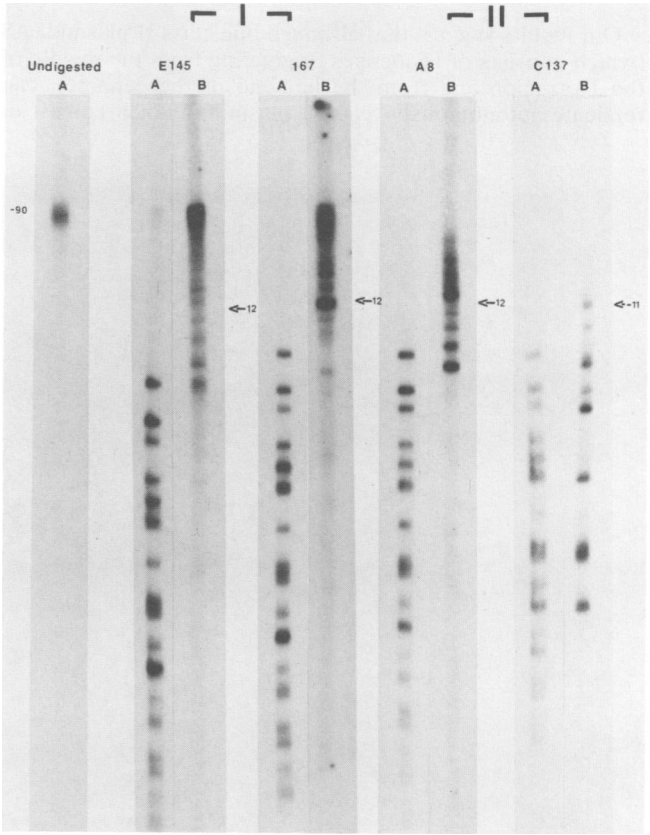

FIG. 5. Class I plasmids replicate and are encapsidated autonomously. The virion DNA obtained as described in the legend to Fig. 4 was digested with *Xho*I (an enzyme that does not cleave the plasmid but cleaves PrV DNA) and electrophoresed into agarose gels. The DNA was transferred to nitrocellulose paper by the method of Southern (30), and filter strips were probed with PrV DNA (A) or pBR325 DNA (B). Also illustrated is the position of genome-size DNA (ca. 90×10^6 daltons), as indicated in the lane marked "Undigested." The arrows indicate the position and size (in megadaltons) of a linear fragment the size of the sum of the sizes of the linear "seed" plasmid and the largest *Xho*I fragment.

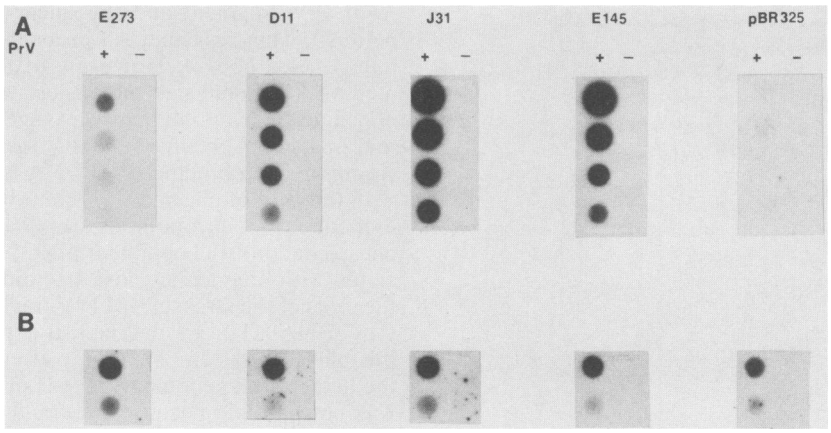

FIG. 6. Amplification of pBR325 sequences in cells cotransfected with PrV DNA and plasmids containing the end fragments of the PrV genome or the junction between these end fragments. RK cells were cotransfected with the indicated plasmid and helper PrV DNA (+) or transfected with the plasmid alone (−). Three days after transfection, intracellular DNA was purified, denatured, serially diluted, and blotted onto nitrocellulose filters. These dot-blots were probed with either (A) nick-translated pBR325 DNA (exposure time, 5 days) or (B) PrV DNA (exposure time, 16 h). The plasmids contained the following inserts: plasmid E273, *Bam*HI fragment 13; plasmid D11, fragment 14′; plasmid J31, the *Bam*HI junction fragment of *Bam*HI fragments 13 and 14′ obtained from concatemeric DNA; plasmid E145, a class I defective DNA fragment.

Our results suggest that although one class II plasmid, A8 (which consists of sequences originating from the middle of the U_L region and from the left end of the genome), can replicate autonomously, class I plasmids (which consist of sequences originating from the middle of the U_L region and from both ends of the PrV genome) can both replicate and be encapsidated autonomously. This finding suggested that recognition signals present on both ends of the PrV

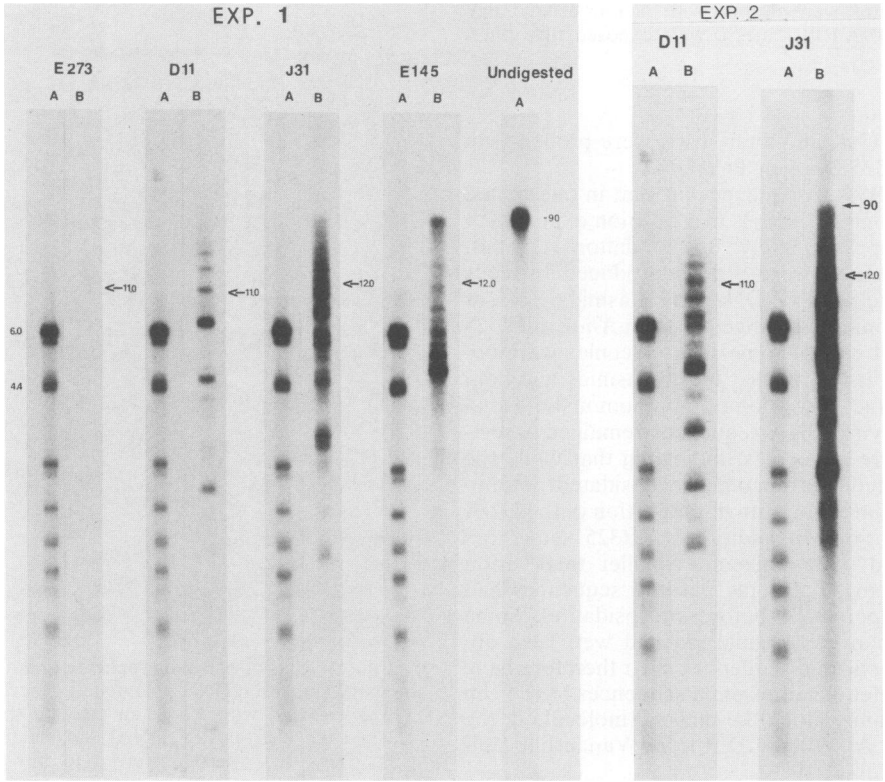

FIG. 7. Replication and autonomous encapsidation of the junction fragment between the two end fragments of the PrV genome. The virion DNA obtained from two different experiments, performed as described in the legend to Fig. 6, was digested with *Sst*I (an enzyme that fails to cleave the plasmids but cleaves PrV DNA), electrophoresed on agarose, and transferred to nitrocellulose filters. Strips were hybridized to nick-translated PrV DNA (A) or pBR325 (B). The arrows indicate the position of the sum of the largest PrV *Sst*I fragment and the linear plasmids.

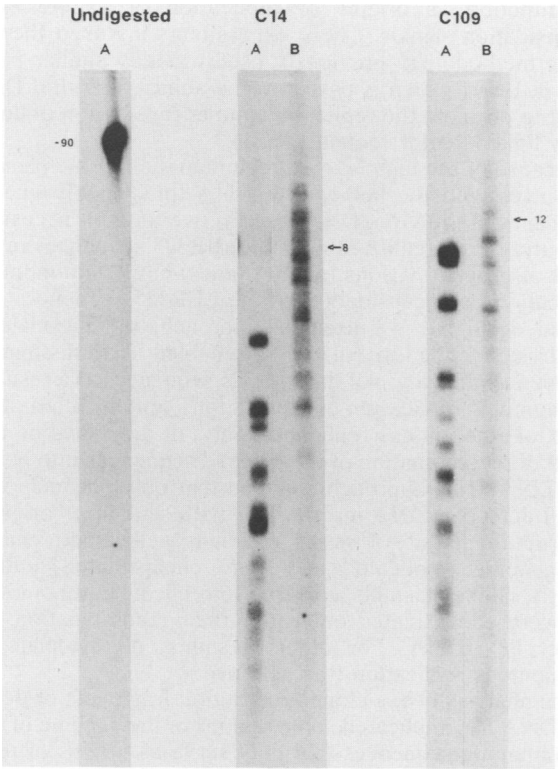

FIG. 8. Sequences in *Bam*HI fragment 13 are necessary but not sufficient for efficient cleavage-encapsidation. RK cells were cotransfected with PrV DNA and plasmid C14 (containing *Bam*HI fragment 15, which includes an origin of replication) or plasmid C109 (containing *Bam*HI fragment 15 linked to *Bam*HI fragment 13). The virions produced by the cells were purified, and the DNA was extracted, digested with *Xho*I and *Sst*I (in the case of C14) or *Sst*I only (in the case of C109), electrophoresed, and transferred to nitrocellulose filters. Strips were probed with nick-translated PrV DNA (A) or pBR325 DNA (B). The arrows indicate the position expected of a fragment the size of the sum of the largest PrV restriction fragment and the linear plasmid.

genome may be necessary for efficient cleavage-encapsidation.

Signals necessary for efficient cleavage-encapsidation are present on both ends of the standard PrV genome. Cloned DNA fragments derived from either end of the standard PrV genome (*Bam*HI fragments 13 [E273] and 14′ [D11] [see map in Fig. 1]), as well as the cloned junction fragment between these two ends (J31) obtained from intracellular concatemeric DNA, were tested for their ability to be amplified when cotransfected into RK cells with helper PrV DNA (Fig. 6).

In cells cotransfected with PrV DNA and plasmid E273 (containing *Bam*HI fragment 13), pBR325 sequences were amplified only to a minimal extent. In cells cotransfected with PrV DNA and plasmid D11 (containing *Bam*HI fragment 14′), amplification of the pBR325 sequences was much more pronounced. In cells cotransfected with plasmid J31 (containing the junction of both end fragments) and PrV DNA, pBR325 sequences were amplified as well as they were in cells cotransfected with plasmid E145 (which replicates and becomes encapsidated autonomously [Fig. 5]).

To determine whether the amplification of the plasmids was the result of autonomous replication, as well as autonomous cleavage-encapsidation, the virion DNA produced by the cells cotransfected with helper PrV DNA and each of the cloned end fragments or the junction fragment were digested with *Sst*I (an enzyme that does not cleave the plasmids being tested but does cleave PrV DNA). The digested DNA was electrophoresed, denatured, and transferred onto nitrocellulose paper by the Southern (30) technique, and filter strips were probed with nick-translated PrV DNA or pBR325 DNA. The results of two experiments are illustrated in Fig. 7.

As expected from the results of the dot-blots (Fig. 6), the virion DNA preparations obtained from cells cotransfected with plasmid E273 (*Bam*HI fragment 13) and helper PrV DNA contained few, if any, pBR325 sequences. In contrast, virions obtained from cells cotransfected with plasmid D11 (*Bam*HI fragment 14′) yielded, after digestion with *Sst*I, a ladderlike array of bands containing pBR325 sequences, some of which were larger than the sum of the largest *Sst*I PrV fragment and the linear plasmid D11 (11×10^6 daltons). This indicates that plasmid D11 replicated autonomously, i.e., that *Bam*HI fragment 14′ contains an origin of replication. However, no detectable pBR325 sequences remained associated with genome-size DNA after digestion with *Sst*I. Therefore, *Bam*HI fragment 14′ (the left end of the genome) does not appear to include all the signals necessary for efficient autonomous cleavage-encapsidation.

DNA from virions produced by cells cotransfected with helper PrV DNA and plasmid J31 (the junction fragment of the two *Bam*HI end fragments 13 and 14′) yielded, after digestion with *Sst*I, some genome-size DNA molecules containing pBR325 sequences. These results indicate that the junction fragment contains an origin of replication (probably within the sequences of *Bam*HI fragment 14′; see above). These results also show that the sequences in *Bam*HI fragment 13 include signals which promote efficient cleavage-encapsidation.

The results described above thus show (i) that *Bam*HI fragment 14′ contains an origin of replication; (ii) that *Bam*HI fragment 13 includes signals that promote cleavage-encapsidation; and (iii) that sequences present in both end fragments of the PrV genome are required for autonomous replication as well as efficient encapsidation of the plasmid. These results, however, do not establish whether *Bam*HI fragment 14′ contains only an origin of replication or whether it also contains signals necessary for cleavage-encapsidation.

To determine whether *Bam*HI fragment 14′ includes signals necessary for cleavage-encapsidation, we constructed a plasmid in which *Bam*HI fragment 13 (which does not include an origin of replication but which does promote packaging [see above]) was linked to *Bam*HI fragment 15, a fragment that originates from the middle of the U_L region (see Fig. 1) and that contains an origin of replication (unpublished results). The resulting plasmid (C109) was tested for its ability to replicate and to be encapsidated autonomously, the rationale being that if the sequences in *Bam*HI fragment 13 were sufficient for efficient encapsidation, plasmid C109 should replicate and be encapsidated autonomously.

The DNA of virions produced by cells cotransfected with plasmid C109 (containing *Bam*HI fragments 13 and 15) or plasmid C14 (containing *Bam*HI fragment 15) and helper PrV DNA was digested with restriction enzymes that do not cleave the plasmids but do cleave PrV DNA. The digested DNA was electrophoresed, denatured, and transferred to nitrocellulose paper, and the filter strips were probed with nick-translated PrV DNA or pBR325 DNA.

Figure 8 shows that the DNA in virions produced by cells

TABLE 1. Summary of the experiments dealing with *cis*-functions necessary for replication and for efficient cleavage-encapsidation[a]

Plasmid	*Bam*HI fragment	Replication	Replication-encapsidation
E273	13	−	−
D11	14′	+	−
J31	13 + 14′	+	+
C14	15	+	−
C109	13 + 15	+	−

[a] The results illustrated in Fig. 7 and 8 are summarized in this Table. See restriction map in Fig. 1 for location of *Bam*HI restriction fragments on the PrV genome.

cotransfected with helper PrV DNA and plasmid C14 (*Bam*HI fragment 15) generated, after double digestion with *Xho*I and *Sst*I (both of which do not cleave the plasmid but do cleave PrV DNA), an array of bands containing pBR325 sequences larger than the sum of the largest PrV DNA fragment and the linearized plasmid. These results show that *Bam*HI fragment 15 does indeed contain an origin of replication (see Fig. 3 for rationale). As expected, the sequences in *Bam*HI fragment 15 do not, however, include cleavage-encapsidation signals, since after digestion with the enzymes, the pBR325 sequences did not remain associated with genome size molecules.

Similarly, virion DNA produced by cells cotransfected with plasmid C109 (which includes the sequences in *Bam*HI fragments 13 and 15) also generated an array of bands containing pBR325 sequences, but, again, no pBR325 sequences remained associated with genome-size molecules after digestion with the restriction enzymes. Thus, even though *Bam*HI fragment 15 includes an origin of replication and fragment 13 includes signals required for cleavage-encapsidation (see above), plasmid C109 was not encapsidated autonomously. Consequently, the sequences in *Bam*HI end fragment 13, although necessary, are not sufficient to allow efficient cleavage-encapsidation. Because the plasmid, which includes *Bam*HI fragments 13 and 14′, promotes autonomous encapsidation of the covalently linked pBR325 sequences, whereas the plasmid, which includes *Bam*HI fragments 13 and 15, does not (despite the fact that both *Bam*HI fragments 14′ and 15 include origins of replication), we conclude that sequences that are present on both ends of the genome, i.e., on both *Bam*HI fragments 14′ and 13, are necessary for efficient recognition by the proteins responsible for cleavage-encapsidation.

Table 1 summarizes the results of the experiments described above. The data show that sequences present near or at both ends of the PrV genome are necessary for efficient cleavage-encapsidation of the PrV DNA.

DISCUSSION

To study the possible *cis* functions for which the DNA of PrV DIPs is enriched, we cloned the *Bam*HI fragments obtained from this DNA. We found that the cloned defective DNA fragments are heterogeneous and that many consist of covalently linked sequences originating from different regions of the genome. These findings confirm our previous conclusions regarding the structure of the genomes of the DIPs (25).

We analyzed the cloned *Bam*HI fragments of the defective DNA (as well as some *Bam*HI DNA fragments derived from the standard PrV genome) for the presence of sequences that can function as origins of replication or as cleavage-encapsidation signals. These experiments involved the use of the methodology previously used to study similar problems in HSV, which is based on the ability of a viral DNA insert to promote the replication and encapsidation of covalently linked vector sequences (31).

Because of the high level of recombination of the plasmid sequences with the helper PrV DNA (presumably via the sequences of the virus DNA inserts), we found it necessary to analyze the organization of the pBR325 sequences in the DNA of progeny virions to determine whether autonomous replication or encapsidation of the pBR325 sequences had indeed occurred. We used an approach, the rationale of which is described in Fig. 3, that enabled us to distinguish between amplification of the pBR325 sequences as a result of autonomous replication-encapsidation, amplification as a result of autonomous replication only, or amplification as a result of recombination of the plasmid sequences into helper PrV DNA. This approach revealed that only plasmids with type I defective DNA inserts, i.e., with sequences originating from the middle of the U_L region as well as both ends of the genome, replicated and were encapsidated autonomously. Some plasmids appeared to replicate autonomously but were encapsidated only after their recombination into helper PrV DNA. For other plasmids, no evidence for autonomous replication was obtained.

The analysis of the cloned restriction fragments of defective DNA has implicated some regions of the genome of PrV as containing sequences that may serve as origins of replication; it also indicated that both ends of the genome may contain signals required for efficient cleavage-encapsidation. We confirmed these findings by using cloned restriction fragments of standard PrV DNA as well as cloned constructs in which different regions of interest of the standard viral genome were linked.

We showed previously by electron microscopy and restriction enzyme analyses that replication of PrV DNA during the first round(s) of replication can be initiated at at least two different locations on the genome, one within or near the inverted repeats and a second in the middle of the U_L region (7, 14). The results of the experiments in which we used cloned restriction fragments of standard PrV DNA described here showed that *Bam*HI fragment 15 (approximate map units 0.455 through 0.460), which originates from the middle of the U_L region, indeed includes an origin of replication. Furthermore, we located another hitherto unsuspected origin of replication within *Bam*HI fragment 14′ (map units 0.000 through 0.009).

The analyses of the cloned defective DNA fragments, as well as of the plasmids containing defined segments of standard DNA, showed that both ends of the genome include sequences that promote efficient cleavage-encapsidation. The finding that cleavage-encapsidation recognition signals may be present on both ends of the genome is not novel. A similar situation exists, for example, in bacteriophage lambda. It has been demonstrated that sequences located on both ends of the lambda genome are necessary for the orderly cleavage of concatemeric DNA (10, 11, 13, 22, 23). Indeed, the replication and encapsidation of the PrV genome (and that of HSV as well) share several features with those of bacteriophage lambda. The incoming parental genomes of both PrV and lambda form circles, and the first round(s) of replication occurs on theta-like structures (14, 27, 36). At later stages of DNA replication, viral DNA is in the form of concatemers in head-to-tail alignment; these structures are precursors to mature linear DNA (4, 5, 20, 28, 29). In both

viruses, the generation of genome-size molecules from concatemeric DNA involves site-specific cleavage, generating the unique ends of the genome, and both cleavage and packaging of the DNA are closely coupled processes (18, 21, 40). Finally, as shown by the results of the experiments described in this paper, cleavage-encapsidation of PrV concatemeric DNA requires signals that are present on both ends of the PrV genome, a situation that is similar to that previously described for bacteriophage lambda.

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