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Genomic Clones of Bovine Parvovirus: Construction and Effect of Deletions and Terminal Sequence Inversions on Infectivity

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Genomic clones of the autonomous parvovirus bovine parvovirus (BPV) were constructed by blunt-end ligation of reannealed virion plus and minus DNA strands into the plasmid pUC8. These clones were stable during propagation in *Escherichia coli* JM107. All clones tested were found to be infectious by the criteria of plaque titer and progressive cytopathic effect after transfection into bovine fetal lung cells. Sequencing of the recombinant plasmids demonstrated that all of the BPV inserts had left-end (3′)-terminal deletions of up to 34 bases. DNA isolated from progeny virions arising from transfected infectious clones was found to be indistinguishable from wild-type DNA by restriction enzyme analysis. Defective genomes could also be detected in the progeny DNA even though the infection was initiated with homogenous, cloned DNA. Full-length genomic clones with 3′ flip and 3′ flop conformations were constructed and were found to have equal infectivity. Analysis of low-molecular-weight DNA isolated from lysates of cells transfected with these clones demonstrated that rescue and replication of BPV DNA could be detected 3 to 8 days after transfection. Expression of capsid proteins from transfected genomes was demonstrated by hemagglutination, indirect immunofluorescence, and immunoprecipitation of [^{35}S]methionine-labeled cell lysates. Use of appropriate antiserum for immunoprecipitation showed the synthesis of BPV capsid and noncapsid proteins after transfection. Independently, a series of genomic clones with increasingly larger 3′-terminal deletions was prepared from separately subcloned 3′-terminal fragments. Transfection of these clones into bovine fetal lung cells revealed that deletions of up to 34 bases at the 3′ end lowered but did not abolish infectivity, while deletions of greater than 52 bases were lethal. End-label analysis showed that the 34-base deletion was repaired to wild-type length in the progeny virus.

Parvoviruses are small, icosahedral viruses with single-stranded DNA genomes. The small size of their genomes (ca. 5 kilobases [kb]) permits the molecular cloning of the entire genome in plasmid vectors. Cloned parvovirus genomes have been shown to be infectious, that is, to give rise to wild-type virus particles after transfection of host cells with recombinant plasmids (14, 19, 30). Infectious molecular clones are required for investigation of the genetics of parvoviruses, providing the double-stranded DNA necessary for in vitro mutagenesis techniques such as deletion analysis and frameshift mutations (13, 38).

Two cloning strategies have been successful in yielding infectious clones of parvoviruses. One is the recombination of seperately cloned halves of genomes. This approach was used by Merchlinsky et al. (19) to generate an infectious clone of minute virus of mice (MVM) and by Laughlin et al. (14) to construct an infectious clone of the dependent parvovirus adeno-associated virus (AAV). For the latter virus, this strategy was used when cloning after addition of *Bgl*II linkers to reannealed plus- and minus-strand DNA proved unsuccessful. Alternatively, Samulski et al. (30) used G-C tailing to insert the duplex AAV type 2 (AAV-2) genome into pBR322.

The overall genomic organization of the autonomous parvovirus bovine parvovirus (BPV) is similar to that of other members of the genus. Certain amino acid sequences and nucleic acid secondary-structure features are highly conserved among all parvoviruses. However, comparison of the putative proteins of the BPV open reading frames with those of other parvoviruses suggests that BPV stands between AAV and the rodent parvoviruses in an evolutionary progression from B19 to AAV to BPV to the rodent parvoviruses (9). The availability of a genomic clone of BPV would allow a dissection, at the molecular level, of the unique features of BPV and, eventually, a comparison of the signals which determine the common and divergent characteristics of members of the family *Parvoviridae*. We report here the construction of infectious clones of the autonomous parvovirus BPV by the novel strategy of blunt-end ligation of reannealed plus- and minus-strand virion DNA, in the absence of linkers, into the multiple cloning site of pUC8. A similar approach was employed by Senapathy and Carter (32) to obtain infectious clones of AAV. Data on the infectivity of clones with combinations of spontaneous terminal deletions and sequence inversions are presented. The expression of viral genes after transfection was also demonstrated.

MATERIALS AND METHODS

Cell culture and virus propagation. BPV was propagated in bovine fetal lung (BFL) cells which were grown in monolayer culture and maintained in culture Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum (complete medium) as described by Parris and Bates (20).

Clone construction and plasmid propagation. Construction and detection of recombinants with pUC vectors (39) was performed by standard methods (18). Some restriction fragments, electrophoresed on low-melting-point agarose gels, were ligated into vectors directly, after the gel chunk was liquified (35). Transformation was done according to the

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high-efficiency procedure of Hanahan (12), using *Escherichia coli* JM107. Plasmid isolations were done by the small- and large-scale procedures of Rodriguez and Tait (26). Plasmid DNA used in transfections was purified by banding once in a cesium chloride-ethidium bromide gradient (18). Recombinant clones were screened by restriction enzyme digestion, and the identity of BPV inserts was verified by Southern blotting with ^{35}S-dATP-labeled nick-translated BPV DNA as the probe.

Transfection of bovine cells. BFL cells (7×10^5 to 8×10^5 per 60-mm-diameter dish) were transfected with recombinant plasmids 24 h after being plated. Transfections were done by using DEAE-dextran essentially as described by Lopata et al. (16). Cells were replenished with fresh, complete medium 4 h before transfection. The medium was aspirated and replaced with 1.5 ml of MEM containing 200 μg of DEAE-dextran (average molecular weight, 500,000) per ml and sample DNA. The cells were incubated for 4 h at 37°C in a CO_2 incubator. The medium was removed, and the cell layers were washed with MEM. A dimethyl sulfoxide shock was performed with 1 ml of 10% dimethyl sulfoxide in MEM per plate for 5 min at room temperature. After removal of the medium, the cell layers were washed with MEM and 5 ml of complete medium supplemented with amphotericin B (Fungizone) (1.25 μg/ml) was added. For plaque assays, an overlay of 1% SeaPlaque agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) with complete medium supplemented with amphotericin B was used.

Analyses of infectivity. Cell layers were observed for up to 10 days for BPV specific cytopathic effect (CPE). Plaques were counted 8 to 10 days after transfection on cell layers stained with 0.1% crystal violet. For indirect immunofluorescence, cells were fixed in −20°C methanol at various times after transfection. The first antibody was an antiserum prepared against purified BPV capsids (rabbit 0118) and the second antibody was fluorescein-conjugated goat anti-rabbit immunoglobulin G (Cooper Biomedical Inc., West Chester, Pa.). For hemagglutination assays, transfected cell layers were scraped into the medium and frozen and thawed three times. The hemagglutination assay was performed in microtiter plates with human type O cells (4). Expression of viral proteins was analyzed by immunoprecipitation of [^{35}S]methionine-labeled lysates (7) prepared from transfected cells. The growth medium was replaced with MEM containing 10% dialyzed serum 12 h before the addition of 20 μCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml, and lysates were prepared 24 h after the addition of the isotope. The serum used as the first antibody was anticapsid antibody (rabbit 0118) or serum from a calf experimentally infected and immunized with BPV (calf 86). Immunoprecipitation products were electrophoresed on 10% sodium dodecyl sulfate gels, treated with Amplify (New England Nuclear), and autoradiographed with an intensifying screen at −80°C. Low-molecular-weight DNA was extracted by a modified Hirt extraction method (34). Virus stocks were prepared from cultures transfected with various clones by freezing and thawing the cell layers in the medium three times. These stocks were used to infect additional cultures for the preparative isolation of progeny viral DNA as previously described (9).

Sequencing and southern blotting. The termini of BPV inserts in recombinant plasmids were sequenced directly by the dideoxy method (31), using ^{35}S-dATP and gradient gels as described by Biggin et al. (6). Forward and reverse primers which anneal just outside the multiple cloning region of pUC8 were used to sequence both termini (39).

DNA in agarose gels was transferred directly to Zeta-Probe cationized membranes (Bio-Rad Laboratories, Richmond, Calif.) by capillary transfer with 0.4 M sodium hydroxide (22). Blots were probed with 1×10^6 to 2×10^6 cpm of BPV virion DNA made radioactive by nick translation with ^{35}S-dATP (specific activity, 10^7 cpm/μg). Blots were washed according to a protocol distributed by the manufacturer.

RESULTS

In this paper, the convention of Armentrout et al. (1) is used: the 3′ terminus of the minus strand of parvovirus DNA is referred to as the 3′ (left) end or map origin.

Cloning of the BPV genome. (i) Cloning by linker addition. An attempt to clone replicative-form (RF) DNA extracted by a modified Hirt procedure from BPV-infected BFL cells after repair with *E. coli* DNA polymerase I Klenow fragment and addition of *Sal*I linkers was unsuccessful. To determine if this restriction was due to blockage of the termini, a similar cloning of the m.u. 0 to 17 and m.u. 92 to 100 *Eco*RI fragments of BPV RF into *Sal*I-cut pUC8 was attempted. No clones with intact 5′ termini were obtained, suggesting that the 5′ end of BPV RF DNA may be blocked, as observed by Merchlinsky et al. (19) for MVM. If the blockage was due to a 5′-terminal protein (23), removal by treatment with BAL 31 exonuclease might allow cloning of the terminal sequences. This approach has been used to prepare a genomic clone from H-1 RF DNA (24) but was not successful in the present study with BPV.

A second approach was the addition of *Sal*I linkers to Klenow-repaired double-stranded viral DNA (reannealed plus and minus strands). In agreement with previous work on AAV-2 (14), we were unable to clone the full-length BPV genome in one step after linker ligation. The double-stranded BPV DNA was checked for accessible termini by separately cloning the terminal *Eco*RI fragments as described above. Clones containing either the 3′ or 5′ *Eco*RI fragments were readily obtained, demonstrating that either end of double-stranded virion DNA may be free.

(ii) Direct blunt-end cloning. A total genomic clone of BPV was obtained by blunt-end cloning of double-stranded BPV virion DNA (Fig. 1). The reannealed plus and minus strands were repaired with Klenow fragment and initially were ligated into the *Sma*I site of pUC8 (Fig. 1A). Screening of 57 white colonies yielded seven clones, designated pGCSma, which had inserts whose restriction fragments migrated to the position of BPV DNA markers on agarose gels and hybridized with radiolabeled BPV DNA upon Southern blotting (data not shown).

The BPV insert cannot be excised intact from the pGC Sma clones, since the inserts are flanked on one side by an *Eco*RI site and BPV contains two sites for this enzyme. To construct clones from which the entire BPV genome could be excised, the *Pst*I site in the multiple cloning region was blunted and destroyed with S1 nuclease. BPV DNA, prepared as described above, was inserted at this site, which is flanked by *Hin*dIII and *Sal*I sites in the multiple cloning region, neither of which is present in BPV (Fig. 1B). Transformants were screened for apparently full-length BPV inserts by digestion with *Sal*I and *Hin*dIII. Screening of 100 colonies gave three clones, designated pGC, with apparently full-length inserts. Digestion of these clones with *Pst*I and *Sal*I or with *Pst*I and *Hin*dIII followed by Southern blotting confirmed the presence of an intact BPV genome (Fig. 2).

The apparently full-length clones were characterized by sequence analysis. This analysis showed that small, sponta-

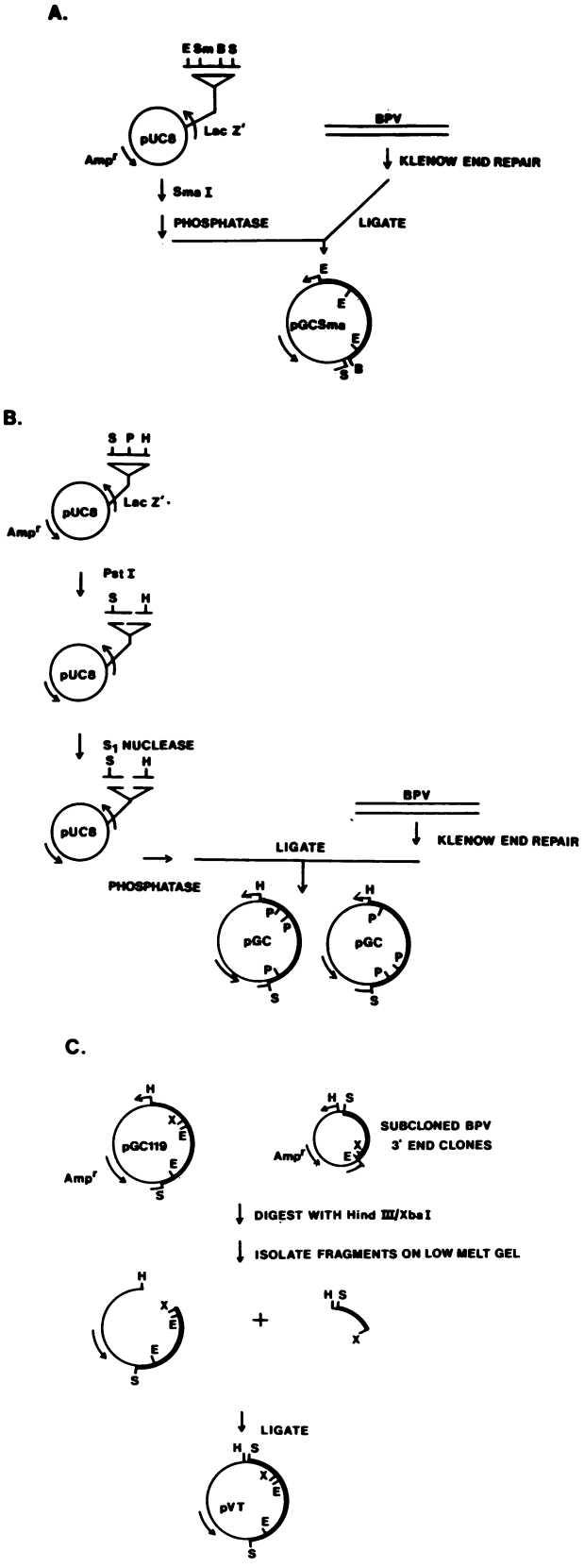

FIG. 1. Strategy for cloning the BPV genome into pUC8. Restriction sites of the vector point outward, while restriction sites of the BPV insert point inward. (A) Clones of the pGCSma series were constructed by ligation into the *Sma*I site of pUC8. (B) Clones of the pGC series were constructed by ligation into the blunted *Pst*I site of pUC8. (C) Clones of the pVT series were constructed by replacing the 3′-terminal *Xba*I fragment of pGC119 with other subcloned BPV 3′-end *Xba*I fragments. Abbreviations for restriction enzyme sites: E, *Eco*RI; Sm, *Sma*I; B, *Bam*HI; S, *Sal*I; P, *Pst*I; H, *Hin*dIII; and X, *Xba*I.

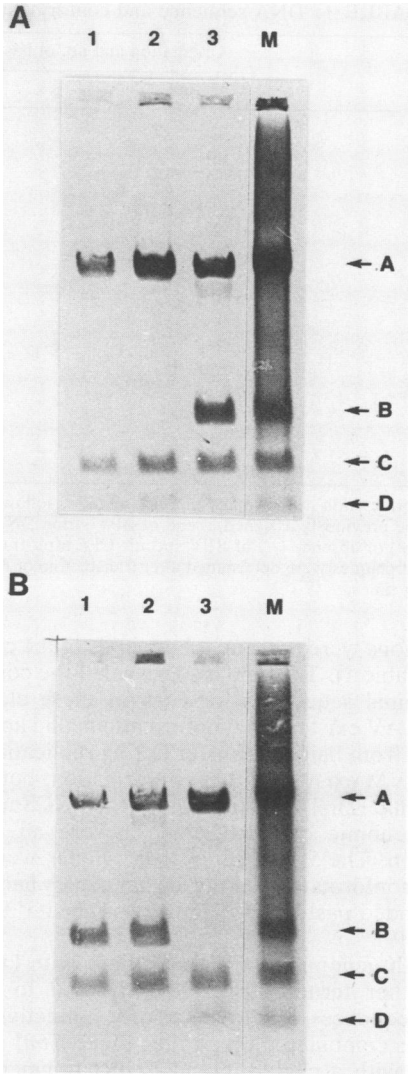

FIG. 2. Restriction enzyme analysis of DNA from BPV genomic clones of the pGC series. The DNA was digested with *Hin*dIII and *Pst*I (A) or with *Sal*I and *Pst*I (B) and separated on a 1.0% agarose gel. The gels were blotted to Zeta-Probe membranes and hybridized with ^{35}S-dATP-labeled nick-translated BPV DNA. Lanes: 1, pGC 119; 2, pGC127; 3, pGC167; M (marker), double-stranded BPV virion DNA digested with the same enzymes. The *Pst*I A (3.88-kb), B (0.87-kb), C (0.48-kb), and D (0.29-kb) fragments of BPV are indicated. *Pst*I-D is the 3′-terminal fragment, and *Pst*I-B is the 5′-terminal fragment.

TABLE 1. DNA sequence and conformation at the termini of BPV genomic clones and infectivity of these clones

Clone	Orientation and no. of bases deleted[a] at:		CPE[b]	No. of plaques (mean ± SD)/ 0.2 μg of DNA[c]
	3′ end	5′ end		
pGCSma17	Flip, −19	Flop, −3	+	ND
pGCSma20	Flip, −3	Flip, −9	+	ND
pGCSma22	Flip, −18	Flip, +8	+	ND
pGCSma32	Flip, −3	Flip, +26	ND	ND
pGCSma41	Flip, −3	ND	ND	ND
pGCSma51	Flip, −9	Flip, ND	ND	ND
pGCSma57	Flip, −25	Flip, ND	ND	ND
pGC119	Flip, −27	Flip, +1	+	14 ± 7
pGC127	Flip, −32	Flip, −7	+	5 ± 2
pGC167	Flip, −14	Flop, +26	+	64 ± 27
pVT501	Flop, 0	Flip, +1	+	65 ± 23
pVT501Δ34	Flop, −34	Flip, +1	+	6 ± 5
pVT502	Flip, 0	Flip, +1	+	50 ± 21
pVT502Δ52	Flip, −52	Flip, +1	−	ND
pVT502Δ79	Flip, −79	Flip, +1	−	ND

[a] Sequence orientation is designated flip or flop and is followed by the number of bases deleted relative to the virion terminus (9). +, Sequence of the clone extends past the previously assigned 5′ end of BPV virion DNA. ND, Not determined.

[b] Presence (+) or absence (−) of BPV specific CPE after transfection of the genomic clone.

[c] Numbers of plaques were determined after transfection of BFL cells with 0.2 μg of plasmid DNA. All samples were tested five times except pGC167, which was tested four times.

neous deletions were present at both ends of the cloned BPV genomes (Table 1). The data also revealed the conformation of the terminal sequences for each of these clones. Both termini of AAV exist in two conformations, flip and flop (17), which arise from hairpin transfer during replication (5). The 5′ end of MVM exists in either conformation, but the 3′ end is uniquely flip (2). The 3′ termini for the pGCSma and pGC series of genomic clones (Table 1) were all in the flip conformation. The 5′ termini of these clones were in either of the two conformations. Four of the clones had 5′ termini which extended past the previously assigned 5′ terminus of BPV DNA (9).

Full-length genomic clones and clones with known deletions at either terminus were constructed to define the terminal sequence requirements for infectivity. Clone pGC119 was combined through the unique *Xba*I site at m.u. 15 of BPV with a cloned 3′-end *Eco*RI fragment, p3′R45, which had previously been sequenced and shown to be intact and in the flip conformation (Fig. 1C). This construction gave a clone, pVT501, which was shown by sequencing to have an unchanged 5′ end but whose 3′ end was now in the flop conformation (Table 1). Apparently, the terminal sequence had undergone an inversion during the cloning and amplification steps. The cloning procedure was therefore repeated, and a clone, pVT502, was obtained which had an undeleted 3′ end in the flip conformation. Three other 3′ *Eco*RI subclones with increasing terminal-deletion sizes were transferred to pGC119 through the *Xba*I site to generate genomic clones with a 34-base-pair deletion (clone pVT501Δ34, flop), a 52-base-pair deletion (pVT502Δ52, flip), and a 79-base-pair deletion (pVT502Δ79, flip).

These genomic clones have proven to be remarkably stable during propagation in *E. coli* JM107. During large-scale preparation of DNA stocks of nine different genomic clones, not a single detectable terminal deletion occurred. The stability of cloned BPV 5′ termini could be due to the absence of the direct repeats found in the MVM 5′ palindrome (8) or to its smaller size. Cloned BPV genomes have not been propagated in *E. coli* strains other than JM107, and it is possible that the properties of JM107, of pUC8, or of this combination of vector and host are responsible for the observed stability of these clones.

Determination of infectivity. Presumptive evidence for the formative of progeny virus would be the development of progressive CPE in transfected cells. CPE was observed in cultures transfected with several genomic clones within 5 to 6 days after transfection (CPE is observed 1 to 2 days after infection with the virus) (Table 1). Maximum transfection was observed with 5 μg of plasmid DNA per 5 ml of medium per 7×10^5 cells, and a decrease in transfection efficiency occurred when more than this amount of DNA was used (data not shown). Saturation of the uptake of plasmid DNA by the cells during transfection probably is not responsible for this inhibition, since such a mechanism should produce a plateau. This phenomenon has not been reported with other parvovirus genomic clones but was observed by Sussman and Milman (37) in the transfection of a plasmid containing the herpes simplex virus thymidine kinase gene. Transfections with DEAE-dextran routinely gave 10-fold-higher efficiency than transfections performed by the calcium phosphate procedure of Perbal (21) (data not shown).

Plaque assays were performed to assess quantitatively the transfection efficiency of the total genomic clones and the effects of variations in terminal structure on infectivity (Table 1). Deletions at the 3′ end of up to 34 bases either had no effect on or reduced but did not abolish infectivity. This is seen most clearly by comparing the plaque production of pVT501 and pVT502 with that of pVT501Δ34 and pGC119, respectively, clones which differ only in the length of the 3′ terminus. Analysis of virion DNA from an infection initiated with a virus stock derived from a transfection with pVT501Δ34 showed that this deletion had been completely repaired. The sizes of end-labeled *Sma*I restriction fragments of this DNA were the same as the sizes of the fragments obtained from wild-type DNA (data not shown). Longer deletions at the 3′ end were lethal. Cell layers transfected with clone pVT502Δ52 or pVT502Δ79 showed no CPE, and no CPE was observed upon blind passage to a second culture. Also, the flip and flop forms of the 3′ end seemed to have equal levels of infectivity as seen by comparing pVT501 with pVT502 and pGC119 with pVT501Δ34.

An effect of plasmid topology on transfection efficiency was noted. Compared with the supercoiled plasmid, release of the genome from pVT501 or pVT502 by digestion with

TABLE 2. Effect of plasmid topology on transfection efficiency[a]

Clone and treatment	No. of plaques/ 0.2 μg of DNA
pGC167	
Sham digested	72, 50
Linearized at 5′ end	10, 9
Linearized at 3′ end	3, 5
Insert released	9, 7
pVT501	
Sham digested	29
Insert released	6
pVT502	
Sham digested	39, 98
Insert released	5, 8

[a] Genomic clones were digested with *Hin*dIII or *Sal*I or both to linearize the construct at one or another margin of the insert or to release the insert. The sham-digested samples were incubated in the absence of restriction endonuclease. Data from two independent experiments are shown.

*Sal*I, which leaves only half of a *Sal*I site attached at each end, decreased the efficiency of transfection 5- to 10-fold (Table 2). This effect was studied in more detail by using plasmid pGC167. Either digestion with *Sal*I or *Hin*dIII, which linearize the plasmid adjacent to the 3′ end (with GCC plus half of a *Hin*dIII site attached) or 5′ end (with half of a *Sal*I site attached) of the BPV insert, respectively, or double digestion with *Sal*I and *Hin*dIII to release completely the unit-length genome resulted in a 5- to 10-fold decrease in plaque titer (Table 2). This is in contrast to the findings of Merchlinsky et al. (19) and Laughlin et al. (14), who observed an increase in infectivity when excised viral genomes were used in transfection assays. Any enhancement of activity due to linearization of transfected BPV DNA molecules could be lowered by nuclease activity in the cells.

Analyses of intracellular and progeny viral DNA. The time course of rescue and replication of the BPV genome was determined by isolating low-molecular-weight DNA from cell layers at various times after transfection (Fig. 3). On blots hybridized with BPV probe, RF and single-stranded DNAs could be demonstrated 3 to 8 days posttransfection with clones pVT501 and pVT502. The pUC8 probe did not hybridize with the progeny DNA, suggesting that newly replicated BPV DNA retained no significant length of plasmid sequences.

To compare progeny DNA from a transfection with that produced by the normal infectious process, transfected cell lysates were amplified by one blind passage to generate a virus stock of sufficient titer to infect cells for isolation of BPV DNA on a preparative scale. The viral DNA was isolated from full virions purified on CsCl gradients and then separated into double- and single-stranded DNA on a neutral sucrose gradient. DNA in each fraction of this gradient was electrophoresed, transferred, and probed with nick-translated BPV DNA. When this blot was compared with one of DNA isolated from a typical infection, the patterns were indistinguishable (Fig. 4). A similar ratio of monomer-length single-stranded and reannealed double-stranded DNAs was seen in the two blots. Viral DNA of subgenomic length, characteristic of defective viral particles, was present (Fig. 4). Apparently, defective genomes arose rapidly during

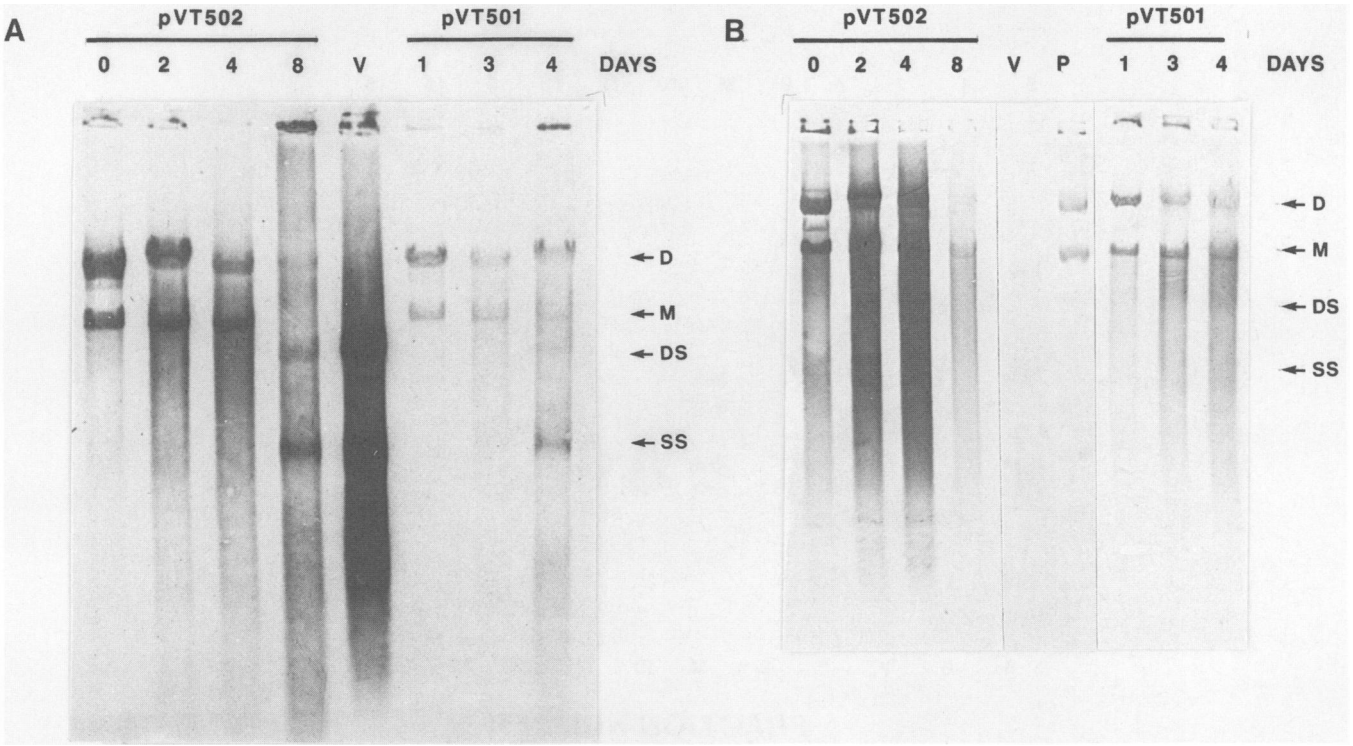

FIG. 3. Analysis of low-molecular-weight DNA after transfection of cell cultures with genomic clones. DNA was isolated from BFL cells by Hirt extraction at various times after transfection with pVT501 or pVT502. The DNA was separated on 1.0% agarose gels, transferred to Zeta-Probe membranes and hybridized with either ^{35}S-dATP-labeled nick-translated BPV DNA (A) or nick-translated pUC8 DNA (B). Markers were BPV virion DNA (lane V) and supercoiled pVT501 (lane P). The positions of the monomer (M) and dimer (D) plasmid DNA bands and the single-stranded (SS) and double-stranded (DS) BPV DNA bands are indicated.

DNA replication even when cloned, initially homogeneous DNA was used to initiate infection. The single-stranded DNA from progeny of transfected cells was replicated in vitro with *E. coli* DNA polymerase I Klenow fragment and digested with restriction enzymes. No differences were seen between transfection-derived and infection-derived DNAs (Fig. 5).

Expression of virally coded proteins. The appearance of BPV-coded proteins after transfection was studied by an indirect immunofluorescence assay using serum prepared against BPV capsids (rabbit 0118) and by hemagglutination, both of which would detect the presence of full or empty virus particles. Clones pGCSma20 (Fig. 6) and pVT501, pVT501Δ34, and pVT502 (data not shown) all gave positive immunofluorescence with anticapsid antibody. After transfection, the pattern of intranuclear fluorescence was indistinguishable from that of cells infected with BPV. The clones which were positive by indirect immunofluorescence were also positive by hemagglutination.

Expression of virally coded proteins was also studied by immunoprecipitation of [^{35}S]methionine-labeled cell lysates with anticapsid antibody (rabbit 0118), as well as antibody which recognizes both capsid and noncapsid proteins (calf 86). No virally coded proteins were detected 2 days after transfection with pVT501 or pVT502 (Fig. 7). By day 5, all three capsid proteins had accumulated in amounts comparable to those produced in a normal infection after 20 h. At this time, the 28-kilodalton (kd) nonstructural protein NP1 and two nonstructural proteins of 75 and 83 kilodaltons were immunoprecipitated with calf 86 serum. These large non-

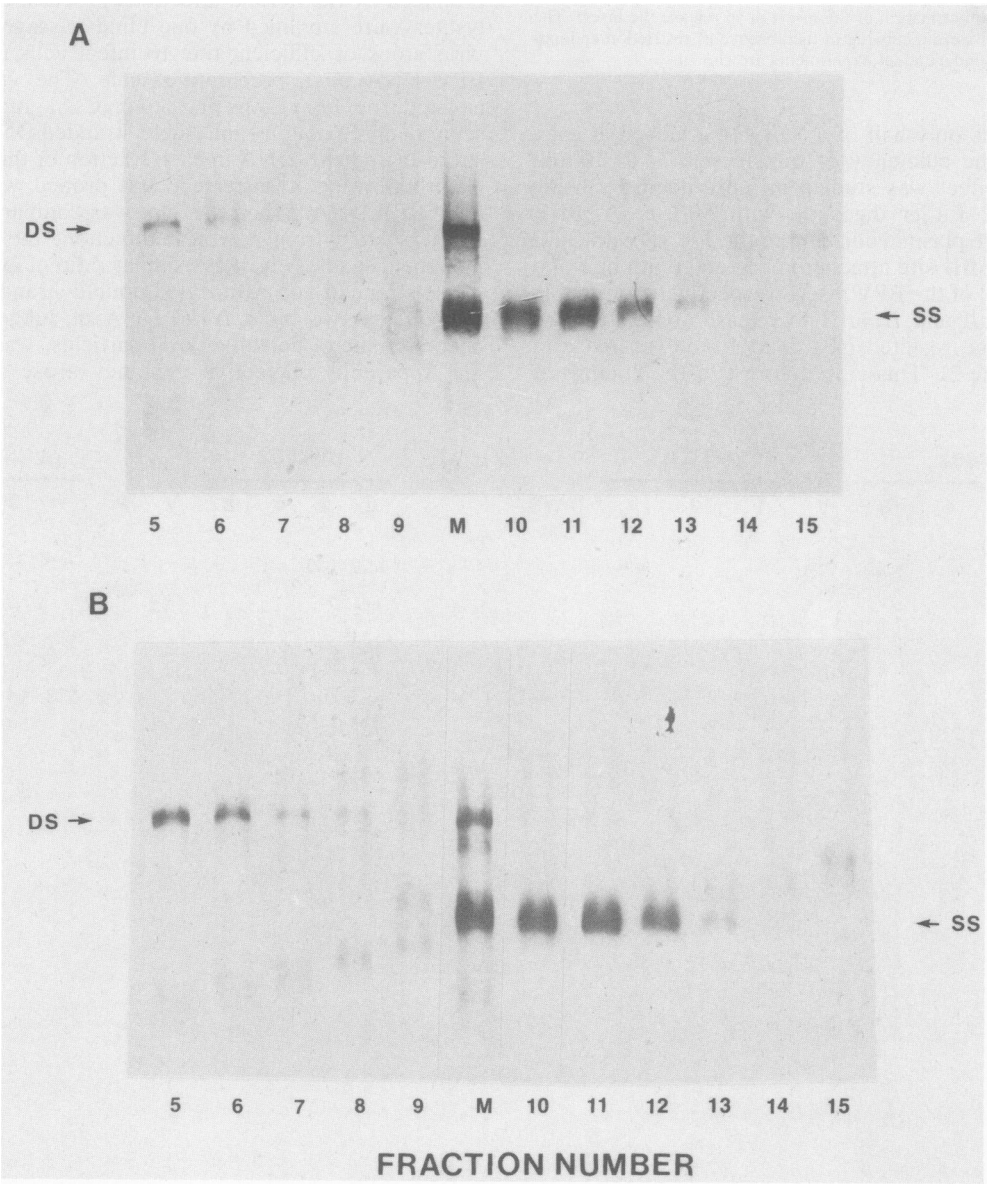

FIG. 4. Sedimentation profile of the DNA from progeny virions of clone pGCSma20. DNA was extracted from CsCl-banded pGCSma20 progeny virions (A) or wild-type virus (B) and fractionated on a neutral high-salt–sucrose gradient. The direction of sedimentation is from left to right. DNA from the fractions was electrophoresed on a 1.0% agarose gel, transferred to Zeta-Probe, and hybridized with ^{35}S-dATP-labeled nick-translated BPV DNA. The marker (lane M) was single-stranded (SS) and double-stranded (DS) BPV virion DNA, as indicated. In addition to double-stranded DNA, subgenomic DNA is seen in fractions 5 through 9.

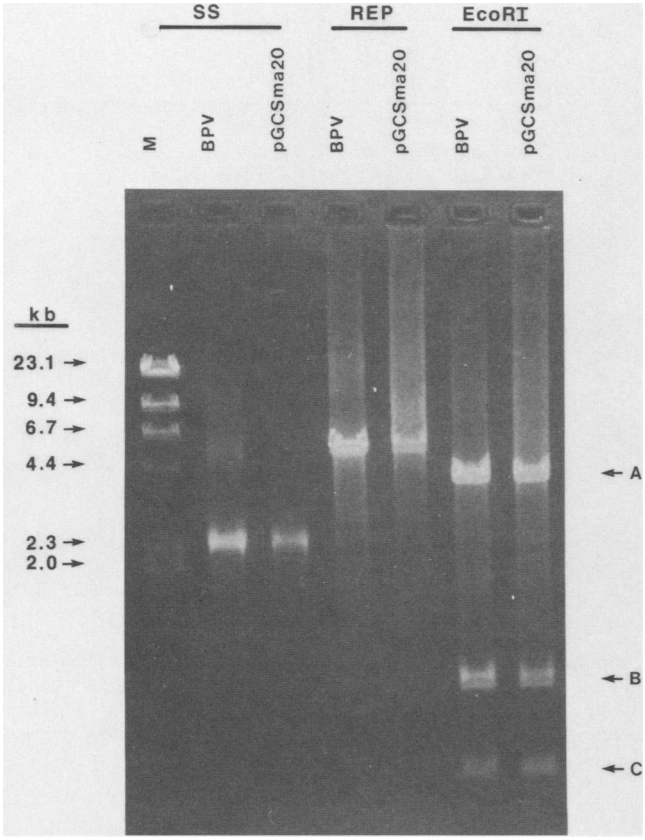

FIG. 5. In vitro replication and restriction enzyme analysis of single-stranded DNA from progeny virions of clone pGCSma20 as compared with wild-type BPV DNA. Single-stranded DNAs were replicated in vitro to duplex form with *E. coli* DNA polymerase I Klenow fragment and digested with *Eco*RI. Single-stranded DNA (SS), in vitro-replicated DNA (REP), and *Eco*RI-digested DNA (*Eco*RI) were separated on a 1.0% agarose gel and stained with ethidium bromide. Lambda DNA digested with *Hin*dIII was used as a size marker (lane M). The sizes of the lambda fragments are indicated. The *Eco*RI A (4.10-kb), B (0.98-kb), and C (0.45-kb) fragments of BPV are indicated.

structural proteins have been shown to be homologous to the nonstructural protein NS1 of about the same molecular weight encoded by MVM and B19 (15).

DISCUSSION

The entire genome of the autonomous parvovirus BPV was cloned in pUC8. The cloning strategy took advantage of the fact that about 10% of BPV virions contain plus-strand DNA, so double-stranded virion DNA could be obtained by annealing and cloned directly into a blunt-cut plasmid. These clones (pGCSma and pGC) contained small spontaneous deletions at the termini. Full-length genomic clones (pVT501 and pVT502) were constructed by specific recombination. All of these genomic clones were infectious after transfection as judged by development of progressive CPE, plaque titers, and the formation of single-stranded and double-stranded progeny DNAs with positions of restriction sites indistinguishable from that of DNA from a normal infection. Other genomic clones containing larger deletions at the 3′ terminus were constructed and found not to be infectious. Expression of BPV proteins from transfected genomes was demonstrated by immunoprecipitation of radiolabeled cell lysates prepared from transfected cells and by the observation of immunofluorescence with an antibody prepared against BPV capsids. A full-length genomic clone with the 3′ terminus in the flop configuration (pVT501) was obtained as a cloning artifact and was found to be as infectious as an otherwise identical clone (pVT502) with the 3′ terminus in the flip configuration.

The strategy which was successful for cloning the BPV genome is dependent on the availability of significant amounts of plus-strand DNA (27). Therefore, it would only be useful for cloning parvoviruses which encapsidate the plus strand, such as AAV (5), B19 (11, 36), lapine parvovirus (J. B. Metcalf, unpublished data), or LuIII (3). Indeed, this strategy has been used to construct a genomic clone for LuIII (N. Diffoot, unpublished data).

Direct cloning of the BPV genome after linker addition to either RF or reannealed virion DNA was unsuccessful. For the RF, the inability to clone the 5′-terminal *Eco*RI fragment

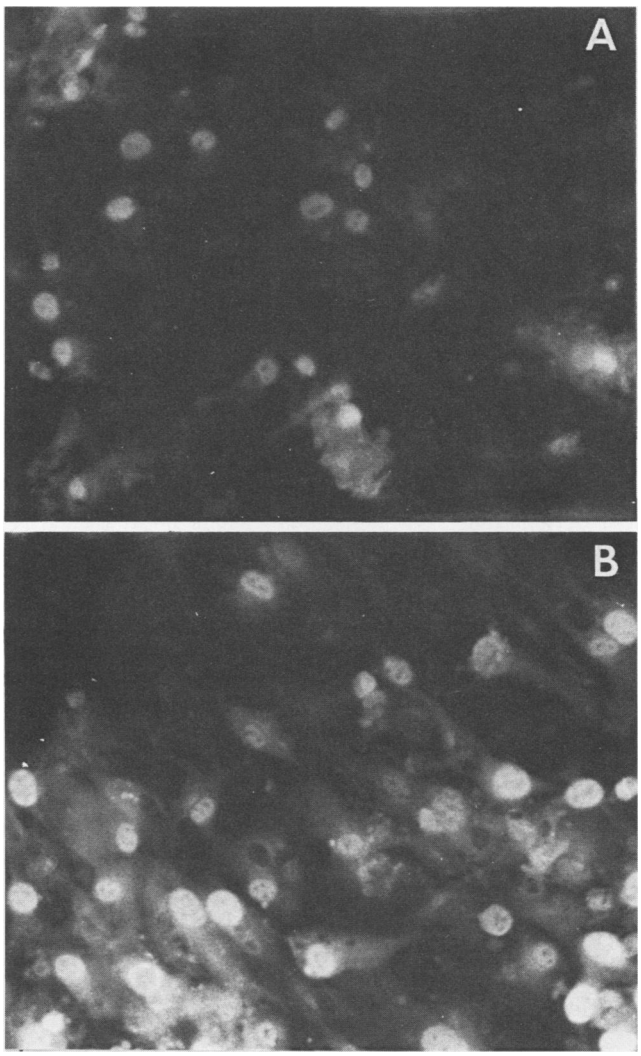

FIG. 6. Production of capsid antigens after transfection of BFL cells with genomic clone pGCSma20 by indirect immunofluorescence. Cells were prepared for immunofluorescence 5 days after transfection with pGCSma20 (A) or infection with BPV (B). The first antibody was anticapsid serum (rabbit 0118), and the second antibody was fluorescein-conjugated goat anti-rabbit immunoglobulin G.

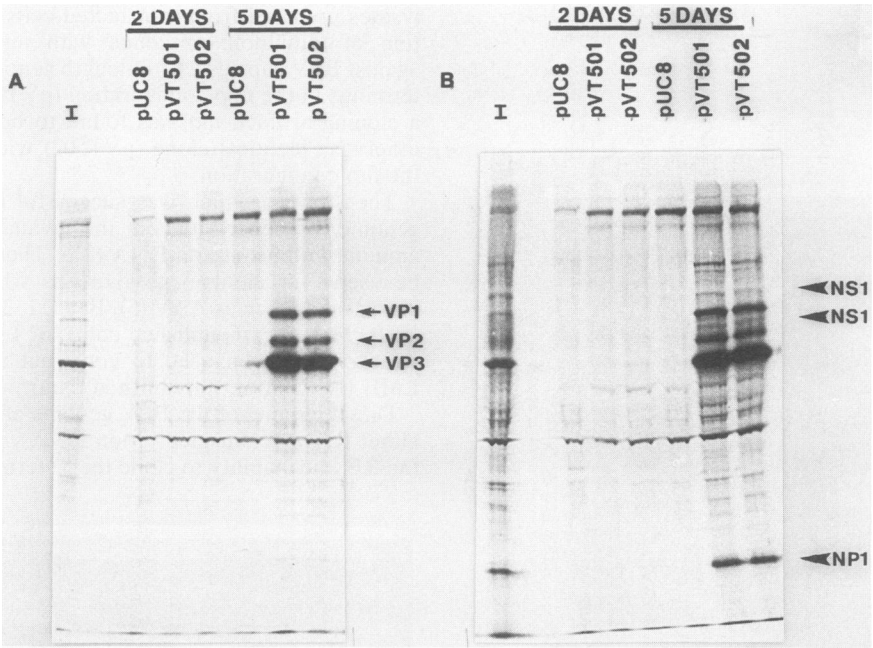

FIG. 7. Demonstration of BPV proteins in transfected cell cultures by immunoprecipitation of radiolabeled cell lysates. Lysates were prepared at 2 and 5 days after transfection and immunoprecipitated with antibody against capsid proteins (rabbit 0118) (A) or antibody which recognizes both capsid and noncapsid proteins (calf 86) (B). Cell layers were transfected with pUC8, clone pVT501, or clone pVT502. In addition, lysates from BPV-infected cell cultures (lane I) were run as markers. The positions of the capsid proteins VP1, VP2, and VP3 are indicated by arrows. The 83- and 75-kilodalton nonstructural proteins NS1 and NP1 are indicated with arrowheads.

suggests that the right end of the RF is not accessible to linker addition. The same phenomenon has been reported for MVM by Merchlinsky et al. (19) and Sahli et al. (28) and has been attributed to the blocking of this terminus by a peptide remnant of the 5′-terminal protein. However, a recent report showed that both the left and right termini of MVM have proteins attached to the 5′ ends (10). The cloning of the 3′ end of BPV means either that BPV lacks a terminal protein here or that there are additional reasons for the different cloning efficiencies of the BPV ends. One explanation might be a predominance in the RF pool of 5′-hairpinned monomer DNA, as implied by the result of Rhode and Klaassen (25), who showed that the *ori* site for rodent parvoviruses lies in the 5′ terminus.

The rate-limiting step for cloning via linker addition is obtaining a molecule with linkers on both termini, which is in turn dependent on the proportion of molecules in the viral DNA pool which have both termini in the extended conformation. Even though both the 3′ and 5′ termini of BPV reannealed plus- and minus-strand virion DNA can be separately subcloned, either terminus may be refractory to linker addition because of fold-back and hydrogen bonding at that terminus. Blunt-end ligation of BPV into the vector in the absence of linkers may be viewed as a two-step reaction in which ligation of one end of the BPV DNA molecule to the vector is the rate-limiting step. Once this has occurred, the second ligation is an intramolecular event which would presumably occur at a much faster rate.

Replication of BPV DNA after transfection resulted in progeny DNA indistinguishable by several criteria from that of a normal infection, including generation of defective genomes (Fig. 4). The time course of a transfection was slower than the time course of an infection. A portion of this delay may be due to the time required for rescue of the genome from the plasmid. Models for the rescue and replication of AAV-2 from genomic clones invoke the occurrence of nicks either in the plasmid sequence near the plasmid-AAV boundary (29) or within the terminal palindrome of AAV-2 (33). Both models postulate a panhandle intermediate which forms by base pairing between the inverted terminal repeats of AAV-2. Since autonomous parvoviruses may not form such a structure, other mechanisms must exist for rescue of these genomes. Rescue of autonomous parvovirus genomes could occur via nicks within the palindromic sequences at each end of the same strand, followed by DNA synthesis initiating at the 3′ hydroxyl group. Therefore, transfection with clones containing deletions at the 3′ end might result in lower infectivity, presumably by decreasing the length of DNA available to form a base-paired hairpin capable of acting as a primer for DNA synthesis. Deletions at the 5′ end generated in the excision could be repaired in subsequent rounds of normal viral DNA synthesis.

Two clones (pGC167 and pGCSma32) have 5′ termini 26 bases past the previously defined mature virion terminus. Data to be presented elsewhere (K. C. Chen, B. C. Shull, M. Lederman, E. R. Stout, and R. C. Bates, submitted for publication) assign these 26 nucleotides to the 5′ terminus, so that the total BPV genomes is 5,517 nucleotides. Infectivity was not affected by the presence or absence of these nucleotides (Table 1). Similarly, the 14-base-pair deletion at the 3′ end of pGC167 had no apparent effect on infectivity. Deletions of up to 34 bases at the 3′ end lowered, but did not abolish, infectivity (Table 1). In this deletion, the 3′ hairpin could be destabilized, and "breathing" of the truncated terminus might lower the rate of initiation of DNA synthesis by a DNA polymerase replication complex. Eventually, this deletion is completely repaired, because the complementary sequences are present. Deletions of 52 and 79 bases at the 3′ end each abolished infectivity. The 52-base deletion leaves only 6 base pairs of the main stem of the palindrome, which

is apparently insufficient for rescue and replication of the BPV genome. The 79-base-pair deletion removes over half of the total 3′ palindrome, which prevents the formation of the hairpin configuration.

A sequence inversion during the construction of pVT502 has resulted in the conversion of a 3′ flip configuration to a 3′ flop configuration. This inversion occurred without deletion of any bases. One occurrence of a sequence inversion during cloning has been reported (25), for the 5′ end of parvovirus H-1. Both BPV clones constructed with 3′ flop ends (pVT501 and pVT501Δ34) were infectious. Even though the 3′ flop conformation discussed here occurred as a cloning artifact, an analysis of BPV RF and virion DNAs has established the presence of 3′ flop in a normal infection (Chen et al., submitted).

Expression of BPV coded capsid proteins has been shown by hemagglutination and indirect immunofluorescence (Fig. 6). Analysis by immunoprecipitation of radiolabeled cell lysates (Fig. 7) has confirmed the synthesis of these proteins as well as the synthesis of the three major noncapsid proteins coded by the virus. These data, taken together with the data on the synthesis of BPV monomer DNA (Fig. 3), the progressive nature of the observed CPE, and the ability to amplify a transfection to generate a virus stock, demonstrate that infectious virions are being produced.

The availability of an infectious genomic clone of BPV will allow the detailed analysis, by site-specific mutagenesis, of the genomic organization of BPV. This will provide information on its evolutionary relationship to other members of the family. It also makes possible the analysis of the function of conserved sequences in proteins and of *cis* signals in the termini involved in rescue and replication.

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