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Nucleotides 1506–1625 of Bovine Papillomavirus Type 1 Genome Can Enhance DNA Packaging by L1/L2 Capsids

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We have previously described a DNA-packaging assay using bovine papillomavirus type 1 (BPV-1) virus-like particles (VLPs) and have identified a region of the BPV genome that assists in packaging. In this study, we identify a specific BPV sequence involved in DNA packaging by BPV-1 VLPs. In the initial screening of BPV-1 genomic sequences essential for DNA packaging, we observed that a plasmid with deletions between nucleotides (nt) 948 and 2113 failed to be packaged into BPV-1 VLPs. However, plasmids containing nt 948 to 2113 were efficiently packaged, suggesting that this 1.2-kb fragment contains a packaging enhancement sequence (PES). Further mapping of the BPV-1 genome showed that this packaging sequence lies between nt 1506 and 1625. Furthermore, this packaging sequence is also recognized by HPV6b VLPs, suggesting that a common packaging mechanism may be used by the two papillomavirus types. Given the phylogenetic difference between these two viral types, it is likely that other papillomavirus types may also use the same packaging mechanism. Identification of the PES has allowed a minimal viral genome sequence to be used in the packaging assay, improving the usefulness of the assay in studying the process of papillomavirus DNA encapsidation.

INTRODUCTION

Papillomavirus (PV) is a small DNA virus that causes a variety of diseases, including some human cancers. The virus does not replicate readily in conventional cultured cells, which makes the study of viral replication difficult. We have developed a novel system for studying papillomavirus DNA encapsidation (Zhao *et al.,* 1998), a major late event in the viral life cycle. This *in vitro* system has allowed us to systematically examine the requirements and conditions for viral DNA encapsidation, studying it in isolation from the other events in the viral replicative cycle, such as cell infection, gene expression, and DNA replication. Our data have indicated that a specific PV DNA sequence is required for plasmid packaging (Zhao *et al.,* 1998). However, this sequence has not yet been determined. A specific region of the viral genome required for DNA or RNA packaging has been reported for a number of viruses. For example in SV40, a 200-bp DNA fragment contains a signal for DNA encapsidation (Oppenheim *et al.,* 1992). In herpesvirus, an AT-rich sequence is essential for cleavage and packaging of DNA (McVoy *et al.,* 1998), and sequences in *pol* are required for the transfer of human foamy virus-based vectors (Erlwein *et al.,* 1998). In papillomaviruses, DNA encapsidation by VLPs at low efficiency has been reported by several groups (Zhou *et al.,* 1993; Roden *et al.,* 1994, 1995, 1996; Unckell *et al.,* 1997; Zhao *et al.,* 1998; Stauffer *et al.,* 1998). DNA encapsidation by PV VLPs *in vitro* has also been studied by Touze and Coursaget (1998) and Kawana *et al.* (1998), with most of these studies confirming that L2 plays a key role in DNA encapsidation or infection (Zhou *et al.,* 1993; Roden *et al.,* 1994, 1995, 1996; Zhao *et al.,* 1998; Stauffer *et al.,* 1998; Kawana *et al.,* 1998). Recently, data reported by Day *et al.* (1998) suggest a role for a nonstructural viral protein, E2, in virion assembly, specifically the recruitment of the viral genome to sites of assembly, through a high-affinity interaction between E2 and specific viral DNA sequences. To date, however, no study focusing on sequences required for specific encapsidation of papillomavirus genomic DNA has been reported. We have, therefore, carried out a series of experiments to define a sequence of the BPV genome involved in DNA packaging.

RESULTS

Fragment 948–2113 of BPV-1 contains a DNA packaging signal

Using a plasmid containing SV40 *ori,* ColE1 *ori,* and the early regions of the BPV-1 genome as a target for packaging, we have established a system for packaging DNA into BPV-1 L1+L2 VLPs, generated with a recombinant vaccinia virus (Zhao *et al.,* 1998). The SV40 *ori* and ColE1 sequences allow the plasmid to replicate efficiently in large T antigen-expressing Cos-1 cells and in bacteria, respectively. This indicated that packaging by BPV-1 VLPs is more efficient if there is PV DNA within the packaged plasmid and demonstrated the size con-

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straints on the packaged plasmid. In this study, we have extended our investigations to define PV sequences involved in DNA packaging into papillomavirus VLPs. Plasmid pSV BPV A was used as a parent plasmid to construct a series of plasmids to map the BPV-1 sequence necessary for DNA packaging (Fig. 1, II). Using available restriction sites within the BPV genome of pSV BPV A, three fragments of 1.0 kb (nt 3460–4450), 2.4 kb (nt 2114–4450), and 3.5 kb (nt 948–4450) were separately deleted. Each of the deleted BPV-1 sequences was replaced by a similar size DNA sequence derived from the β-gal gene to make the plasmids about 8 kb in length (Fig. 1, II).

These three plasmids, together with pSV BPV A as a positive control, were tested for their replication efficiency in Cos-1 cells and packaging into VLPs. Southern blot analysis showed that the four plasmids replicated at similar rates in Cos-1 cells (Fig. 2, inset). When the packaging efficiencies of these four plasmids by BPV-1 VLPs were compared in three separate experiments, we found that plasmids with a deletion of the 1.0-kb fragment from nt 3460 to 4450 and of the 2.4-kb fragment from nt 2114 to 4450 produced similar numbers of bacterial colonies, comparable to those produced with the positive control plasmid pSV BPV A (data not shown). However, deletion of 3.5 kb from nt 948–4450 resulted in a 10-fold reduction in heavy VLPs (VLPs containing mostly full-size plasmids) and a 25-fold reduction in light VLPs (VLPs containing mostly truncated plasmids) (Fig. 2). These results suggested that the 1.2-kb fragment of the BPV-1 genome (from nt 945 to 2113) contained the sequence for DNA packaging into VLPs.

We also measured both the total amount of VLPs and the amounts of VLPs that contained plasmids in the heavy (Fig. 1, fraction 4) and light (Fig. 1, fraction 8) fractions. The L1 protein of nonfractionated VLP preparations and that of the heavy and light VLP preparations were quantified by immunoblotting and densitometric analysis, with defined quantities of purified L1 protein serving as the standard. A total of $(3.18 \pm 1.21) \times 10^7$ VLPs were purified from 1×10^6 Cos-1 cells. The amounts of VLPs that contained plasmids were calculated from the transformation assay using DNA prepared from VLP fractions. It is assumed that one packaged VLP contains one plasmid copy that can be either full-size or truncated and which can form one colony after the plasmid is purified and used to transform *Escherichia coli*. The efficiency of transformation was determined using a standard plasmid. About 3.9% $[(1.24 \pm 0.24) \times 10^6]$ of pooled VLPs (heavy and light) contained plasmid DNA, with 1.4% $[(4.43 \pm 0.55) \times 10^5]$ of heavy particles containing plasmid when pSV BPV A was used for packaging. But only 0.3% $[(1.1 \pm 0.04) \times 10^5]$ of pooled VLPs and 0.1% $[(4.06 \pm 1.52) \times 10^4]$ of heavy VLPs contained plasmid when pSV BPV D was used for packaging.

Packaging enhancing sequence is located in a 400-bp fragment (nt 1336–1725) of the BPV-1 genome

Based on the results from the initial sequence mapping experiments, we further investigated whether a shorter sequence within the 1.2-kb fragment of the BPV-1 genome contains a signal for DNA packaging into VLPs. To this end, we used five pairs of primers (Table 1) to produce two 800-bp and three 400-bp BPV fragments by PCR, covering the whole 1.2-kb fragment identified above. These fragments were inserted into pSV BPV D at the *Eco*RI site to produce a new series of plasmids (Fig. 1, III). These newly constructed plasmids, together with pSV BPV A as a positive control and pSV BPV D as a negative control, were further tested for their replication efficiency in Cos cells and their packaging into VLPs. Again, Southern blot analysis indicated that all five plasmids replicated equally well in Cos cells (Fig. 3, inset). At this time, only the VLP suspension from Fraction 4 was used for DNA preparation and analysis of packaging efficiencies. When the BPV-1 VLP packaging efficiencies of the seven plasmids were compared in six separate experiments, we found that two plasmids containing the 800-bp sequence from nt 948 to 1725, or from nt 1336 to 2113, produced similar numbers of plasmid-containing VLPs (data not shown), comparable to those produced by the positive control plasmid pSV BPV A. The negative control plasmid produced only a tenth as many plasmid-containing VLPs, indicating that both 800-bp regions of the BPV-1 genome contained the DNA packaging enhancing sequence (data not shown).

However, the three plasmids containing a 400-bp sequence from nt 1725 to 2113, nt 1336 to 1725, or nt 948 to 1335 showed different packaging efficiencies (Fig. 3), although they replicated equally well in Cos cells (Fig. 3, inset). Plasmid with the addition of the 400-bp sequence from nt 1725 to 2113 could not be packaged into VLPs, indicating that this region does not contain a DNA packaging enhancing sequence. Although the plasmid with the 400-bp sequence from nt 948 to 1335 showed improved packaging efficiency (three times higher than that of the negative control), the packaging efficiency was below that achieved by the positive control group, indicating that the actual DNA packaging enhancing sequence may lie outside this fragment. Further testing of the plasmid containing a 400-bp sequence from nt 1336 to 1725 revealed that it contained a packaging sequence as it produced plasmid-containing VLPs at a similar efficiency as the positive control plasmid (Fig. 3). These results suggested that the BPV-1 fragment from nt 1336 to 1725 enhances DNA packaging into BPV-1 VLPs.

The 120-bp (nt 1506–1625) fragment of BPV-1 enhances DNA packaging

To determine whether the BPV sequence that enhances DNA packaging is much shorter than the 400-bp

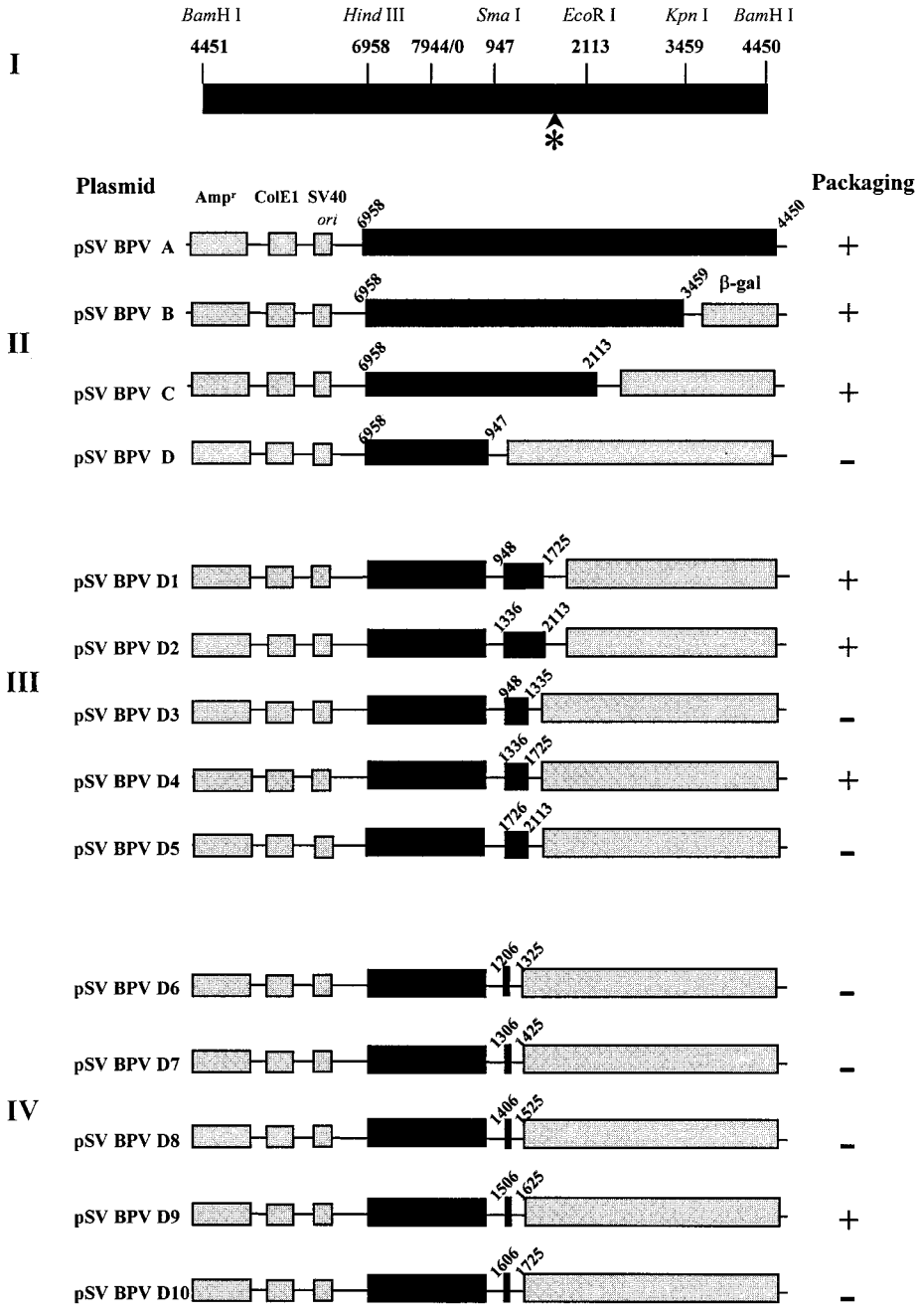

FIG. 1. Plasmids used in the packaging assay. (I) BPV sequence. The enzyme sites are the clone sites used to construct the different plasmids. The asterisk with an arrow indicates the position of a DNA sequence that assists in packaging. (II) Four plasmids used for initial mapping of BPV sequences essential for DNA packaging into VLPs. pSV BPV A, without L1/L2, was used as a parent plasmid to construct the three other plasmids. pSV BPV B resulted from pSV BPV A with a deletion of nt 3460 to 4450, replaced with 1 kb of the β-gal gene. pSV BPV C resulted from pSV BPV A with a deletion of nt 2114 to 4450, replaced with 2.4 kb of the β-gal gene. pSV BPV D resulted from pSV BPV A with a deletion of nt 948 to 4450, replaced with 3.4 kb of the β-gal gene. (III) Five plasmids, pSV BPV D1, pSV BPV D2, pSV BPV D3, pSV BPV D4, and pSV BPV D5, used for further mapping of BPV sequence essential for DNA packaging into VLPs. pSV BPV D was used to construct the five plasmids with an insertion of about 800 or 400 bp produced by PCR of the BPV genome within a 1.2-kb sequence from nt 948 to 2113. Each fragment was digested with *Eco*RI and cloned into pSV BPV D. 2.6 kb of the β-gal gene was cloned into pSV BPV D1 and pSV BPV D2 with the addition of an approximately 800-bp BPV fragment at the *Hin*dIII site. 3.0 kb of the β-gal gene was cloned into pSV BPV D3, pSV BPV D4, and pSV BPV D5 with the addition of an approximately 400-bp BPV fragment at the *Hin*dIII site. (IV) Five plasmids, pSV BPV D6, pSV BPV D7, pSV BPV D8, pSV BPV D9, and pSV BPV D10, used for fine-mapping of the BPV sequence essential for DNA packaging into VLPs. pSV BPV D was again used to construct these five plasmids with insertion of a 120-bp fragment produced by PCR of BPV genome within a 500-bp sequence from nt 1206 to 1725. Each fragment was digested with *Eco*RI and cloned into pSV BPV D. 3.2 kb of the β-gal gene was separately cloned into these plasmids.

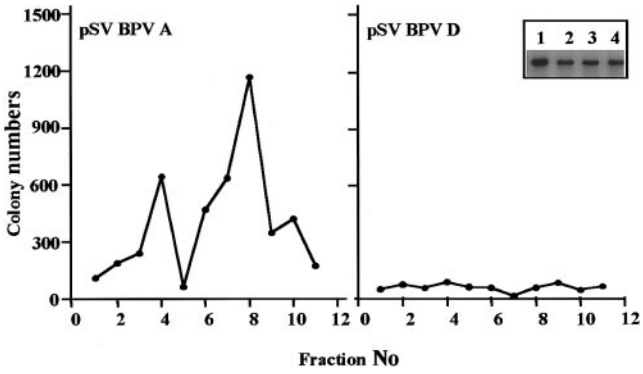

FIG. 2. Packaging of pSV BPV A and pSV BPV D by VLPs, measured as the number of *E. coli* colonies produced by DNA extracted from VLP fractions in a CsCl gradient, as described under Materials and Methods. Cos-1 cells were separately transfected with pSV BPV A and pSV BPV D and infected with rVV BPV-1 L1/L2. Data points are the average of three separate experiments. (Inset) DNA replication of four plasmids transfected into Cos-1 cells was detected by Southern blot analysis: (1) pSV BPV A, (2) pSV BPV B, (3) pSV BPV C, and (4) pSV BPV D.

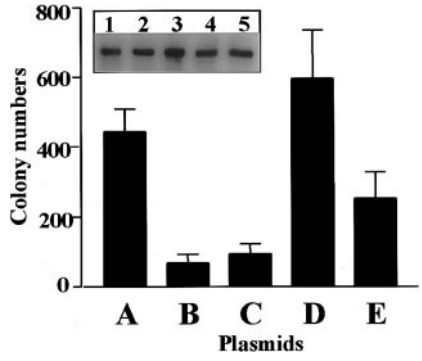

FIG. 3. Packaging of five plasmids by VLPs, measured as the number of *E. coli* colonies produced by DNA extracted from VLP suspensions, as described under Materials and Methods. Cos-1 cells were separately transfected with five plasmids and infected with rVV BPV-1 L1/L2. Data are the average of six separate experiments. (A) pSV BPV A was used as a positive control; (B) pSV BPV D was used as a negative control; (C) pSV BPV D5; (D) pSV BPV D4; (E) pSV BPV D3. (Inset) DNA replication of five plasmids transfected in Cos-1 cells was detected by Southern blot analysis: (1) pSV BPV A, (2) pSV BPV D, (3) pSV BPV D3, (4) pSV BPV D4, and (5) pSV BPV D5.

fragment between nt 1336 and 1725, we conducted a fine-mapping study. We used 10 primers (Table 1) to produce five fragments by PCR that covered the BPV sequence nt 1206 to 1725. Each fragment contained 120 bp, overlapping its neighbors by 20 bp. These five fragments were digested with *Eco*RI and separately cloned into plasmid pSV BPV D to produce five new plasmids (Fig. 1, IV). To ensure that these five plasmids have a size similar to that of the positive control plasmid pSV BPV A, a 3.2-kb fragment of the β-gal gene was cloned into pSV BPV D at the *Hin*dIII site (Fig. 1, IV). The five plasmids were further tested in the packaging assay, along with pSV BPV A and pSV BPV D4 as two positive controls and pSV BPV D as a negative control. Southern blot analysis showed no difference in replication efficiency among these plasmids in Cos cells (Fig. 4, inset). Again, the VLP suspension from Fraction 4 was used for DNA preparation and the packaging assay. Packaging assays clearly indicated that four plasmids containing a BPV-1 fragment from nt 1206 to 1325, 1306 to 1425, 1406 to 1525, or 1606 to 1725 were not packaged into VLPs (Fig. 4, I), although Western blotting indicated that BPV L1 capsid protein was expressed equally in Cos-1 cells (Fig. 4, II). How-

TABLE 1

Oligonucleotide Primers Used for Amplification of BPV Fragments by PCR

No.	Fragment	Size (bp)	Oligonucleotide primers
1	948–1725	777	5′-CGG AAT TCG GGG CAG GTG TAG AAC TGT CTG
			3′-CGG AAT TCG TAA AGC ACC ATG TTT AAG TGT AGC
2	1336–2113	777	5′-CGG AAT TCA GCT GGG GCT CTT TAA ATC
			3′-CGG AAT TCC CTT TTA GCC AGA GCT TTA AAG
3	948–1335	387	5′-CGG AAT TCG GGG CAG GTG TAG AAC TGT CTG
			3′-CGG AAT TCT AAA AAC TGT AGC ATT TTT AGA
4	1336–1725	389	5′-CGG AAT TCA GCT GGG GCT CTT TAA ATC
			3′-CGG AAT TCG TAA AGC ACC ATG TTT AAG TGT AGC
5	1726–2113	387	5′-CGG AAT TCC TGA GTG GAT ACG GGC GCA AAC
			3′-CGG AAT TCC CTT TTA GCC AGA GCT TTA AAG
6	1206–1325	120	5′-CCG GAA TTC AAG CTT GAA GCT AAC CGT GTT CTT ACG CCC
			3′-CCG GAA TTC AGC ATT TTT AGA TTT AAC AAG CTG
7	1306–1425	120	5′-CCG GAA TTC AAG CTT TTG TTA AAT CTA AAA ATG CTA CAG
			3′-CCG GAA TTC CCC ATT GCT GAT TAG TGG TCT
8	1406–1525	120	5′-CCG GAA TTC AAG CTT GAC CAC TAA TCA GCA ATG GGT GCT GGC
			3′-CCG GAA TTC TCA TGA GAT CTT TTT TGC ATC TGC ATC TGC
9	1506–1625	120	5′-CCG GAA TTC AAG CTT ATG CAA AAA AGA TCT CAT GAA GGA GGA
			3′-CCG GAA TTC CTC TTC TCT TAC ATT TAG CGT GTT TGC
10	1606–1725	120	5′-CCG GAA TTC AAG CTT CGC TAA ATG TAA GAG AAG AGT GTT
			3′-CCG GAA TTC GTA AAG CAC CAT GTT TAA GTG TAG CGG

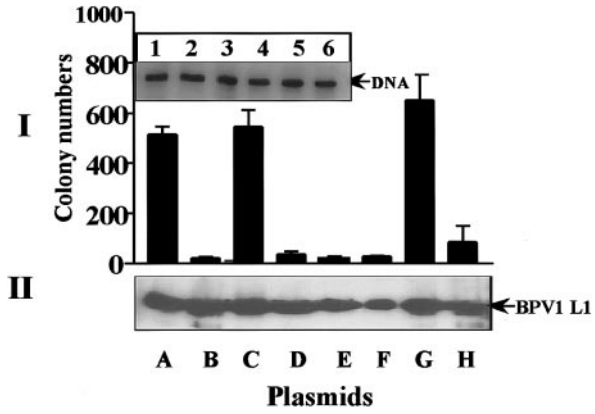

FIG. 4. Packaging assay of (I) eight plasmids by VLPs, measured as the number of *E. coli* colonies produced by DNA extracted from VLP suspensions, and Western blotting assay; (II) VLP suspensions, as described under Materials and Methods. Cos-1 cells were separately transfected with eight plasmids and infected with rVV BPV-1 L1/L2. Data for the packaging assay are the average of six experiments. (A) pSV BPV A and (C) pSV BPV D4 were used as two positive controls; (B) pSV BPV D was used as a negative control; (D) pSV BPV D6; (E) pSV BPV D7; (F) pSV BPV D8; (G) pSV BPV D9; and (H) pSV BPV D10. (Inset) DNA replication of six plasmids transfected into Cos-1 cells was detected by Southern blot analysis: (1) pSV BPV A, (2) pSV BPV D6, (3) pSV BPV D7, (4) pSV BPV D8, (5) pSV BPV D9, and (6) pSV BPV D10.

ever, the plasmid containing nt 1506–1625 of the BPV-1 genome was packaged into VLPs, as were the two positive control plasmids (Fig. 4, I). These results suggested that a 120-bp (nt 1506–1625) fragment of BPV is important for DNA packaging.

BPV sequence containing the DNA packaging sequence is recognized by HPV 6b L1 and L2 capsid proteins

To examine whether the BPV-1 packaging signal can be recognized by other PV VLPs, we tested whether pSV BPV D4 could be packaged by HPV6b VLPs. Plasmid pcDNA6bE1 was constructed for the packaging assay (Fig. 5, I): it contains nt 715–2778 from HPV 6b, which probably includes a DNA packaging sequence. Plasmids pSV BPV A and pSV BPV D were again used as positive and negative controls, respectively. An immunoblotting assay indicated that HPV6b L1 protein was expressed in Cos-1 cells (Fig. 5, III). Packaging assays showed that pSV BPV D was not packaged into HPV6b VLPs (Fig. 5, II), whereas pcDNA6bE1 was efficiently packaged, indicating that there is a DNA packaging sequence in 6bE1 corresponding to the packaging enhancing sequence present in pSV BPV A and pSV BPV D4, which were also packaged by HPV6b VLPs (Fig. 5, II).

DISCUSSION

In the present study, pSV BPV A, containing the entire BPV genome except for the L1 and L2 genes, was used as a positive control plasmid to examine DNA packaging sequences. We observed that deletions of sequences covering the E5, E4, E3, E2, and E8 gene regions did not affect DNA packaging efficiency by BPV VLPs, indicating that these regions are not involved in DNA packaging. The plasmid pSV BPV D, with a deletion of the E1 gene, failed to be effectively encapsidated, even though it contained the E6 and E7 gene regions and the long control region (LCR) sequences, suggesting that the E1 gene region is involved in BPV DNA packaging. This observation is also supported by the experiment using pcDNA6bE1.

Studies carried out by Touze and Coursaget (1998) and Kawana *et al.* (1998) have suggested that *in vitro* DNA encapsidation by HPV VLPs is independent of the HPV DNA sequence. However, they did not set out to com-

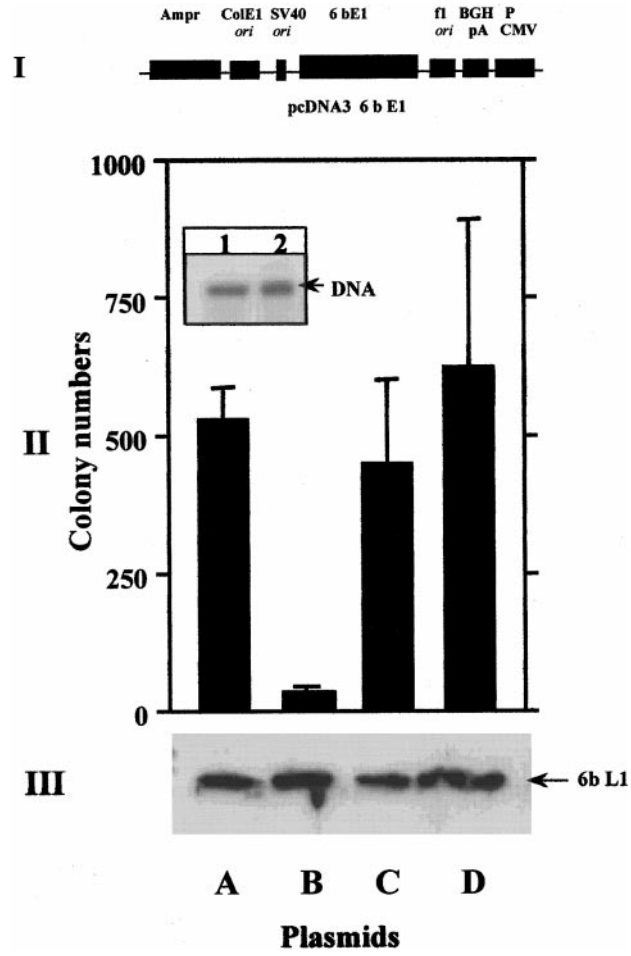

FIG. 5. (I) Plasmid pcDNA3 6b E1. (II) Packaging of four plasmids by HPV 6b VLPs, measured as the number of *E. coli* colonies produced by DNA extracted from VLP suspensions. (III) Western blotting assay of VLP suspensions, as described under Materials and Methods. Cos-1 cells were separately transfected with four plasmids and infected with rVV HPV 6b L1/L2. Data for the packaging assay are the average of four experiments. (A) pSV BPV A and (C) pSV BPV D4 were used as positive controls; (B) pSV BPV D was used as a negative control; (D) pcDNA3 6b E1. (Inset) DNA replication of two plasmids transfected into Cos-1 cells was detected by Southern blot analysis (1) pSV BPV A and (2) pcDNA3 6b E1.

pare plasmids with and without PV sequences. Also, the plasmids used in these studies were smaller than the 7.9-kb PV genome. Recently Stauffer *et al.* (1998) also concluded that DNA encapsidation by HPV 18 VLPs is independent of the HPV DNA sequence after comparing three plasmids, with one containing only the long control region of the viral genome, but again without using plasmids that contained the rest of the genome. In our previous study, we determined whether plasmids containing various PV sequence were packaged into VLPs (Zhao *et al.,* 1998). We have found that 8.2 times as many VLPs (1.34 g/ml) were produced when a plasmid containing 5.4 kb of BPV sequence was used than when plasmid pcDNA/purβ, which lacked the PV sequences, was used. In the present study, we observed that plasmid pSV BPV D, containing only the long control region of BPV, resulted in a 10-fold reduction in packaging efficiency, similar to that found with pcDNA/purβ. Results obtained by Stauffer *et al.* (1998) and by us in the present study indicate that the PV LCR does not contain a packaging signal, leading Stauffer *et al.* (1998) to conclude that DNA encapsidation by HPV 18 VLPs is independent of HPV DNA sequences. Furthermore, plasmids pSV BPV D4 and pSV BPV D9, which did not contain any intact ORFs, were efficiently packaged into BPV VLPs in the present study, indicating that no viral protein is acting *in trans* during the packaging. Based on the results obtained in the present study, together with our previous results (Zhao *et al.,* 1998), we can conclude that DNA encapsidation by BPV VLPs is enhanced by a specific DNA sequence in the papillomavirus genome and that a low percentage of VLPs can trap plasmid that does not contain PV sequences. We have called this 120-bp sequence a packaging enhancement sequence (PES). Since the focus of our work is the study of DNA packaging by BPV, the potential infectivity of VLPs that packaged the various plasmids was not determined.

It is not clear whether any element within the PES is conserved among other papillomavirus types. Previous studies have shown that various types of adenoviruses use different packaging sequences (Robinson and Tibbetts, 1984; Hearing *et al.,* 1987). In the present study, however, the sequence important for BPV DNA packaging was also recognized by HPV 6b VLPs. Considering the phylogenetic difference between these two papillomavirus types, it is likely that other papillomavirus types may use the same packaging mechanism. In addition, results obtained in the present study have shown that the LCR of the BPV genome alone did not increase packaging efficiency. However, it may be argued that there is a possibility that the LCR is necessary in combination with the 120-bp sequence to support efficient packaging because the LCR is present in the plasmid construct, and it has been observed in bacteriophage f6 that packaging segment S must be in combination with segment L to support DNA packaging (Onodera *et al.,* 1998). Based on the results obtained from the experiments using pcDNA 6b E1, this possibility may be ruled out because no LCR is present in this plasmid, which was efficiently packaged into HPV 6b VLPs.

Data on *cis* sequences of viral genomes required for DNA or RNA packaging into VLPs have been found in several viruses (Hearing *et al.,* 1987; Grable and Hearing, 1992; Oppenheim *et al.,* 1992; Zimmermann and Hammerschmidt, 1995; Kaye *et al.,* 1995; Schmid and Hearing, 1998; Heinkelein *et al.,* 1998). Adenovirus (Ad) packaging is dependent on the presence of a *cis*-acting sequence (nt 194–380) in the viral genome (Hearing *et al.,* 1987; Grable and Hearing, 1992; Schmid and Hearing, 1998). In SV40, a *cis*-acting DNA signal for encapsidation is present within a 200-bp DNA fragment (Oppenheim *et al.,* 1992). Zimmermann and Hammerschmidt (1995) reported that a *cis*-acting element is essential for cleavage of the DNA concatamers and encapsidation of the virion DNA in Epstein–Barr virus. Two *cis*-acting elements are recognition signals for encapsidation in HIV (McBride and Panganiban, 1996; McBride *et al.,* 1997) and in human cytomegalovirus (Bogner *et al.,* 1998). So far, there is no evidence whether any *cis*-acting sequence of BPV is involved in DNA packaging. An early study by Lusky and Botchan (1984) identified two *cis*-acting sequences termed PMS (plasmid maintenance sequences) in BPV, which function to maintain the genome as an episome in transformed and tumor cells. PMS-2 was mapped to a 140-bp segment between nt 1514 and 1654 within the E1 opening reading frame (Lusky and Botchan, 1984). In the present study, pSV BPV D9 contained the BPV PMS-2 analyzed by Lusky and Botchan and was efficiently packaged. However, a later publication from this group indicated the difficulty in repeating the experiment to define the BPV PMS sequences, which they concluded were probably artifacts (Yang *et al.,* 1991). Also, Nallaseth and DePamphilis (1994) reported that BPV contains *cis*-acting sequences that overlap the PES that we have identified and which suppress origins of DNA replication, but their effect on DNA packaging was not assayed. Further investigation is required to more precisely define the functions of this region.

The BPV PES is AT-rich (more than 60% A+T), similar to many other viral packaging signals, and the 120-bp size is similar to those reported in feline leukemia virus (FeLV), Epstein–Barr virus, and adenovirus. In FeLV, for example, a 107-nucleotide sequence comprises a packaging signal, and deletion of this sequence resulted in an approximately 200-fold reduction of packaged virions (Burns *et al.,* 1996). A sequence of 159-bp, including 11-bp elements, contains the signal essential for DNA packaging in Epstein–Barr virus (Zimmermann and Hammerschmidt, 1995), and the sequence nt 194–356 facilitates encapsidation in adenovirus type 5 (Hearing *et al.,* 1987). However, much shorter sequences required for DNA packaging have been reported for other viruses. For example, only 24 bp in the 3′ region of

gene 16 is a preferred packaging sequence in phage T4 (Lin and Black, 1998). Two 6-bp, AT-rich sequences required for specific DNA packaging have been identified in human cytomegalovirus (Bogner *et al.,* 1998). The 120-bp size of the PES of BPV represents the minimal size that can be defined using our approach. More precise mapping of specific elements within the PES will require using a series of short oligonucleotides rather than the enzyme-cleaved fragments used in this study. Moreover, this approach may be complicated by the existence of multiple elements within the PES that enhance packaging. Further studies of the mechanisms of papillomavirus DNA encapsidation using this assay are now under way in our laboratory.

MATERIALS AND METHODS

Recombinant vaccinia viruses

BPV-1 L1+L2 and HPV 6b L1+L2 recombinant vaccinia viruses (rVVs) were constructed as described previously (Zhou *et al.,* 1992). All the viruses were grown in CV-1 cells and plaque-purified three times.

Construction of recombinant plasmids for packaging

Plasmid pSV BPV-1 L1/L2 (referred to as pSV BPV A, Fig. 1, II), which contains the entire BPV-1 genome except for the L1 and L2 open reading frames, has been shown to contain sequences essential for DNA packaging by BPV capsids (Zhao *et al.,* 1998). This plasmid was used as a parent plasmid to construct a series of plasmids with deletions of BPV-1 sequences of up to 3.5 kb for the initial sequence mapping experiments (Fig. 1, II). A plasmid pSV BPV D containing a β-gal gene (Fig. 1, II), derived from pSV BPV A with a 3.5-kb deletion of BPV from nt 948 to 4450, was used as a template to construct another two series of plasmids for fine sequence mapping experiments. It was found that pSV BPV D does not contain a sequence enhancing DNA packaging in initial sequence mapping experiments. Eight pairs of oligonucleotide primers (Table 1) were used to amplify 10 fragments of the BPV genome covering nt 948 to 2113. These 10 fragments produced by PCR were digested with *Eco*RI and cloned into *Eco*RI-cut pSV BPV D to construct 10 plasmids. Three fragments of the β-gal gene, with sizes of 2.6, 3.0, and 3.2 kb, were individually cloned into these 10 plasmids at the *Hin*dIII site, giving plasmids of a size (about 8 kb) similar to that of the positive control plasmid pSV BPV A (Fig. 1, III and IV). All 10 plasmids were equally efficient at transforming *E. coli,* with 1 ng of plasmid DNA producing about 1000 colonies.

DNA transfection and VLP preparations

Cos-1 cells, grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), were brought up to 70% confluence in 150-cm^2 flasks. Cells were washed in OPTI-MEM medium (Gibco) and transfected with 10 μg of plasmid DNA using Lipofectamine (Gibco) according to the supplier's instructions. After transfection, cells were grown for 36 h in DMEM supplemented with 10% FBS. The transfected cells were then infected with rVVs and grown at 37°C for 24 h and finally at 22°C for a further 24 h before use. For comparison of DNA transfection and replication of different plasmids, cells were collected, pelleted by centrifugation, and resuspended in lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.2% Triton X-100). Plasmid DNA was prepared by the Hirt (1967) method and then digested with *Dpn*I and a single-cut enzyme, electrophoresed on a 1% agarose gel, blotted onto a membrane, and probed with ^{32}P-labeled DNA.

For preparation of VLPs, cells were pelleted by centrifugation and washed with phosphate-buffered 0.15 M sodium chloride (PBS), pH 7.4, containing 2 mM phenylmethylsulphonyl fluoride (PMSF). Cell pellets were resuspended in 5 ml of PBS containing PMSF and homogenized in a dounce homogenizer with tight-fitting pestle for 10 min on ice. The nuclei released were collected by centrifugation at 3000 rpm at 4°C for 15 min and resuspended in 10 ml of PBS with PMSF. This nuclear suspension was sonicated for 40 s on ice, and the lysates were laid over 20% sucrose/PBS and pelleted by centrifugation at 26,000 rpm for 2 h using a Beckman SW28 rotor. The VLP pellets were resuspended in 11.5 ml of PBS containing 5.5 g CsCl and centrifuged in a Beckman SW41 rotor at 40,000 rpm at 21°C for 20 h. Fractions were collected from the resulting gradient and were used for the analysis of encapsidated plasmid DNA.

Extraction and transformation of encapsidated plasmid DNA

To prepare the encapsidated DNA from VLP fractions, 100 μl of VLP suspension from each CsCl gradient fraction was dialyzed as described by Zhao *et al.* (1998). The dialyzed VLP suspensions were then added to 5 units of DNase and 5 units of RNase (Pharmacia) in 0.4 M Tris–HCl buffer containing 60 mM MgCl, to bring the mixture to 20 mM Tris–HCl and 3 mM MgCl at pH 7.0, and digested at 37°C for 30 min. Three hundred microliters of 2% sodium dodecyl sulfate (SDS) and 500 μl of phenol were added to each VLP suspension with gentle inversion. The VLP–SDS–phenol mixtures were held in a water bath at 65°C for 30 min. The aqueous phase containing DNA was extracted with phenol–chloroform and chloroform. DNA was precipitated with 50 μl of 3 M sodium acetate and 2 vol of 100% ethanol at −70°C for 2 h, pelleted by centrifugation at 14,000 rpm for 10 min, and resuspended in 20 μl of TE buffer. Two microliters of DNA was used to transform 40 μl of *E. coli* DH-5 α cells with a Gene Pulser (Bio-Rad). The transformed *E. coli* cells were spread on ampicillin-containing LB plates and incubated at 37°C for 16 h. Ampicillin-resistant colonies were counted to measure the efficiency with which plasmid DNA was packaged into VLPs.

Immunoblotting of L1 capsid protein in VLP suspensions

Three hundred microliters of nonfractionated VLP suspension was precipitated with 1.2 ml acetone at −70°C for 4 h, and the protein pellets were collected by centrifugation at 14,000 rpm for 15 min. These pellets were resuspended in 50 μl of 1× Laemmli buffer (Laemmli, 1970) and boiled for 5 min, and 10 μl of protein suspension was separated on a 10% (w/v) SDS–PAGE gel and electrotransferred onto nitrocellulose membranes (Bio-Rad). Blots were washed with PBS for 10 min and then blocked in PBS containing 5% nonfat milk for 1 h. A polyclonal anti-BPV1 L1 antibody (Dako) was used to probe the capsid proteins. The blots were then incubated with HRP-conjugated, anti-rabbit antibody (Silenus, Australia) at room temperature for 4 h and developed by enhanced chemiluminescence (ECL, Amersham, Australia).

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REFERENCES

Bogner, E., Radsak, K., and Stinski, M. F. (1998). The gene product of human cytomegalovirus open reading frame UL56 binds the *pac* motif and has specific nuclease activity. *J. Virol.* **72,** 2259–2264.

Burns, C. C., Moser, M., Banks, J., Alderete, J. P., and Overbaugh, J. (1996). Identification and deletion of sequences required for feline leukemia virus RNA packaging and construction of a high-titer feline leukemia virus packaging cell line. *Virology* **222,** 14–20.

Day, P. M., Roden, R. B., Lowy, D. R., and Schiller, J. T. (1998). The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J. Virol.* **72,** 142–150.

Erlwein, O., Bieniasz, P. D., and McClure, M. O. (1998). Sequences in *pol* are required for transfer of human foamy virus-based vectors. *J. Virol.* **72,** 5510–5516.

Grable, M., and Hearing, P. (1992). *cis* and *trans* requirements for the selective packaging of adenovirus type 5 DNA. *J. Virol.* **66,** 723–731.

Hearing, P., Samulski, R. J., Wishart, W. L., and Shenk, T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* **61,** 2555–2558.

Heinkelein, M., Schmidt, M., Fischer, N., Moebes, A., Lindemann, D., Enssle, J., and Rethwilm, A. (1998). Characterization of a *cis*-acting sequence in the *Pol* region required to transfer human foamy virus vectors. *J. Virol.* **72,** 6307–6314.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26,** 365–369.

Kawana, K., Yoshikawa, H., Taketani, Y., Yoshiike, K., and Kanda, T. (1998). *In vitro* construction of pseudovirions of human papillomavirus type 16: Incorporation of plasmid DNA into reassembled L1/L2 capsids. *J. Virol.* **72,** 10298–10304.

Kaye, J. F., Richardson, J. H., and Lever, A. M. (1995). *cis*-acting sequences involved in human immunodeficiency virus type 1 RNA packaging. *J. Virol.* **69,** 6588–6592.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lin, H., and Black, L. W. (1998). DNA requirements in vivo for phage T4 packaging. *Virology* **242,** 118–127.

Lusky, M., and Botchan, M. R. (1984). Characterization of the bovine papilloma virus plasmid maintenance sequences. *Cell* **36,** 391–401.

McBride, M. S., and Panganiban, A. T. (1996). The human immunodeficiency virus type 1 encapsidation site is a multipartite RNA element composed of functional hairpin structures. *J. Virol.* **70,** 2963–2973.

McBride, M. S., Schwartz, M. D., and Panganiban, A. T. (1997). Efficient encapsidation of human immunodeficiency virus type 1 vectors and further characterization of *cis* elements required for encapsidation. *J. Virol.* **71,** 4544–4554.

McVoy, M. A., Nixon, D. E., Adler, S. P., and Mocarski, E. S. (1998). Sequences within the herpesvirus-conserved *pac1* and *pac2* motifs are required for cleavage and packaging of the murine cytomegalovirus genome. *J. Virol.* **72,** 48–56.

Nallaseth, F. S., and DePamphilis, M. L. (1994). Papillomavirus contains *cis*-acting sequences that can suppress but not regulate origins of DNA replication. *J. Virol.* **68,** 3051–3064.

Onodera, S., Qiao, X., Qiao, J., and Mindich, L. (1998). Isolation of a mutant that changes genomic packaging specificity in phi6. *Virology* **252,** 438–442.

Oppenheim, A., Sandalon, Z., Peleg, A., Shaul, O., Nicolis, S., and Ottolenghi, S. (1992). A *cis*-acting DNA signal for encapsidation of simian virus 40. *J. Virol.* **66,** 5320–5328.

Robinson, C. C., and Tibbetts, C. (1984). Polar encapsidation of adenovirus DNA: Evolutionary variants reveal dispensable sequences near the left ends of Ad3 genomes. *Virology* **137,** 276–286.

Roden, R. B., Weissinger, E. M., Henderson, D. W., Booy, F., Kirnbauer, R., Mushinski, J. F., Lowy, D. R., and Schiller, J. T. (1994). Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. *J. Virol.* **68,** 7570–7574.

Roden, R. B., Hubbert, N. L., Kirnbauer, R., Breitburd, F., Lowy, D. R., and Schiller, J. T. (1995). Papillomavirus L1 capsids agglutinate mouse erythrocytes through a proteinaceous receptor. *J. Virol.* **69,** 5147–5151.

Roden, R. B., Greenstone, H. L., Kirnbauer, R., Booy, F. P., Jessie, J., Lowry, D. R., and Schiller, J. T. (1996). *In vitro* generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *J. Virol.* **70,** 5875–5883.

Schmid, S. I., and Hearing, P. (1998). Cellular components interact with adenovirus type 5 minimal DNA packaging domains. *J. Virol.* **72,** 6339–6347.

Stauffer, Y., Rai, K., Masternak, K., and Beard, P. (1998). Infectious human papillomavirus type 18 pseudovirions. *J. Mol. Biol.* **283,** 529–536.

Touze, A., and Coursaget, P. (1998). *In vitro* gene transfer using human papillomavirus-like particles. *Nucleic Acids Res.* **26,** 1317–1324.

Unckell, F., Streeck, R. E., and Sapp, M. (1997). Generation and neutralization of pseudovirions of human papillomavirus type 33. *J. Virol.* **71,** 2934–2939.

Yang, L., Mohr, I., Li, R., Nottoli, T., Sun, S., and Botchan, M. (1991). Transcription factor E2 regulates BPV-1 DNA replication in vitro by direct protein–protein interaction. *Cold Spring Harbor Symp. Quant. Biol.* **56,** 344.

Zhao, K.-N., Sun, X.-Y., Frazer, I. H., and Zhou, J. (1998). DNA packaging by L1 and L2 capsid proteins of bovine papillomavirus type 1. *Virology* **243,** 482–491.

Zhou, J., Sun, X.-Y., Davies, H., Crawford, L., Park, D., and Frazer, I. H. (1992). Definition of linear antigenic regions of the HPV16 L1 capsid protein using synthetic virion-like particles. *Virology* **189,** 592–599.

Zhou, J., Stenzel, D. J., Sun, X.-Y., and Frazer, I. H. (1993). Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *J. Gen. Virol.* **74,** 763–768.

Zimmermann, J., and Hammerschmidt, W. (1995). Structure and role of the terminal repeats of Epstein–Barr virus in processing and packaging of virion DNA. *J. Virol.* **69,** 3147–3155.