MOLECULAR CLONING AND ANALYSIS OF THE GENOME OF BOVINE PARVOVIRUS

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(ABSTRACT)

The genome of bovine parvovirus (BPV) has been cloned by blunt end ligation of double stranded virion DNA into the plasmid pUC8. The resulting genomic clones were infectious after transfection into bovine fetal lung (BFL) cells. Sequencing of the plasmids demonstrated that deletions were common at both ends of the cloned BPV genome. Deletions of up to 34 bases at the 3′ end lowered but did not abolish infectivity, while a deletion of 52 bases eliminated infectivity. End label analysis demonstrated the repair of deletions of up to 34 bases at the 3′ end or 35 bases at the 5′ end to the wild type length. Mutually inverted sequence orientations of the palindromic termini, known as the flip and flop forms, can occur during replication of parvovirus DNA. Cloning of BPV terminal sequences permitted the identification of the 3′ flop sequence inversion as a natural component of BPV DNA. This is the first report of sequence inversions within the 3′ end of an autonomous parvovirus. Clones with the 3′ flop or flip conformations were equally infectious. Wild type virion DNA was shown to have predominantly the 3′ flip conformation but a significant amount of 3′ flop was also detected. At the 5′ end, both the flip and flop sequence conformations were identified in nearly equal amounts. The progeny virion DNA from transfection of genomic clones had the same ratio of flip to flop as did wild type at both the 3′ and 5′ ends, regardless of the starting terminal conformations of the genomic clone. These data suggest that, while sequence inversion occurs at both termini during BPV DNA replication, some mechanism exists for the preferential replication of the 3′ flip conformation. Replicative form DNA from BPV infected cells had the same ratio of flip and flop at each end and the same termini as virion DNA. A set of deletion and frameshift mutants affecting each of the coding regions of BPV was constructed using one of the genomic clones. None of these mutants was infectious when transfected

into BFL cells, which demonstrates that all three of the major open reading frames are essential for the production of infectious virus.

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INTRODUCTION

The viruses in the family Parvoviridae have linear single stranded DNA genomes which replicate in the nucleus of host cells. Replication of autonomous parvoviruses requires host cell factor(s) present during S-phase, while the replication of defective parvoviruses requires co-infection by a helper virus. The ends of the viral genome are unusual in having self-complementary sequences which can form hairpin structures. Autonomous parvoviruses have nonidentical palindromes at their termini, whereas the defective parvoviruses have identical terminal palindromes contained within a longer inverted repeat. Replication of viral DNA is initiated by self-primed synthesis from the 3′ hairpin resulting in formation of a double stranded replicative form (RF) DNA. The RF DNA serves as the template for displacement synthesis of progeny single stranded DNA which is concommitantly encapsidated.

The complete nucleotide sequence of adeno-associated virus-2 (AAV-2), parvovirus H-1, and minute virus of mice (MVM) have been published. From these studies several generalizations can be made about the genomic organization of parvoviruses. Analysis of the minus strand reveals two major open reading frames (ORF) but none in the plus strand. The left ORF encodes two or more noncapsid proteins while the right ORF encodes proteins found in the capsid. The initiation sites for the mRNAs are associated with TATA boxes and the 3′ ends with AATAAA signals. With all three of these viruses the mRNAs are 3′ co-terminal, using a common polyadenylation signal at

about map unit 95. The right ORF constitutes an overlapping transcription unit in which two or three capsid proteins are encoded from different mRNA's. The capsid proteins share the carboxy terminus and differ in the amino terminus. Recently, the complete nucleotide sequence of bovine parvovirus (BPV) was published. BPV shows the expected left and right ORF organization which, based on comparison of sequences, appears to encode noncapsid and capsid proteins, respectively. With BPV there is an additional ORF located in the middle of the genome, the mid ORF. BPV has poly A signals both at map unit 60 and 98, suggesting differences in transcription patterns from other parvoviruses for which the sequence has been determined.

The sequences of the terminal palindromes of the MVM genome have been studied in detail. Comparison of the termini of RF and viral DNA of MVM revealed that the left terminus of MVM exists in a unique conformation while the right end exists in two mutually inverted orientations. Sequence inversions can occur at the termini of parvovirus genomes by the process of hairpin transfer in which a palindromic sequence is nicked on one strand and transferred to the other strand by repair synthesis. The lack of sequence inversions at the left end of MVM suggests that hairpin transfer is not used in processing the left palindrome. MVM encapsidates almost exclusively the minus strand. AAV, which has identical inverted repeats, shows sequence inversions at both ends of the genome and plus and minus strands are encapsidated with equal frequency. In contrast, BPV is intermediate between MVM and AAV with the encapsidation of about 10% plus strands. However, detailed sequence analysis of BPV replication intermediates has not been reported.

BPV has nonidentical palindromes which are similar in secondary structure but not in primary nucleotide sequence with MVM. The presence of sequence inversions at the 5′ terminus of viral DNA of BPV has been established but definitive analysis of the 3′ end has not been presented. The terminal palindromes are clearly essential in the replication of MVM and AAV-2, since deletions abolish replication. The effect of terminal deletions on the replication of BPV has not been reported. The occurrence of identical ends in AAV-2 is thought to be responsible for the encapsidation of both plus and minus strands. The mechanism by which a significant fraction of plus strands becomes encapsidated in BPV, which has nonidentical ends, is not clear and replication models developed for MVM replication do not adequately address BPV replication.

Parvoviruses are heavily dependent on the host cell for their replication. They have been proposed as probes of eukaryotic DNA replication because of their small genome size and thus limited coding capacity. However, recent studies demonstrate that parvovirus genomes are densely packed with information. Multiple nonstructural proteins as well as capsid proteins are encoded which may interact in complex ways in the viral replication cycle. Thus, parvoviruses despite their small size and predicted simplicity present a great deal of scientific challenge.

Detailed analysis of the genetic organization of parvoviruses requires systems in which mutations can be introduced and the phenotypes of the resulting mutants studied. The genomes of AAV-2 and MVM have been cloned into bacterial plasmids and the cloned genomes were infectious, that is gave rise to virions, after transfection into host cells. These clones permitted the genetic analysis of AAV-2 using frame shift and deletion mutants. A genomic clone of AAV-2 was used to demonstrate the occurrence of sequence inversions at both of the identical ends during replication. Further, correction during replication of a deletion within one of the terminal palindromes of AAV-2 was demonstrated, showing that it was not necessary to have both palindromes fully intact.

The overall objective of this work was to define and analyze the genetic functions and replication cycle of BPV. The specific objectives were:

1. Construct and characterize a series of complete genomic clones and determine the infectivity of these clones after transfection into bovine fetal lung (BFL) cells.

2. Determine the structure of the terminal palindromes of BPV virion and RF DNA and study the effects of variations in terminal sequence on the replication of BPV.

3. Construct a series of deletion and frameshift mutations using genomic clones and conduct preliminary characterization of these mutants.

LITERATURE REVIEW

PROPERTIES AND TAXONOMY

Family Parvoviridae includes animal viruses with linear single stranded DNA genomes of molecular weight 1.5 to 2.0 x 10^6 (Siegl et al., 1985). Infectious virions have a density of 1.39 to 1.42 g/cm^3. All members have nonenveloped particles 18-26 nm in diameter and of icosahedral symmetry. The virions are stable at pH values ranging from 3 to 9, withstand heating to 56°C for 60 min and are unaffected by lipid solvents. Hemagglutination (HA) is exhibited by parvoviruses with the exception of Aleutian disease virus (ADV) and goose parvovirus (GPV). This is due to the interaction of the viral capsid with red blood cell glycoprotein(s). Parvoviridae replicate in the nucleus of infected cells. The three genera currently recognized are Parvovirus, Dependovirus and Densovirus.

Members of genus Parvovirus, referred to as autonomous parvoviruses, infect a variety of mammalian hosts. Infection requires the presence of specific host cell surface receptors to mediate binding and internalization of virions (Linser et al., 1977). Internalization is probably by way of coated pits (Linser et al., 1979). Further limitations on virus replication are imposed by the need for progression of the host cell through S-phase (Rhode, 1973) and the need for a factor(s) which

depends on the differentiated state of the cell (Spalholz and Tattersall, 1983). Parvovirus infections are characterized by extensive cytopathic effect (CPE) and cell lysis.

Members of genus Dependovirus, referred to as defective parvoviruses, alos infect mammalian cells. Dependoviruses are dependent for completion of replication on co-infection by a helper adenovirus or herpes virus. Thus, both Parvoviruses and Dependovirus are dependent on "helper" factors with Parvovirus requiring host cell factor(s) and Dependovirus requiring helper virus factor(s). Genus Dependovirus is more commonly known as Adeno-Associated virus (AAV). AAV has the capability of integrating into the host genome upon entry into a cell which is not co-infected with a helper virus. Upon subsequent infection by a helper virus the AAV genome is rescued and replicated. Productive AAV infections do not result in CPE.

Genus Densovirus (DNV) includes single stranded DNA viruses which infect insects in the orders Diptera, Lepidoptera, and Orthoptera. The Densoviruses do not require a helper virus and replication occurs in the nucleus, resulting in formation of dense intranuclear inclusions. The taxonomy of DNV is chaotic due to the lack of standardized tests to determine viral relatedness (Siegl et al., 1985). Host range and tissue tropism have often been used as criteria for naming species. Such biological properties may have lead to incorrect classifications (Siegl et al., 1985).

DNA sequence data is available on members of all three parvovirus genera. Within genus Parvovirus complete sequence data are available on H-1 (Rhode and Paradiso, 1983), MVM (Astell et al., 1983), B19 (Shade et al., 1986), and BPV (Chen et al., 1986b) and partial sequence data on feline panleukopenia virus (FPLV) (Carlson et al., 1985) and canine parvovirus (CPV) (Rhode, 1985). Comparison of these sequences demonstrates that MVM, H-1, FPV and CPV form a closely related group. B19 and BPV are each different from the MVM cluster and from each other. The evolutionary relationship B19 <—> AAV <—> BPV <—> MVM was derived from comparison of the putative amino acid sequences of the open reading frames (ORFs) (Chen et al., 1986b). A region of sequence homology is found conserved between AAV-2 and members of genus Parvovirus. This left ORF region shows 60% homology among AAV-2, BPV, and MVM. The left ORF conserved region encodes nonstructural proteins. A partial sequence of Bombyx DNV has been published (Bando et al., 1987). A sequence homologous to the left ORF conserved region

was found. This region encompassed 300 nucleotides and within the conserved region substitutions were more common at the third positions of codons. The wide conservation of the left ORF homology implies that all three genera of Parvoviridae share a common ancestor. A virus known as RA-1, isolated from the synovial fluid of a person with rheumatoid arthritis, has been tentatively classified as a parvovirus. A partial sequence of this virus was found to have significant homology with BPV (Van Leeuwen, 1986). These data provide strong support for inclusion of RA-1 in genus Parvovirus. This also suggests that a wider grouping of BPV related viruses may exist.

A recent taxonomic study by Mengeling et al. (1986), using data based on indirect immunofluorescence (IFA) and serum neutralization, demonstrates immunological relatedness of several parvovviruses. Common antigens were demonstrated for the group MVM, Kilham rat virus (KRV), porcine parvovirus (PPV), CPV, and feline parvovirus (FPV). The cross reacting antigen(s) was thought to be due to recognition by the antibodies of a nonstructural protein(s). This conclusion was based on the observation that antisera prepared by infection of the natural host were more widely reactive than antisera prepared against purified virions. These data are in agreement with the nucleotide sequence data discussed above which demonstrates conservation of sequences in the left ORF which encodes nonstructural proteins. BPV and GPV were not related by IFA to each other or to any of the other viruses tested.

HOST RANGE AND PATHOLOGY

Tumor cell lines have been the source of many parvovirus isolates. Tumor cells present a population of S phase cells which is very suitable for parvovirus infections. KRV, the prototype of genus Parvovirus, was isolated from a rat cell line and the widely studied parvovirus H-1 was isolated from a human tumor named HEP1 by injection of tissue homogenates into newborn hamsters (Siegl, 1984a). In many cases the natural host of a parvovirus is not known. Parvovirus

LuIII, isolated from a human cell line known as Lu106, has been isolated once only, and a natural host is not known.

Parvoviruses frequently cause latent infection of their host and such latently infected animals may shed parvoviruses into their feces. BPV, lapine parvovirus (LPV) and minute virus of canines (MVC) were all isolated from feces of apparently healthy animals. The mechanism by which latent infection by autonomous parvoviruses is established is not clear. Latent infections usually occur in the presence of a strong antibody response (Siegl, 1984a). Clearly, perpetuation of the virus in latent infections escapes the humoral immune response of the host.

A number of parvoviruses are associated with serious and economically important diseases. GPV causes a hepatitis in goslings which has up to 95% mortality. FPV causes feline ataxia and feline enteritis. Feline ataxia affects newborn kittens who present a susceptible population of dividing brain cells. Aleutian disease of mink, caused by ADV, is an immune complex disease. Infection with ADV leads to a chronic viremia. Antibodies provoked to ADV fail to neutralize the virus so circulation of antigen antibody complexes ensues (Porter, 1981). Deposition of immune complexes in various tissues, including the kidneys, leads to the pathological changes.

The phenomenon of host range variation is well illustrated by CPV (Siegl, 1984b). A world wide outbreak of canine enteritis and myocarditis began in 1978 which was shown to be caused by a parvovirus, CPV. Upon detailed investigation CPV was found to be a variant of FPV. Serological tests clearly demonstrated the relatedness of FPV and CPV. Restriction analysis of replicative form (RF) DNA from infected tissue is a useful means of differentiating FPV and CPV. It is thought that CPV may have arisen during attenuation of FPV for use in feline vaccines. The extreme pathogenicity CPV illustrates the potential of parvoviruses to cause serious disease in a new host.

MVM provides a useful model for the study of host range in cell culture. Two strains of MVM exist. The prototype, which is designated MVM(p), infects fibroblasts. An immunosuppressive strain, designated MVM(i), preferentially infects lymphocytes. In a study of these strains Spalholz and Tattersall (1983) found that both strains bind to the same cell surface receptor which is found on both mouse fibroblasts and lymphocytes. Uptake of ^{125}I labeled virions

could be demonstrated in both the permissive and restrictive cells for each strain. In the restrictive cell viral DNA replication was blocked. This intracellular restriction is unusual as susceptibility to virus infection is usually determined at the cell surface by the presence or absence of viral receptors.

VIRION STRUCTURE

Parvoviruses have been determined by electron microscopy to have 32 capsomeres, suggesting a triangulation number of T = 3 (Karaski, 1966). A recent study of KRV empty capsids by neutron scattering has suggested a T = 1 structure with 60 subunits (Wobbe, 1984). This structure is in better agreement with the molecular weight of KRV empty capsid and with data from MVM suggesting about 60 molecules of viral protein per capsid. The proteins of the KRV empty capsid were found to have two domains. An inner domain of highly basic residues is thought to form a postively charged inner shell capable of neutralizing DNA phosphate. A second hydrophobic domain is thought to provide protein-protein interactions to stabilize an outer shell. Tattersall et al. (1977) have suggested, based on peptide mapping, that the N-terminal region of the minor capsid protein VP-I of MVM, which is highly basic, serves a charge neutralization function. Cross linking studies on H-1 suggest that the minor capsid protein forms a cluster which may constitute a single capsomer (Paradiso, 1983).

The protein composition of MVM full particles consists of three proteins VP-1 to 3 of 83, 64, and 61 kd, respectively (Tattersall et al., 1976). Empty capsids on the other hand have only VP-1 and VP-2. The basis for this difference was shown to be due to a proteolytic cleavage of VP-2 in full but not empty capsids to form VP-3 (Tattersall et al., 1977). The same situation prevails in H-1. A highly conserved glycine rich sequence is found at the proteolytic site. Rhode (1985a) has noted that the high glycine content would cause the amino terminus of VP-2 to exist in a random coil. The random coil might be the substrate for proteolytic cleavage (Carlson et al., 1985). The gly-rich homology is conserved in BPV which has little overall sequence homology with MVM or

H-1. AAV-2, which does not undergo proteolytic cleavage of a capsid protein, does not have the conserved glycine rich sequence (Chen et al., 1986b). These data support the idea that the gly-rich conserved sequence is functionally important.

BPV full virions have four proteins VP-1 to 4 of 80, 72, 62, and 60 kd. All four of these proteins are found as in vitro translation products of BPV specific RNA (Lederman et al., 1983). Sequence data suggests that BPV in fact encodes three proteins and that VP-4 is generated by proteolytic cleavage of VP-3 at the gly-rich conserved site (Chen et al., 1986b). LPV virions also have four capsid proteins (Matsunaga and Matsuno, 1983). Empty LPV capsids contained little or no VP-4 and VP-4 was not detected by immunoprecipitation of ^{35}S-methionine labeled proteins from LPV infected cells. VP-4 was suggested to be generated by proteolysis of VP-3. The function of the "extra" capsid protein of BPV and LPV is not known. Based on comparison of molecular weights of the capsid proteins of BPV and other parvoviruses it seems likely that VP-2 constitutes the "extra" capsid protein.

The finding of empty capsids is a constant feature of parvovirus infections. This demonstrates that capsid formation does not require DNA-protein interactions. Empty capsids do differ in structure from full capsids as shown by the differing susceptibility of full and empty capsids to proteolytic cleavage and by the different effects of protein cross-linking agents on empty and full capsids (Paradiso, 1983).

Several studies demonstrate that virions undergo a maturation process. Myers and Carter (1980) performed pulse-chase studies on AAV-2 to study the encapsidation process. They found that ^{35}S-methionine label is found first in empty capsids and chases into mature virions. A capsid assembly intermediate was isolated which had a density equivalent to mature virions but sedimented at 60S on a neutral sucrose gradient rather than 110S of mature virions. The DNA of this intermediate was sensitive to DNase, suggesting a more open structure than mature virions. The assembly intermediate could be chased into mature virions over several hours. There was no evidence for a pool of excess free progeny single strands.

Muller and Siegel (1983a and 1983b) have developed a system for the in vitro synthesis and encapsidation of single stranded viral DNA using parvovirus LuIII. Nuclei from LuIII infected cells

gently lysed with the detergent Brij 58 were found to incorporate label into RF DNA which chased into mature 110S virions. A nucleoprotein complex sedimenting at 70 to 100S was isolated and characterized as an assembly intermediate. Electron microscopy of these complexes revealed capsids with a linear strand of DNA attached. The DNA in these complexes was largely sensitive to DNase digestion and contained double stranded RF DNA and molecules with mobility intermediate between single stranded and double stranded monomer. This is consistent with involvement of preformed capsids with RF DNA to direct displacement synthesis of single stranded progeny DNA. Rhode (1976) has reported that a mutation in the capsid protein coding region abolishes synthesis of progeny single stranded DNA.

GENOME STRUCTURE OF PARVOVIRUSES

A definitive trait of parvoviruses is the occurrence of palindromes at the 3′ and 5′ termini of the genome. Dependoviruses have ends which are inverted terminal repeats about 145 bases long (Lusby et al., 1980). Within this inverted terminal repeat is a palindrome of 125 bases which is capable of forming a T shaped structure. In contrast to the Dependoviruses the terminal palindromes of autonomous parvoviruses are not identical. The left end (3′ end of minus strand) can fold into a T shaped structure. The shape of the folded left palindrome is the same as AAV-2 despite the lack of nucleotide sequence homology between these viruses. Unpaired bases occur in the 3′ palindrome of all autonomous parvoviruses sequenced to date (Astell et al., 1979). The right palindrome (5′ end of minus strand) can fold into a simple U-shaped structure. MVM and H-1 both have unpaired bases near the axis of the 5′ palindrome while BPV has a fully base paired stem. Densonucleosis viruses are thought to have inverted terminal repeats resembling AAV (Nakagaki and Kawase, 1980).

As previously noted, the genomes of a number of parvoviruses have been sequenced, allowing a comparison of the genomic organization. A major feature is the occurrence of two separate

ORFs. This has been found in AAV and autonomous parvoviruses MVM, H-1 and BPV, as well as in Bombyx DNV. For each of these parvoviruses the right ORF encodes two or three capsid proteins while the left ORF encodes nonstructural proteins. With the exception of the Bombyx DNA genome, all mRNA's are transcribed from the minus strand. Bombyx DNV has an ORF of significant size on the plus strand which may code for a protein (Bando et al., 1987). A shorter ORF occurs within the right end of the left ORF of MVM and H-1 (Astell et al., 1983; Rhode and Paradiso, 1983). This mid ORF only partially overlaps the main left ORF in BPV and probably encodes the nonstructural protein NP-1 (Chen et al., 1986b).

TATA like sequences occur near map unit 4 and 38 in all parvoviruses which have been sequenced (Chen et al., 1986b). The promoter near map unit 38 (P38) in MVM, H-1 and AAV-2 encodes the capsid proteins and P4 encodes nonstructural proteins. An "extra" promoter, located at map unit 18 of AAV-2, has recently been shown to encode nonstructural proteins (Mendelson et al., 1986). The sequence GGPyCAATCT (the CCAAT box) has been identified as a promoter element about -80 nucleotides from the cap site. A CCAAT box is found in the appropriate position of some parvovirus promoters but is not a consistent feature. For example, a CCAAT sequence is found near P38 of parvovirus H-1 but not in MVM. Since these two viruses are highly related (> 70% homology) this would argue against a significant role for this CCAAT sequence. A more consistent feature near parvovirus promoters is the occurrence of GC rich sequences at -50 to -70 and -80 to -100 (Chen et al., 1986b).

Formation of the mature 3' end of mRNA requires processing. Eukaryotic genes lack strong transcription stop signals so transcription apparently stops at various weak stop signals (Birnstiel et al., 1985). The mature 3' end is then formed by an endonucleolytic cleavage followed by the addition of a poly A tail of about 150-200 bases. The sequence AATAAA, which occurs about 20 nucleotides upstream of the poly A addition site, is necessary but not sufficient for 3' end processing (McDevett et al., 1984). Clusters of G/T rich sequences (Birnstiel et al., 1985), a consensus sequence CAPyTG (Berget 1984), or stem and loop secondary structures (Woychik et al., 1984) are variously reported to occur in the region downstream from the AATAAA signal.

The genomes of H-1 and MVM have AATAAA signals only in the vicinity of map unit 95. Apparently all the mRNA's are co-terminal and use the same set of poly A signals. The occurrence of three AATAAA signals in MVM and two in H-1 is of interest. Such clustering of polyadenylation signals could provide redundancy in the 3' end processing mechanism. AAV-2 has AATAAA signals at map 60 and 96. As no mRNA's have been mapped to the map unit 60 region, AAV-2 appears to use only the map 96 signal. BPV has six AATAAA signals at map units 2.5, 60.0, 61.6, 64.6, 74.8, and 98.5. The map unit 2.5 signal is certainly not functional as no mRNA's could terminate there. The cluster of three signals near map 60 is reminiscent of the clustering seen near map unit 95 in MVM and H-1. G/T clusters occur downstream of the map unit 60.0 and 61.6 signals and CACTG occurs after the map unit 64.6 signal. The map unit 98.5 almost certainly is required for processing of mRNA's in the right ORF. The cluster of signals near map 60 could be involved in the processing of left ORF transcripts.

Relative to the rodent parvoviruses, BPV shows several differences in genome structure. BPV is larger than the rodent parvovirus group, 5500 vs. 5100 bases. Much of the size difference seems to occur in the region of the mid ORF which in BPV only partly overlaps the left ORF. BPV shows a different promoter structure in having an apparent promoter at map unit 12. Finally, BPV shows a more complex system of Poly A addition sites.

PARVOVIRUS TRANSCRIPTION

AAV-2 encodes six mRNAs, with a spliced and unspliced mRNA originating from promoters at map units 5, 18, and 38 (Laughlin et al., 1979). The spliced mRNAs all use a common splice from map unit 41 to 49. All six mRNAs are 3' co-terminal at map unit 96. Janik et al. (1984) have demonstrated that the two transcripts originating from P38 encode the three capsid proteins. This was done using recombinant plasmids which contained portions of the AAV-2 genome. A plasmid containing map unit 31-97 was capable of directing the synthesis of all three capsid proteins when

co-transfected with adenovirus DNA. Removal of P38 abolished capsid protein synthesis. A recent paper reports the N-terminal sequences of AAV-2 capsid proteins B and C (Becerra et al., 1985). Capsid protein C was confirmed to initiate from an ATG at nucleotide 2810 on the unspliced mRNA originating from P38. Capsid protein B also appears to to be encoded by the unspliced mRNA originating at P38 and is thought to initiate translation at a threonine codon, ACG. If this finding is verified it would be the first known case of initiation at a non-methionine codon in a eukaryotic system. Capsid protein A is thought to originate within the intron and so is probably encoded by the 2.6 kb unspliced RNA. Overall, this scheme uses two mRNA's to generate three carboxyl co-terminal capsid proteins.

A recent paper has identified a set of four nonstructural (NS) proteins from the P4 and P18 promoters of AAV-2 (Mendelson et al., 1986). An 18 amino acid synthetic peptide was synthesized from the region of the common intron. Antibodies against this peptide identified four proteins designated Rep 40, 52, 68, and 78. The two smaller proteins are assumed to originate from P18 and the larger two from P5. This organization of the left ORF of AAV-2 resembles the right ORF in generating a nested set of proteins with partially shared amino acid sequence.

Among the autonomous parvoviruses the RNA splicing pattern of MVM has been studied in the greatest detail. Based on nucleotide homology, the splicing pattern of MVM appears to hold true for H-1 and the other viruses related to MVM. Three transcripts designated R1, R2, and R3, have been mapped in MVM (Pintel et al., 1983). These transcripts are 3′ co-terminal at map unit 95 and have a common intron from map units 46 to 48. Transcripts R1 and R2 originate from P5. R1 is spliced only at the common intron while R2 has an additional large splice from map unit 9.5 to 38.5. Transcript R3 originating from P38 is the most abundant. The original S1 mapping data demonstrated that the 5′ leader of the R3 transcript is heterogenous in length, presumably due to different splicing patterns at the common intron. A recent study has confirmed this. Morgan and Ward (1986) synthesized cDNA's using an end labeled oligonucleotide which hybridized near the intron junction. Two specific bands of the expected size for R3 transcripts were eluted from a polyacrylamide gel and sequenced by the Maxam-Gilbert method. Two splicing patterns were confirmed by this method. Four possible splicing patterns were predicted for this region by a

computer search for consensus splicing signals. Oligonucleotides were synthesized which hybridized to each predicted splice junction. Hybridization of these probes to viral mRNA under stringent conditions revealed that three splice patterns were used at the common intron. The three patterns occurred in all three RNA transcipts.

The coding functions of the MVM and H-1 transcripts have been assigned by several techniques. Transcript R3 has been shown to encode the two capsid proteins of MVM by construction of an MVM-bovine papilloma virus chimera (Labienic-Pintel and Pintel, 1986). The MVM sequence of this plasmid contained only P38 and the right ORF but encoded both capsid proteins in the usual ratio. This data agrees with hybrid arrested translation (HART) data in H-1 (Rhode and Paradiso, 1983). The work of Morgan and Ward (1986) demonstrates that the small capsid protein VP-2 arises from the most frequent splice pattern which removes the in phase ATG used to initiate VP-1, the larger capsid protein. The origin of VP-2 in H-1 has been established by direct protein sequencing and is in agreement with the RNA mapping data.

A large nonstructural protein has been identified in MVM and H-1 of molecular weight 83,000 on polyacrylamide gels (Cotmore et al., 1983; Paradiso, 1984). HART data shows that this protein originates from transcript R1. A stop codon in the ORF encoding NS-1 occurs before the common intron so the C-terminus of this protein does not vary (Morgan and Ward, 1986). Transfection experiments using chloramphenicol acetyltransferase (CAT) expression plasmids under the control of P38 of H-1 demonstrate that NS-1 functions as a *trans*-acting transcriptional activator of P38 (Rhode, 1985b). A small noncapsid protein NS-1 has been identified in MVM by immunoprecipitation of in vitro translation products using mRNA from MVM infected cells (Cotmore et al., 1983). Transcript R2 has been shown to encode NS-2. Cotmore and Tattersall (1986) constructed bacterial fusion proteins containing specified regions of the left ORF of MVM. Antibodies against these fusion proteins revealed that NS-2 shares the amino terminal coding region of NS-1 and then is spliced onto a short open reading frame which overlaps the carboxy terminus of NS-1. The function of NS-2 is unknown.

Preliminary S1 mapping data is available for BPV (Burd, 1982). The major transcripts are a 2.6 kb family originating near map unit 14 and splicing onto the right ORF. These RNA's almost

certainly encode the capsid proteins of BPV based on their location in the right ORF. The predicted amino acid sequence for the right ORF of BPV contains several homologies with the capsid proteins of MVM and H-1 (Chen et al., 1986b). The 2.6 kb family is the most abundant BPV mRNA, which is consistent with the assignment of encoding the capsid proteins. A 1.05 kb family of mRNAs originates near map unit 14 and splices from about map unit 16 to 43.5. These mRNAs terminate at map unit 59 and thus probably use the Poly A addition signals located in that region. The map 43 to 59 region is the mid ORF of BPV and could encode a nonstructural protein about the size of NP-1 in BPV. Recently, a nonstructural protein of BPV of 76 kd has been identified (Lederman et al., 1987). A 2.25 kb transcript which originates at map unit 14 and contains the left ORF may encode this protein. The 2.25 kb transcript has a small intron near map unit 40 and terminates at map unit 60. A set of unspliced mRNAs originating at map unit 14 with the same 5′ terminus but different 3′ termini was also found. These mRNAs are contained within the left ORF and thus presumably are involved in the translation of noncapsid proteins.

These preliminary data make clear that the transcription of BPV differs from the MVM pattern. Since MVM is often taken as a paradigm for the autonomous parvoviruses, it is important to note these differences. BPV shows spliced mRNA's terminating at map unit 60 and sequencing data reveals the presence of Poly A addition signals at this location. The BPV capsid mRNAs do not appear to originate from a promoter near map unit 38. Overall, a more complex pattern of mRNA formation and splicing is evident in BPV.

PARVOVIRUS REPLICATION

A proposed replication model for autonomous parvoviruses is given in Fig. 1 (Hauswirth, 1984). Overall, replication can be divided into three steps. Step one is the conversion of input single strand DNA to a double strand parental RF. Step 2 is the amplification of parental RF. Step 3 is the formation of progeny single stranded DNA.

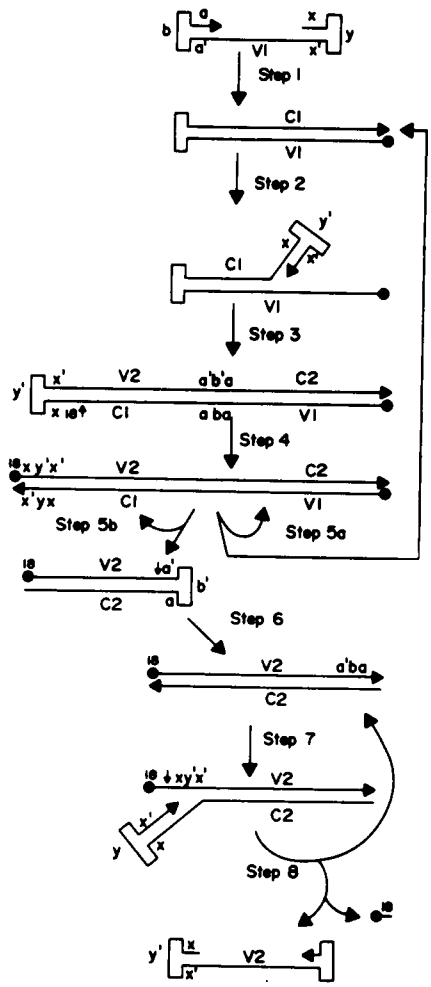


Figure 1. A model for the replication of autonomous parvoviruses.

Conversion of single stranded input DNA is thought to occur by self priming from the 3′ end of the minus strand. This is supported by the ability to replicate single stranded viral DNA in vitro using *E. coli* DNA polymerase I Klenow fragment. Cloning of S1 nuclease resistant fragments from single stranded BPV DNA demonstrates that the terminal palindromes do exist in hairpinned conformation (Chen et al, .1986b).

Amplification of parental RF is thought to occur by a complex series of steps possibly using a dimer intermediate. Dimer RF is an invariable finding of intracellular RF pools and pulse chase data shows that it chases into monomer RF. The dimer can be formed by hairpin folding of the 3′ end of parental RF and continued replication. Repair of the hairpin is proposed to occur by a process called hairpin transfer, after a proposal first made by Cavalier-Smith (1974). A nick made on the complementary strand permits displacement synthesis. Hairpin transfer results in the occurrence of two mutually inverted terminal sequences, designated flip and flop (Hauswirth, 1984).

Resolution of the dimer intermediate is depicted here as occurring by displacement of the V2/C2 hairpin monomer by asymmetric synthesis initiated at the 3′ end of V1/C1. This asymmetric replication is proposed to be mediated by a protein covalently attached to the 5′ end of V2. The designation "18" in the figure at step 4 indicates that the nick site occurs 18 bases beyond the mature virion terminus. This is supported by the finding that the 5′ terminus of MVM RF DNA is 18 bases longer than the terminus of viral DNA (Astell et al., 1985).

The V2/C2 hairpin monomer has an inverted 3′ hairpin relative to the input. Hairpin transfer (step 6) results in reforming the input 3′ conformation. Sequence analysis of MVM virion and RF DNA established that the 3′ end exists in a unique conformation while the 5′ end exists in flip and flop conformations. Preservation of the 3′ end conformation necessitates the use of a dimer replication intermediate (Astell et al., 1985). Models with only monomer length RF DNA intermediates predict sequence inversions at both the 3′ and 5′ ends.

The final step, progeny single stranded DNA synthesis, is thought to occur by displacement synthesis. There is evidence that displacement synthesis is mediated by interaction of empty capsid with the single stranded DNA. No free pool of single stranded progeny DNA can be found in AAV-2 (Myers and Carter, 1980) or MVM (Richards et al., 1977) infected cells, which is consistent

with the concomitant synthesis and encapsidation of progeny single stranded DNA. Muller and Siegl (1983b) were able to demonstrate that LuIII progeny virion DNA appeared in DNase resistant 110S particles after a 30 second pulse label. The existing replication models are largely based on data from MVM which encapsidates almost exclusively minus strands. Modifications of this model may therefore be necessary to accomodate the encapsidation of significant amounts of plus strand by viruses such as BPV and LuIII.

GENOMIC CLONING AND GENETIC ANALYSIS OF PARVOVIRUSES

The genomes of AAV-2 (Samulski et al., 1982; Laughlin et al., 1983) and MVM (Merchlinsky et al, 1983) have been cloned into plasmid vectors. Transfection of the clones into host cells results in the rescue and replication of the viral genome and the production of infectious progeny virus. Samulski et al. (1982) note that integration into the host cell genome is a regular part of the replication cycle of AAV and that cloned genomes may be rescued from the plasmid by the same mechanism used for integrated genomes. MVM, an autonomous parvovirus, is not thought to integrate into host cell DNA so the mechanism of rescue of cloned MVM DNA cannot be explained in the same way. Merchlinsky et al. (1983) suggest that rescue involves enzyme activities which are involved in the processing of dimer replication intermediates during normal replication.

Genomic clones have been particularly useful in genetic analysis of AAV-2. A series of deletion and frame shift mutants were constructed and used to define several mutant phenotypes (Hermonat et al., 1984; Tratschin et al., 1984a). Deletions or frameshifts within the left ORF abolished the rescue and replication of AAV DNA and were designated *rep-*. Deletions within the right ORF did not prevent the rescue and replication of viral DNA but capsid protein synthesis was abolished. These mutants were also defective in producing progeny single stranded DNA,

probably due to the role of the capsid proteins in the displacement synthesis of progeny single stranded DNA. The right ORF mutants were designated *cap*-. A third class of mutant designated *lip*-, for low infectious particles, (Hermonat et al., 1984) or *inf*- (Tratschin et al, 1984a). was associated with mutations in the middle of the genome. To date, the genomic clone of MVM has not been used in a similar genetic analysis probably due to lower transfection efficiency.

Chapter I

INFECTIOUS GENOMIC CLONES OF BOVINE PARVOVIRUS

INTRODUCTION

Parvoviruses are small icosahedral viruses with single stranded DNA genomes. They are classified as defective or autonomous based on the requirement for a helper virus to undergo lytic infection (Siegl et al., 1985). The palindromic termini of parvovirus genomes are folded into stable hairpin structures which provide *cis* signals necessary for replication and encapsidation (Faust and Ward, 1979). The terminal palindromes of AAV, a defective parvovirus, are identical while the palindromes of autonomous parvoviruses are nonidentical. The termini of AAV exist in either of two sequence inversions, designated flip and flop, which arise from hairpin transfer during replication (Samulski et al., 1982). A schematic representation of sequence inversion is given in Fig. 1. The 5′ end of MVM exists in flip and flop forms but the 3′ end has been shown to have a unique sequence orientation (Astell et al., 1985). The autonomous parvovirus BPV has a genome of 5491

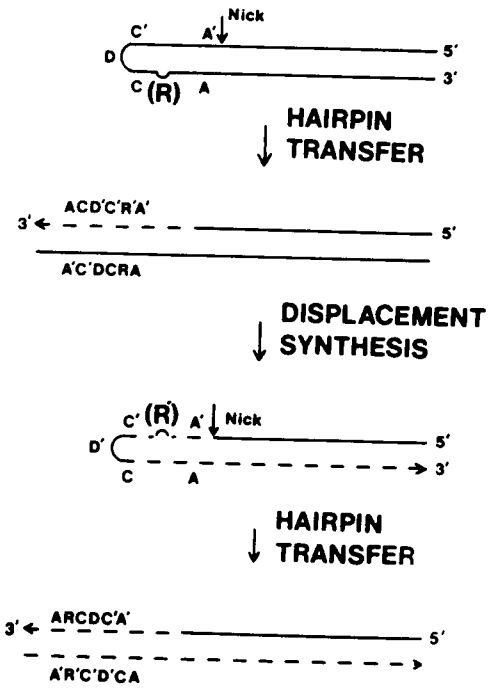


Figure 1. The origin of the flip and flop sequence conformations: The process of hairpin transfer consists of nicking one strand near the border of the palindrome to expose a 3′ OH. DNA synthesis then leads to transfer of the palindrome to the daughter strand with inversion of the sequence. Further DNA synthesis results in formation of a new hairpin duplex. Note that the unpaired base sequence R in the flip sequence is replaced by its complement R′ on the opposite side of the hairpin. If a restriction nuclease site occurs as part of such a looped out region the flip and flop conformations of the extended duplex will cut at different sites (Rhode and Klaassen, 1982).

nucleotides (Chen et al., 1986b) which shows little overall homology with the DNA sequence of other parvoviruses. Thus, the study of BPV has the potential to enlarge our understanding of parvoviruses.

The small size of parvovirus genomes 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 (Samulski et al., 1982; Laughlin et al., 1983 and Merchlinsky et al., 1983). Cloning of the genome of a parvovirus permits the easy isolation of large amounts of DNA by transformation of *E. coli* with the recombinant plasmid. Infectious molecular clones have been useful in investigating the genetics of AAV-2 by providing the double stranded DNA necessary for in vitro mutagenesis techniques such as deletion analysis and frame shift mutations (Hermonat et al., 1984; Tratschin et al., 1984a).

Merchlinsky et al. (1983) attempted to clone the monomer RF DNA of MVM and obtained clones which had intact 3′ ends but were deleted at the 5′ end. Using overlapping clones of the left side of the genome from RF DNA and a right end clone from in vitro replicated virion DNA they were able to construct an infectious clone of MVM in the plasmid pBR322. An alternative approach for the cloning of parvovirus genomes is the use of reannealed plus and minus strand virion DNA. AAV encapsidates equal numbers of plus and minus strands. Among the autonomous parvoviruses encapsidation of plus strands ranges from about 1% in MVM to 50% in LuIII. BPV encapsidates about 10% plus strands. AAV-2 infectious clones have been prepared from reannealed virion plus and minus strands. Samulski et al. (1982) used GC-tailing to insert the intact duplex AAV-2 genome into pBR322. The resulting plasmid, pSM620, was found to be infectious. Laughlin et al. (1983) attached BglII linkers to the genomic termini in an attempt to clone the genome in one step. However, this approach did not yield full length clones. They were able to clone the two halves of the genome using the single HindIII site and BglII linkers. These two plasmids were then recombined through the HindIII site to yield the infectious genomic clones pAV1 and pAV2.

In this report we present data on the construction of molecular clones of the autonomous parvovirus BPV which are infectious and do not undergo deletions within the terminal palindromes

during propagation in the widely used *E. coli* host JM107. The cloning strategy described here uses reannealed plus and minus strand virion DNA and blunt ended cloning into the multiple cloning site of pUC8. The availability of protocols for plasmid sequencing and forward and reverse primers (Vieira and Messing, 1982) allows for rapid verification of the 3′ and 5′ end nucleotide sequences of individual clones. This approach for clone construction will be useful in the development of infectious genomic clones of other parvoviruses which encapsidate both strands.

MATERIALS AND METHODS

Cell Culture and Virus Propagation

BPV was propagated in either bovine fetal lung (BFL) cells or buffalo lung (BUL) cells grown in monolayer culture and maintained in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS) as described by Parris and Bates (1976).

Clone Construction and Plasmid Propagation

Ligation of insert DNA to vectors of the pUC series (Vieira and Messing, 1982) was performed by standard methods (Maniatis et al., 1982). Vectors were routinely treated with calf intestinal phosphatase to reduce the background of nonrecombinant clones. Some ligations were performed using isolated restriction fragments in melted low melt agarose gel chunks as described by Struhl (1985). Transformation was done according to the high efficiency procedure of Hanahan (1983) using *E. coli* strain JM107. Transformed cells were plated on LM plates containing ampicillin, 50 μg/ml, the Lac inducer IPTG and the chromogenic Lac substrate Bluo-Gal (BRL,

Gaithersburg, MD) for detection of recombinant clones by insertional inactivation (Vieira and Messing, 1982). Plasmid isolations were done using the small and large scale procedures of Rodriguez and Tait (1983). Plasmid DNA used in transfections was purified by banding once in a cesium chloride-ethidium bromide gradient (Maniatis et al., 1982). Recombinant clones were screened by restriction digestion and the identity of BPV inserts was verified by Southern blotting using ^{35}S-dATP labeled nick translated BPV DNA as the probe.

Transfection of Bovine Cells

BFL or BUL cells were seeded at 7 to 8 x 10^5 per 60 mm petri plate and used after 24 h for transfection with recombinant plasmids. Transfections were done by either the DEAE-dextran procedure essentially as described by Lopata et al. (1984) or by the calcium phosphate ($CaPO_4$) procedure as described by Perbal (1984). Briefly, 4 h prior to transfection the medium on the cell cultures was replaced with fresh medium. For the DEAE-dextran method the medium was then aspirated and replaced with 1.5 ml of MEM containing 200 µg/ml of DEAE-dextran (average MW 500,000) and sample DNA as indicated in individual experiments. $CaPO_4$ precipitates were formed by mixing sample DNA and sufficient salmon sperm carrier DNA to make 10 µg of total DNA in 450 µl HeBS (21 mM Hepes buffer; 140 mM NaCl; 5 mM KCl; 0.82 mM Na_2HPO_4; 5.5 mM dextrose; pH 7.05) and then adding 50 µl of 1.25 M $CaCl_2$ dropwise with shaking. After 20 min at room temperature the precipitates were added to the cell culture medium. 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 DMSO shock was performed using 1 ml per plate of 10% DMSO in MEM (DEAE-dextran method) or 20% DMSO in MEM ($CaPO_4$ method) for 5 min. After removal of the medium the cell layers were again washed with MEM and 5 ml of fresh MEM with 10% FCS and supplemented with fungizone (1.25 µg/ml) was added. For plaque assays an overlay of 1% Seaplaque (FMC Corporation, Rockville, ME) low melt agarose with MEM containing 10% FCS and supplemented with fungizone (1.25 µg/ml) was used.

Analysis of Infectivity

Cell layers were observed for up to 10 days for BPV specific cytopathic effects (CPE). Plaques were counted 8 to 10 days after transfection after staining cell layers with 1% crystal violet. Indirect immunofluorescence staining (IFA) was done using coverslip cultures. The coverslips were removed from the dishes at various times after transfection, rinsed with PBS and fixed in -20°C methanol. In this procedure the first antibody was an antiserum prepared against purified BPV capsids (rabbit 0118) and the second antibody was fluorescein conjugated goat antirabbit antibody. For hemagglutination (HA) assays, transfected cell layers were scraped into the medium, and frozen and thawed three times to disrupt the cells. The HA assay was performed according to the microtiter method using human type O cells (Bates et al., 1972). Viral proteins were analyzed by immunoprecipitation of ^{35}S-methionine labeled proteins from transfected cell lysates using rabbit 0118 serum or serum from a calf experimentally infected and immunized with BPV (calf 86). Calf 86 serum recognized both capsid and noncapsid proteins of BPV. For labeling the proteins, the medium was changed to MEM with 10% dialyzed FCS 12 h prior to the addition of 20 μCi/ml of ^{35}S-methionine. Cell lysates were prepared by the method of Bloom et al. (1982) 24 h after the addition of the radioactive label. Low molecular weight DNA was extracted by a modified Hirt extraction method which employs proteinase K (Siegl and Gautchi, 1978). Virus stocks were prepared from cultures transfected with various clones by freezing and thawing the cell layers and medium three times. These stocks were used to infect additional cell cultures for the preparative isolation of progeny viral DNA as previously described (Chen et al., 1986b).

Plasmid Sequencing

The termini of BPV inserts in recombinant plasmids were sequenced by the dideoxy method with ^{35}S-dATP as the label (Sanger et al., 1977). Plasmid DNA, 1-2 μg, was denatured with sodium

hydroxide and precipitated before annealing to primer. Forward and reverse primers which anneal just outside the multiple cloning region of pUC8 were used to sequence both termini (Vieira and Messing, 1982). Annealing of primer was done for 15 min at 37°C with about 30 ng of primer in tris-magnesium buffer (10 mM Tris-HCl; 10 mM $MgCl_2$; pH 8.3). Sequencing reactions were then run using 1-2 units of Klenow fragment, 10 μCi ^{35}S-dATP per reaction with nucleotide mixtures which had been empirically optimized. Reactions were run for 15 min at 42°C. After addition of all four unlabeled deoxynucleotides, samples were again incubated for 15 min at 42°C, followed by the addition of stop mixture (deionized formamide with 20 mM EDTA, 0.03% xylene cyanol FF and 0.03% bromphenol blue). Electrophoresis was performed on 0.4 mm buffer gradient gels (Biggin et al., 1983) run at constant power of 60 watts. Gels were dried without fixing and autoradiographed for 1-5 days at room temperature.

Southern Blotting with Alkaline Transfer

DNA in agarose gels was transferred directly to Zeta-Probe (BioRad, Richmond, CA.) cationized membranes by capillary transfer with 0.4 M NaOH (Reed and Mann, 1985). After transfer, blots were rinsed briefly in 2X SSC, placed into a heat sealable bag containing 9.5 ml of 4X SSPE (0.72 M NaCl; 0.04 M sodium phosphate, pH 7.0; 0.04 M EDTA), 0.5% nonfat dry milk, 1% SDS and 50% formamide and prehybridized at 42°C for 2 hours. Probe (1-2 x 10^6 CPM) was denatured in 0.5 ml deionized formamide for 10 min at 60°C, added to the prehybridization buffer and hybridized 18-24 h at 42°C. Probes were prepared by nick translation of BPV virion DNA using ^{35}S-dATP as the label. Specific activity of about 10^7 cpm/μg was usually obtained. Blots were washed at room temperature for 5-15 min each in solutions containing 0.1% SDS and SSC concentrations of 2x, 1x, 0.5x, and 0.1x successively. A stringency wash was performed in 1% SDS, 0.1X SSC for 30 min at 50°C. Blots were autoradiographed at room temperature.

RESULTS

In this dissertation the convention of Armentrout et al. (1978) is used: the 3′ terminus of the minus strand is referred to as the 3′ end or map origin.

Cloning of the BPV Genome

I first attempted to clone RF DNA extracted by a modified Hirt procedure from BPV-infected BFL cells. RF DNA was repaired using *E. coli* DNA Polymerase I Klenow fragment, ligated to SalI linkers and inserted into SalI digested pUC8. We were unable to obtain any full length clones by this method. RF DNA was checked for free termini by attempting to clone the terminal EcoRI fragments. BPV has two EcoRI sites which occur at map 17 and 92. RF DNA was ligated to SalI linkers, digested with SalI and EcoRI, and then ligated to pUC8 which had been digested with SalI and EcoRI. Screening by restriction digestion and agarose gel electrophoresis of 200 clones yielded four clones with 3′ ends, designated p3′R, and none with full length 5′ ends. Most clones contained inserts which did not correspond in size to either the 3′ or 5′ ends and these were not further analyzed. These data suggested that the 5′ end of BPV RF DNA may be blocked as was observed for MVM (Merchlinsky et al., 1983). If the blockage is due to a 5′ terminal protein, treatment with Bal 31 exonuclease may remove such a protein and allow cloning of the terminal sequences. This approach was used to prepare an infectious clone from H-1 RF DNA (Rhode, 1985b) which is known to have a terminal protein (Revie et al., 1979). However, I was unable to isolate any full length clones by this method.

The second approach I utilized was the addition of linkers to double stranded viral DNA (reannealed plus and minus strands). The DNA was repaired with Klenow fragment, ligated to SalI linkers, and inserted into SalI digested pUC8. In agreement with previous work on AAV-2 (Laughlin et al., 1983), we were unable to clone the full length BPV genome in one step following

linker ligation. The double stranded BPV DNA was checked for accessible termini by separately cloning the terminal EcoRI fragments. After ligation of SalI linkers, the BPV DNA was digested with SalI and EcoRI, and then ligated to pUC8 which had been digested with EcoRI and SalI. Clones containing either the 3′ or 5′ EcoRI fragments, designated p3′V and p5′V, were readily obtained, which demonstrated that the genomic ends of double stranded virion DNA are free.

The third approach I used was direct blunt end cloning of double stranded BPV virion DNA to vector. The cloning strategy is outlined in Fig. 2. BPV DNA was isolated from virions and purified on neutral sucrose gradients as previously described (Chen et al., 1986b). The double stranded DNA fraction (reannealed plus and minus strands) was repaired with Klenow fragment to maximize the number of blunt ends available for cloning. Initially, this DNA was ligated into the blunt ended SmaI site of pUC8 (Fig. 2a). Screening of 57 white colonies yielded seven clones, designated pGCSma, which had inserts that migrated to the position of BPV DNA markers on agarose gels (Fig. 3). Each of these seven clones were confirmed to have BPV inserts by Southern blotting. This construction yields clones in which the insert is flanked at one end by restriction sites which are not present in the BPV genome but at the other end the BPV insert is flanked by an EcoR1 site from the vector. Since BPV has two EcoRI sites, the pGCSma clones cannot be digested to release an intact BPV insert. In order to construct clones from which the BPV insert could be excised intact, I took advantage of the fact that BPV lacks both HindIII and SalI sites which are present in the multiple cloning site of pUC8 (Fig. 2b). These sites are separated by a PstI site. The pUC8 vector was digested with PstI and then blunted by digestion with S1 nuclease. The effectiveness of the S1 nuclease digestion was monitored by the appearance of white colonies after ligation and transformation of a portion of the vector. BPV DNA prepared as described above was ligated to the blunted PstI site and transformants were screened for apparently full length BPV inserts by digestion with SalI and HindIII. Screening of 100 colonies gave three clones, designated pGC, with apparently full length inserts. Digestion of these clones with PstI and SalI or PstI and HindIII was used to confirm the presence of an intact BPV genome and to determine the orientation of the inserts within the vector (Fig. 4).

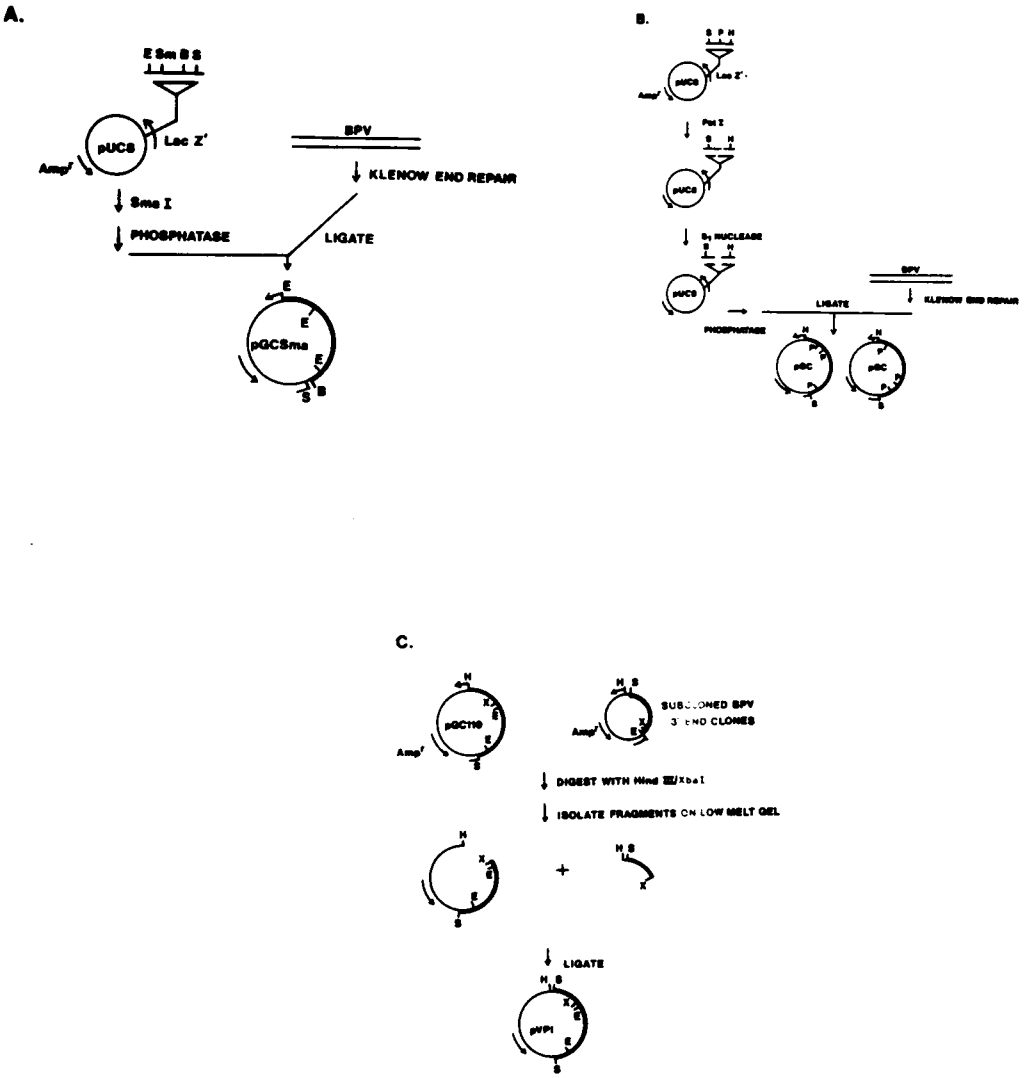


Figure 2. Strategy for cloning the BPV genome into pUC8: Restriction sites of the vector point outward while restriction sites of the BPV insert point inward. (A) Clones of the pGCSma series were constructed by ligation into the SmaI site of pUC8. (B) Clones of the pGC series were constructed by ligation into the blunted PstI site of pUC8. (C) Clones of the pVPI series were constructed by replacing the 3′ terminal XbaI fragment of pGC119 with other subcloned BPV 3′ end XbaI fragments. Abbreviations for restriction enzyme sites are as follows: EcoRI (E); SmaI (Sm); BamHI (B); SalI (S); PstI (P); HindIII (H); and XbaI (X).

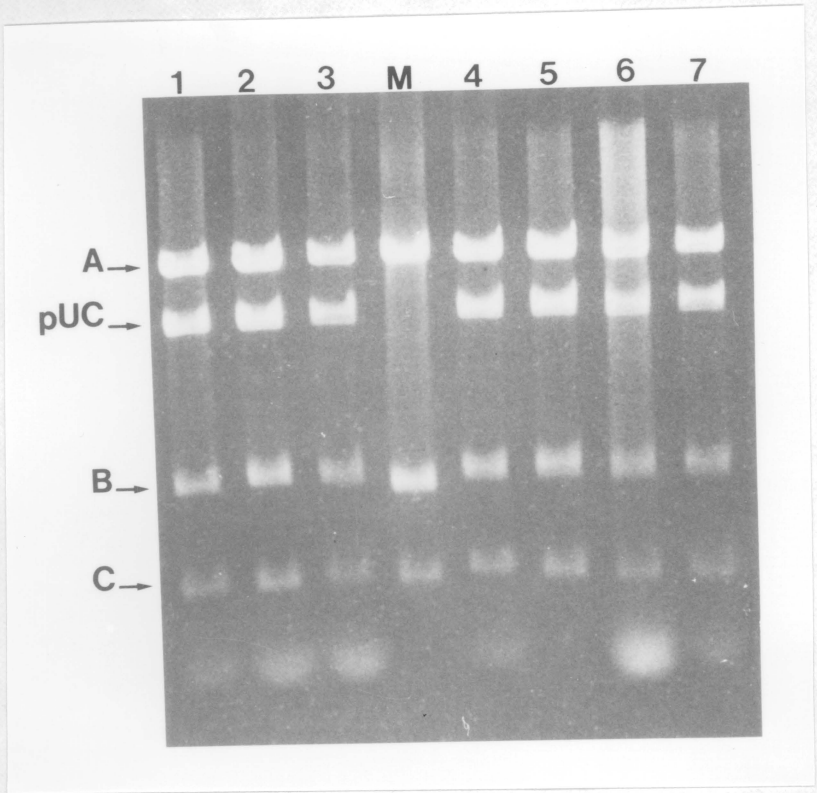


Figure 3. Restriction enzyme analysis of DNA from the pGCSma clones: The DNA was digested with SalI and EcoRI, separated on a 1.0% agarose gel and stained with ethidium bromide. Lanes 1-7 contain pGCSma17, 20, 22, 32, 41, 51, and 57, respectively. The marker (M) was double stranded BPV virion DNA digested with the same enzymes. The positions of pUC8 and the EcoRI A (4.10 kb), B (0.98 kb), and C (0.45 kb) fragments of BPV are indicated.

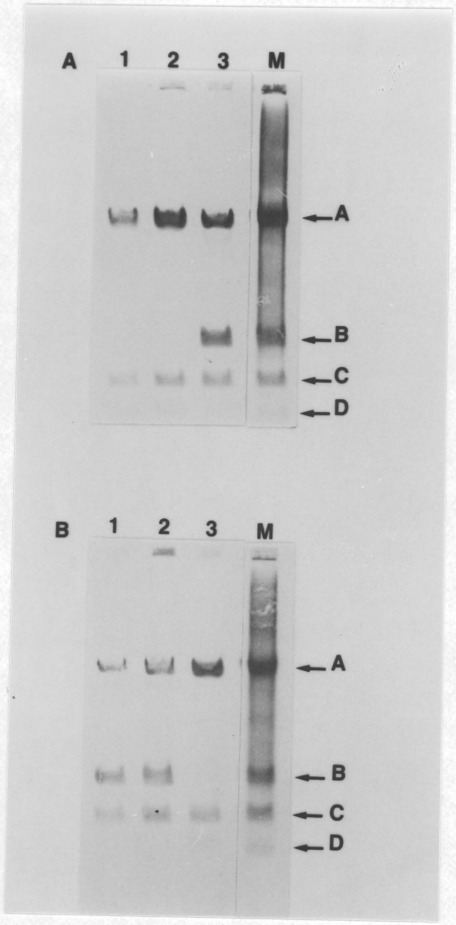


Figure 4. Restriction enzyme analysis of DNA from the pGC clones: The DNA was digested with HindIII and PstI (A) or SalI and PstI (B) and separated on a 1.0% agarose gel. The gels were blotted to Zeta-Probe membranes and hybridized with ^{35}S-dATP labeled nick translated BPV DNA. Lanes 1 contain pGC119, lanes 2 contain pGC127 and lanes 3 contain pGC167. The marker (M) was double stranded BPV virion DNA digested with the same enzymes. The PstI A (3.88 kb), B (0.87 kb), C (0.48 kb), and D (0.29 kb) fragments of BPV are indicated. PstI D is the 3′ terminal fragment and PstI B is the 5′ terminal fragment.

The apparently full length clones were characterized by sequence analysis using the dideoxy method. This analysis showed that deletions were present at either end of the cloned BPV genomes (Table 1). The data also reveals the conformation of the terminal sequences for each of these clones. The 3′ terminus for all of the clones in the pGCSma and pGC series was in the flip orientation. Two of the clones, pGCSma17 and pGC167, were found to have the 5′ terminus in the flop conformation. The existence of the 5′ flop conformation in BPV DNA was previously demonstrated when the size of end labeled 5′ terminal NdeI fragments was measured (Chen et al., 1986b). Three of the clones have 5′ termini which extend past the previously assigned 5′ terminus of BPV DNA (Chen et al., 1986b).

Clone pGC119, which has a 3′ deletion of 27 bases, was used in the construction of a genomic clone with an intact 3′ end. The source of the intact 3′ end was a previously cloned EcoRI 3′ end fragment, p3′R45. This clone was digested with HindIII and XbaI and ligated to pGC119 digested in the same way (Fig. 2c). BPV DNA has a single XbaI site at map unit 15 while there are none in pUC8. This construction gave a clone, pVPI1, with an intact 3′ end and the BPV genome flanked at both ends by a SalI site (Fig. 2c). When pVPI1 was sequenced at both termini the 5′ end was unchanged, as expected, but the 3′ end had an undeleted sequence in the flop conformation. The EcoRI subclone, p3′R45, used as the donor for the intact 3′ end was sequenced and unambiguously determined to be in the 3′ flip conformation. Thus, the terminal sequence had apparently undergone an inversion during the cloning steps. The cloning procedure was repeated and a clone, pVPI2, was obtained which had an undeleted 3′ end in the flip conformation. During characterization of 3′ EcoRI subclones, I isolated a single clone, p3′V8, which was in the 3′ flop conformation and had a 34 base terminal deletion. This subclone was transferred to pGC119 as above to yield clone pVPI1Δ34. Two clones deleted at the 3′ end by 52 and 79 bases, respectively, were constructed by ligating deleted 3′ EcoRI subclones to pGC119 to give pVPI2Δ52 and pVPI2Δ79. The 3′ termini of these clones were confirmed by sequencing to be in the flip conformation.

Table 1. Summary of DNA sequencing of the termini of BPV genomic clones and determination of infectivity.

Clone	3′ end[1]	5′ end[2]	CPE[3]	plaques per 0.2 μg ± S.D.[4]
pGCSma17	flip, -19	flop, -3	pos	nd[5]
pGCSma20	flip, -3	flip, -9	pos	nd
pGCSma22	flip, -18	flip, +8	pos	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	pos	14 ± 7
pGC127	flip, -32	flip, -7	pos	5 ± 2
pGC167	flip, -14	flop, +26	pos	64 ± 27
pVPI1	flop, 0	flip, +1	pos	65 ± 23
pVPI1Δ34	flop, -34	flip, +1	pos	6 ± 5
pVPI2	flip, 0	flip, +1	pos	50 ± 21
pVPI2Δ52	flip, -52	flip, +1	neg	nd
pVPI2Δ79	flip, -79	flip, +1	neg	nd

[1]Sequence orientation is designated as flip or flop followed by the number of bases deleted relative to the virion terminus (Chen et al. 1986b).

[2]Terminology is same as for the 3′ end. Here a + sign indicates that the sequence of the clone extends past the previously assigned 5′ end of BPV virion DNA.

[3]Denotes the presence or absence of BPV specific cytopathic effect after transfection of the genomic clone.

[4]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. Results are given followed by the standard deviation.

[5]Not determined.

Determination of Infectivity

Several parameters were used to assess the infectivity of the genomic clones. IFA provides evidence for synthesis of viral proteins. Clones pGCSma17, pVPI1, pVPI1Δ34, and pVPI2 all gave positive IFA results (Fig. 5). When transfections were done using $CaPO_4$ the pattern of intranuclear fluorescence was indistinguishable from that of cells infected with BPV. However, transfections in the presence of DEAE-dextran resulted in cytoplasmic fluorescence in both sample and control cultures. In spite of this nonspecific background, specific nuclear fluorescence was readily distinguishable. HA detects the presence of full or empty virions. The clones which were positive by IFA were also positive for HA, with titers greater than 1:4096. Positive results by IFA and HA establish expression of the viral genome but do not prove that infectious virus is being produced. Presumptive evidence for the formation of progeny virus is the observation of CPE in cells transfected with a genomic clone. CPE occurred in cell cultures transfected with each of the four genomic clones described above within 5-6 days after transfection while CPE is observed in 1-2 days after infection with the virus. This lag in the appearance of CPE after transfection of genomic clones presumably reflects the time required for rescue of the BPV genome from the recombinant plasmid. Several other genomic clones were found to yield CPE upon transfection (Table 1).

Plaque assays were performed to assess quantitatively the effects of variations in terminal structure on infectivity (Table 1). Clones pVPI1, pVPI2 and pGC167 all have similar infectivity. Evidently the 14 base deletion at the 3′ end of pGC167 had little effect on infectivity. Alternatively, the shorter 5′ end of both clones pVPI1 and pVPI2 relative to pGC167 could lower infectivity. Clones pGC119, pGC127 and pVPI1Δ34 all show clearly reduced infectivity relative to clones with intact 3′ ends. That this reduction in infectivity was due to the 3′ end deletions is strongly suggested by comparing the infectivity of pVPI1 and pVPI2 with the infectivity of pVPI1Δ34 and pGC119, respectively. These clones differ only in the structures of the 3′ end. Clearly, these deletions at the 3′ end of up to 34 bases reduced but did not abolish infectivity. Also, the flip and flop forms of the

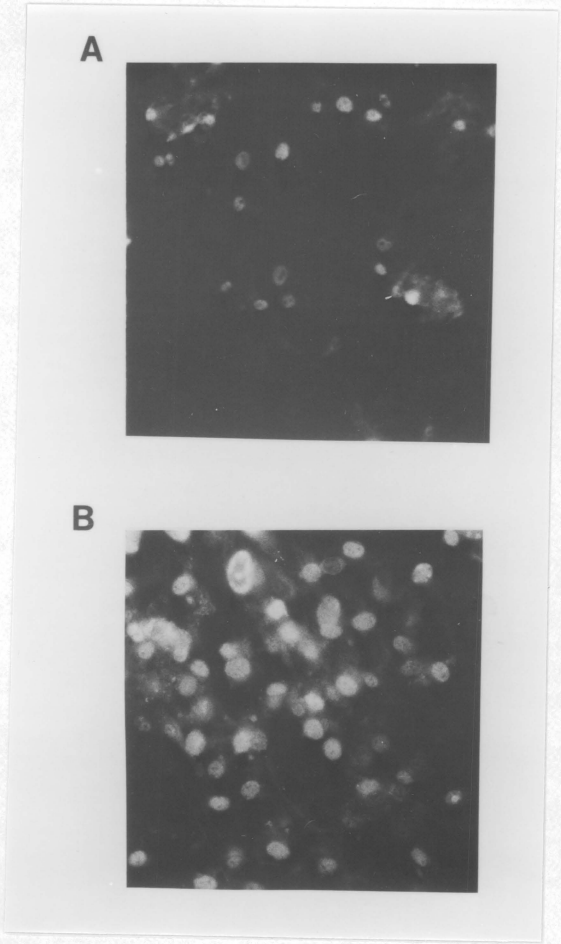


Figure 5. Capsid antigen production from clone pGCSma20: Cell cultures were analyzed by indirect immunofluorescence after (A) transfection with pGCSma17 or (B) infection with BPV. Transfection was done by the calcium phosphate method and cells were prepared for immunofluorescence at 5 days. The first antibody was anticapsid serum (rabbit 0118) and the second antibody was fluorescein conjugated goat antirabbit serum.

3′ end seemed to have equal levels of infectivity as seen by comparing pVPI1 and pVPI2 as well as pGC119 and pVPI1Δ34. Clones pVPI2Δ52 and pVPI2Δ79 were not infectious. Cell layers transfected with either of these plasmids showed no CPE. These transfected cell layers were frozen and thawed three times and used as virus stocks to infect fresh cell layers. No CPE was seen on these second cell layers demonstrating that little or no virus production occurred from either of these mutant plasmids.

I consistently obtained a maximum level of transfection when about 5 μg of plasmid DNA was used. Greater concentrations of DNA in transfections were associated with a sharp decrease in plaque titers. The effect of plasmid topology on transfection efficiency was studied by transfecting plasmids after digestion with restriction enzymes. Plasmids pVPI1 and pVPI2 were digested with SalI which releases the intact BPV insert. Efficiency of transfection decreased 5 to 10 fold when compared to that obtained with supercoiled plasmid (Table 2). Plasmid pGC167 was used to study this in more detail. Single digestion with SalI or HindIII was used to open the plasmid at the 3′ or 5′ end of the BPV insert, respectively. Double digestion with SalI and HindIII was used to release the unit length BPV genome from the plasmid. When each of the three forms of plasmid was transfected and compared to transfections with supercoiled plasmid, a 5 to 10 fold decrease in plaque titer was observed (Table 2). Thus, all forms of linearized BPV recombinant plasmid showed lower infectivity than supercoiled plasmid. This in contrast to the findings of Merchlinsky et al. (1983) and Laughlin et al (1983) who observed an increase in infectivity when excised viral genomes were used in transfection assays of MVM and AAV-2 clones.

Analysis of Intracellular DNA and Progeny Viral DNA

The time course for the rescue and replication of the BPV genome was determined by isolating low molecular weight DNA from cell layers at various times after transfection using the Hirt extraction procedure. The low molecular weight DNA was electrophoresed on 1.0% agarose gels and transferred to Zeta-Probe as described in Materials and Methods. Input recombinant plasmid

Table 2. Plaque assay of BPV genomic clones after digestion with restriction enzymes.[1]

Clone	plaques/0.2 μg[2]
pGC167	
mock cut	72, 50
5′ end cut	10, 9
3′ end cut	3, 5
5′/3′ ends cut	9, 7
pVPI1	
mock cut	29
5′/3′ ends cut	6
pVPI2	
mock cut	39, 98
5′/3′ ends cut	5, 8

[1]Genomic clones were digested with conbinations of HindIII and/or SalI to digest the BPV insert at one or both ends as indicated. Mock plasmid control was subjected to the same procedure with the restriction enzyme omitted.

[2]Plaques were determined after transfection of BFL cells with 0.2 μg of plasmid.

DNA, detectable with either nick translated pUC8 or BPV probes, was seen to decline steadily after transfection (Fig. 6). On blots hybridized with BPV probe, RF and single stranded DNA could be demonstrated as early as 3 days when cells were transfected with clone pVPI1 but not until 8 days with pVPI2. The pUC8 probe did not hybridize with the viral DNA bands which suggests that newly replicated virion DNA retains no significant length of plasmid sequences. The length of infection required to demonstrate viral DNA synthesis may be related to the transfection efficiency and the rate of secondary infection by progeny virions. Upon daily observation of the transfected cultures CPE involving about 10% of the cell layer was seen at day five. Thus, amplification of viral DNA synthesis by secondary infection from the transfected cells probably contributes to the detection of viral DNA after transfection.

Cell layers transfected with genomic clones were frozen and thawed three times to release progeny virus. These crude cell lysates from transfected cells were used to infect BFL cells for preparation of virus stocks which were then used to infect a sufficient number of cells for the isolation of preparative amounts of viral DNA. The viral DNA was purified as described in Materials and Methods. Following separation on a neutral sucrose gradient, DNA from each fraction was electrophoresed on an agarose gel, transferred to Zeta-Probe and hybridized with nick translated BPV DNA. When this blot was compared to a blot of DNA isolated from wild type BPV, the band patterns were indistinguishable (Fig. 7). A similar ratio of monomer length single stranded and double stranded DNA is seen in the two blots. The single stranded DNA from progeny of transfected cells was replicated in vitro with Klenow fragment and digested with EcoRI. No differences were seen between the transfection derived DNA and DNA of wild type virus (Fig. 8). The above data taken together establishes that rescue and replication of the cloned BPV DNA occurs after transfection. Also, viral DNA of subgenomic length characteristic of defective viral genomes was present in fractions 5 through 9 (Fig. 7). Apparently, defective genomes arise rapidly during DNA replication even when cloned, and therefore initially homogeneous, DNA is used to initiate infection.

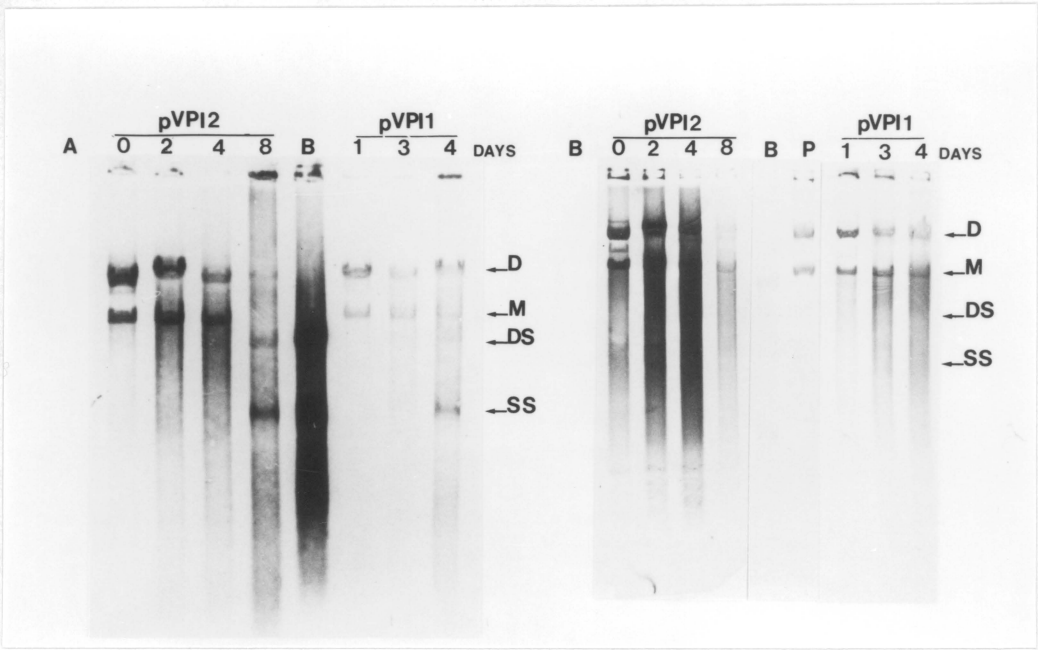


Figure 6. Analysis of low molecular weight DNA after transfection: DNA was isolated from BFL cells by Hirt extraction at various times as noted after transfection with pVPI1 or pVPI2. The DNA was separated on 1.0% agarose gels, transferred to Zeta-Probe membranes and hybridized with either ^{35}S-dATP labeled nick translated BPV DNA (A) or nick translated pUC8 DNA (B). Markers were BPV virion DNA (B) and supercoiled pVPI1 (P). The positions of the monomer (M) and dimer (D) plasmid bands and the single stranded (ss) and double stranded (ds) BPV bands are indicated.

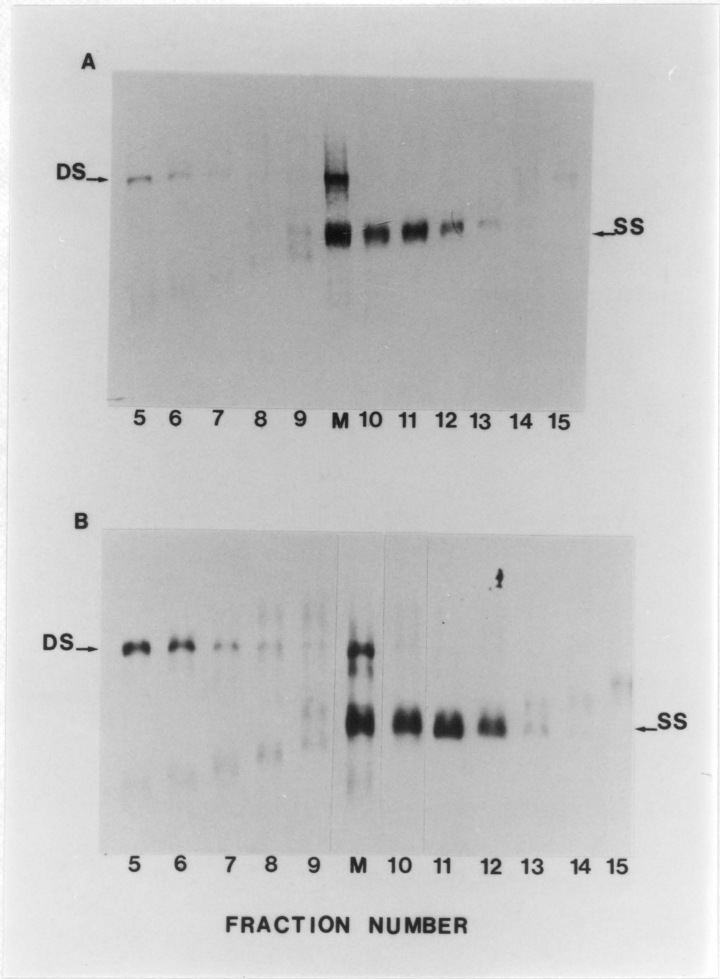


Figure 7. Sedimentation profile of DNA from progeny virions: DNA was extracted from CsCl banded pGCSma20 progeny virions (A) or wild type virus (B) and fractionated on a neutral high salt sucrose gradient. DNA from the fractions was electrophoresed on 1.0% agarose, transferred to Zeta-Probe and hybridized with ^{35}S-dATP labeled nick translated BPV DNA. The marker (M) was single and double stranded BPV virion DNA. The positions of the single stranded (ss) and double stranded (ds) BPV bands are indicated. The gradient fractions are noted at the bottom. The direction of sedementation is from left to right.

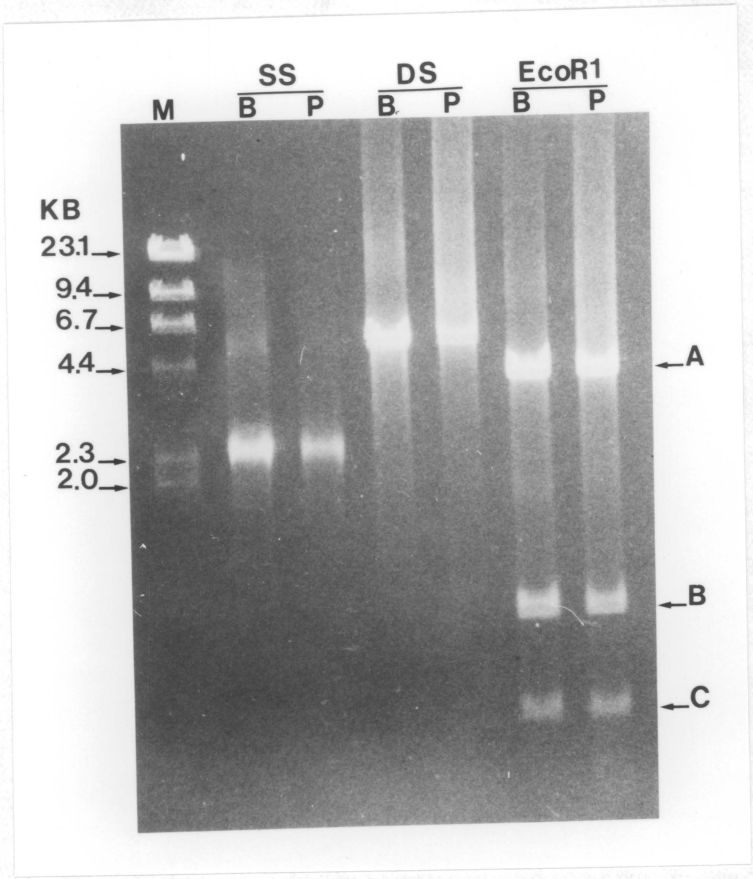


Figure 8. Restriction enzyme analysis of progeny virion DNA: Single stranded virion DNA was purified by neutral high salt sucrose gradient centrifugation. Progeny DNA from clone pGCSma20 (P) was compared with DNA isolated from wild type BPV (B). Single stranded DNA was replicated in vitro to duplex form using *E. coli* DNA polymerase I Klenow fragment and a portion of the replicated DNA was digested with EcoR1. Single stranded DNA (SS), in vitro replicated DNA (Rep), and EcoRI digested DNA (EcoRI) were separated on a 1.0% agarose gel and stained with ethidium bromide. Lambda DNA digested with HindIII was used as a size marker (M). The sizes of the lambda fragments are indicated in kilobases. The EcoRI A (4.10 kb), B (0.98 kb), and C (0.45 kb) fragments of BPV are indicated.

Analysis of Viral Proteins after Transfection

The appearance of BPV encoded proteins after transfection was studied by immunoprecipitation of ^{35}S-methionine labeled proteins using either serum against BPV capsid proteins (rabbit 0118) or serum recognizing both capsid and noncapsid proteins (calf 86). The most abundant capsid protein, VP-3, could be detected at 2 days after transfection using either calf 86 or rabbit 0118 serum (Fig. 9). At day 5 all three capsid proteins had accumulated in amounts comparable to a normal infection harvested after 20 h. Two days after transfection the 28 kd nonstructural protein NP-1 could be immunoprecipitated from cell lysates with calf 86 serum. Also, a 75 kd nonstructural protein could be demonstrated with calf 86 serum 5 days after transfection. This nonstructural protein has recently been shown to be homologous to nonstructural proteins of about the same molecular weight which are encoded by MVM and B19 (Lederman et al., 1987). Secondary infection by progeny virions leads to observable infection involving 10 to 15% of the cells by 5 days after transfection. It is possible that the large nonstructural protein of BPV is in fact synthesized earlier in the transfected cells but remains undetectable until amplified by spread of the infection to other cells. There are also four bands with molecular weights greater than the large capsid protein, VP-1, which are immunoprecipitated by calf 86 serum but not by rabbit 0118 serum.

DISCUSSION

The isolation under annealing conditions of double stranded BPV DNA from virions is a consistent feature of our purification scheme. That this double stranded DNA is reannealed plus and minus strands rather than RF DNA which might copurify with the virions is demonstrated by the cloning of terminal fragments. The duplex fraction of DNA from virion purification readily yielded subclones separately containing the left and right palindromes. By contrast, the screening

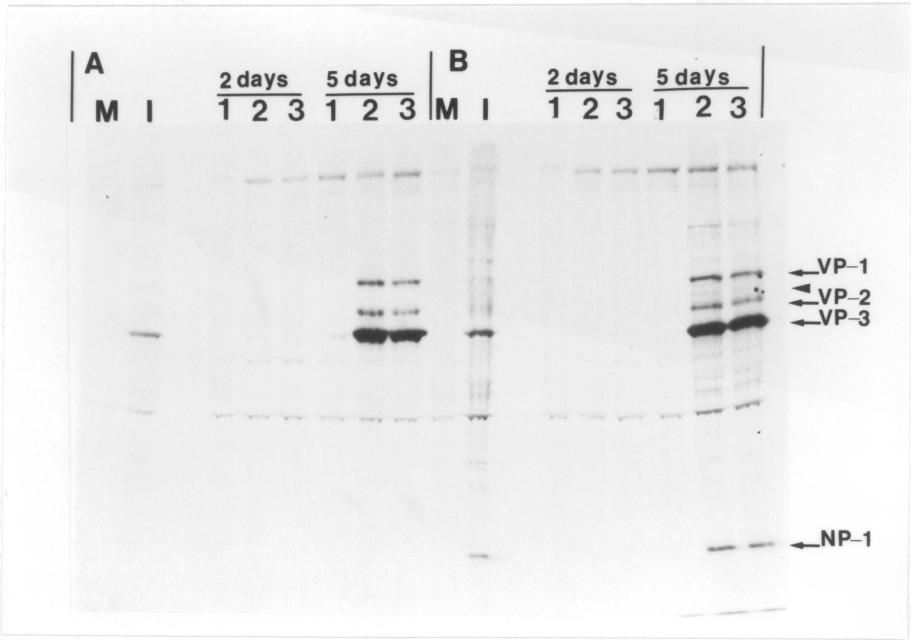


Figure 9. Immunoprecipitation of viral proteins after transfection: BFL cells transfected with clones pVPI1 or pVPI2 were labeled with 20 μCi/ml of ^{35}S-methionine in the presence of 10% dialyzed fetal calf serum. Cell lysates were prepared by the method of Bloom et al. (1982). Immunoprecipitation products were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels, treated with Amplify (New England Nuclear) and exposed with an intensifying screen at -80°C. Lysates were immunoprecipitated using 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 as a negative control (lanes 1), clone pVPI1 (lanes 2) or clone pVPI2 (lanes 3). In addition, lysates from mock infected (M) and BPV infected (I) cell cultures were run as markers. The positions of the capsid proteins VP-1, VP-2, and VP-3 and the nonstructural protein NP-1 are indicated by arrows. The recently identified 75 kd nonstructural protein of BPV (Lederman et al., manuscript in preparation) is marked with an arrow head.

of over 200 recombinants from the cloning of terminal EcoRI fragments of in vivo RF DNA failed to yield any intact 5′ palindromic sequences while four clones containing the 3′ end were isolated from the same RF DNA preparation. The inability to isolate clones containing the right terminus of MVM from RF DNA has been reported by Merchlinsky et al. (1983) and Sahli et al. (1985). They attributed this to blocking of the right terminus by a peptide remnant of the 5′ terminal protein. A recent report showed that both the left and right termini of MVM have terminal proteins attached to the 5′ ends (Chow et al., 1986). Thus, if blocking of the right terminus of RF DNA is caused by terminal protein attachment, then a significant proportion of the left termini must be free of terminal protein. Also, these cloning data suggest that an as yet unidentified terminal protein in BPV is attached to the 5′ end of RF DNA. Another factor which could contribute to the failure to obtain clones containing the right palindrome of BPV RF DNA is the possibility that much of the right end is in the hairpin form. The fraction of BPV RF DNA which is in the extended form has not been determined.

The construction of infectious genomic clones of BPV was readily accomplished by direct blunt end ligation. This clearly shows that the termini of the cloned double stranded virion DNA was not blocked. Despite this, cloning of these molecules by SalI linker addition failed. Laughlin et al. (1983) also were unable to clone AAV-2 virion DNA directly into a plasmid following linker addition. They were able to isolate two subgenomic clones by the ligation of BglII linkers to the termini followed by digestion with HindIII, which cuts once within the AAV-2 genome, and ligation to pBR322 digested with BglII and HindIII. Likewise, we were able to obtain subclones of the BPV genome by ligation of SalI linkers to double stranded virion DNA and cloning of the terminal EcoRI fragments into pUC8. The efficiency of linker ligation is dependent on the proportion of viral DNA molecules in which both termini exist in the extended conformation. The data on linker ligation suggest that while either end of viral DNA is accessible to linker ligation, few molecules have both ends accessible. Blunt end ligation of BPV into the vector may be viewed as a two step reaction where ligation of one end of the BPV DNA molecule to the vector is the rate limiting step. Once ligation of one end has occurred the second ligation event is an intramolecular ligation which would presumably occur at a much faster rate. The second ligation could be de-

pendent on spontaneous interconversion of the hairpin conformation to the extended conformation. During the ligation of linkers to viral DNA both necessary ligations should have equal probability, that is, the attachment of a linker at one end of the genome is not affected by the prior attachment of a linker at the other end.

Plasmid sequencing has given detailed information about the termini of BPV genomic clones. There was much size heterogeneity at both termini among the clones tested for infectivity (Table 1). This demonstrates that a unique terminal sequence is not required at either end of the genome for rescue and replication. Deletions of up to 34 bases at the 3′ end lowered but did not abolish infectivity (Table 1). The 3′ end deletions could lower infectivity by destabilizing the 3′ hairpin configuration which is required for replication. Breathing of the 3′ hairpin due to lower stability would lower the rate of initiation of replication by a DNA polymerase replication complex. The 14 base pair deletion at the 3′ end of pGC167 has no apparent effect on infectivity which is consistent with the postulated effect of deletions on 3′ hairpin stability. Deletions of 52 and 79 bases at the 3′ end each abolished infectivity. The 52 base deletion leaves only six base pairs of the main stem of the palindrome. Evidently this structure is not sufficient for rescue and replication of the BPV genome although a base paired 3′ OH can exist at the end of this truncated palindrome. The 79 base pair deletion removes over half of the total 3′ palindrome which prevents the formation of the hairpin primer configuration.

Models have been proposed for the rescue and replication of the genome of AAV-2 from genomic clones (Samulski et al., 1983; Senapathy et al., 1984). These models invoke the occurrence of nicks either in the plasmid sequence near the plasmid-AAV boundary (Samulski et al., 1983) or within the terminal palindrome of AAV-2 (Senapathy et al., 1984), followed by displacement synthesis. Both models postulate a panhandle intermediate which forms by base pairing between the inverted terminal repeats of AAV-2. Since autonomous parvoviruses do not have inverted terminal repeats capable of forming such an intermediate the AAV models cannot account for the rescue of cloned autonomous parvovirus genomes. Rescue of autonomous parvovirus genomes could occur via nicks within the palindromic sequences at each end of the same strand, followed by displacement synthesis from the 3′ end. This would result in a deletion at the 5′ end which could be repaired

in subsequent rounds of normal viral DNA synthesis. If this model of rescue is correct, deletions at the 3′ end of cloned BPV genomes could lower the infectivity after transfection by decreasing the length of DNA within which the critical 3′ nick could occur and still leave a base paired hairpin capable of acting as a primer for DNA synthesis.

Clone pGC167 is interesting because the 5′ sequence is 26 bases longer than the putative virion terminus. Clones pGC119 and pGCSma22, and pGCSma32 also have 5′ termini which extend past the putative 5′ terminus of BPV. The virion DNA terminus was established by the sequencing of cloned S1 nuclease resistant fragments of single stranded virion DNA and by measuring the length of end labeled NdeI restriction fragments of in vitro replicated viral DNA (Chen et al., 1986b). A problem with the end label analysis is the possibility of incomplete replication of viral DNA to the fully extended form. End label analysis of double stranded virion DNA showed that the 5′ terminus is actually 26 bases longer than originally assigned (Chapter 2). Among the S1 nuclease resistant clones was one which terminated at 26 bases past nucleotide 5491, the same 5′ terminus found in clones pGC167 and pGCSma32. These data demonstrate that the 5′ terminus occurs at nucleotide 5517.

In contrast to previous reports we found that supercoiled plasmids gave higher transfection efficiency than excised BPV inserts. Laughlin et al. (1983) found that cleavage of their AAV-2 genomic clone had a differential effect on DNA replication and the yield of infectious virus. Linearization without excision of the AAV-2 insert increased the yield of virus by 2 to 3 fold but did not affect the replication of DNA. Excision of the AAV insert increased infectious particle yield by 3 to 10 fold and also increased the yield of DNA by 2 to 3 fold. It was suggested that this differential effect of topology on virus yield and DNA replication relates to the production of capsid protein, which is not required for RF DNA replication. More efficient transcription of the linearized template was proposed to be the basic mechanism of the topology effect. This conflicts with data showing that transcription of supercoiled DNA occurs at a higher rate than linearized DNA (Harland et al., 1983; Weintraub et al., 1986). The basis for our finding of higher infectivity with supercoiled plasmid could be due to differences in the plasmid vector pUC8 vs pBR322 which was used by Laughlin et al. (1983) and Merchlinsky et al. (1983). Plasmid pUC8 is a pBR322 derivative

with much of the original plasmid sequences deleted (Vieira and Messing, 1982). Possibly, vector sequences in pBR322 could inhibit some crucial or rate limiting step in the rescue of the viral genome. The well documented "poison sequence" in pBR322 is an example of this type of mechanism (Lusky and Botchan, 1981). An alternative possibility is that linear DNA is considerably less stable than circular DNA in BFL cells. In this case, any enhancement of infectivity due to linearization of transfected DNA molecules could be masked by lowered stability caused by, for example, high nuclease activity in BFL cells.

We have routinely observed a transfection efficiency of about 40-80 plaques per 0.2 μg of plasmid DNA using plasmids with intact 3′ termini. The number of plaques appeared to be only approximately proportional to amount of DNA transfected. This is significantly higher than the level obtained with the MVM clone which gave 3 plaques per μg when transfected as supercoiled plasmid (Merchlinsky et al., 1983). We observed a decrease in transfection when greater than 5 μg of DNA was used per 60 mm plate. 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 saturation rather than a decrease in plaque titer. This phenomenon has not been reported with other parvovirus genomic clones but was observed by Susman and Milman (1984) in the transfection of a plasmid containing the herpes simplex thymidine kinase gene.

The BPV genomic clones have proven to be remarkably stable to deletions within the termini during propagation in *E. coli* JM107. During large scale preparation of DNA, stocks of nine different genomic clones, I have not found a single detectable terminal deletion. In contrast, AAV-2 was reported to undergo terminal deletions at a low but significant rate during propagation in *E. coli* strain HB101 (Samulski et al., 1982). MVM clones were reported to undergo rapid deletion of the 5′ palindrome while the 3′ palindrome was stable during propagation in *E. coli* strain LE392 (Boissy and Astell, 1985). A mechanism involving slipped mispairing between direct repeats during replication has been proposed to account for deletions within the cloned 5′ terminus of MVM during passage in *recA* + strains of *E. coli* (Boissy and Astell, 1985). The stability of cloned BPV 5′ termini could be due to a lack of direct repeats in the pattern of the MVM 5′ palindrome. Also, the 5′ palindrome of BPV is shorter than MVM (173 vs 206 bases) which could contribute some-

what to its stability toward deletion. Finally, I have not compared the stability of BPV cloned palindromes in other *E. coli* strains and cannot rule out the possibility that properties of JM107 contribute to the observed stability of our clones. While I have not observed any terminal deletions, I have documented the occurence during cloning of a sequence inversion at the 3′ end of the genome which resulted in the conversion of a 3′ flip to a 3′ flop sequence. This inversion occurred without deletion of any bases. One previous occurrence of a sequence inversion during cloning of the 5′ end of parvovirus H-1 has been reported (Rhode and Klaassen, 1982). Both clones constructed with 3′ flop ends were infectious but the relevance of the 3′ flop sequence to wild type BPV DNA remains to be established.

Progeny viral DNA could be recovered directly from transfected cell layers 3 to 8 days after transfection (Fig. 6). Plasmid containing sequences declined steadily during this time, suggesting that replication of the plasmid cannot be initiated from BPV origin(s) of replication. This agrees with data obtained using the MVM genomic clone (Merchlinsky et al., 1983). Fractionation of the DNA of progeny BPV virions on a neutral high salt sucrose gradient demonstrated the presence of a range of encapsidated DNA which in addition to monomer length double and single stranded DNA included probable defective genomes. The data of Fig. 7 demonstrate that a population of defective forms comparable to that found in wild type infections accumulates rapidly after transfection with a genomic clone.

Immunoprecipitation of labeled proteins demonstrated the synthesis of both nonstructural and capsid proteins after transfection of BPV genomic clones. The major capsid protein, VP-3, could be detected 2 days after transfection and all three capsid proteins had accumulated in great abundance 5 days after transfection when CPE was first evident in the cell layers (Fig 9). The nonstructural protein NP-1 was detected using calf 86 serum 2 days after transfection. The early appearance of NP-1 suggests a possible regulatory role for this protein in the temporal expression of the BPV genome. Nonstructural proteins of 75 and 83 kd have recently been identified in lysates of BPV infected cells and shown to be homologous to the NS-1 proteins of MVM and B19 (Lederman et al., 1987). A nonstructural protein of 75 kd was detected using calf 86 serum 5 days after transfection. An 83 kd nonstructural protein was not detected in either the transfected cell

layers or BPV infected control lysate. Nonstructural proteins of about this size have been detected for several other parvoviruses (Bloom et al., 1982; Cotmore et al., 1983; Molitor et al., 1985 and Cotmore et al., 1986). Molitor et al. (1985) identified a nonstructural protein, designated NS-1, in PPV infected cells of 84 kd which appeared prior to the capsid proteins at the time of initiation of DNA synthesis. They suggested that NS-1 may play a role in DNA synthesis. Rhode (1985b) has demonstrated that NS-1 of parvovirus H-1 functions as a *trans*-activator of transcription from the capsid protein promoter. A functional role for the 76 kd nonstructural protein of BPV has not yet been found.

BPV appears to be a useful model system in the study of the parvovirus family. Molecular clones of the BPV genome are stable upon propagation in *E. coli* JM107 and manipulation of the terminal sequences is readily accomplished. These properties should be useful in the further dissection of the functional properties of the BPV palindromes.

Chapter II

ANALYSIS OF THE TERMINI OF BOVINE PARVOVIRUS

INTRODUCTION

Parvoviruses are small icosahedral viruses with single stranded DNA genomes which have terminal palindromes. It is likely that the *cis* signals for replication and encapsidation reside in the hairpin termini of the viral genomes (Faust and Ward, 1979). However, different signals may reside at the different termini of autonomous parvoviruses, each playing a distinct role in the replication process. The terminal hydroxyl of the 3′ hairpin is used to initiate replication while the origin of replication of the parental RF molecules lies within the 5′ terminus (Rhode and Klaassen, 1982). Merchlinsky et al. (1983) found that deletion of either palindromic terminus of an infectious clone of MVM prevented rescue and replication. Certain deletions within one terminus of AAV-2 prevented rescue and replication while for others deletion in both termini were fatal (Samulski et al., 1983). It was possible to restore function by replacing the sequence deleted from the hairpin with

an unrelated but palindromic sequence which allowed restoration of the T shaped conformation (Lefebvre et al., 1984).

Selective encapsidation of defective genomes which retain the palindromic termini routinely occurs during propagation of parvoviruses (Faust and Ward, 1979). Two major classes of defective genomes of MVM were identified. Both palindromic termini were present in type I genomes, but only the 5′ palindromic terminus was retained in type II genomes. Both type I and II genomes were encapsidated, while only type I was replication competent. Since type II genomes were encapsidated, any *cis* signals necessary for encapsidation must reside in the 5′ terminus.

An important feature of several DNA replication models for autonomous parvoviruses is the occurrence in both RF and viral DNA of a unique 3′ end structure and the occurrence of equal frequencies of two conformations, designated flip and flop, at the 5′ end. The occurrence of only one conformation at the 3′ end of MVM, both in virion DNA and in RF DNA, has been demonstrated (Astell et al., 1985), but the generality of the MVM model for all autonomous parvoviruses remains to be determined. Another feature of MVM DNA structure is the occurrence of an 18 base extension at the 5′ end of RF DNA relative to virion DNA. The existence of a similar extension in the RF DNA of other autonomous parvoviruses has not been confirmed.

In this report I demonstrate the presence of 3′ flop conformation in both virion and RF DNA of the autonomous parvovirus, BPV. End label analysis of virion DNA from the progeny virions of transfected genomic clones demonstrates that the flip and flop conformations are interconvertable but the 3′ flip conformation accumulates preferentially. Analysis of the 5′ end of BPV demonstrates the occurrence of flip and flop conformations in approximately equal amounts in both virion and RF DNA. The 5′ end of RF DNA does not differ from that of virion DNA. These data suggest that the replication of BPV differs in several respects from that of the rodent parvoviruses, MVM and H-1.

MATERIALS AND METHODS

Clone Construction

Clones were constructed by standard methods (Maniatis et al., 1982) using plasmids pUC8 and pUC18. Vectors were routinely treated with calf intestinal phosphatase to reduce the background of nonrecombinant clones. BPV DNA was subjected to an end repair reaction using Klenow fragment to maximize the number of flush ends. Monomer intracellular RF DNA, isolated by a modified Hirt extraction procedure (Siegl and Gautschi, 1978), was used as the starting material for clones of the series p3'R. These clones were constructed by adding SalI linkers to the termini of monomer RF and digesting with EcoRI which cleaves BPV at map unit 17 and 92. The terminal EcoRI fragments were cloned into pUC8 which had been digested with EcoRI and SalI. Clones designated p3'V and p5'V were constructed by the same procedure but using double stranded (reannealed plus and minus strands) BPV virion DNA as the starting material. Clones designated p3'Xba and p5'Xba were constructed using double stranded virion DNA. Virion DNA was digested with XbaI, which digests BPV once at map unit 15, and ligated to pUC18 which which had been digested with SalI and XbaI. In each of these series the designation 3' denotes a left end fragment while 5' denotes a right end fragment. The above clones were analyzed by restriction digestion of DNA isolated by a minilysate procedure (Rodriguez and Tait, 1983) followed by electrophoresis on 1.0% agarose. Inserts were confirmed as originating from BPV by transfer to Zeta-Probe membranes and hybridization with ^{35}S-dATP labeled nick translated BPV DNA. Additionally, the termini of most of the BPV inserts were confirmed by plasmid sequencing.

Terminal SmaI clones were constructed by digesting double stranded recombinant virion DNA with SmaI and ligating to SmaI digested M13mp18 phage RF DNA. Single stranded M13 DNA was isolated by standard methods (Messing, 1983) and sequenced by the dideoxy method using ^{35}S-dATP as the label (Biggin et al., 1983).

End Label Analysis

Double stranded (reannealed plus and minus strands) virion DNA was isolated from either progeny virions of cell cultures transfected with genomic clones or wild type virions. RF DNA was isolated from cells infected with wild type BPV. DNA, 1-2 μg per 50 μl reaction, was 3′ end labeled using 50 μCi of ^{35}S-ddATP (New England Nuclear, Boston, MA) and 20 units of terminal deoxynucleotidyl transferase (New England Nuclear, Boston, MA) in a cacodylate buffer (100 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM DTT, 1 mM $CoCl_2$, pH 7.6). After incubation at 37°C for 2 h, DNA was precipitated and the pellets were washed three times with 70% ethanol. The labeled DNA was then digested with SmaI or NdeI and the labeled fragments were analyzed on a sequencing gel using an M13mp18 sequence reaction as size marker. Sequencing gels were run as described in the previous chapter. Single stranded virion DNA (minus strand) was also end labeled as above, digested with HhaI and analyzed as above.

Computer analysis.

The computer program of Pustell and Kafatos (1984) was obtained from International Biotechnologies Inc. (New Haven, CN). Homologies were sought between the left and right palindromes of several parvoviruses as well as between the corresponding palindromes of various parvoviruses.

RESULTS

Sequence Inversions Demonstrated in Cloned BPV Termini

In vivo RF DNA and double stranded (reannealed plus and minus strands) virion DNA were used to generate clones of the 3′ and 5′ terminal EcoRI fragments of BPV. Representative clones with their extent of deletion are listed in Table 1. No 5′ end clones were obtained from in vivo RF DNA, as was previously observed (Chapter 1). However, both 3′ and 5′ clones were obtained using double stranded virion DNA as the starting material. Surprisingly, a clone, p3′V8, was found to have a flop conformation in the 3′ terminus, as shown in Fig. 1. This is the first observation of a sequence inversion within the 3′ palindrome of an autonomous parvovirus. Sequence data of clones containing BPV termini shows that the 3′ flop conformation is a minor form while at the 5′ end both the flip and flop conformations occur commonly (Table 1 and Table 1, Chapter 1).

Since we have previously demonstrated that a sequence inversion within the 3′ terminus of BPV can occur during cloning (Chapter 1), it was necessary to use an alternate cloning strategy to confirm the presence of 3′ flop in the native virion DNA. The analysis of flip and flop forms of parvoviral palindromes is greatly facilitated by the occurrence of restriction enzyme sites which occur in unpaired regions of the folded palindrome (Rhode and Klaassen, 1982). When a restriction site occurs as part of a looped out region within a folded hairpin of single stranded DNA, the site can occur in one of two positions in double stranded DNA as the result of hairpin transfer during replication. The single SmaI site of BPV lies within an unpaired base region of the 3′ hairpin and in double stranded DNA can occur at 44 or 106 nucleotides in the flip and flop forms, respectively (Figs. 1, 2). The cloning of a fragment of about 100 nucleotides after digestion with SmaI would provide direct evidence for the existence of the 3′ flop conformation in virion DNA. When a set of SmaI terminal clones was sequenced, one with a 96 base insert was found (Table 1). This clone, M13Sma199, had the expected sequence for a 3′ flop conformation but with a ten base pair terminal

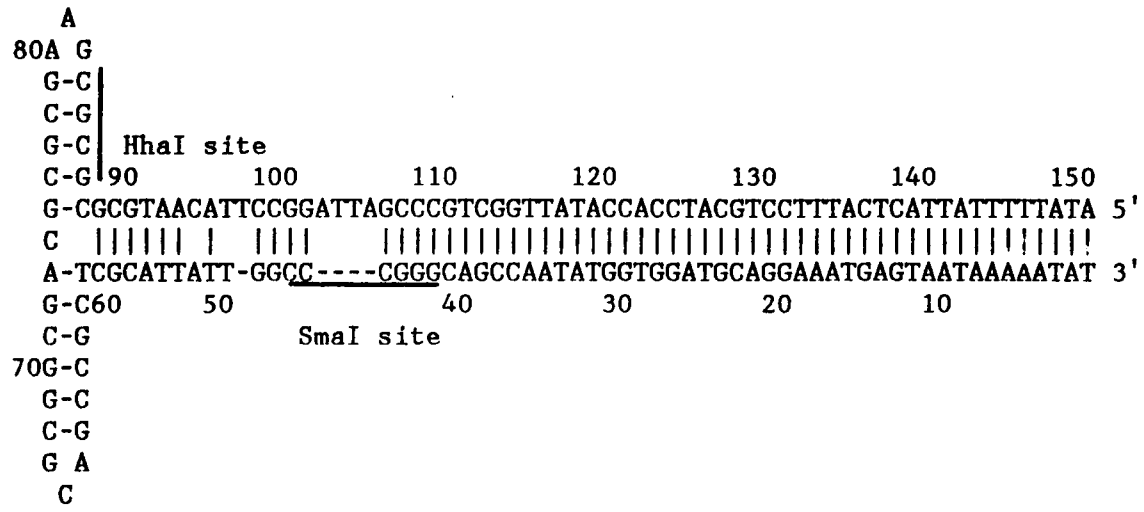
Table 1. Summary of DNA sequencing data of subgenomic clones of BPV which contain terminal sequences.

Clone	3′ end[1]	5′ end[1]
p5′Xba213		flip, -11
p5′Xba219		flop, 0
p3′Xba208	flip, -17	
p3′Xba240	flip, nd[2]	
p3′Xba242	flip, -29	
p3′R5	flip, -35	
p3′R45	flip, 0	
p3′R78	flip, -52	
p3′R81	flip, -79	
p3′V1	flip, -19	
p3′V5	flip, -26	
p3′V7	flip, -32	
p3′V8	flop, -34	
M13Sma186	flip, -13	
M13Sma189	flip, -22	
M13Sma199	flop, -10	

[1]Sequence orientation is given as flip or flop followed by the nunber of bases deleted relative to the mature virion 3′ or 5′ terminus. The mature 5′ terminus is taken as nucleotide 5491 (Chen et al., 1986b).

[2]Not determined.

(A) flip conformation



(B) flop conformation

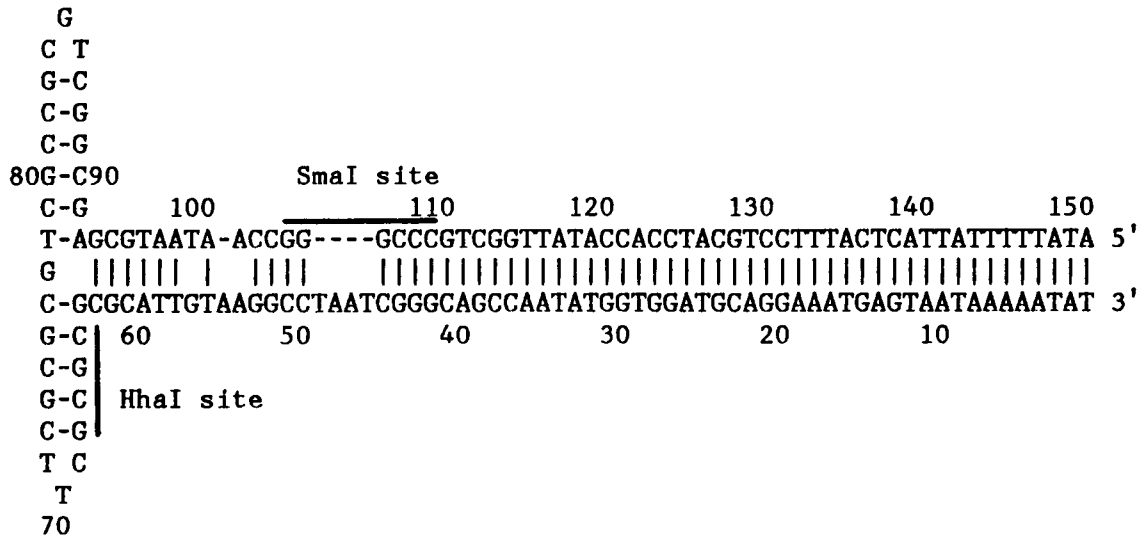


Figure 1. Flip and flop conformations of the BPV 3′ hairpin (minus strand): The SmaI and HhaI restriction enzyme sites are indicated.

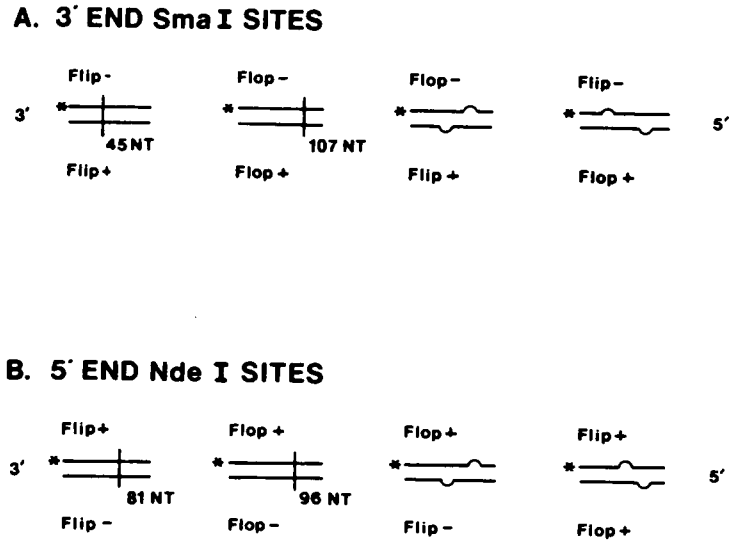


Figure 2. BPV restriction enzyme sites affected by sequence inversions: Reannealing of BPV virion DNA plus and minus strands results in double stranded molecules which may be fully duplex or contain unpaired bases in the terminal palindromes due to the annealing of strands with different terminal sequence conformations. In this diagram, which depicts the terminal palindromes of BPV, unpaired base regions are represented as loops. The plus and minus strands are indicated and * denotes the addition of ^{35}S-ddATP to the 3′ ends for analysis of the length of terminal restriction fragments. Depicted are the 3′ terminal palindrome with the location of the SmaI sites (A) and the 5′ terminal palindrome with the location of the NdeI sites (B). Restriction digestion of fully duplex molecules will yield fragments of the indicated length, which includes the extra base added by end labeling.

deletion. The complete nucleotide sequence of the 3′ flop conformation is shown in comparison to the 3′ flip sequence in Fig. 1. Two other clones with BPV terminal SmaI inserts were sequenced and both were in the flip conformation. These data provide direct proof by cloning of the existence of the 3′ flop conformation in BPV virion DNA. Sequence inversion during cloning could not give rise to the cloned 96 base pair fragment since the size of this insert was defined by restriction digestion prior to cloning. Sequence inversion during cloning could convert a flip sequence to flop only when the cloned fragment is longer than the invertable sequence.

Demonstration of 3′ Flop Conformation by End Label Analysis

End labeling followed by digestion with a restriction enzyme which digests at different positions in the flip and flop conformations gives direct visualization of the flip and flop conformations and permits an estimate of the ratio of the two forms. The flip and flop conformations at the 3′ end can be demonstrated using either SmaI or HhaI (Figs. 1, 2a). The availability of infectious genomic clones with varied terminal structures has also provided an opportunity to follow the fate of specific cloned sequences after transfection.

End label analysis of the 3′ end of double stranded (reannealed plus and minus strands) DNA from wild type virus by SmaI digestion gave a major band at 42 nucleotides and minor bands at 40, 41, and 43 nucleotides (Fig. 3). The occurrence of multiple bands strongly suggests that the position of the 3′ terminal nucleotide varies over a 4 base range. The major band corresponds well with the expected size of 45 nucleotides for the 3′ flip conformation. A minor but distinct band occurs at 103 nucleotides which corresponds well with the expected value of 107 nucleotides for the 3′ flop conformation. The measured distance between the SmaI sites corresponding to the flip and flop conformations is 61 nucleotides, which is within one base of the expected value of 62. The occurrence of the band corresponding to 3′ flop conformation in wild type virion DNA provides direct evidence for the existence of this conformation in BPV viral DNA and confirms the isolation of 3′ flop by cloning. The relative intensities of the bands corresponding to flip and flop demon-

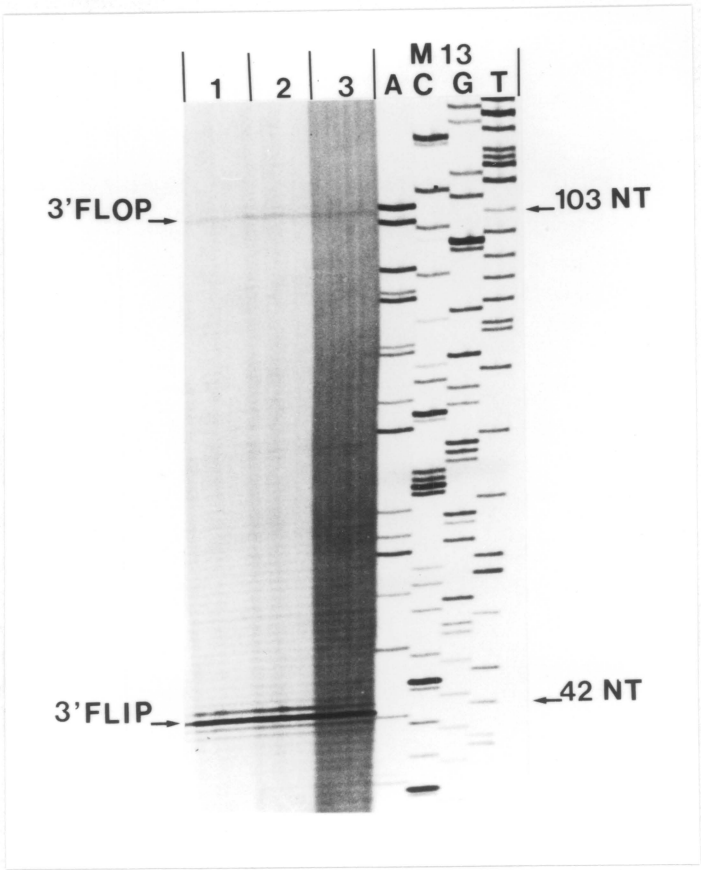


Figure 3. Sequence inversions in the 3′ end of BPV virion DNA: Double stranded virion DNA (reannealed plus and minus strands) was purifiied from cell cultures infected with progeny virions of transfected cell cultures. DNA was labeled on the 3′ OH of the plus and minus strands using terminal transferase and ^{35}S-ddATP as described in Materials and Methods. Samples were then digested with SmaI and run on a polyacrylamide sequencing gel with a set of M13mp18 sequencing reactions as a size marker (M13). Lane 1 contains pVPI2, lane 2 contains pVPI1 and lane 3 contains wild type BPV DNA. The position of the bands corresponding to the flip and flop conformations are indicated on the left and the sizes of these bands in nucleotides, as derived from the known sequence of M13mp18 DNA, are indicated on the right.

strate that the flop conformation accumulates to a much lesser extent in virion DNA than the flip conformation. Progeny double stranded virion DNA from the transfection of genomic clones pVPI1 and pVPI2, both of which have undeleted 3′ ends, gave bands which corresponded exactly in position to those of the wild type DNA (Fig. 3). Progeny DNA of clones pVPI1 (3′ flop) and pVPI2 (3′ flip) have identical ratios of 3′ flip and flop forms (Fig. 3). This suggests that both conformations of the 3′ end may be derived from the other but that the flip isomer accumulates preferentially in viral DNA regardless of the conformation present in the clone used for transfection.

The single stranded DNA in a number of autonomous parvoviruses contains an HhaI site within one of the arms of the hairpin (Astell et al., 1979). Examination of the sequence of BPV DNA reveals the presence of an HhaI site in one of the arms of the 3′ hairpin (Fig. 1). End label of single stranded BPV virion DNA (minus strand) followed by digestion with HhaI should give bands of 66 and 77 nucleotides corresponding to 3′ flop and flip, respectively. When samples of minus strand BPV DNA were analyzed in this way, prominent bands were obtained at 63 and 79 nucleotides which correspond well with the expected lengths of 66 and 77 for flop and flip, respectively (Fig. 4). There were a number of secondary bands observed.

Astell et al. (1979) in performing a similar analysis of MVM found that HhaI tends to make single stranded cuts within the duplex region containing the HhaI recognition site. This can give rise to two fragments corresponding to digestion at either end of the HhaI recognition site. Weaker bands at nucleotides 71 and 84 agree well with the expected values of 73 and 84 predicted for cleavage at the distal side of the HhaI site. Bands at 77,78, and 80 result from variation in the position of the terminal nucleotide, as noted above.

The intensity of the bands corresponding to flip and flop after digestion with HhaI was nearly equal, in contrast to the previous data using SmaI digestion which suggested that 3′ flop occurs at much lower frequency than 3′ flip. The unexpected increase in the intensity of the band corresponding to the 3′ flop conformation could be due to contamination of the single stranded DNA used with double stranded virion DNA. Double stranded virion DNA in the flip orientation has an HhaI fragment which co-migrates with the 66 nucleotide flop fragment of single stranded virion DNA. To test this possibility single stranded virion DNA purified by neutral sucrose gradient

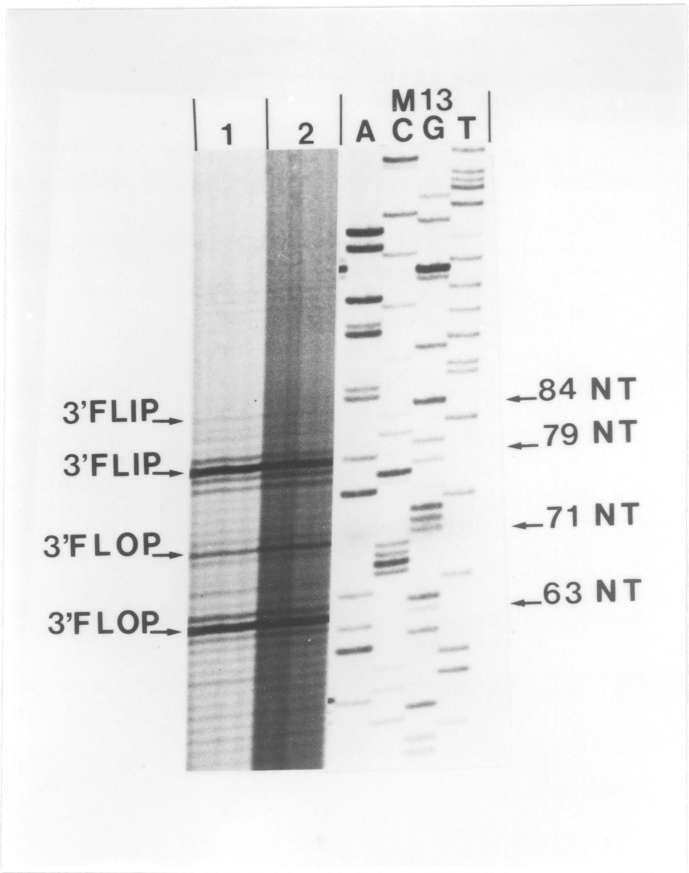


Figure 4. Analysis of the 3′ end of single stranded BPV virion DNA: Single stranded viral DNA was end labeled using terminal transferase, digested with Hha1 and analyzed on a sequencing gel as described in Materials and Methods. Lane 1 contains progeny virion DNA from transfection of clone pVPI1 and lane 2 contains wild type BPV virion DNA. Bands corresponding to the 3′ flip and flop sequence inversions are indicated on the left and the corresponding sizes in nucleotides are indicated on the right.

centrifugation was further purified by extraction from an agarose gel. When this more highly purified single stranded DNA was analyzed by end label and HhaI digestion the intensity of the 79 nucleotide band corresponding to the flip conformation was considerably more intense than the 64 nucleotide band corresponding to the flop conformation (Chen, K. C., personal communication). These data provide further proof that both the flip and flop conformations exist in BPV virion DNA and that the flip conformation greatly exceeds the flop one.

End Label Analysis of the 5′ Terminus

The flip and flop conformations at the 5′ end can be demonstrated by end labeling of double stranded virion DNA, followed by digestion with NdeI (Fig 2b). NdeI digestion of 3′ end labeled double stranded (reannealed plus and minus strands) virion DNA gave bands of 77 and 93 nucleotides (Fig. 5) both of which are significantly longer than the predicted size of 55 and 70 nucleotides (Chen et al., 1986b). Based on data discussed in the previous chapter, the 5′ terminus was reassigned to nucleotide 5517. Using the reassigned 5′ end of BPV DNA, the expected sizes of the flip and flop fragments would be 81 and 96 nucleotides, which is in reasonable agreement with the observed values. The distance between the measured flip and flop sites is 16 nucleotides which is in good agreement with the predicted value of 15. The flop band is slightly more intense than the flip band, suggesting that more 5′ flop DNA is encapsidated. The difference in intensity of the 5′ flip and flop bands is considerably less than seen at the 3′ end. A three base variation in the position of the 5′ terminal nucleotide is implied by the three bands associated with the flop position.

The 5′ end of in vitro replicated minus strand virion DNA was compared to the terminus of double stranded (reannealed plus and minus strands) virion DNA (Fig. 5). A series of bands were seen, none of which were as long as that given by double stranded virion DNA. The multiple band pattern and shorter size of the end labeled fragments from in vitro replicated virion DNA are con-

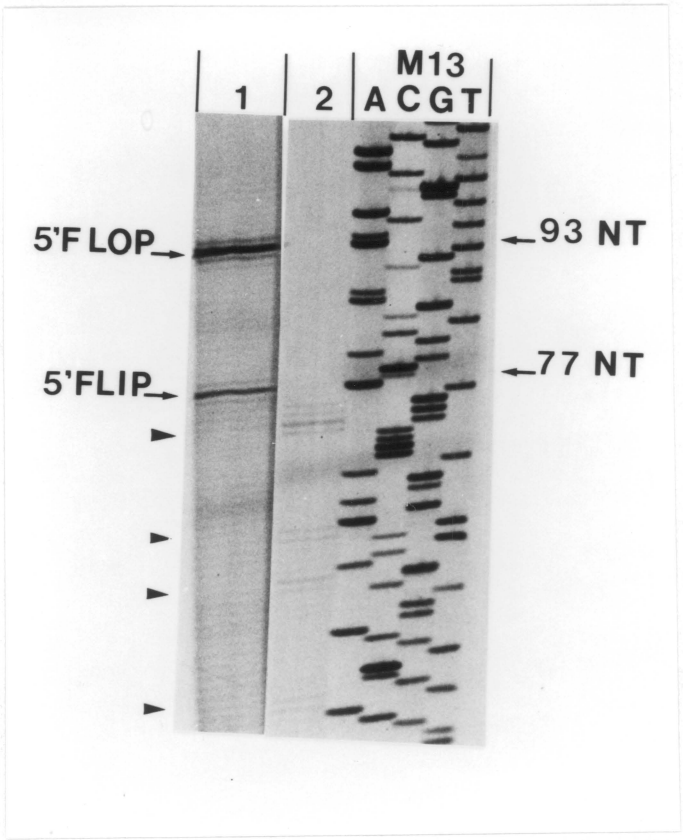


Figure 5. Analysis of the 5′ termini of BPV virion DNA: Single stranded (minus strand) virion DNA was replicated in vitro using *E. coli* DNA polymerase I Klenow fragment and all four deoxynucleotides. Double stranded virion DNA and in vitro replicated DNA were end labeled, digested with NdeI and electrophoresed on a sequencing gel as described in Materials and Methods. Lane 1 contains double stranded virion DNA and lane 2 contains in vitro replicated DNA. The flip and flop bands of double stranded virion DNA are indicated by arrows and the positions of a series of doublet bands given by in vitro replicated DNA are indicated by arrowheads. Sizes of the designated bands are indicated on the right.

sistent with incomplete replication of the 5′ hairpin by Klenow fragment, as previously reported (Cotmore and Tattersall, 1984).

Demonstration of the Repair of Terminal Deletions

The end label protocol also provides an excellent tool for following the fate of deleted termini after rescue and replication from transfected plasmids as well as determining the ratio of flip and flop at each end of the progeny viral DNA. Clone pVPI1Δ34 is deleted at the 3′ end by 34 bases and at the 5′ end by 25 bases. The progeny viral DNA has clearly reverted to wild type in the location of both termini and in the ratio of flip to flop (Fig. 6). Clone pVPI1Δ34 has a 3′ flop structure and provides additional data on the preferential accumulation of the 3′ flip conformation after transfection with a 3′ flop clone. Clone pVPI1Δ34 progeny virion DNA was isolated from both bovine fetal lung (BFL) cells and buffalo lung (BUL) cells and there was no difference in any of the measured parameters associated with cell type. The progeny virion DNA of clone pGCSma20 with a 3′ deletion of 3 bases and 5′ end deletion of 35 bases was also was tested by end label analysis. Again, both ends were repaired to wild type length and the ratio of flip to flop at each terminus was indistinguishable from wild type virion DNA (data not shown).

Terminal Structure of BPV RF DNA

In vivo RF DNA isolated from BPV infected BFL cells by the Hirt method was compared to double stranded virion DNA by end label analysis (Fig. 7). RF DNA shows the same 3′ and 5′ end bands and the same proportion of flip to flop as double stranded virion DNA. Clearly, the 3′ flop form is present in both the RF DNA pool and in encapsidated viral DNA in the same proportions. These findings were confirmed in a second analysis with a different preparation of BPV RF DNA (data not shown). In contrast to MVM, there is no extension of the 5′ end of BPV

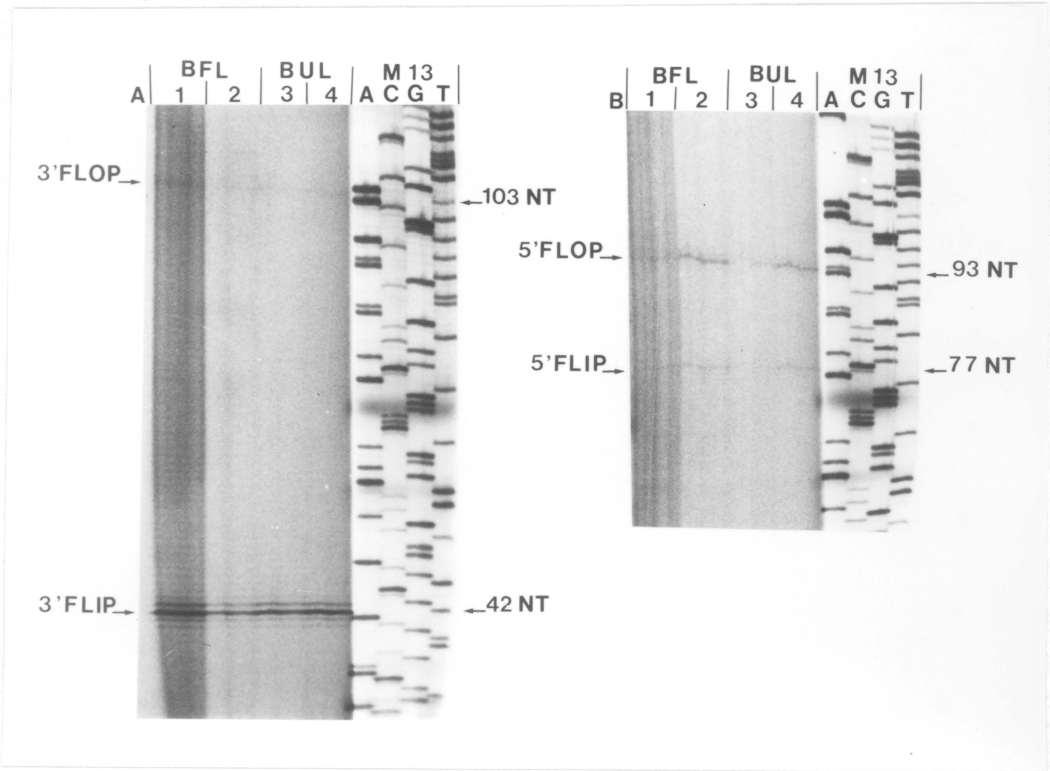


Figure 6. Analysis of the progeny virion DNA of clone pVPI1Δ34: Progeny virions from BFL cells transfected with pVPI1Δ34 were used to infect either BFL or BUL cells. Double stranded virion DNA purified from each of these cell types was then analyzed for flip and flop sequence inversions by end labeling and digestion with SmaI for analysis of the 3′ end (A), or with NdeI for analysis of the 5′ end (B), as described in Materials and Methods. The cell type from which virion DNA was isolated is indicated. Lanes 1 and 3 contain wild type BPV virion DNA and lanes 2 and 4 contain progeny DNA from pVPIΔ34. Bands corresponding to the flip and flop sequence inversions are indicated on the left and the corresponding sizes in nucleotides are indicated on the right.

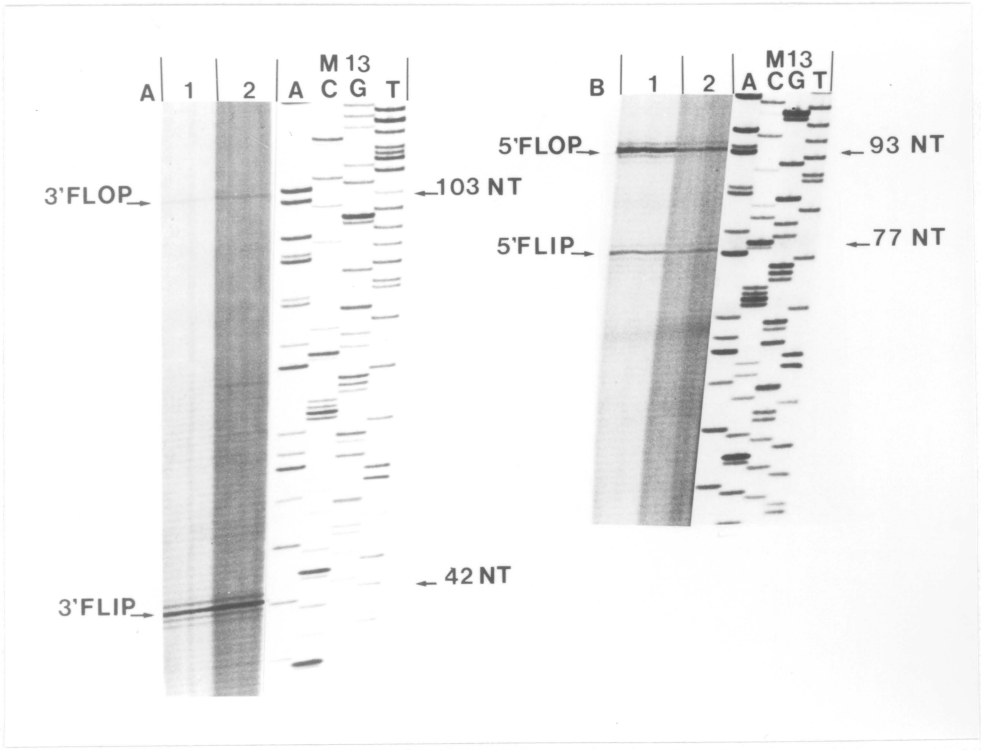


Figure 7. Analysis of in vivo BPV RF DNA for sequence inversions: RF DNA was isolated from BFL cells infected with BPV by Hirt extraction and analyzed for flip and flop sequence inversions by end labeling and digestion with SmaI for analysis of the 3′ end (A), or with NdeI for analysis of the 5′ end (B), as described in Materials and Methods. Lanes 1 and 2 contain double stranded virion DNA from wild type BPV and lanes 3 contain RF DNA. Bands corresponding to the flip and flop sequence inversions are indicated on the left and the corresponding sizes in nucleotides are indicated on the right.

RF DNA compared to encapsidated DNA and the 3′ end of BPV RF DNA does not have a unique conformation.

Homologies Among Parvovirus Palindromes

Since the terminal palindromes of autonomous parvoviruses are important in replication, conserved features may provide clues to functionally important sequences. Accordingly, a computer search was made for conserved features within the terminal palindromes of BPV and MVM which have very little overall sequence homology.

When the left palindromes of BPV and MVM were compared, two short blocks of homology were found (Fig. 8). One of these blocks consists of most of the two arms the 3′ hairpin. Clearly, the primary sequence of this portion of the palindrome is under some evolutionary constraint. On the main stem a short region of homology occurs around the start of the palindrome. This homology contains the sequence ATTTTTA which is identified as a possibly important *cis* signal. When the 5′ end palindromes of BPV and MVM were compared, no significant regions of homology were detected.

A comparison of the left and right termini of BPV was made to detect possible repeated *cis* signals at the ends of the genome. A striking region of homology was detected (Fig. 9a) which is centered about the ATTTTTA sequence and shows 76% matching over a 55 base stretch when three single base gaps are introduced. The conservation of the AT_5A signal within this homology is especially striking. Within the conserved larger sequence at the BPV termini is a 16 base sequence which shows 81% homology (Fig. 9b). This sequence is located within the palindromes at each end of the BPV genome.

(A) DNA homology within the "T" portion of the 3' palindrome.

```
  60          70          80
3' CGC CGACGCGGCGACGCGCGAAGC 5' BPV
   ||| ||||||| || |||| |||||
3' CGCGCGACGCG CG CGCG GAAGC 5' MVM
      50          60
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(B) DNA homology at the terminus of the palindrome.

```
    145   150  155
3' ATTTTTA TATCTCG 5' BPV
   ||||||| ||| |||
3' ATTTTTACTAT TCG 5' MVM
  110  115   120
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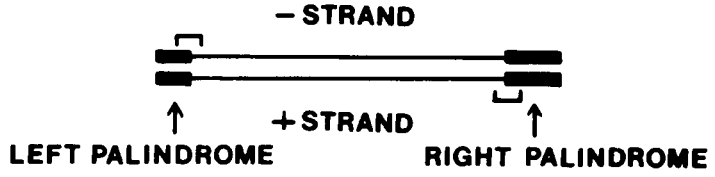
Figure 8. **DNA sequence homologies between the 3′ palindromes of BPV and MVM:** Upper sequences are BPV and lower sequences are MVM. Minus strand sequences are given.

(A) Sequence homology which occurs at the border of the palindromes.

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                    terminal palindrome
      120         130         140         150         160
3' TTATACCACCTACGTCCTTTACTCA TTATTTTTATATCTCGGACACTAAT TCACT 5'
   || ||||||||   ||  ||| |||  |||||||||||| |||  |||  || |||||
3' TTCTACCACCTTTCTCACTTAGTCACATATTTTTATATTTCGC CACATATCTCACT 5'
 5380       5370       5360       5350       5340       5430
                    terminal palindrome
```

(B) Sequence homology within the terminal palindromes.

```
       130
3' CTACGTCCTTTACTCA 5'
   ||||  ||||| ||||
3' CTACCACCTTT CTCA 5'
    5375       5365
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Figure 9. **DNA sequence homologies between the termini of BPV:** Upper lines give sequence from the minus strand of the left end of BPV, while the bottom lines gives plus strand sequence from the right end. (A) These sequences are approximately centered at the terminus of each palindrome, as depicted in the schematic representation of the BPV genome in duplex form. (B) A homology which occurs within the sequences given in (A) when a different alingment is used. These sequences occur within the terminal palindromes.

DISCUSSION

Terminal deletions of the palindromes have occurred in nearly all BPV clones which have been characterized (Table 1; Chapter 1, Table 1). The size of these deletions, which may be as large as 79 nucleotides, greatly exceeds the heterogeneity in position of the terminal nucleotide of BPV, as demonstrated by end labeling experiments. This suggests that the terminal deletions found in BPV clones arise during cloning. Similar deletions, with sizes up to 113 nucleotides, were found during the cloning of the defective parvovirus AAV-2 (Samulski et al., 1983). The position of the terminal nucleotide of BPV varies by four nucleotides at the 3′ end and by three nucleotides at the 5′ end (Figs. 3, 5). This is close to the three base variation observed at the termini of AAV-2 (Fife et al., 1977) and the three base variation in the position of the 3′ terminal nucleotide of MVM (Astell et al., 1979). The occurrence of similar variation in position of the terminal nucleotide in three different parvoviruses suggests that this is a general property of parvoviruses.

BPV genomic clones with deletions of up to 34 bases at the 3′ end or 35 bases at the 5′ end give rise to progeny virus in which the DNA is repaired to wild type length. Similar observations were made with AAV-2 (Samulski et al., 1983). Repair to wild type sequence was demonstrated after transfection of a clone with a 113 base deletion in the left end and a 9 base deletion in the right end. Repair of the shorter deletion can occur simply through the course of self-primed DNA synthesis, provided the deletion leaves enough of the hairpin to form a functional primer. The larger deletion in AAV-2 was proposed to be repaired by a gene correction mechanism which uses a single stranded circular molecule formed by base pairing between the inverted terminal repeats. This mode of repair is possible in AAV-2, which has identical palindromes, but cannot occur during BPV DNA replication because the terminal palindromes of BPV are not identical. Correction of terminal deletions at each end of BPV probably occurs through self-primed synthesis. A deletion of 52 nucleotides at the 3′ end of BPV was fatal to replication (Chapter 1). This is shorter than the 113 nucleotide deletion of the 3′ end of AAV-2 which was found to be repaired. The smaller size of

tolerable deletions in BPV is probably a consequence of having nonidentical termini which prevents repair by an AAV-like gene correction mechanism.

End label experiments and cloning data both suggest that the 5′ terminus of BPV extends past the published value of 5491 (Chen et al., 1986b). Sequencing data reveals four clones with sequences extending past the base 5491 position (Table 1; Table 1, Ch. 1). End label analysis of the 5′ end of double stranded virion DNA shows bands of 77 and 93 nucleotides for flip and flop, respectively (Fig. 5) compared to expected values of 55 and 70. The original assignment of the 5′ end was based partly on the end label analysis of in vitro replicated minus strand DNA. End label and NdeI digestion gave bands of 55 and 70 nucleotides which were consistent with placement of the 5′ terminus at nucleotide 5491. Reexamination of the end label products of in vitro replicated minus strand DNA reveals a number of bands in addition to those at nucleotides 55 and 70 (Fig. 5), which suggests incomplete replication of the hairpin and consequent underestimate of the length of the terminal NdeI fragments. Cotmore and Tattersall (1985) have noted that Klenow preparations differ in the ability to replicate the 5′ hairpin to a fully extended form. The other method used to assign the 5′ terminal nucleotide was the sequencing of S1 nuclease resistant fragments of single stranded (minus strand) virion DNA. Multiple clones were isolated which terminated at nucleotide 5491. One of these clones, however, has the same 26 base extension found in clones pGCSma32 and pGC167 (Chen, K. C., personal communication). Based on the concurrence of these 3 clones, and end label analysis, the revised 5′ terminus of BPV is placed at nucleotide 5517.

The 5′ terminus of the RF DNA of MVM has been demonstrated to be 18 bases longer than virion DNA (Astell et al., 1985). This 18 base extension is largely removed prior to encapsidation but some encapsidated DNA retains the extension and the associated terminal protein (Chow et al., 1986). Such an unprocessed RF extension cannot account for the BPV clones which terminate past nucleotide 5491 because the 5′ end label data clearly reveal that BPV RF DNA has the same terminus as virion DNA (Fig. 7). These data demonstrate that the RF DNA extension of MVM is not a universal feature of autonomous parvovirus replication and indicates differences in the replication of the 5′ ends of BPV and MVM.

The 10 base terminal sequence 5′ ATATTTTTAT 3′ of the reassigned 5′ end of BPV is identical to the terminus of the 3′ end and is also part of a larger sequence conserved between the left and right palindromes of BPV (Fig. 9). This sequence is closely related to the sequence 5′ AATATTTTT 3′ which has been shown to be a nuclear matrix attachment site (Cockerill and Garrard, 1986). BPV DNA is known to become attached to the nuclear matrix (Briggs, 1983). The AT_5A sequence is not conserved at the borders of the 5′ palindrome of MVM although the sequence GT_5A occurs 15 bases from the terminus. The occurrence of a conserved sequence around the border of the palindromes of MVM was noted by Chow et al. (1986). They showed that the sequence around the putative nicking sites at each end of the MVM genome could be represented as a set of five direct or indirect repeats of an 11 base consensus sequence. The conserved sequences covered 60 bases at the 3′ end and 54 bases at the 5′ end and were approximately centered on the putative nick sites. This is very close to the size of the conserved sequences at the 3′ and 5′ ends of BPV and here too the sequences are approximately centered on the nick sites. Conserved motifs around the terminus of the palindromes could form recognition sites for enzymes which are involved in hairpin transfer processing of RF DNA. Cleavage of MVM RF DNA during replication occurs at the sequences CTATTC and CTTATC for the 5′ and 3′ ends, respectively, which are related to the cleavage sites of several recombinase enzymes (Hogan and Faust, 1986). It has been proposed that the sequences TGAAC, TGACCAAC and TGAA(C/A)C, which occur on each side of the borders of the palindromes of H-1 and MVM, are recognition signals for processing of the termini during viral replication (Rhode and Klaassen, 1982). A computer search indicated that there are no sequences related to these in the termini of BPV.

The occurrence of the 3′ flop conformation in BPV virion DNA has been proven by sequence analysis of primary clones (Table 1) and by end labeling experiments (Fig 3). Both these methods show that 3′ flop is a minor but significant species in BPV DNA. The occurrence of sequence inversions in viral DNA has been shown to occur after transfection of cells with cloned AAV-2 (Samulski et al., 1982). Here, we show that sequence inversions arise at both termini after transfection of BPV clones. The occurrence of sequence inversions at both ends of AAV-2 was expected because the termini of AAV-2 are identical. BPV, however, has nonidentical termini and

this is the first report of the occurrence of sequence inversions at both ends of an autonomous parvovirus.

The occurrence of 3′ flop in BPV suggests differences in the replication of the 3′ end of BPV compared to MVM. Based on the occurrence of a unique 3′ end in MVM DNA and the abundance of dimer in MVM RF preparations, a replication model has been proposed in which an obligatory dimer replication intermediate is used (Astell et al., 1985). Processing of the dimer is proposed to occur in a way which results in conservation of the input 3′ end and produces equal proportions of 5′ flip and flop. The occurrence of both flip and flop conformations at the 3′ end of BPV demonstrates that this model does not explain the replication of BPV DNA. AAV DNA is proposed to replicate via monomer intermediates with hairpin transfer occurring at both the 3′ and 5′ ends (Berns and Hauswirth, 1984). This results in equal proportions of flip and flop at both termini. The much greater abundance of 3′ flip than 3′ flop in BPV virion and RF DNA suggests that BPV DNA does not replicate according to the AAV model. Sequence inversion clearly occurs at the 3′ end of BPV DNA but since the flip greatly exceeds flop, some mechanism must exist for the preferential replication of the flip conformation at the level of RF DNA. In vivo RF DNA has the same ratio of 3′ flip to flop as does virion DNA (Fig. 7), which demonstrates that the predominance of the 3′ flip conformation in virion DNA is not due to more efficient encapsidation of the flip form from the intracellular RF DNA pool. Even when transfection is done with plasmids which have a 3′ flop conformation the progeny virion DNA contains predominantly the flip conformation. This rules out a replication mechanism in which the viral DNA population contains a preexisting mixed of 3′ flip and flop DNA molecules which are replicated with complete retention of the starting 3′ end conformation. Since the 3′ flip and flop conformations of BPV have the same amount of base paired secondary structure, it seems unlikely that any factors such as stability of the hairpin structures could account for the differences in replication of the two conformations. The unpaired four base sequence ATTA on the main stem of the hairpin should lead to bending of the stem with respect to the arms which would make the overall geometry of the flip and flop conformations different (Fig. 1). This difference in geometry could mediate differential binding of some factor(s) during DNA replication. The flip and flop conformations at the 5′ end should have identical sec-

ondary structure and overall geometry, differing only in the sequence of the 17 base central loop. Differential replication of the 5′ end therefore seems unlikely. There was, however, a small but consistent difference seen in the proportion of flip to flop at the 5′ end of BPV RF and virion DNA. The significance of this observation is unclear.

The high degree of sequence conservation at the borders of the hairpins of BPV suggests a possible mechanism for the encapsidation of both strands of BPV. Since encapsidation appears to require binding of empty capsids in or near the palindromes, the conserved sequences could mediate binding to either strand to generate an encapsidation complex. In this model, binding would occur on the minus strand of one palindrome and on the plus strand of the other palindrome. Recent binding studies have shown that BPV capsid proteins VP-2 and VP-3 bind strongly to a restriction enzyme fragment containing the 3′ terminal 290 nucleotides of the BPV genome and VP-3 also binds weakly to a fragment containing the 5′ terminal 450 nucleotides (Lederman et al., 1987). These observed differences in binding affinity at the two ends suggest a possible basis for the unequal encapsidation of progeny DNA strands. Since the minus strand is the major strand encapsidated, it is likely that capsid binding at the 3′ end mediates its encapsidation. Parvovirus LPV encapsidates plus and minus strands in about the same proportion as BPV (Shull, B. and R. C. Bates, unpublished data). Sequencing of cloned LPV DNA identified a clone containing the AT_5A sequence found conserved at the BPV termini (Shull, B. C., and J. B. Metcalf, unpublished data) which showed some homology with the 5′ terminus of BPV (Fig. 10). The possible occurrence of the AT_5A sequence at the 5′ end of a second autonomous parvovirus which encapsidates a significant amount of plus strand strengthens the possiblity that it represents part of a signal involved in encapsidation.

Parvovirus LuIII encapsidates approximately equal numbers of plus and minus strands. The sequence of the palindromes of LuIII has recently been determined (Shull et al., 1987). LuIII does not have identical ends, in agreement with previous data (Bates et al., 1984). This rules out an AAV-like mechanism in which both strands are encapsidated because the ends are identical. The sequence of the LuIII palindromes shows only minor differences from MVM, which encapsidates predominately minus strands. Further, there are no conserved sequences between the palindromes

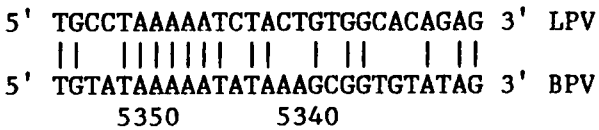

Figure 10. Sequence homology between the 5′ palindrome of BPV and LPV: LPV sequence is on the upper line and BPV minus strand sequence is on the lower line.

of LuIII which could function as repeated *cis* signals such as that found in BPV. It appears unlikely that the encapsidation of plus strands by LuIII can be explained solely by *cis* signals in or near the terminal palindromes since LuIII and MVM, which differ greatly in encapsidation behaviour, have nearly identical palindromes. Either the *cis* encapsidation signal(s) lie outside the terminal palindromes or the capsid proteins of the two viruses have differences in binding to encapsidation signals which lead to the different encapsidation patterns.

The replication cycle of AAV-2 shows symmetry in the occurrence of flip and flop conformations at both termini and in the encapsidation of equal amounts of plus and minus strands. These symmetries are both thought to be mediated by the inverted terminal repeats. The terminal repeats of AAV-2 are 145 nucleotides long of which the first 125 are palindromic. Thus, the terminal repetition extends 20 bases past the palindrome, suggesting that this region is important in the replication of AAV-2. The conserved sequence at the borders of the palindromes of BPV constitute a partial inverted terminal repeat which extends 19 bases beyond the border of the palindromes (Fig 9). Although the termini of BPV are nonidentical, the terminal conserved sequence could contain repeated signals involved in hairpin transfer and encapsidation. That both these processes are only partially symmetrical could reflect the imperfect conservation of sequences at the termini. If this idea is correct, *cis* signals important in both encapsidation and hairpin transfer during replication would occur within the 55 base conserved sequence.

Chapter III

SITE DIRECTED MUTAGENESIS OF BPV

INTRODUCTION

The ability to clone parvoviral genomes makes it possible to construct site directed mutations to study the genetic functions of these viruses. Genomic clones have been exploited to study the genetic functions of AAV-2 using a series of deletion and frame shift mutants (Hermonat et al., 1984; Tratschin et al., 1984a) This work permitted definition of three classes of mutations associated with the left and right ORFs. The *rep* mutants, which were defective for DNA replication, were associated with mutations in the left ORF. The *cap* mutants, which mapped to the right ORF, did not produce infectious virus due to failure to produce capsid proteins. A defect in the map 48 to 55 region was associated with a low yield of infectious virus particles despite high level DNA and capsid antigen synthesis. This mutation, called *lip*, involves the coding region for the amino terminal portion of the minor capsid protein, VP-1. Recent work demonstrates that the left ORF of AAV-2 encodes four different proteins which have functions in the replication of viral DNA (Mendelson et al., 1986) yet the mutation data on AAV-2 defined only one complementation group within the

left ORF. All mutations in the left ORF abolished DNA replication and could not be complemented by other left ORF mutants. This is probably due to the overlapping structure of the left ORF transcripts of AAV-2. That is, each frameshift or deletion mutation introduced alters multiple proteins. The construction of point mutations introduced by oligonucleotide directed mutagenesis may be useful in further dissection of the genetic functions of AAV-2, as well as other parvoviruses.

Attempts to perform a similar mutational analysis with the MVM genomic clone have been less successful due to the low transfection efficiency of this clone (Pintel et al., 1984). The effect of deletion of terminal sequences on rescue and replication of MVM using clones lacking either the left or right palindrome was studied (Merchlinsky et al., 1983). It was found that both palindromes must be present in the same molecule for rescue and replication of viral DNA to occur. Studies of AAV-2 clones with various deletions within the terminal palindromes revealed that correction of deletions could occur provided the sequence was not deleted in both palindromes. A gene correction mechanism was proposed to account for this (Samulski et al., 1983). Together, these data define *cis* and *trans* genetic functions.

Several types of specialized expression plasmids have been constructed for the study of parvovirus genomes. Pintel et al. (1984) constructed a chimeric MVM-bovine papilloma virus (BPpV) plasmid which expressed MVM capsid proteins in mouse C127 cells. Following up on this work, Labienic-Pintel and Pintel (1986) constructed a BPpV-MVM chimera which contained only the capsid coding region and associated promoter of MVM. This plasmid encoded both capsid proteins of MVM in the usual ratio and empty capsids assembled in the transfected cells. These data established that the right ORF encodes the capsid proteins and that no genetic function of the left ORF is necessary for regulation of the ratio of the two capsid proteins. A similar conclusion was reached by Janik et al. (1984) who demonstrated the production of all three capsid proteins of AAV-2 after transfection of a clone containing the right ORF and associated promoter. Again, the capsid proteins were produced in the same ratio as during normal infection. Expression vectors using the chloramphenicol acetyl transferase (CAT) gene have been constructed using H-1 (Rhode, 1985b) and AAV-2 (Tratschin et al., 1986). In both cases the CAT gene was put under control of the viral capsid protein gene promoter. H-1 constructs containing the left ORF region, which en-

codes the large nonstructural protein N1, supported higher CAT expression than plasmids lacking lacking this function. Increased CAT expression from the capsid promoter could be obtained in *trans* by co-transfection of plasmids separately containing the CAT gene and the N1 gene of H-1. Similarly, CAT gene expression could be enhanced in *trans* by left ORF gene products of AAV-2. A final type of clone has been constructed by Hermonat and Muzyczka (1984) using AAV-2. A gene for neomycin resistance was substituted for the capsid protein coding region. This plasmid was transfected into host cells in the presence of adenovirus and used to isolate a stock of recombinant virus carrying the neomycin resistance gene. This recombinant stock was used to transduce human cell lines to neomycin resistance.

Studies on *cis* functions of the BPV genome and the structure of the terminal palindromes after rescue and replication from genomic clones were reported in the first two chapters of this work. The purpose here is to construct and characterize a set of deletion and frameshift mutations in the coding regions of BPV. These mutants will be analyzed after transfection into BFL cells for rescue and replication of the BPV genome and for production of viral proteins.

MATERIALS AND METHODS

Construction of Mutant Genomes

Plasmid pVPI1 was used for the construction of a set of frameshift and deletion mutants. Frameshift mutants were constructed at restriction enzyme sites which occur once in the BPV genome and do not occur in the pUC8 parent plasmid. For restriction sites producing 5′ overhangs the plasmid was linearized and the ends filled in with DNA polymerase I Klenow fragment and all four deoxynucleotides. The resulting blunt ended linear molecules were then ligated with T4 DNA ligase. For restriction enzyme sites which yield blunt ends the linearized plasmid was ligated to 8

base ClaI linkers (5′ CATCGATG 3′; Pharmacia, Piscataway, NJ). ClaI does not digest either BPV or pUC8, so the appearance of a ClaI site provided a convenient marker for linker insertion. Deletion mutations were constructed using restriction enzyme sites which occur once or twice in BPV and are absent from pUC8. Plasmid was digested with the enzyme(s) and the resulting fragments were separated on 1% low melt agarose gels. The fragment of interest was excised from the gel. The gel chunk was melted by heating at 70°C for 5 min, mixed with T4 ligase reaction mixture and incubated overnight at 15°C (Struhl, 1985). The ligation mixtures were then melted and mixed with 10 volumes of Tris-HCl, $MgCl_2$, $CaCl_2$ buffer (10 mM each, pH 8.0) (Struhl, 1985). Transformation with ligation mixtures was done by the Hanahan procedure (1983) using *E. coli* strain JM107 or DH5α. Large preparations of plasmids were purified by the procedure of Rodriguez and Tait (1983) and banded once on a cesium chloride-ethidium bromide gradient (Maniatis et al., 1982).

Transfection and Analysis of Mutant Plasmids

Monolayers of BFL cells were transfected as already described using the DEAE-dextran method with DMSO shock. In some experiments the calcium phosphate precipitate method was used as described by Perbal (1985). Cells were seeded at 7-8 x 10^5 cells per 60 mm plate. Transfection of cells in suspension was based on the method of Milman and Herzog (1981). Cells were detached from glass bottles by trypsinization, resuspended in MEM supplemented with 200 μg/ml DEAE-dextran and distributed to 17x100 mm polypropylene tubes at 10^6 cells/tube. DNA, 0.2 to 1 μg, was added and the tubes were incubated at 37° C for 1 h. The cells were washed once with MEM and shocked for 5 min with 10% DMSO, washed again and seeded into 60 mm culture dishes in 5 ml MEM plus 10% FCS. For plaque assay, cells were allowed to attach overnight, then the media was aspirated and replaced with MEM plus 10 % FCS in 1% low melt agarose. Transfected cell layers were observed for BPV specific CPE. Proteins were analyzed by HA, IFA staining and immunoprecipitation as previously described (Chapter 1). DNA was isolated from

transfected cells for detection of viral DNA by Southern blotting either by the Hirt procedure, as previously described (Chapter 1), or by a procedure for isolation of whole cell DNA. For whole cell DNA, cells were lysed in the culture dishes using 200 µl of 10mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.6% SDS. The lysates were transferred to micro centrifuge tubes and digested with proteinase K (100 µg/ml) at 60°C for 8 hrs. The DNA was extracted with 1:1 phenol:chloroform and then chloroform, recovered by ethanol precipitation and redissolved in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. DNA samples were analyzed by Southern blotting with alkaline transfer to Zeta-Probe membranes, as previously described. Probes were nick translated BPV DNA labeled with either ^{35}S-dATP or ^{32}P-dATP, as noted.

RESULTS

Construction of Mutant Clones

Table 1 lists the mutations constructed and their genomic locations. Frameshift mutations are denoted as pVPI1FS followed by the name of the restriction site used in the construction. For convenience these are referred simply as FS restriction site. Mutants FSXba and FSBst were both constructed by filling in the 5′ overhang resulting from restriction digestion and ligating to circular plasmid. This results in a frameshift in both cases. Mutants FSHpa, FSNae, and FSBal were all formed by ligation of ClaI linkers after digestion with the indicated restriction enzyme. Analysis of the complete nucleotide sequence of BPV reveals two BalI restriction sites located at map units 39 and 54 (Chen et al., 1986b). Repeated attempts to digest pVPI1 DNA with BalI resulted in linearization, suggesting that one of the sites does not digest. To establish which of the two potential BalI sites was being digested, a double digest of pVPI1 was made with HpaI and BalI. This resulted in a fragment of 1.6 kb which demonstrates that the BalI site at map unit 54 was digested.

Table 1. Summary of the structure of frameshift and deletion mutants of the BPV genome.

plasmid	map position	reading frame
FSXba	15	left
FSHpa	24	left
FSNae	30	left
FSBal	54	middle
FSBst	85	right
ΔHpa/Nae	24-30	left
ΔHpa/Bal	24-54	left and middle
ΔKpn	44-90	left, middle and right
ΔBan	48-69	left, middle and right

Digestion at the map 39 BalI site would yield a fragment of 0.84 kb. Four deletion mutations, designated pVPI1Δ restriction site(s), were constructed. The ΔKpn and ΔBan mutants were constructed by simple digestion and religation, as each of these enzymes digests BPV twice and does not digest pUC8. The map unit 24 to 30 region was deleted in ΔHpa/Nae by taking advantage of the fact that each of these restriction sites occurs only once in the BPV genome. Similarly, the map unit 24 to 54 region was deleted in ΔHpa/Bal. HpaI, NaeI and BalI all produce blunt ends so no end repair reactions were necessary during preparation these deletion mutations. The positions of the mutants in relation to the genome of BPV are shown in Fig. 1.

All mutations were confirmed by restriction enzyme digestion. The frameshift mutants were checked for loss of the cognate restriction site and, where appropriate, for the appearance of a ClaI site. Deletion mutants ΔKpn and ΔBan were screened by digestion with the cognate enzymes. In both cases digestion resulted in linearization of the plasmids, confirming loss of the internal BPV restriction fragment. All four Δ mutants were analyzed by digestion with EcoR1 and SalI. SalI releases the entire BPV insert and EcoR1 digests BPV at map units 17 and 92. The Δ mutants occur within the 4.2 kb EcoR1 A fragment. The appropriate shortening of the EcoRI A fragment was demonstrated on all four Δ mutants when analyzed by agarose gel electrophoresis.

Transfection and Analysis of Mutant Clones

Infectivity of the mutant clones was analyzed by transfection into BFL cells using the DEAE-dextran method. No CPE was observed in cell layers transfected with any of the deletion or frameshift mutants (Table 2). Since all these mutants occur in coding regions, this is not unexpected. Cell layers transfected with several of the mutants were tested for capsid production by HA. This technique detects both full and empty capsids and might be expected to show positive results with mutations not affecting the capsid coding region, the right ORF. HA was negative on cell layers transfected with the ΔKpn, ΔBan, FSXba or FSBst mutations.

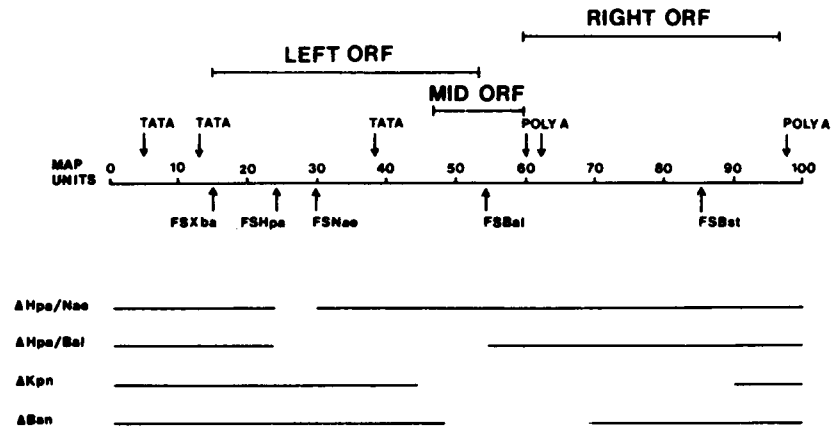

Figure 1. Structure of BPV deletion mutations: The genome of BPV is represented in map units with the 3' end at the left. Above this line the three major open reading frames of BPV are depicted along with the position of consensus promoter sequences (TATA) and polyadenylation sites (poly A). Arrows below the genomic map line denote the position of frame shift mutants. The deletion mutants are depicted at the bottom with gaps in the lines depicting the postition of the deletions.

Table 2. Summary of analysis of mutant plasmids after transfection.

Plasmid	CPE	HA	IFA	Immunoppt	DNA
FSXba	neg	neg	neg	neg	neg
FSHpa	neg	nd	pos[1]	nd	neg
FSNae	neg	nd	nd	nd	nd
FSBal	neg	nd	nd	nd	nd
FSBst	neg	nd	pos[1]	neg	neg
ΔHpa/Nae	neg	nd	neg	nd	neg
ΔHpa/Bal	neg	nd	neg	nd	neg
ΔKpn	neg	neg	neg	neg	neg
ΔBan	neg	neg	neg	nd	neg

[1]These results were obtained on coverslips transfected by the DEAE-dextran method in Leighton tubes as described in Materials and Methods.

For a more sensitive analysis of viral protein production from mutant plasmids transfected cell layers were analyzed by IFA and immunoprecipitation. There was no demonstrable production of viral antigen by any of the four Δ mutants when transfected cell layers were analyzed by IFA. FSXba and FSBst also gave negative IFA. Positive results were obtained by IFA on the FSHpa and FSBst mutants when transfection was carried out in Leighton tubes, as will be discussed below. No viral proteins were detected when mutants ΔKpn, FSXba and FSBst were ananyzed for viral protein production by immunoprecipitation. An antiserum (calf 86) which recognizes both capsid and noncapsid proteins of BPV was used in this analysis so it is unlikely that viral specific proteins were not detected due to the limited specificity of the antibody.

Next, experiments were run with the aim of detecting viral DNA synthesis in cell layers transfected with mutant plasmids. DNA was isolated by Hirt extraction from cell layers transfected with mutant plasmids and the DNA was analyzed by Southern blotting. No BPV DNA replication could be detected after transfection with any of the deletion mutants. Since each of the mutant plasmids involve at least part of the left ORF, the failure to detect viral DNA replication could be due to disruption of virally encoded function(s) required for DNA replication (Hermonat et al., 1984). Transfections with frameshift mutants FSXba, FSHpa and FSBst were also analyzed by Hirt extraction. Again, no viral specific DNA synthesis could be demonstrated for any these mutant plasmids even though ^{32}P labeled probe was used for maximum sensitivity. The FSBst mutant would be expected to support viral DNA replication since the mutation involes only the right ORF (Hermonat et al., 1984). Comparison was made of the yield of viral DNA after transfection with the complete genomic clone pVPI1 using the Hirt extraction and whole cell DNA methods. Southern blots of viral DNA indicated that both methods gave comparable recovery of viral DNA. This suggests that failure to detect viral DNA synthesis from any of the mutant plasmids is not due to poor recovery of DNA in the Hirt extraction procedure.

Leighton Tube Transfection

Failure to detect replication of viral DNA or synthesis of viral proteins could reflect either failure of the methods to detect low levels of expression or complete suppression of activity of the cloned genomes by the mutations. The latter possibility seemed unlikely in view of data obtained with the AAV-2 mutant clones (Hermonat et al., 1984 and Tratschin et al., 1984a). More likely, low efficiency of transfection limits the population of cells expressing viral products and, since all the mutations prevent formation of infectious progeny virus, amplification of activity by secondary infection is abolished. Accordingly, efforts to improve the transfection efficiency were undertaken.

Leighton tubes are 13 x 130 mm test tubes with a flattened chamber at the bottom for small scale cell culture. Leighton tubes provide an excellent chamber for performing IFA analysis because a cover slip occupies the entire culture surface. Cell cultures of BFL cells were seeded at 10^5 cells per tube and transfected with infectious and mutant plasmids by the DEAE-dextran method. Coverslips from these transfections were stained with appropriate BPV antisera. Strongly positive results were obtained on pVPI1 and FSBst and a weaker positive result was obtained of FSHpa. These data definitely established that expression of viral protein(s) occurs from mutant plasmids. Further, the two mutants giving positive results are left and right ORF mutants, which demonstrates expression from two different coding regions by mutant genomes.

In the above transfection media pH during the 4 h DEAE-dextran incubation exceeded pH 8.0. BFL cells are sensitive to high pH and this was reflected in a variable loss of cells in the Leighton tubes. Some tubes retained confluent cell layers while others lost most of the cell layer. The samples showing strong fluorescence on IFA had all retained confluent cell layers. The cell layer transfected with ΔHpa/Nae was very poor and gave a negative IFA result. Since this deletion occurs in the same region of the genome as FSHpa, which gave a positive IFA result, it would be expected to give a positive result as well. A second transfection was attempted using Leighton tubes in which media pH during the transfection step was maintained at 7.4 to 7.6 to prevent cell damage. The cell layers were uniformly healthy in appearance but IFA staining of cells transfected with the

infectious plasmid pVPI1 were negative. This result suggested that the media pH during transfection is critical. Higher pH appears to improve transfection per se but also causes cell damage. An optimum compromise pH might be found at which cell damage is minimal and transfection is maximal.

Suspension Transfection

Transfection of cells in suspension was tried according to the method of Milman and Herzberg (1981) in a second effort to improve transfection efficiency. This method gave very poorly reproducible cell lawns. Most plates had subconfluent cell layers after overnight growth. One plate in which a confluent and apparently healthy cell layer was obtained was a plaque assay transfected with pVPI1. This plate gave a very low plaque response (9 plaques/0.2 μg vs 65 plaques/0.2 μg expected). These results indicate that suspension transfection is not a useful technique with BFL cells.

Transfection of Transformed Cell Lines

Tumor cell lines often provide susceptible host cells for parvovirus infections. Transformation of cells by SV-40 can greatly increase susceptibility to parvovirus infection due to an increase in viral DNA replication (Chen et al.,1986a). These observations formed the basis for an experiment using two transformed cell lines. Newborn human kidney cells (NBE) are a line of SV-40 transformed cells commonly used as a host for parvoviruses LuIII and H-1. BFL-T cells are a line of SV-40 transformed bovine fetal lung cells (Bates R. C., unpublished data). NBE and BFL-T cells were transfected by the DEAE-dextran method with pVPI1 and mutant plasmids FSHpa and FSBst. IFA stain for BPV antigens was negative on both cell lines after transfection with pVPI1 and the mutant plasmid FSBst. Southern blotting for viral DNA was negative in both cell lines

after transfection with pVPI1 and the mutant plasmids FSHpa and FSBst. Both cell lines were also exposed to BPV virions and neither cell line gave rise to hemagglutinating virus, suggesting that NBE and BFL-T cell lines are not hosts for BPV infection.

Co-Transfection Experiments

Simultaneous transfection of two mutant plasmids can give rise to expression of viral products by either complementation between the two mutants or by recombination to produce infectious progeny virus. Each of four combinations of mutants which were co-transfected into BFL cells by the DEAE-dextran method gave rise to CPE 8 to 10 days after transfection (Table 3). The appearance of CPE was delayed by 3 to 5 days compared to transfection with infectious genomic clones and CPE occurred as localized plaque-like areas. The occurrence of plaques clearly demonstrates the spread of virus from primary transfected cells to neighboring cells.

Hirt preparations from BFL cell layers co-transfected with FSBst x FSHpa and FSBst x ΔHpa/Nae were analyzed by Southern blotting (Fig. 2). Viral DNA bands of single stranded and double stranded BPV DNA are seen after transfection with pVPI1. In addition, an intense diffuse band of viral DNA is seen below the single stranded BPV band in the pVPI1 lane and in the co-transfection lanes. This DNA provides direct evidence for rescue and replication of the viral genome during co-transfection. No replication of viral DNA was seen when the three mutant plasmids used in the co-transfection experiment were transfected singly (Fig. 2).

DISCUSSION

The construction of frameshift and deletion mutants using genomic clone pVPI1 was readily accomplished by taking advantage of restriction enzyme sites which occur one or two times in BPV

Table 3. Co-transfection of mutant plasmids[1].

plasmid 1	plasmid 2	CPE	DNA
FSXba	FSBst	Pos	nd[2]
FSHpa	FSBst	Pos	Pos
ΔHpa/Nae	FSBst	Pos	Pos
FSHpa	FSBal	Pos	nd

[1]About 1 μg of each of the two indicated plasmids were co-transfected into BFL cells by the standard DEAE-dextran procedure.

[2]Not determined.

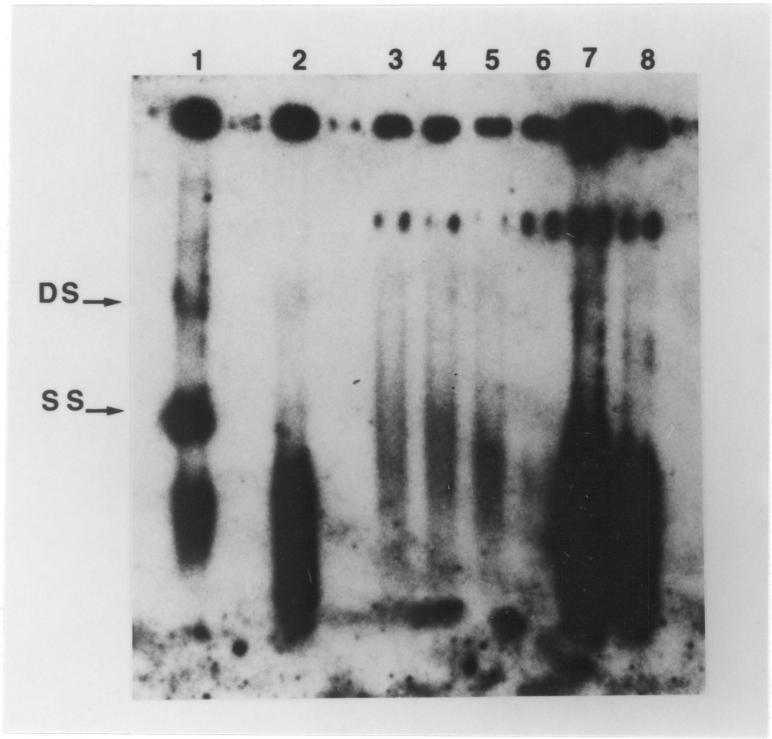

Figure 2. BPV viral DNA produced after co-transfection of mutant plasmids: Mutant plasmids were transfected either singly or co-transfected in pairs into BFL cells by the DEAE-dextran method. Low molecular DNA was extracted by the Hirt procedure and the resulting DNA was analyzed by Southern blotting with ^{32}P-dATP labeled BPV virion DNA as the probe. Lane 1 contains BPV virion DNA as a marker. Lane 2 contains the DNA extracted from cells transfected with the infectious genomic clone pVPI1. The remaining lanes contain DNA from cells transfected with: lane 3, pUC8 (negative control); lane 4, FSHpa; lane 5, FSBst; lane 6, ΔHpa/Nae; lane 7, FSHpa and FSBst co-transfection; lane 8, ΔHpa/Nae and FSBst co-transfection.

and not in pUC8 (Fig. 1). Frameshift mutants which singly affect each of the three major ORFs of BPV were constructed. Among the deletion mutants, ΔHpa/Nae affects only the left ORF, while the other three affect multiple ORFs (Table 1). The left ORF of BPV is thought to encode at least two nonstructural proteins of molecular weights 75,000 and 82,000 (Lederman et al., 1987) which probably function in the replication of viral DNA. The mid ORF probably codes for the non-structural protein NP-1 of molecular weight 28,000. NP-1 has been suggested to play a role in the late stages of BPV DNA replication (Robertson, 1983). The right ORF encodes the three capsid proteins. The promoters from which mRNAs encoding these various proteins originate is not yet determined.

None of the mutants was infectious after transfection, as judged by the absence of CPE. This demonstrates that functions in each of the ORFs of BPV are essential for productive viral infection. The frameshift mutations, which selectively abolish the function(s) of one ORF only, demonstrate this point clearly. This finding is a reflection of the dense packing of information in the parvovirus chromosome. A similar mutational analysis of AAV-2 showed that mutations in both the left and right ORF abolished productive infection. One class of mutation in the middle of the genome was associated with the formation of a low level of infectious progeny virus. This class of mutations affected the minor capsid protein, VP-1 (Hermonat et al., 1984; Tratschin et al., 1984).

Despite the lack of progeny virion formation by most of the AAV-2 mutants, assay of intracellular viral DNA revealed replication by mutants which affected only the right ORF. Assays for DNA replication after transfection of various BPV mutant plasmids were all negative (Table 2). The FSBst mutation, which affects only the right ORF, would be expected to support DNA replication by analogy with AAV-2. Analysis for viral protein after transfection with several mutant plasmids was negative by HA and immunoprecipitation, but a positive IFA result was obtained on two mutants (Table 2). This result demonstrates that expression of the left and right ORFs can occur separately even though both functions are required for progeny virion formation. The positive IFA result on FSHpa was obtained using an antiserum which recognizes only capsid proteins (rabbit 0118), while the FSBst result was obtained using an antiserum which recognizes both non-structural and capsid proteins (Calf 86). The specificity of the rabbit 0118 serum suggests that capsid

protein production was detected from the FSHpa mutant. Since calf 86 serum reacts with both nonstructural and capsid proteins the identity of the proteins produced by the FSBst mutation is not clear. The transcription of viral DNA could occur either from the unreplicated input plasmid or from viral DNA after rescue and replication. The detection of capsid proteins of AAV-2 from nonreplicating plasmids has been demonstrated by Janik et al. (1984). It seems likely that the FSHpa mutation does not undergo rescue and replication, since this is a left ORF mutation.

Co-transfection of four combinations of mutant plasmids resulted in the formation of infectious progeny virus (Table 3). Progeny virion DNA was detected by Southern blotting for two of these transfections. The pairs of mutant plasmids used affect different ORFs and might, therefore, be expected to exhibit complementation. Complementation was exhibited when left and right ORF AAV-2 mutants were co-transfected (Hermonat et al., 1984; Tratschin et al., 1984a). Recombination between the co-transfected AAV-2 genomes was observed to occur at a very low level. The mechanism of virus production in the present experiments cannot be determined without further work. Recombination could readily lead to the formation of wild type virion DNA with subsequent infection by the resulting virus. Alternatively, complementation between the mutant genomes might lead to the separate encapsidation of mutant genomes. Co-infection of adjacent cells could then give rise to the observed CPE.

A major problem in the analysis of the mutant genomes of BPV is the low transfection efficiency routinely obtained. This problem has also hampered analysis of mutants of the MVM genomic clone (Pintel et al., 1984). The successful analysis of AAV-2 mutant genomes indicates a higher transfection efficiency. This may, in part, reflect the fact that AAV-2 can undergo integration and excision from the host cell genome as a normal part of its replication cycle. Excision of AAV-2 from recombinant plasmids probably occurs by the same mechanism which is normally used to rescue the genome from an integrated state and appears to be highly efficient. BPV and MVM, on the other hand, have not evolved a strategy of integration into the host cell chromosome and the mechanism for rescue of the cloned genome may be less efficient than that of AAV-2. Since the mutant BPV genomes do not produce progeny virus it is necessary to detect activity in the original

population of transfected cells. When the transfection efficiency is low, a negative result cannot be used to conclude lack of function due to the mutation.

Experiments aimed at improving the transfection efficiency have not been very successful. Initial experiments demonstrated that the DEAE-dextran method with DMSO gives higher transfection efficiency than the calcium phosphate method, in agreement with other work (Sussman and Milman, 1984). For this reason the DEAE-dextran method has been used here. DEAE-dextran exerts a variable degree of toxicity toward different cell types (Lopata et al., 1984). The BFL cells used here seem to vary in response to DEAE-dextran treatment, as judged by the amount of non-specific CPE seen after transfection. At times the BFL cells have become highly sensitive to DEAE-dextran with a concomitant drop in transfection efficiency and this has hampered attempts to optimize transfection. Lowering the DEAE-dextran concentration or shortening the time of exposure lowers the nonspecific CPE but also reduces transfection efficiency. BUL cells are a useful alternate host cell for BPV infection but BUL cells have proven even more sensitive to the toxic effects of DEAE-dextran than BFL cells. Two SV-40 transformed cell lines were tested as potential hosts for transfection with BPV genomic clones but neither cell line was permissive for BPV infection.

CONCLUDING REMARKS

In this dissertation I have used the technique of blunt end cloning to construct two series of genomic clones of the autonomous parvovirus BPV. Series pGCSma consists of seven clones in the blunt SmaI site of pUC8. In this series of clones the BPV insert is flanked at one terminus by a vector EcoRI site. Since BPV has two EcoRI sites, it is not possible to remove the insert intact by restriction enzyme digestion. The pGC series was constructed by blunt end ligation into the PstI site which was made blunt by S1 nuclease digestion. This positioned the SalI and HindIII sites of the vector at the termini of the BPV insert. Since neither of these sites is present in BPV, the insert may be manipulated at either end. Sequencing demonstrated that all of these primary clones were deleted at the 3′ end and most were deleted at the 5′ end. Clone pGC119, which has a 3′ end deletion of 27 bases and a 5′ end deletion of 25 bases, was used to construct a series of clones with various 3′ ends. These clones, designated pVPI, included both 3′ flip and flop conformations and 3′ end deletions ranging from 0 to 79 bases.

Infectivity of the genomic clones was tested after transfection into BFL cells. Clones pGCSma17, 20 and 22 as well as pGC119, 127, and 167 were all infectious as jugded by the appearance of BPV specific CPE. Further analysis of transfected cell layers by HA, IFA, immunoprecipitation of labeled proteins using BPV specific antisera and Southern blotting to detect

viral DNA all confirmed that virions indistinguishable from wild type were produced from the genomic clones.

Plaque assays were used to quantify the infectivity of various genomic clones in the pGC and pVPI series. These data demonstrated that the 3′ flip and flop conformations were equally infectious. Clones with deletions of 27 to 34 bases were infectious but had a ten fold reduction in plaque titer compared to clones with an intact 3′ end. A 3′ end deletion of 12 bases caused no reduction in plaque titer. The two largest 3′ end deletions tested were 52 and 79 bases and neither of these plasmids was infectious. Evidently, the region between bases 34 and 52 of the 3′ end of BPV defines a critical region necessary for rescue and replication. Base 52 occurs prior to the two arms of the hairpin and just inside a region of the hairpin containing unpaired bases. It is possible that deletions which fall within this region prevent the formation of a stable hairpin and thereby prevent initiation of replication. The region between bases 34 and 52 should be a useful target for further mutational analysis. Plasmids with deletions of up to 35 bases at the 5′ end were infectious and a 5′ end deletion of 25 bases seemed to cause no quantitative decrease in infectivity. Terminal deletions which did not abolish replication were repaired to wild type length at both the 3′ and 5′ termini, as shown by end label analysis of progeny virion DNA. End label analysis also demonstrated that wild type BPV DNA has a variation in position of the terminal nucleotide of four bases at the 3′ end and three bases at the 5′ end. Similar findings were obtained with AAV-2 (Hermonat et al., 1983). Deletions at one terminus of AAV-2 were repaired to wild type length and the heterogeneity in position of the terminal nucleotide was the same as wild type DNA. The heterogeneity of terminal nucleotide position seems to be a general property of parvoviruses, having been found in AAV-2, BPV, MVM (Astell et al., 1979) and LuIII (Shull et al., 1987). It has been suggested that the nicking enzyme which is thought to be involved in processing the palindromic termini does not have absolute site specificity but cuts with different probability within a range of several bases to generate the terminal heterogeneity (Berns, 1984).

The occurrence of both the flip and flop sequence inversions at the 3′ end of wild type BPV DNA was confirmed by two methods. BPV virion DNA was digested with SmaI which cuts once within the 3′ palindrome. Since the SmaI site occurs as part of an unpaired base region of the

hairpin, it can be found in different positions in the flip and flop conformations as a result of hairpin transfer. This will lead to two terminal restriction fragments of 44 and 106 nucleotides corresponding to the flip and flop conformations, respectively. Cloning of the terminal SmaI fragments lead to the isolation of a clone in the 3′ flop conformation. This method of cloning prevented the possibility of artifacts such as sequence inversion within the 3′ palindrome by bacterial recombination enzymes during cloning. End label analysis of double stranded virion DNA provided an independent confirmation of the occurrence 3′ flop and demonstrated that it is present at much lower levels than the predominant flip conformation. End label analysis of in vivo RF DNA revealed that 3′ flop was present at about the same ratio to flip as in virion DNA. Analysis of progeny DNA from cell cultures transfected with various genomic clones demonstrated that the final ratio of flip to flop was constant and independent of the starting conformation of the plasmid.

Analysis of the 5′ end of progeny virion DNA demonstrated that the flip and flop forms occur with approximately equal frequency and clones with either form seem equally infectious. The 5′ terminus was reassigned to nucleotide 5517 based on end label analysis and cloning data. The reassigned terminus may be of functional significance as it occurs in a region which has considerable homology with the 3′ terminus. This region of homology includes a sequence AT_5A which is conserved at the 3′ terminus of all autonomous parvoviruses sequenced to date. Clearly, this sequence is an excellent target for site directed mutagenesis. Comparison of the termini of in vivo RF DNA and double stranded virion DNA by end label analysis revealed that they have identical termini and the same proportion of flip to flop. This is in contrast to MVM which has an 18 base extension of the 5′ end of RF DNA relative to virion DNA (Astell et al., 1985). This extension of the RF DNA is thought occur by nicking of the plus strand at a point 18 bases beyond the mature virion terminus during DNA replication. The nicking enzyme may remain attached as the terminal protein, which is removed during encapsidation by cleavage of the 18 base extension (Astell et al., 1985).

Construction of frameshift and deletion mutations was readily accomplished using the genomic clones. Mutations in each of the three major ORFs abolished infection, which demonstrates that products from each of these coding regions are essential. Attempts to further analyze the

phenotypes of the mutant genomes were largely unsuccessful. DNA rescue and replication could not be demonstrated after transfection of the right ORF mutant FSBst, which by analogy with AAV-2, would be expected to undergo rescue and replication. Assays for proteins by HA and immunoprecipitation were negative. Positive IFA was obtained on the mutants FSHpa and FSBst after transfection in Leighton tubes during which the cells were exposed to pH greater than 8.0 during incubation with DEAE-dextran. This result demonstrated that expression of the mutant plasmids does occur after transfection from both the left and right ORFs. The failure to demonstrate activity reproduceably after transfection is almost certainly due to the low routine efficiency of transfection. The DEAE-dextran transfection method has been more efficient than the calcium phosphate method but results vary considerably. A major problem in reproducable transfection is the variable sensitivity of the BFL cells to DEAE-dextran. Attempts to use alternate host cells, including two SV-40 transformed cell lines, were unsuccessful.

As an alternative to the use of deletion and frameshift mutations, construction of CAT expression plasmids could be used to study promoter functions. In this case activity and control of a putative promoter can be done by the measurement of CAT activity in transfected cells. The use of such a reporter enzyme provides an inherent amplification of activity from the transfected plasmid which can offset low transfection efficiency. This approach has been used to study the capsid gene promoter of the autonomous parvovirus H-1 (Rhode, 1985b). A second possible approach in the genetic analysis of BPV is the construction of bovine papilloma virus chimeras. Since bovine papilloma virus plasmids are capable of extrachromosomal replication, passenger molecules undergo amplification. This approach has been used to express the capsid proteins of MVM in the absence of left ORF function (Labienic-Pintel and Pintel, 1986).

This work demonstrates that the replication of BPV DNA differs from proposed models based mainly on MVM. First, BPV does not have a unique 3′ conformation in either virion or RF DNA. Second, the 5′ end of BPV RF DNA is not extended relative to virion DNA. Third, BPV encapsidates a significant amount of the plus strand. These differences demonstrate that a number of features of the MVM replication model are not universal to autonomous parvoviruses. The replication of BPV in some respects resembles that of AAV in that both show flip and flop se-

quence inversions at both termini and encapsidation of both strands. These properties are attributed to the fact that AAV has identical termini. BPV, however, does not have identical termini although a 55 base conserved region occurs at the termini which could account for the partially symmetric properties in its replication. This would suggest that signals for hairpin transfer and encapsidation occur in the conserved terminal sequence. Site directed mutations within this region could be constructed to study the effects on encapsidation and the ratio of flip to flop at each terminus.

A major puzzle in the replication of BPV is the mechanism by which the ratio of 3′ flip to flop is established and maintained. The occurrence of 3′ flop suggests some processing of the 3′ end by hairpin transfer but does not explain the preferential accumulation of the 3′ flip conformation in both RF and virion DNA. Since the flip form becomes dominant after transfection with clones having either 3′ flip or flop it is clear that the flip conformation replicates preferentially. It is possible to envision models in which the input conformation of the 3′ end is largely but not fully conserved. An example of this would be that most input molecules are replicated via a dimer intermediate with retention of the input 3′ conformation, as is proposed for MVM. If a portion of the replicating molecules were replicated via monomer intermediates with sequence inversion, then the observed unequal ratio of 3′ flip to flop might occur. However, if this model were correct then transfection of a 3′ flop clone should result in the accumulation of progeny viral DNA containing predominantly 3′ flop conformation. The data clearly show that 3′ flip always accumulates as the dominant conformation. It is interesting to note that transfection with clones having 3′ flip conformation also leads to the formation of 3′ flop. This demonstrates that each form of the 3′ end can give rise to the other.

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