CIS AND TRANS SIGNALS FOR THE REPLICATION OF BOVINE PARVOVIRUS

by

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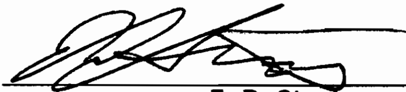
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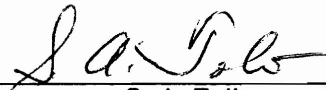
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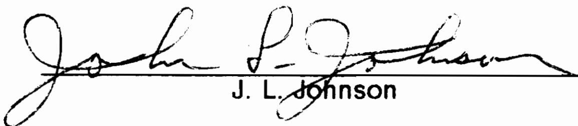
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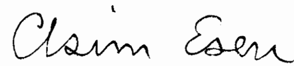
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(ABSTRACT)

The *cis* and *trans* signals important in BPV replication were identified using a transient replication assay, the mobility shift assay, and a comparison between the BPV and LPV genomes. Replication of deleted BPV genomic clones, which contain the natural left (3′ OH end of the viral minus strand) and right (5′ PO_4 end of the viral minus strand) BPV termini, defined the minimum size of the BPV origin of replication (*ori*) to be the terminal 171 nucleotides of each terminus. Clones containing duplicate termini or altered left ends were also shown to replicate. The BPV *ori* was determined to have two domains identified by a computer analysis of homologus regions between these termini. Three proteins were identified that bind to the left terminal 171 nucleotides in the hairpin conformation. Inhibition of the formation of the DNA-protein complexes with competitor DNA localized two potential binding sites that correspond to the domains mentioned above. Two of the DNA-protein complexes were formed by BPV-coded proteins as determined by inhibition of the complex by anti-BPV antibodies. The third complex resulted from binding of a host cell S-phase protein that is a likely candidate for the S-phase factor required for autonomous parvovirus replication. The BPV *ori* thus appears to function by binding both cellular and viral proteins for the initiation of DNA synthesis from the hairpined termini. The comparison of the BPV and LPV genome sequence suggest that the genomic organization of LPV may be more like BPV than that of the rodent parvovirus minute virus of mice; and therefore, LPV may contain similar *cis* signals.

Acknowledgements

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Table of Contents

INTRODUCTION 1

Characteristics and Taxonomy of Parvoviridae 1

Structure 2

Biology 3

Organization of the Genome 5

Parvovirus Replication Models 8

Replication of Eukaryotic DNA 15

OBJECTIVES 19

REPLICATION OF BPV MINI-GENOMES 21

Introduction 21

Materials and Methods 23

Construction of deleted clones 23

Cell culture and viruses 27

Transfections 27

Isolation and Detection of Replicated Chimeric DNA 27

RESULTS 28

Structure of chimeric replicons 28
Replication of Deleted BPV Clones 31
Replication of a Clone with Duplicate Left Termini 34
Replication of a Clone with Duplicate Right Termini 37
Replication of Genomic Clones with Altered Termini 39
Replication of BPV / AAV Chimeras in BFL Cells 39
DISCUSSION 41
The Termini of BPV Act Alone as a Replicon 41
The Minimal Sequences Sufficient For BPV Replication. 43
Termini Interact with Replication Proteins 46

PROTEIN RECOGNITION OF BOVINE PARVOVIRUS TERMINAL DNA SEQUENCES 48
Introduction 48
MATERIALS AND METHODS 50
Cell Cultures 50
Nuclear Extracts 50
Preparation of Radiolabeled DNA 52
Mobility Shift Assay 53
Competitor DNA Preparation 54
RESULTS 54
Terminal Fragment Conformation 54
Identification of Viral DNA-Protein Interaction 57
Cellular factor binds BPV terminus 60
Identification of Viral Proteins Binding to the BPV terminus 60
Specific Inhibition of DNA-Protein Complex 62
Sequences Important in DNA-Protein Complex Formation 65
DISCUSSION 66
Viral Protein Recognizes Termini 68

Cellular Protein Recognizes Termini . . . 70
Role of DNA Binding by Viral and Cellular Protein in Replication . . . 72

GENOME HOMOLOGIES BETWEEN BPV AND LPV . . . 75
Introduction . . . 75
Methods . . . 76
Cell Culture and Virus Infection . . . 76
Polyacrylamide Gel Electrophosesis and Immunoblotting . . . 77
Isolation and Cloning of LPV . . . 77
DNA/DNA Hybridization . . . 78
PCR Amplification of LPV Termini . . . 78
Results . . . 80
Comparison of Proteins by PAGE and Immunoblotting . . . 80
Comparison of Virion DNA . . . 80
DNA/DNA Hybridization . . . 83
Amino Acid Sequence Similarities . . . 87
Restriction Enzyme Analysis . . . 89
Discussion . . . 91

SUMMARY . . . 93

LITERATURE CITED . . . 97

List of Illustrations

Figure 1. AAV replication model 10

Figure 2. Modified rolling hairpin model of replication 11

Figure 3. Kinetic hairpin transfer model of replication 14

Figure 4. Structure of BPV deletion and chimeric clones 24

Figure 5. Terminal sequences of the left and right ends of BPV and AAV 26

Figure 6. Hybridization analysis of selected deletion and chimeric clones 30

Figure 7. Replication of dl 3-94l_2 by cotransfection with SSV16 32

Figure 8. Replication of dl 13-89 by cotransfection with pVT501 DNA 33

Figure 9. Replication of dl 6-95 by cotransfection with pVT501 DNA 35

Figure 10. Replication of a clone with duplicate left termini. 36

Figure 11. Replication of a clone with duplicate right termini 38

Figure 12. Replication of genomic clones with altered termini 40

Figure 13. Replication of pAB53 and pAB33 by cotransfection with pVT501 42

Figure 14. Two homologous regions of the left and right termini of BPV 45

Figure 15. Conformation of terminal fragment 55

Figure 16. Binding of BPV infected-cell proteins to the BPV left terminus 58

Figure 17. Altered mobility of the left terminus due to protein binding 59

Figure 18. Cellular protein binds the BPV left terminus 61

Figure 19. Antiserum inhibition of C2 and C3 complexes 63

Figure 20. Specific competition using hairpinned termini 64

Figure 21. Specific competition using double strand termini 67

Figure 22. Sequence of the left termini of BPV and AAV and the right termini of BPV . . . 69
Figure 23. Comparison of proteins from mock and infected cell lysates 81
Figure 24. Detection of protein homology by immunoblotting 82
Figure 25. Comparison of virion DNA by agarose gel electrophoresis 84
Figure 26. Size comparison of LPV and BPV viral DNA 85
Figure 27. DNA/DNA cross hybridization 86
Figure 28. Amino acid sequence similarities between LPV and BPV 88
Figure 29. Southern blot of LPV DNA and PCR product clones 90

Chapter I

INTRODUCTION

Characteristics and Taxonomy of Parvoviridae

The family Parvoviridae is divided into 3 genera: genus Parvovirus - those viruses that replicate autonomously within their host cell; genus Dependovirus - those viruses that require a helper virus to replicate within their host cell, and genus Densovirus - those viruses that replicate within insect cells. As implied by their classification, the host range of the viruses differs as does their requirement for helper functions, but they all replicate in the nucleus of cells in the S-phase of the cell cycle. These viruses have a single strand (ss) DNA genome which, by convention (Armentrout et al., 1978) is referred to as the virus antisense (minus) strand so that the left terminus is the 3′ hydroxyl end and the right terminus is the 5′ phosphate end. The size of the genomes does not vary much, with adeno-associated virus (AAV) being 4,675 bases in length (Srivastava et al., 1983) and bovine parvovirus (BPV) being 5517 bases long (Chen et al., 1986). The capsid is naked, icosahedral in shape, and is ap-

proximately 22 nm in diameter. The mature infectious virion has a density of 1.41 g/ml as measured by CsCl centrifugation and sediments at 110S (Siegl et al., 1985).

Structure

Much of the basic classification of the members of the parvovirus family is based on the virion structure as detected by its antigenic properties. The virion can be detected in several basic serological tests.

All of the parvoviruses can agglutinate red blood cells (Siegl, 1984a). The hemagglutinin of the virion reacts with a receptor on cells of numerous blood types particularly human type O. The receptor is thought to be N-acetyl neuraminic acid. This common feature makes the hemagglutination test a good method for detection and quantification of autonomous parvoviruses, but it is not useful for classification. The autonomous parvoviruses can be easily delineated by hemagglutination inhibition (HI) tests due to the non-cross-reactivity of polyclonal antisera specific for each virus (Siegl, 1984a). Based on HI tests, there are 16 distinct groups or serotypes of parvoviruses (Cotmore and Tattersall, 1987). Hemagglutination is likely a function of the overall virion structure. A detailed picture of the capsid has been difficult to obtain. The most recent studies on rat virus (RV) using small angle neutron scattering suggest that the capsid has a triangulation number of 1 and only 60 protein subunits (Wobbe et al., 1984).

This small capsid is composed of 3 or 4 capsid proteins. Some viruses such as BPV (Lederman et al., 1983; Chen et al., 1986) and AAV (Srivastava et al., 1983; Janik et al., 1984) and likely LPV (Matsunaga and Matsuno, 1983) code for each of the proteins separately. The three capsid proteins coded for by BPV are VP1 (80 kDa), VP2 (72 kDa), and VP3 (62 kDa). The AAV and LPV proteins are slightly larger. The fourth BPV capsid protein VP4 arises from VP3 by proteolytic cleavage. The majority of other autonomous parvoviruses are similar to minute virus of mice (MVM) and RV in that they code for 2 capsid proteins, VP1 (~83 kDa) and VP2

(~64 kDa) (Tattersal et al., 1976), with the third capsid protein, VP3, being generated by proteolytic cleavage of VP2 (Tattersall et al., 1977). Interesting variations to this pattern are that of Aleutian mink disease virus (ADV) and the human parvovirus B19. The molecular weight of the ADV capsid proteins varies depending upon whether the virus is a natural isolate or a cell culture adapted one (Bloom, 1982). Also, the major capsid protein of B19 are 84 kDa and only 58 kDa (Cotmore et al., 1986).

Biology

The basic classification of parvoviruses is also based on the biology of the viruses. The pathogenicity of the viruses is varied and *in vivo* they have been termed "viruses in search of a disease" (Siegl, 1984a). The diseases they do cause are most severe and even fatal in the young and new born of the species they infect. Recent reviews by Cotmore and Tattersall (1987) and Siegl and Tratschin (1987) describe clearly the early studies by Kilham, Margolis, and Toolan with the rodent parvoviruses and the severe abnormalities caused by experimental infection with MVM, RV, and H-1. Based on the prevalence of anti-parvovirus antibodies, widespread infections have also been noted for parvoviruses such as MVM (Parker et al., 1970), RV (Robey et al., 1968), BPV (Storz et al., 1972), LPV (Matsunaga et al., 1977; Metcalf et al., 1989), and CPV (Siegl, 1984b). With CPV, porcine parvovirus, and BPV, all of which cause severe enteritis, the prevalence of the virus and the mode of transmission, either by fecal contamination or transplacentally, has caused concern over the possibility of epidemics (Siegl, 1984). Some reports indicate also that B19 infection can become widespread within a localized population.

Data from the infectivity, pathogenicity, and cell culture isolation of parvoviruses led to the proposal that there is an absolute requirement for the S phase of the cell cycle for virus proliferation (Tennant et al., 1969). *In vitro* evidence suggests that virus entry into the cell occurs at any time (Wolter, et al., 1980), but data from infection of synchronized cells with

MVM and cells blocked in their cell cycle (Ward and Dadachanji, 1978; Tattersall, 1972; Wolter et al., 1980) demonstrate that virus replication depends on an S-phase event.

This S-phase cell factor may also be extremely host cell specific for each parvovirus. This is demonstrated by the cell tropism in infected cell cultures of several parvoviruses. For example, B19 only replicates in erythroid and not myeloid progenitor cells (Ozawa et al., 1986). MVM preferentially replicates in cells of different differentiated states (Tattersall, 1978; Tattersall and Bratton, 1983). The proliferation of LPV in cell culture may be limited by similar cell tropism (Metcalf et al., 1989).

The exact nature of the S-phase factor is undetermined. The basic requirement for a helper virus to complete the AAV infectious cycle implies that some replication requirement is supplied or activated by the adenovirus or herpesvirus. However, recent data (Yakoboson et al., 1987 and 1989) demonstrates that AAV can also replicate without helper virus in UV irradiated or hydroxyurea synchronized HeLa cells. Therefore, when the S phase factor is present in all of the host cells, AAV replication proceeds similarly to autonomous parvovirus replication. In the absence of this factor or a helper virus, AAV DNA will integrate into the host cell chromosome. The decision of replication versus integration may be made in a manner similar to the way lambda decides lysis versus lysogeny as suggested by Labow and Berns (1988).

Besides integration, AAV can inhibit the plating efficiency of cells when it is used to infect non-permissive host cells (Winocour et al., 1988). This phenomenon is apparently due to the disruption of the cell cycle. Data on the infection of non-permissive or resting host cells with an autonomously replicating virus, MVM, suggest that transformation of these cells with various agents allows replication of the viral genome (Cotmore and Tattersall, 1987; Siegl and Tratschin, 1987). In contrast, some transformed cells are not sensitive to parvovirus infection (Van Hille et al., 1989). This observation may be due to the decreased expression of NS-1 (see below) in these cells (Van Hille et al., 1989) or perhaps to a cellular DNA binding protein that is turned on by the transformation process and down regulates parvovirus replication (Avalosse et al., 1989).

Organization of the Genome

The organization of the genomes of the members of the parvovirus family is very similar. The termini consist of palindromic sequences which can form secondary cruciform or panhandle structures. The remainder of the genome is divided into two major open reading frames (ORFs). The actual DNA sequence and mRNA transcription map differs between members and suggest that parvoviruses have evolved to code for proteins of similar function by slightly different mechanisms.

The first autonomous parvoviruses to be sequenced completely were MVM (Astell et al., 1983) and H-1 (Rhode and Paradiso, 1983). The viruses share ~70% homology across the entire genome (Banerjee et al., 1983). Recently the sequence of the entire LuIII genome has been determined and is highly homologous to MVM (Diffoot et al., 1989, unpublished data). There is less homology between this rodent-like group of viruses and other parvoviruses. The evolutionary relationship B19 ←→ AAV ←→ BPV ←→ MVM was suggested by Chen et al. (1986) based on amino acid sequence homologies from the ORFs.

In general parvoviruses have 2 large ORFs. The left ORF (the ORF closest to the 3′ OH end of the virus minus strand) codes for the nonstructural proteins. The right ORF codes for the capsid proteins (Carter et al., 1984). All of the transcripts appear to co-terminate at a poly A site located at approximately map unit (mu) 95. The poly A tail addition may be more complicated in BPV, B19, and ADV because a poly A site in the middle of the genome may be active (Burd, 1982; Ozawa et al., 1987; Alexandersen et al., 1988). The major exception to this pattern appears to be members of the Densoviruses which code for proteins from both strands of the genome (Tijssen, 1989).

The rodent-like parvoviruses (MVM, H-1, RV, CPV, PPV) appear to code for two NS proteins, NS-1 and NS-2. The larger protein, NS-1, in MVM has a molecular weight of 83,000 (Cotmore et al., 1983). The NS-2 protein has a molecular weight of 25,000 (Cotmore et al., 1983). Three mRNA transcripts have been identified for MVM (R1, 2, and 3)(Pintel et al., 1983).

The R1 and R2 transcripts originate from the promoter at map unit (mu) 5 (P5) and code for the NS proteins. The R3 transcript codes for the two capsid proteins (Labienic-Pintel and Pintel, 1986) and originates from the P38 promoter. Rhode (1985) has demonstrated that this promoter in H-1 is positively regulated through a trans activation response sequence (*tar*) by the NS-1 protein. Cotmore and Tattersall (1988) demonstrated that NS-1 also can bind the right end of MVM; and, thus, it may have a role in replication. All of the transcripts of ADV appear to originate from a promoter at map unit 3 and it has two additional transcripts that appear to code for very small NS proteins.

Studies on AAV-2 have also shown that the NS proteins are important in transactivation (Tratschin et al., 1986) of the major AAV promoter, P40, which codes for the capsid proteins (Hermonant et al., 1984; Tratschin et al., 1984). The left ORF of AAV-2 actually codes for four NS proteins (Mendelson et al., 1986) with molecular weights of 72,000, 68,000, 52,000, and 48,000. These proteins are coded for by four different mRNAs. The larger two are coded for by transcripts originating at P5 and the smaller two from transcripts originating at P18. Mutations in the infectious genomic clone of AAV-2 in the left ORF are replication negative (*rep*$^-$) (Hermonant et al., 1984; Tratschin et al., 1984).

A third group of parvoviruses including B19, BPV, and perhaps LPV due to some gross protein similarities to BPV (Matsunaga and Matsuno, 1983), have a slightly different genomic organization. Both B19 (Ozawa et al., 1987) and BPV (N. Diffoot unpublished data) appear to encode numerous mRNAs originating from the leftward most promoter in the genome. B19, however, codes for only two capsid proteins from the right ORF and 3 NS proteins of molecular weights 77,000, 52,000, and 34,000 (Ozawa and Young, 1987). The right ORF of BPV codes for 3 relatively large capsid proteins (Chen et al., 1986; Lederman et al., 1983). The two large NS proteins of BPV with molecular weights of 75,000 and 83,000 and the NS proteins of B19 are immunologically related (Lederman et al., 1987) and share similar nuclear localization signals; the region identified by the amino acid sequence KKTGKRN in BPV (Lederman et al., 1986; Ozawa and Young, 1987). This conserved sequence is just to the amino terminal side of a 12 amino acid sequence that is believed to be an ATP binding site and is conserved among all

parvoviruses. The similarities between these NS proteins and those of MVM and AAV-2 suggest that the NS proteins all have similar functions in transactivation and replication. Importantly, BPV has no *tar* sequence up stream of P38 which, may imply that this NS protein has a slightly different function (Chen et al., 1986).

BPV and the human parvovirus RA-1 appear to be the only parvoviruses with a large middle ORF (Chen et al., 1986). This ORF likely codes for the highly basic NP-1 protein. Other small NS proteins have been detected in parvoviruses including MVM (Cotmore et al., 1983) and LPV (Matsunago and Matsuno, 1983).

Besides the conservation of the function of some of the large NS proteins, one feature which appears to be conserved in all parvoviruses are the unique palindromic nucleotide sequences present at the termini. The palindromes are usually imperfect in nature. In AAV-2, the termini are 145 nt long and are identical in sequence (Srivastava et al., 1983). They can form T-shaped hairpins that can exist in two alternative sequence orientations (Lusby et al., 1980). One orientation is termed flip and the other, which is its reverse complement, is flop. The left and right ends of autonomous parvoviruses, except for B19 (Deiss et al., 1980), are not identical. The left end can form a T-shaped hairpin ranging in size from 125 nt for MVM (Astell et al., 1979) to 165 nt for BPV (Chen et al., 1986). The right end can form a slightly longer U-shaped hairpin (Rhode and Paradiso, 1983; Chen et al., 1986).

The termini are faithfully replicated by a mechanism of "hairpin transfer" (see below). Early evidence that the termini served as origins of replication come from pulse chase studies identifying the origins of complementary strand DNA synthesis (Hauswirth and Berns, 1977) and studies on defective viral genomes (Faust and Ward, 1979; Carter and Laughlin, 1984). More recent data on deletions in the termini of the infectious AAV-2 genomic clones identify *cis* signals necessary for replication (Samulski et al., 1983; Senapathy et al., 1984; Hermonant et al., 1984). Shull et al. (1988) have also shown that deletions within the BPV terminus inhibit the ability of the infectious BPV genomic clone to replicate. Further molecular evidence from work with integration vectors of AAV-2 demonstrates that both the *ori* and sequences necessary for encapsidation lie with the termini (McLaughlin et al., 1988).

The specific DNA sequence of the "core" *ori* has not been identified. Rhode and Klaasen (1982) suggest it consist of an AT-rich repeat adjacent to the right terminal repeat of H-1. DNA sequence alone may not define the *ori*. Deletion and insertion mutations within the AAV-2 termini have demonstrated that the conformation of the termini is important in replication (LeFebvre et al., 1984; Bohenzky et al., 1988). This is significant because the sequence similarities are minimal between the AAV termini and the left terminus of the autonomous parvoviruses. Perhaps conformation and not the actual DNA sequence has been preserved among parvoviruses and allows a common replication function.

Parvovirus Replication Models

The basic mechanism for replication of linear DNA with palindromic ends was proposed by Cavalier-Smith (1974) as a model for eukaryotic chromosome replication. The model suggests that the resolution of a terminal hairpin occurs by a single-stranded nick followed by self-priming DNA synthesis. The parvovirus termini appear ideally suited for this mechanism and the replication of the viruses had been claimed to be a model of chromosome replication. However, recent evidence suggest that eukaryotic telomeres are replicated by a "telomerase" which is a ribonucleotide-protein (Morin, 1989). Regardless of how parvoviruses serve as models for eukaryotic DNA replication, three parvovirus replication models have been proposed. Each model has 3 basic steps: 1) conversion of ss DNA to ds DNA; 2) amplification of ds replicating form (RF) DNA; 3) encapsidation.

The hairpin transfer model (Hauswirth and Berns, 1977) has been proposed as the model for AAV replication (Fig. 1; reprinted from Hauswirth and Berns, 1984). The single-stranded genome is converted to a template for replication (Form I or IV) with the hairpin termini serving as a primer for complementary strand DNA synthesis by host cell DNA polymerases. Amplification of the closed end molecule to generate RF molecules requires a site specific

endonuclease. This "nickase" would produce a single-strand break at the closed end at a site on the genome internal to the terminal repeat. The nick generates a free 3'OH on the parental viral strand which serves as a primer for elongation of the viral strand by synthesis of the reverse complement of the original palindrome. This basic mechanism explains the heterogeneous terminal sequence observed for AAV-2 (Lusby et al., 1980). Recently the AAV *rep* 68 protein was found to bind specifically to the terminal hairpin (Im and Muzyczka, 1989). It was further demonstrated that in the presence of ATP this protein can nick the hairpin and allow synthesis of the complementary strand of DNA (Snyder et al., 1990). Once the resolution of the hairpin is complete, the open end RF molecule can be amplified by continued hairpin formation, DNA synthesis, and terminal resolution.

Progeny genomes are generated by displacement of ss DNA during elongation. The signals for encapsidation are unknown. It is still proposed (Cotmore and Tattlerall, 1987) that the termini are recognized by capsid proteins. As mentioned, this recognition probably occurs within 275 nt of the end of AAV (Mclaughlin et al., 1988). For AAV, the signal must be present at both ends because strands of both plus and minus polarity strands are packaged and are infectious (Samulski et al., 1988).

The rolling hairpin model (Fig. 2; reprinted from Chen et al., 1989) is based on data about the replication of the rodent-like viruses. This model suggests a mechanism to account for the asymmetry of the replication of the rodent-like viruses. The asymmetry is evident in the fact that the MVM encapsidates 99% minus strand DNA and that only the right end palindrome has both flip and flop sequence orientations (Astell et al., 1983). The model was first proposed by Tattersall and Ward (1976) and latter modified (Astell et al., 1985; Cotmore and Tattersall, 1987).

In this model, single-stranded virion DNA is converted to ds RF DNA through hairpin formation at the left end and synthesis of a complementary strand DNA using host cell polymerases (Gunther et al., 1984; Kollek et al., 1982). When the replication of H-1 was examined by pulse-chase experiments (Tseng et al., 1978), no Okazaki fragments were detected. This suggests that no lagging strand DNA synthesis is occurring; and thus, the hairpin

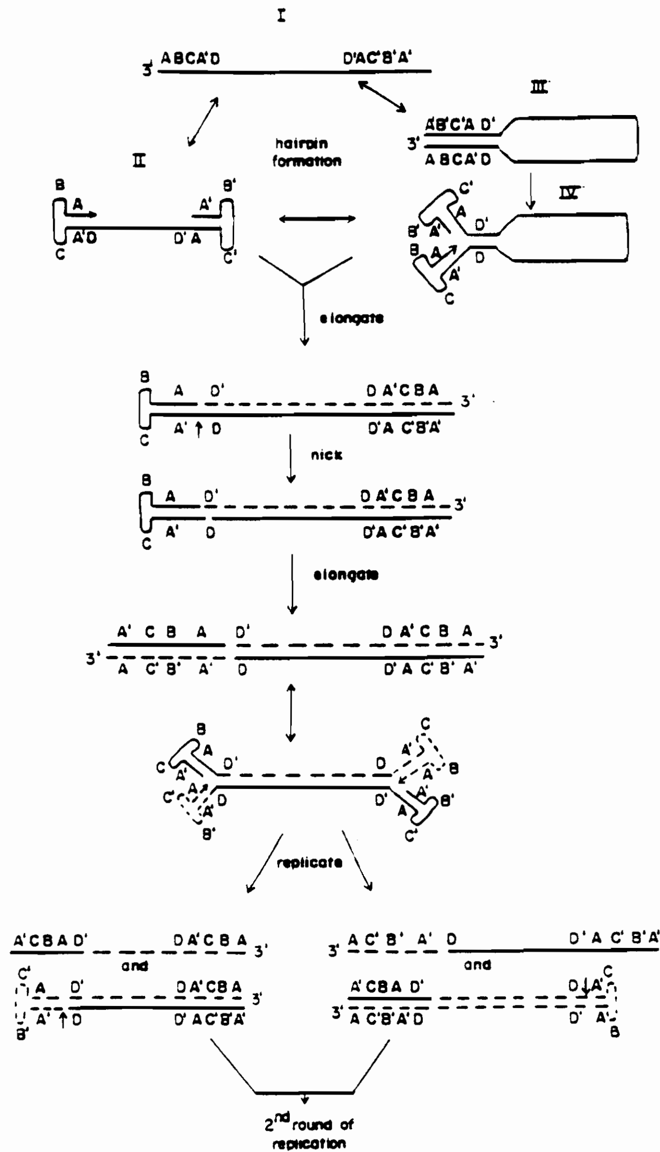


Figure 1. AAV replication model: Proposed mechanism for replication of AAV (reprinted from Hauswirth and Berns, 1984).

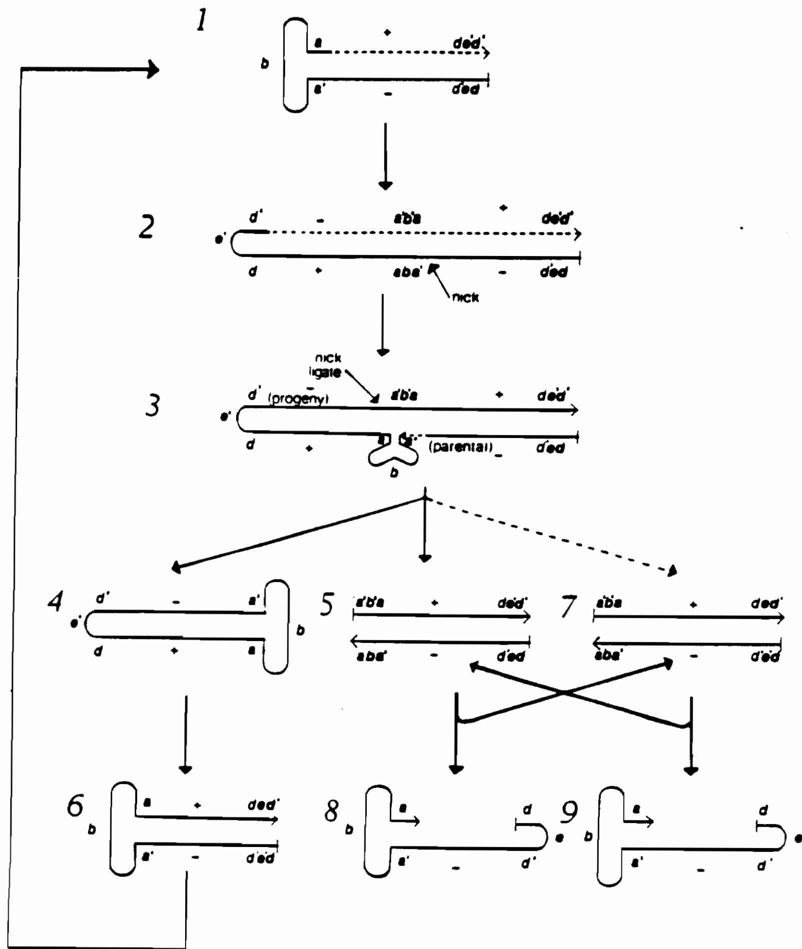


Figure 2. Modified rolling hairpin model of replication: Proposed mechanism of replication for MVM and other autonomous parvoviruses (reprinted from Chen et al., 1989).

terminus is likely the primer for DNA synthesis in parvovirus replication. Displacement of the right end hairpin occurs to yield a closed end duplex RF molecule as with AAV. This molecule contains 18 extra nucleotides not found in virion DNA (Astell et al., 1985); and thus, is probably generated by the proposed mechanism which allows repair of the terminus. Cotmore and Tattersall (1989) have identified a covalently closed ss DNA circular RF molecule which could be generated in step 4 or by a ligation reaction instead of displacement synthesis in step 1.

Amplification of the closed end ds RF involves more steps than proposed in the AAV model. Instead of nicking occurring at the left end after step 1, DNA synthesis begins from the right end using the hairpin as a primer. Elongation of the DNA yields a dimer molecule (step 2). The dimer generates a circular monomer RF (step 4) which can be reamplified and one of two open end molecules (5 and 7) which differ in the sequence of the right end. These molecules are proposed to be generated by cleavage by a terminal topisomerase-like molecule (Cotmore and Tattersall, 1987) which recognizes the sequence GAAnnACCAAC (Astell et al., 1982 , 1985). This nickase would create a single-strand break to the right side of the middle left end at the sequence CTTATCA. The open ended molecules are then generated by displacement DNA synthesis from the internal left end. Concomitantly, the terminal protein may bind to the free 5′ end generated by the internal nick. As the displacement synthesis occurs, the 5′ protein may slide along the genome and recognize a second nick site where a second strand break occurs. This newly created 3′ OH could then be ligated to the 5′ end bound by the protein. While there is evidence for proteins being bound to the 5′ terminus (Cotmore and Tattersall, 1988 and 1989; Revie et al., 1979; Astell et al., 1982), no direct evidence for a protein with endonucleolytic or topoisomerase activity has been obtained for MVM or the other rodent-like viruses. Lastly, encapsidation of only minus strand DNA occurs during displacement DNA synthesis from molecules 5 and 7.

The modified rolling hairpin model cannot account for the replication of BPV or LuIII. Both of these viruses have nonidentical termini (Chen et al., 1986; Diffoot et al., 1989) and those of LuIII are almost identical to those of MVM. However, BPV encapsidates 90% minus and 10% plus strand DNA; and both termini display both flip and flop sequence orientations

(Chen et al., 1988). LuIII encapsidates 50% of each strand polarity and also has both flip and flop orientations at each terminus (Diffoot et al., 1989).

A third model, the kinetic hairpin transfer model, proposed by Chen et al. (1989), takes into consideration the polarity of virion DNA and sequence orientations at the termini of most parvoviruses. The model is simple because it suggests that parvovirus replication proceeds predominantly through four monomer ds RF (Fig 3. step 4, 6, 7, 8; reprinted from Chen et al., 1989). The conversion of the ss virion DNA to a ds RF molecule, again, is proposed to occur through a self-priming event that utilizes the host cell replication proteins. It has been demonstrated that there is no BPV virion-associated DNA polymerase (Pritchard et al., 1978) but the ss viral DNA does serve as a template for the Klenow fragment and eukaryotic DNA polymerase γ and α (Bates et al., 1980; Pritchard et al., 1981; Robertson et al., 1983). The conversion of BPV ss DNA to ds RF is sensitive to aphidicolin *in vivo* (Robertson et al., 1984) and *in vitro* (Bates et al., 1980; Pritchard et al., 1981) and, therefore, the reaction probably involves DNA pol α. However, DNA polymerase δ and α have similar sensitivity to polymerase inhibitors (Lehman and Kaguni,1989).

The closed end replicative intermediate (step 2) is amplified to generate all four possible monomer RF molecules (step 3, 4, 5, 6) by successive cycles of terminal resolution (step 2 and 3), hairpin formation (step 3′), and DNA synthesis. No BPV protein which binds to the termini or causes nicks has been identified. This model proposes that the amplification of a particular RF is based on the differential kinetics of hairpin formation and complementary strand synthesis (step 3′). The differential kinetics are probably due to differential protein recognition of the flip and flop sequence orientation at the terminus.

Encapsidation probably occurs by a mechanism similar to that already mentioned. The model does suggest that packaging is not a selective process. As with AAV and MVM the packaging signal is unknown. Still, Lederman et al. (1987) demonstrated that VP2 of BPV can bind to the first 225 nt of the left terminus under stringent conditions. There is some evidence that capsid proteins may be important in DNA synthesis (Robertson et al., 1984; Buller and Rose, 1978).

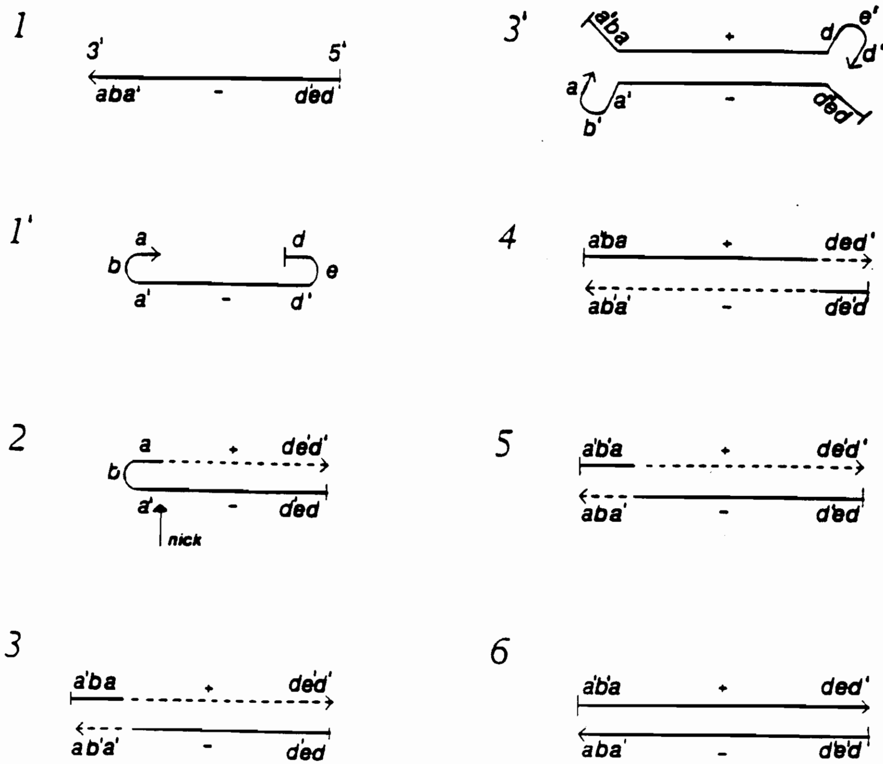


Figure 3. Kinetic hairpin transfer model of replication: Proposed unified mechanism for replication of parvoviruses (reprinted from Chen et al., 1989).

Replication of parvoviruses probably involves mechanisms proposed by each model. For replication to occur, an initial recognition event must occur. The initiation probably involves the interaction of viral and cellular that bind to a terminal hairpin. The formation of this protein-DNA complex probably allows the synthesis of complementary DNA, and resolution of the hairpin. Amplification of the RF molecules may involve the same or different set of proteins. These events seem quite reasonable, in regard to what is known about eukaryotic viral DNA and cellular DNA replication.

Replication of Eukaryotic DNA

Eukaryotic chromosomes are replicated by initiating DNA synthesis at numerous replicons. These initiation events are also important to the replication of viral DNA because their exploitation could allow a small viral genome to be preferentially replicated.

Recent reviews (Campbell, 1986; Kornberg, 1988; Laskey et al., 1989; Stillman, 1989) describe some of the basics of eukaryotic DNA replication. DNA replication occurs during the S phase of the cell cycle and appears to be regulated by specific cellular proteins. The basics of replication may be similar to prokaryotic DNA replication

In prokaryotes, the advancing replication fork is probably led by one or more helicase-like proteins (Kornberg, 1982; 1988). The free single strand DNA is covered with single-stranded DNA binding proteins which allow the primasome complex to attach and synthesize a short ribonucleotide primer. This primer is then extended by DNA polymerase III holoenzyme which may form a dimeric molecule (Kornberg 1988; McHenry et al., 1988) that catalyzes concurrent DNA synthesis on both strands through a 180° loop in the lagging strand.

The event that controls replication is the unwinding of the helix at the origin. Initiation occurs when an "open" complex is formed with the *dna*A proteins. The *dna*A protein complex binds tightly to four 9-mer repeats and causes the sequential melting of 3 AT rich 13-mer re-

peats (Bramhill and Kornberg, 1988a; 1988b). Formation of the primasome occurs by the binding of the *dna* B, C and *dna* G (primase) proteins to the open complex region.

One of the most thoroughly studied eukaryotic virus replication systems is that of adenovirus. The cell free replication of the 36 kilobase ds DNA adenovirus genome (Challberg and Kelly, 1979) has allowed the identification of both the cellular and viral proteins necessary for viral replication. The viral proteins required are a single-stranded DNA binding protein, a 140 Kd (Adpol) DNA polymerase, and a 80 Kd preterminal protein (pTP) (Stillman, 1983; 1989). Three cellular factors, NF I, II, and III, are also required for replication. The first event of replication is the binding of the pTP to dCMP in a reaction catalyzed by AdPol (Lichy et al., 1981; 1982) requiring the first 18 nt of the genome terminus (Guggenhiemer et al., 1984). The pTP-CMP complex primes DNA synthesis which is stimulated by NF I and NF III (Nagata et al., 1982; Rosenfeld et al., 1987). *In vitro*, 25% of the adenovirus genome can be replicated with just these components (Stillman, 1983); but NF II, a topoisomerase-like molecule, is required for complete replication (Nagata et al., 1983).

The NF I factor increases replication approximately 10 fold and recognizes nt 21-43 of the adenovirus terminus (Rosenfeld et al., 1987). The consensus binding site, TGG (A/C)-N_5-GCCA, (Gronostajski et al., 1984) is similar to that of the transcriptional activator, CTF (Jones et al., 1987). In fact, CTF and NF-I are probably the same protein and recognize the consensus binding site TTGGCT-N_3-AGCCAA (Jones et al., 1987; Santoro et al., 1988). Similarly, NF II interacts with nt 38-55 of the adenovirus terminus to increase replication 3 fold (Rosenfeld et al., 1987); and its probably the same protein as the octamer transcription factor I (O'Neill et al., 1988).

Perhaps an even more applicable model for eukaryotic chromosomal DNA replication is that of SV40 because *in vivo* it replicates like a mini-chromosome organized into nucleosomes. As with adenovirus, *in vitro* replication of the 5,340 bp SV40 genome (Li and Kelly 1984; 1985) has allowed the identification of cellular and viral proteins required for replication. Only nuclear extracts from cells naturally permissive to SV40 will function in the *in vitro* replication of SV40 (Li and Kelly, 1985). Besides the nuclear extracts, the only viral protein required for

replication is the large T antigen which recognizes 3 binding sites. Each site contains repeated units of the sequence GCCTC contained within the 180 bp origin (McKnight and Tjian, 1986; Smale and Tjian, 1986). The T antigen has different binding affinities to each site. Sites I and II are required for replication with the spacing between them being important for their effect on replication (Smale and Tjian, 1986; James and Tjian, 1985). Alteration of the spacing between these sites suggests that protein-protein interactions are important in SV40 DNA replication. The T antigen binds to a variety of cellular proteins including transcriptional activators like AP-2 (Stillman, 1989), oncogene products like p53 (Wang et al., 1989), and DNA pol α (Smale and Tjian, 1986). The interaction with cell proteins and the ability to unwind the DNA helix at the origin (Dean et al., 1987) probably explain T antigen's function in the initiation and elongation of SV40 replication.

These two models suggest that the following generalized description of viral DNA replication. A specific DNA sequence, the origin, which may also be involved with transcriptional activation, is recognized by viral proteins. Other initial events probably include the unwinding of the DNA helix and interaction between viral and cellular proteins. One such cellular protein which activates the unwinding of the SV40 origin has been identified (Roberts and D'Urso, 1988). This pre-priming complex is recognized by the replicating enzyme complex. Elongation proceeds in a bidirectional fashion catalyzed by a replisome of dimeric polymerases possibly DNA pol α and δ (Perlich and Stillman, 1988; Laskey et al., 1989).

Chromosomal replication likely proceeds in a fashion similar to that mentioned above but on a larger scale. The recent review by Laskey et al., (1989) highlighted many aspects of eukaryotic replication. DNA replication probably occurs due to the accumulation of positive initiator factors at the onset of S-phase which is triggered by cell cycle factors such as cyclin and mitosis promoting factor (Cross et al., 1989). Replication then begins synchronously at specific origins. As mentioned, the replication fork at each replicon consists of a dimeric polymerase. The replisome includes proteins like PCNA (Bravo et al., 1987) which increases the processivity of polymerase δ (Lehman and Kaguni, 1989), a ss DNA binding protein (RF-A), and RF-C which coordinates the movement of replication (Laskey et al., 1987). As with viral

replication, there may be coordination between transcription and replication (DePamphilis, 1988) of chromosomes due to the movement of the replication fork and the transcription machinery (Brewer, 1988). Chromatin assembly appears to be directed by several proteins (Laskey, 1989) one of which, chromatin assembly factor (CAF-1)(Smith and Stillman, 1989), assembles nucleosomes on replicated DNA. As with initiation, termination is a specific event with *cis*-acting DNA sequences perhaps limiting each origin to a single round of replication. The ends of chromosomes are replicated by a specific enzyme, telomerase. In the last analysis, DNA replication in any system maybe more similar than dissimilar.

OBJECTIVES

One of the broad goals of virus research is to define the events involved in viral replication. Several years ago, an *in vitro* system for replicating BPV DNA was developed to begin to answer this question. However, the lack of homogeneous viral specific DNA and of detailed genetic evidence about parvoviruses limited the amount of information that these experiments could provide.

The details of the biology of BPV and parvoviruses in general are now available to allow a detailed investigation of BPV replication. The infectious genomic clone of BPV, pVT501, allows the isolation of large quantities of specific viral DNA. The mutation of these genomic clones and the ability to assay their function by transfecting cells has defined regions of the termini as critical to replication.

The function of the nonstructural proteins in replication and transactivation of transcription is becoming clearer. The regions of the BPV genome which code for these proteins has been determined. The larger nonstructural proteins may affect replication by interacting with the genomic termini. However, there is no evidence that begins to describe the mechanisms by which these proteins act.

Interestingly, though, little is known about how the parvovirus genome is encapsidated or how the capsid proteins may function. Also there has been little progress toward an

understanding of the replication requirements provided to parvoviruses by actively dividing cells.

Still, the development of three detailed models of parvovirus replication and the very specific predictions of Chen's kinetic hairpin transfer model provide a framework to ask specific questions about BPV replication. Each model predicts several sites of DNA synthesis initiation and elongation involving DNA-protein interactions. The conversion of ss DNA to ds RF predicts the interaction of both viral and cellular proteins with the left and right ends to generate closed end monomer RF. The resolution of the closed end terminus also requires a specific group of proteins. The amplification of RF molecules and production of ss DNA virion suggests DNA-protein interaction takes place to initiate DNA synthesis.

The developing model of eukaryotic DNA replication describes 3 basic steps to the initiation of DNA synthesis which may be applicable to parvovirus replication. First, proteins specifically recognize the origin of replication. Second, these "initiator" protein(s) interact with other factors and DNA polymerases. Lastly, DNA is replicated during the S-phase of the cell cycle due to the accumulation of positive and/or negative regulatory proteins.

Thus it seems reasonable to begin to address the question of the nature of the signals involved in the initiation of BPV replication. Specifically, my dissertation will address the following questions:

1. What are the minimal *cis* signals required for BPV replication?
2. What proteins interact with the minimal specific DNA sequences required for replication?
3. How similar are the *cis* and *trans* replication signals between BPV and LPV?

Chapter II

REPLICATION OF BPV MINI-GENOMES

Introduction

Bovine Parvovirus (BPV) is an autonomously replicating virus with a 5517 base single stranded (ss) DNA genome (Chen et al., 1988). From DNA sequence analysis, it is clear that the BPV genome is organized in a similar fashion to other autonomous parvoviruses (Chen et al., 1986). BPV has nonidentical terminal palindromes believed to form a T-shaped hairpin at the left end (3′ terminus of the viral minus strand) and a U-shaped hairpin at the right end.

Data on several parvoviruses indicate that the terminal regions have *cis* signals required for replication. Early evidence for both MVM (Faust and Ward, 1979) and AAV (De la Maza and Carter, 1980) investigating naturally occurring defective viruses suggest that the origin of replication (*ori*) lies within approximately 250 nucleotides (nt) of one or both of the genomic terminus for each virus. Recombinant deletion mutants of AAV which contain only its termini were efficiently replicated and packaged (McLaughlin et al., 1988). AAV genomes in which all of the coding sequences are intact but the termini have been deleted cannot replicate

(Samulski et al., 1983). Studies with BPV (Shull et al., 1988) showed that deletion of the first 34 or 52 nucleotides of the left end of the genomic clone significantly diminished the infectivity of the clone. Similarly, deletion of either terminus in the MVM genomic clone prevented rescue and replication of the clone (Merchlinsky et al., 1983). The exact sequence of the termini may not be the only signals for replication as the secondary conformation of the terminal hairpin is also important (Lefebvre et al., 1983; Bohensky et al., 1988).

Two models of autonomous parvovirus replication have been proposed; the kinetic hairpin transfer model (Chen et al., 1989) and the modified rolling hairpin model (Astell et al., 1985; Cotmore and Tattersall, 1987). Both models require replication to initiate from the viral ss DNA via a self priming event that involves the formation of a hairpin and DNA synthesis from the 3′ hydroxyl terminus. This closed end double stranded (ds) DNA molecule is resolved in one step (kinetic hairpin transfer model) or two steps through a dimer ds DNA molecule (modified rolling hairpin model) into an open ended monomer ds DNA RF. Replication of the RF molecule also originates at the 3′ end of either the plus or minus strand and again requires the formation of a hairpin. In support of the kinetic hairpin transfer model, evidence on the exact sequence (i.e. "flip" or "flop") of cloned BPV (Chen et al., 1988) and LuIII (Diffoot et al., 1989) termini and the packaging of specific ratios of plus and minus strand viral DNA for both viruses suggests that the terminal *ori*'s initiate replication at different rates.

The rates of replication are probably determined through an interaction between the parvovirus *ori* and various replication proteins as in other eukaryotic viruses (Stillman, 1989). The nonstructural (NS) proteins coded for by the left open reading frame (ORF) of parvoviruses affect replication by acting in *trans* to recognize the *cis* replication signals. Mutations in the left ORF of adeno-associated virus (AAV) inhibit replication (*rep*$^-$) but can be complemented, indicating that the *rep* proteins function in *trans* (Hermonant et al., 1984; Tratschin et al., 1984). One of these proteins, *rep*68, binds to the AAV terminus (Im and Muzyczka, 1989; Ashkotrab and Srivastava, 1989). The large NS protein (NS-1) of MVM also plays a role in replication by binding to the 5′ phosphate terminus of ds RF molecules (Cotmore and Tattersall, 1988). Based on immunological cross reactivity between BPV NS proteins and those of MVM and

B19, one or both of the large BPV NS proteins (75 kDa or 83 kDa) probably has functions similar to those of MVM (Lederman et al., 1987)

In order to identify the specific BPV DNA sequences required for replication of the viral genome, chimeric "mini-genomes" containing various combinations of the terminal regions of BPV were constructed and used to transfect bovine fetal lung cells. To address whether the sequence or the conformation of the termini direct the replication of BPV, chimeras containing an AAV terminus and a BPV terminus were constructed. The results of transient replication assays using these chimeras demonstrate that the BPV *ori* is contained completely within the terminal 291 nt of the left terminus and 249 nt of the right terminus. Further, the *cis* signals sufficient for replication are contained solely within each terminus. Also the termini of AAV cannot be interchanged with those of BPV. Therefore, the replication machinery appears to be sequence specific and not merely conformation specific.

Materials and Methods

Construction of deleted clones

Deleted clones and chimeric clones containing duplicate BPV termini were constructed by excising specific fragments from the genomic clones pVT501, (Shull et al., 1988) 503, or 504 and re-ligating the remaining fragment. The clones used for replication assays are diagrammed in Figure 4. In the genomic clone pVT503, the entire BPV genome present in pVT501, flanked by Sal I linkers, was ligated into the Sal I site of a pUC8 vector which has the Aat II site deleted.

The pVT504 plasmid is similar to 503 except that the orientation of the BPV fragment with respect to the multiple cloning site is reversed. This plasmid also contains a 65 bp deletion (nt

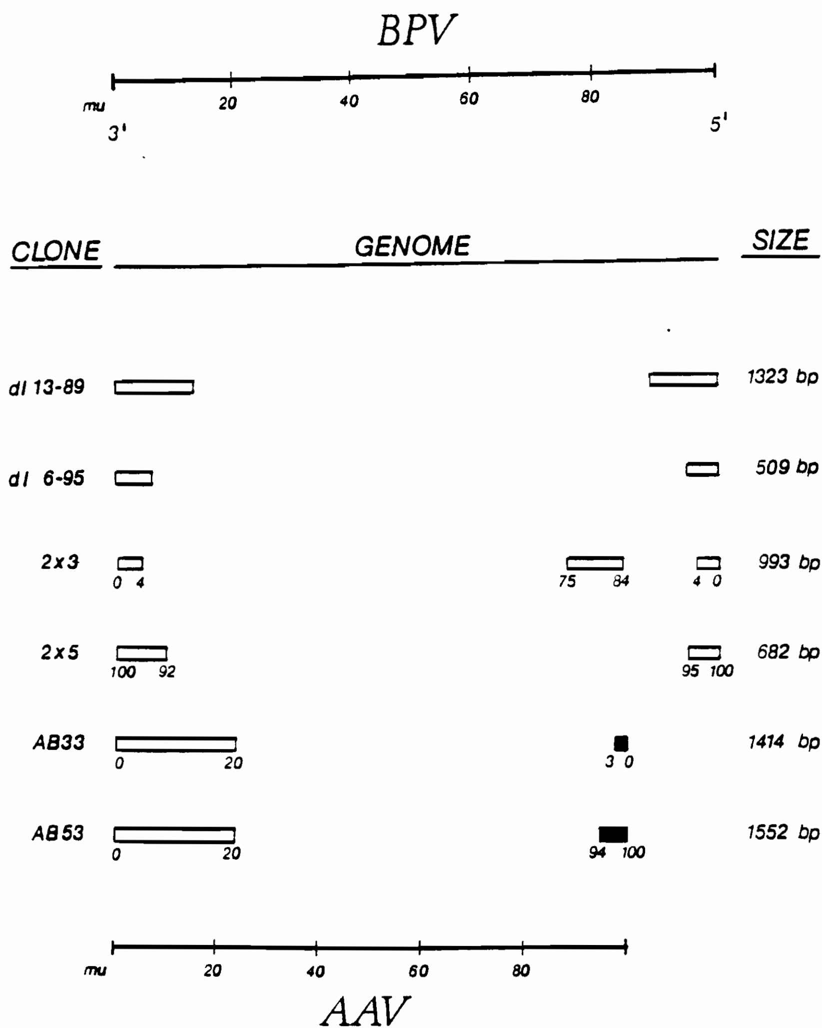


Figure 4. Structure of BPV deletion and chimeric clones: The heavy horizontal line represents the 5,517 bp genome of BPV (top) and the 4,675 bp genome of AAV (bottom) on a scale of 100 map units. The portion of each genome contained in each clone is represented by the boxed areas. The entire length of the viral DNA insert is listed for each clone.

53-118) at the left terminus of the BPV fragment (Fig. 5). The clones dl 13-89 and dl 6-95 have the BPV fragment between the Xho I sites and the Nhe I and Aat II sites removed, respectively. The plasmid dl 6-95 has the same 65 bp deletion in its left terminus as is found in pVT504.

The chimeric clone pVT2 x 3 contains two inverted BPV left termini from nt 1 to the Pst 1 site at nt 291. It also contains a small stuffer fragment, nt 4165 to nt 4645 of the BPV genome, and the 65 bp deletion in one of its termini. This clone was constructed by isolating the Hind III and Pst I cut pVT504 vector fragment containing the left terminus and ligating it to fragments generated from a Hind III - Pst I digest of pVT501. The plasmid pVT2 x 5 contains two inverted BPV right termini from nt 5517 to the Nhe I site at nt 5060 on one end and nt 5517 to the Aat II site at nt 5268 at the other end. It was created by digesting pVT503 with Aat II and filling in the end with the Klenow fragment. These molecules were digested with Hind III and the vector fragment containing the right terminus was ligated to the Hind III to Nhe I fragment of pVT504 that had the Nhe I site also repaired with the Klenow fragment.

Chimeric clones containing an AAV terminus and a BPV terminus were constructed using pVT501 and the AAV clones dl 3-94 or dl 3-94I_2 which are derived from the AAV genomic clone pSM620 (McLaughlin et al., 1988). The clone pAB33, which contains a left terminus from BPV and AAV, was constructed by first subcloning the AAV sequences from dl 3-94 into the Pst I site of pUC8. The right AAV terminus was removed and the left terminus of pVT501 was ligated in its place as the Hind III to Bgl II fragment of pVT501. The clone pAB53 was constructed by first subcloning the right AAV terminus of dl3-94I_2 into pUC8 as a Pst I fragment. The Eco RI to Bgl II fragment of this clone that contains the terminus was ligated to a pUC8 vector containing the Hind III to Bgl II left terminus fragment of BPV.

Restriction endonucleases were purchased from Boheringer Mannheim and Bethesda Research Laboratories. DNA fragments were purified by electroelution or directly from agarose gels by binding to silica (Geneclean, Bio 101). All recombinant plasmids were obtained by transformation of *Escherichia coli* JM107 or DH5α by the procedure of Hanahan (1983) and DNA was isolated by alkaline lysis (Rodriguez and Tait, 1983). Plasmid DNA was

(A) BPV minus strand, left (3') terminus, flip conformation

```
        A
   30—A G
      G C
      C G
      G C                          120                                160
      C G          |                |                 |                |
      G CGCGTAA^C A^TT CCGG^ATTA GCCCGTCGGTTATACCACCTACGTCCTTTACTCATTATTTTTATATCTCGGACAC--5'
      C
      A TCGCATT_A T_T -GGCC----CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT   3'
      G C-                      |                |
      C G                      40
      G C
      G C
      C G
      G A
       C
```

(B) BPV minus strand, right (5') terminus, flip conformation

```
            5360                     5400
              |              |          |              |
3'--GAAATATAAAAATATGTGACTAAGTGAGAAAGGTGGTAGAAAATCAACATAAGAATCAATAGTTATTTCCGCGCTTCGCGGTAT^TTTTTA
                                                                                              A
   5'  ATATTTTTATACACTGATTCACTCTTTCCACCATCTTTTAGTTGTATTCTTAGTTATCAATAAAGGCGCGAAGCGCCATA_TGACAT
        |             |              |              |              |
      5517                         5480                          5440
```

(C) AAV minus strand, left (3') or right (5') terminus, flip conformation

```
     A
    A A
    G C
    C G
    C G
    C G
    G C-80
    G C
    G C                               120
    C G              |                 |                 |
    G CCGGAGTCACTCGCTCGCTCGCGCGTCTCTCCCTCACCGGTTGAGGTAGTGATCCCCAAGGA--5'
    C
    C GGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAA   3
    G C |
   -G C 40
    G C
    C G
    G C
    A T
    C G
    C G
    T T
     T
```

(D) dl 6-95, left terminus (deletion of nt 54-118 from (A))

```
     C  53  119                                    160
    C G                                             :
    C GTTATACCACCTACGTCCTTTACTCATTATTTTTATATCTCGGACAC--5'
    G
    G CAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT   3'
    G C
    C G
     A—40
```

Figure 5. Terminal sequences of the left and right ends of BPV and AAV: The sequence depicted is that of the minus strand in the proposed secondary structure of the viral ss DNA.The numbering starts with nucleotide number 1 as the first base of the left end.

routinely purified by banding once in a cesium chloride - ethidium bromide gradient (Maniatis et al., 1982).

Cell culture and viruses

Bovine fetal lung (BFL) cells and HeLa cells were grown in monolayer culture and maintained in Eagle's Minimal Essential Medium (MEM) supplemented with 10% supplemented calf serum as described by Parris and Bates (1976). Human adenovirus 2 virus stocks were prepared from freeze-thaw cleared lysates of HeLa cell cultures.

Transfections

Subconfluent (80-90%) BFL cells in 100 mm dishes were cotransfected with recombinant plasmids. Typically, 10 - 15 μg of DNA was added per plate, or in concentrations specified in individual experiments 24 hours after initial seeding of the plates. Transfections were done using DEAE Dextran as described previously (Shull et al.,1987). Alternatively, subconfluent (60-75%) HeLa cells in 100 mm dishes were cotransfected with wild type and deleted AAV clones using the DEAE-Dextran procedure described by Tratschin et al (1984). Wild type human adenovirus 2 was used to inoculate cotransfected HeLa cells. The virus was added directly to the transfecting medium at a multiplicity of infection of 5 to 10 (Tratschin 1984).

Isolation and Detection of Replicated Chimeric DNA

Low-molecular-weight DNA was extracted from cells using a modified Hirt procedure (Robertson et al., 1984) when the cells displayed visible cytopathic effects (i.e. 50 - 70 percent

of the cells infected) or at 7 days post transfection. Cells were scraped into the culture media and pelleted. The cell pellet was washed once with and then suspended in TE (10mM Tris-HCl pH 7.5, 10mM Na_2 EDTA) and the cells lysed by the addition of SDS and Proteinase K to final concentrations of 0.6% and 0.3%. After incubation at room temperature for one hour, NaCl was added to give a final concentration of 1M and the mixture held on ice overnight. The cell debris was removed by centrifugation for 10 minutes at 4°C for BFL cells or 1 hour for HeLa cells. The supernatant was extracted once with phenol-chloroform and once with chloroform. The DNA was precipitated with ethanol at -20°C for at least 4 hours.

Recovered DNA was analyzed for the presence of specific viral DNA molecules by electrophoresis in agarose gels and transfer to Zeta-Probe membranes (Bio Rad, Richmond, CA) using 0.4 M NaOH. Southern blots were hybridized under stringent conditions (50% formamide, 0.76 M Na^+, 42°C) with 2 to 4 x 10^6 cpm of ^{32}P-labeled DNA. The probe was prepared according to the method of Feinberg & Vogelstein (1983) and denatured in formamide at 55°C for 10 min prior to its addition to the hybridization buffer. The membranes were washed at room temperature for 4 - 10 min each in 0.1% SDS and SSC concentrations of 2x, 1x, 0.5x, and 0.1x successively. A final stringency wash was performed in 1% SDS and 0.1x SSC for 30 min at 50°C.

RESULTS

Structure of chimeric replicons

The structures of the deleted BPV clones and the chimeric clones containing either duplicate BPV termini or a BPV terminus and an AAV terminus are shown in Figure 4. The clones were constructed to be devoid of any DNA sequences that could possibly code for a

complete viral protein. Each also contains a unique internal restriction enzyme site for possible use as a cloning vector. Figure 6 illustrates the unique restriction digest pattern of several of the chimeras and demonstrates that each clone can be uniquely identified based on its hybridization to probes containing specific BPV sequences. The duplicate end clones, pVT2 x 3 and pVT2 x 5, can be identified based on their hybridization to left and right end specific probes (compare panel A and C, lanes 4 and 5). The pVT2 x 5 clone can be uniquely identified by its hybridization to the Aat II fragment (mu 85-95) of BPV. Importantly, the other clones except for pVT501 do not hybridize to this probe because they either do not contain a right end or because they do not contain DNA sequence internal to mu 95. The dl 3-97 clone (lane 3) was created using polymerase chain reaction mutagenesis (see Chapter 2) from pVTC350. It was included in the assay due to its size and to emphasize the fact that deleted pVT501 clones can be distinguished from the duplicate end chimeras. The exact sequence of all of the clones was confirmed by dideoxy sequencing (Sanger et al., 1977).

During the cloning of these terminal viral sequences into pUC plasmids in *Escherichia coli* JM107 and 109 hosts, it was extremely difficult to recover molecules with complete terminal sequences. Deletions occurred most frequently in the left terminus of BPV. The deletions consistently removed the majority of nucleotides between nt 45 and nt 120 which eliminates all of the nucleotides proposed to form the cross-arms of the T shaped hairpin at the left terminus. The instability of cloned palindromes in *E. coli* has been reported previously (McFadden, et al., 1988; McLaughlin et al., 1988). The use of *E. coli* strains that have deletions in several of the genes for recombination which allow the stable propagation of cloned palindromes, JC 8111 (Boissy and Astell, 1985) and DB 1256 (Delange et al., 1986), proved unsuitable for our purposes due to the low number of transformants recovered. However, DH5α cells which are *rec*A minus were acceptable hosts. Once clones with intact termini was recovered, they were stably maintained throughout this study regardless of which *E. coli* strain was used for propagation. This is apparently not the case with the termini of AAV (McLaughlin et al., 1988).

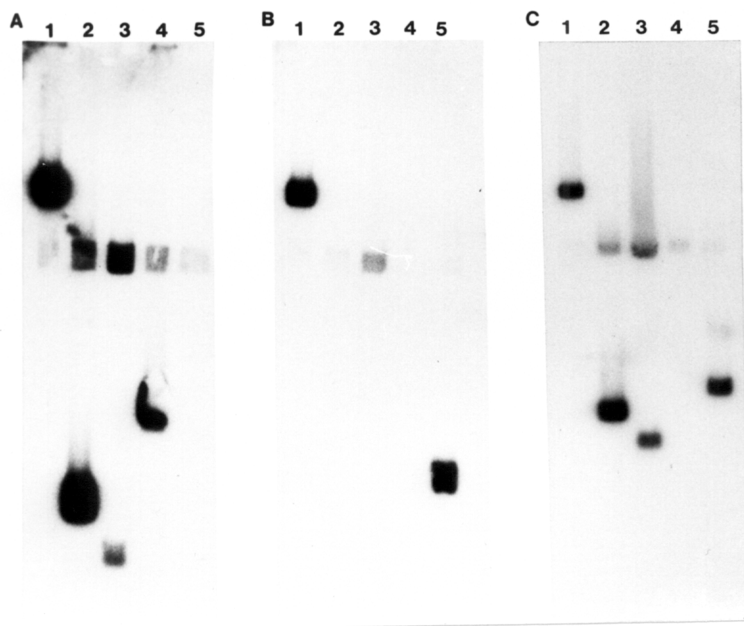


Figure 6. Hybridization analysis of selected deletion and chimeric clones: Panels A and B are Southern blots of duplicate agarose gels probed with the ^{32}P-labeled left terminus Hind III - Xba I fragment of BPV from pVT501 (panel A) or the Aat II fragment (mu 85 to 95) of BPV from pVT501 (panel B). The Sal I digests of the following plasmid DNA were separated on a 1.5 % agarose gel. Lane 1, pVT501; lane 2, dl 6-95; lane 3, dl 3-97; lane 4, pVT2 x 3; lane 5, pVT2 x 5. In panel C, the same DNA samples were electrophoresed on a 1% Agarose gel; and the Southern blot was probed with ^{32}P-labeled Sal I fragment of pVT2 x 5.

Replication of Deleted BPV Clones

To determine whether clones containing just the termini of BPV could act as a replicon, BFL cells were cotransfected with both the BPV infectious genomic clone, pVT501 (Shull et al., 1988), and the deleted mini-genomes. This type of assay for transient replication of DNA molecules containing origins of replication is fairly common. The general usefulness of the assay in detecting the replication of a "mini-genome" is demonstrated by the assay in Figure 7. The AAV clone dl 3-94I_2 that is known to contain the AAV *ori* was used to cotransfect HeLa cells, also infected with adenovirus, with the AAV genomic clone pSSV16; an isolate of psub201(+) (Samulski et al., 1987; a gift from N. Muzyczka). As expected, the dl 3-94I_2 fragment replicated as a monomer ds DNA fragment of 2569 nt (Fig. 7, arrowhead) as judged by its similar migration distance to the 2690 bp marker fragment. To determine which DNA was replicated in the eukaryotic cell, DNA from transfected cells was digested with Dpn I which only recognizes DNA methylated on both strands. Any DNA fragment present in the odd numbered lanes from 5 - 13 that is also present in the even numbered lanes from 6 - 14 is resistant to Dpn I digestion and must have been replicated in the eukaryotic cells.

In a similar assay, BFL cells were cotransfected with pVT501 and dl 13-89 (Fig. 8). The arrowhead denotes the viral DNA insert of dl 13-89 that is rescued from the pUC plasmid in cells cotransfected with pVT501 and dl 13-89. The fragment is not present in cells transfected with either clone alone (Fig. 8, lanes 2, 3). As the molar ratio of pVT501 to dl 13-89 approaches 1:1, the dl 13-89 fragment is increasingly rescued (lanes 4-7). The dl 13-89 fragment migrates as a monomer length ds fragment of 1,323 bp based on its co-migration with the marker (lane 9) which is the viral DNA excised from the plasmid with Sal I. A monomer ds fragment is the expected form of replicating molecules that would be isolated using the modified Hirt procedure when the CPE was less than 70 percent. The arrow denotes the same dl 13-89 which is Dpn I resistant and as such is replicated (lanes 16 and 17). The amount of Dpn I resistant monomer BPV RF (asterisk lane 12) in cells transfected only with pVT501 is approximately 10

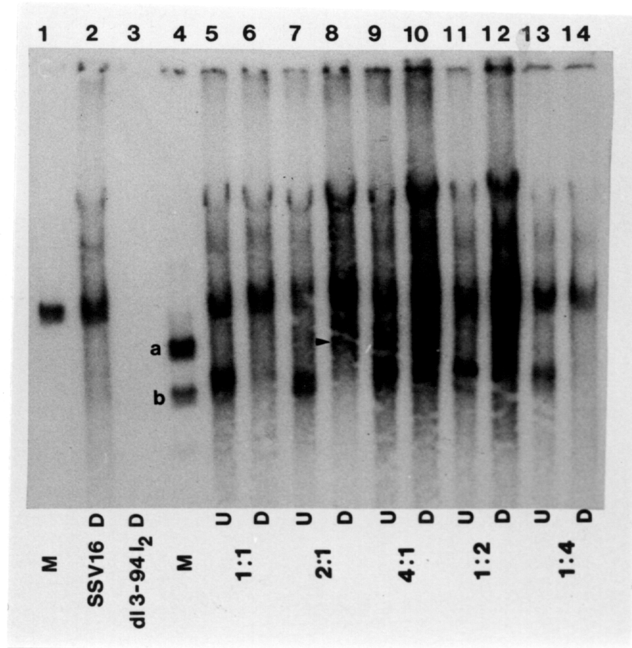


Figure 7. Replication of dl 3-94I_2 by cotransfection with SSV16: DNA was electrophoresed on a 1% agarose gel and transferred to a Zeta Probe membrane and probed with the ^{32}P-labeled 4,675 bp AAV fragment from the SSV16 subclone of psub201(+) (Samulski et al., 1987). Markers are: lane 1, 15 ng isolated Xba I fragment from SSV16; lane 4, 25 ng of Pvu II and Eco RI digested SSV16. The size of the 3 AAV fragments are (a) 2690 bp, (b) 1700 bp, and (c) 218 bp (not visible at this exposure). Lanes 2 and 3 contain DNA from cells transfected with SSV16 and dl 3-94I_2 respectively. Lanes 5 - 14 contain DNA from cells transfected with varying concentration of SSV16 and dl 3-94I_2 DNA so that the molar ratio of SSV16 to dl 3-94I_2 was as indicated. In lanes labeled with a D, the DNA was treated with Dpn I. The DNA was not treated with a restriction enzyme in lanes labeled with a U. The arrowhead indicates the rescued and replicated 2569 bp dl 3-94I_2 AAV fragment.

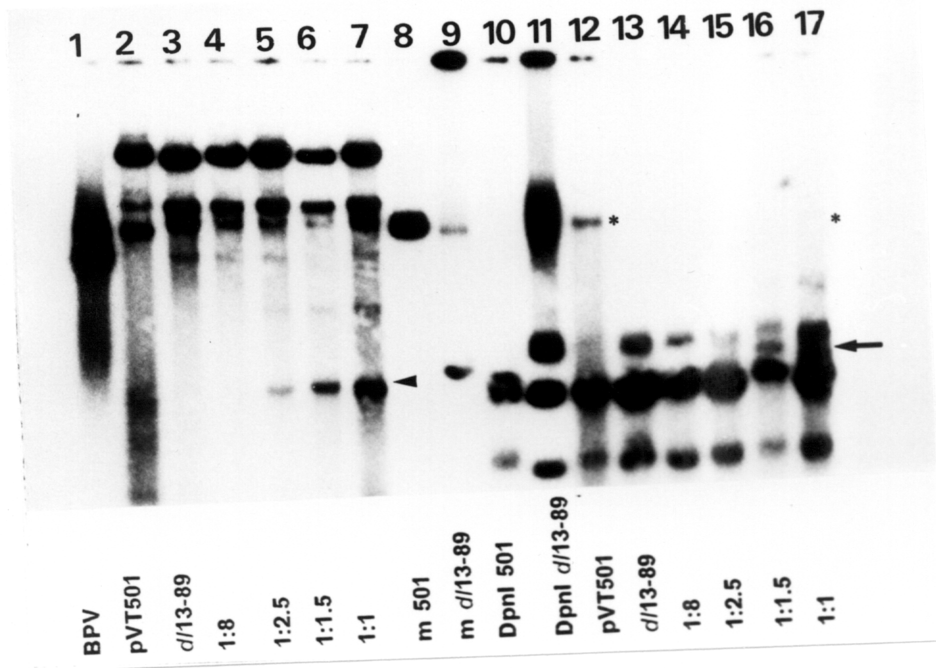


Figure 8. Replication of dl 13-89 by cotransfection with pVT501 DNA: DNA was electrophoresed on a 1% agarose gel, transferred to Zeta Probe, and probed with the ^{32}P-labeled 1,323 bp dl 13-89 BPV fragment. Lanes 2-7 contain undigested DNA from cells transfected with different concentrations of pVT501 DNA and a constant concentration of dl 13-89 DNA. Lanes 12-17 contain the same DNA digested with DpnI. Lanes 2 and 12 contain the DNA from cells transfected with 10 ug of pVT501. Lanes 3 and 13 contain DNA from cells transfected with 12 ug of dl 13-89. The molar ratio of pVT501 to dl 13-89 DNA was 1:8 in lanes 4 and 14, 1:2.4 in lanes 5 and 15, 1:1.6 in lanes 6 and 16, and 1:1.2 in lanes 7 and 17. Markers are: lane 1, 7.5 ng each of ds and ss BPV viral DNA; lane 8, 20 ng pVT501 digested with SalI which to excise the full length (5491 bp) BPV genome; lane 9; 20 ng of *dl*13-89 digested with SalI to excise the 1,323 bp BPV fragment; lane 10; DpnI-digested pVT501; lane 11; DpnI-dl 13-89 plasmid DNA. The arrowhead indicates the rescued 1,323 bp dl 13-89 BPV fragment and the arrow indicates that fraction of the rescued fragment which is actually replicated. The asterisk denotes replicated full length ds BPV DNA.

fold greater than in cells cotransfected with pVT501 and dl 13-89 (asterisk lane 17). The replication of dl 13-89 thus appears to interfere with the replication of full length BPV.

Similar results are seen for the replication of dl 6-95 (Fig. 9). A ds monomer length fragment is rescued (arrowhead) and replicated (arrow) as judged by its resistance to digestion with Dpn I. The slightly slower mobility of the replicated fragment as compared to the marker is likely due to the high salt composition of the Hirt DNA mixture as full length BPV isolated from transfected cells (lane 4) migrates more slowly than the full length BPV marker (lane 6). Interestingly, this replication occurs in spite of the 65 bp deletion present in the left terminus of dl 6-95 (Fig. 5) which removes the nucleotides necessary to form the proposed T-shape conformation at the left end. However the complementary nucleotides which remain could from a "mini-T" structure. This structure may allow the deleted left end to function as an *ori*. That dl 6-95 is efficiently replicated is highlighted by its interference with the replication of BPV (asterisks, compare lanes 9 and 10). This interference suggest that the deleted clones are competing for replication factors that are essential for replication of full length BPV.

Replication of a Clone with Duplicate Left Termini

The parvovirus replication models and the data in Figures 8 and 9 suggest that both termini function as an *ori*. To determine the *cis* sequences necessary for BPV replication, the replication of the chimeric clones containing duplicate inverted BPV termini was assayed in the basic transient replication assay. The Southern blot in panel A of Figure 10 illustrates that the monomer length (993) bp viral DNA insert of pVT2 x 3 is rescued and replicated as shown by the resistance of the fragment to Dpn I digestion (lane 3) and the absence of a same sized fragment in DNA from cells transfected with either pVT2 x 3 or pVT501 alone (lanes 2 and 4). When the blot containing undigested duplicated termini fragments was stripped and reprobed with a BPV fragment specific for the right terminus no hybridization was seen (Fig. 10, panel B).

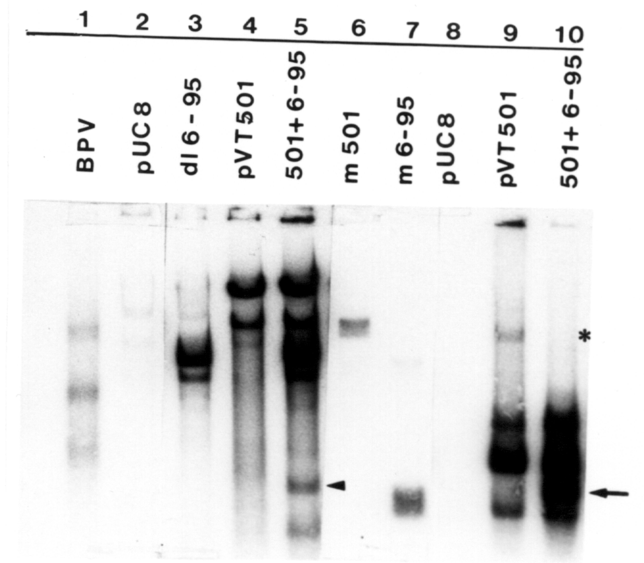


Figure 9. Replication of dl 6-95 by cotransfection with pVT501 DNA: DNA was probed with the ^{32}P-labeled 509 bp dl 6-95 BPV fragment. Lanes 2-5 contain undigested DNA from cells transfected with the following plasmids: lane 2, 15ug pUC8; lane 3, 6.5ug dl 6-95 and 8.5ug pUC8; lane 4, 10.2ug pVT501; lane 5, a 1:1.6 molar ratio of pVT501 to dl 6-95. Lanes 8-10 contain the same DNA as in lanes 2,4, and 5 respectively but digested with Dpn I. The markers are: lane 1, ds and ss BPV viral DNA; lane 6, pVT501 digested with Sal I; lane 7, dl 6-95 digested with Sal I to excise the 509 bp BPV fragment.

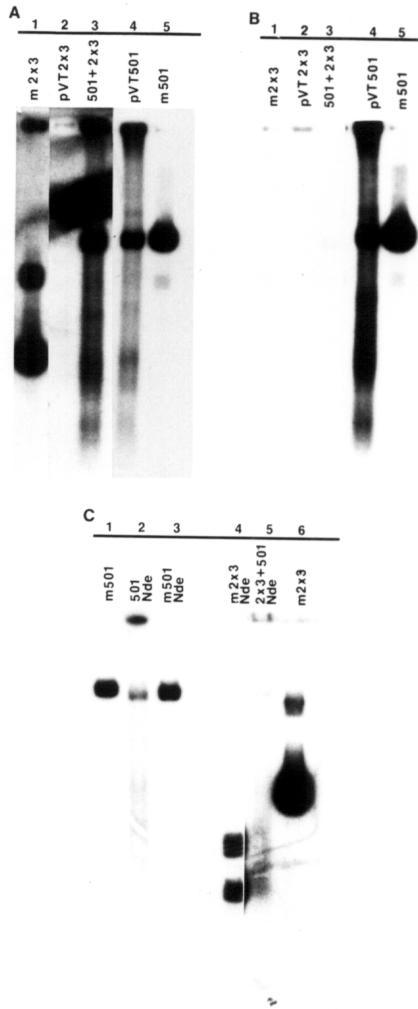


Figure 10. Replication of a clone with duplicate left termini.: Panel A and B are the same Southern blot. In panel A, the DNA was hybridized to the ^{32}P-labeled Sal I to Cla I, left terminus, of pVTC350; and in panel B, the probe was the right end Aat II fragment of BPV (mu 85 to 95) from pVT501. The DNA was isolated from cells transfected with the plasmids indicated and digested with Dpn I. The markers in lanes 1 and 4 are the Sal I BPV fragment of pVT2 x 3 (993 bp) and pVT501 (5491 bp) respectively. In panel C, samples of the same DNA used in panel A and B was digested with Nde I where indicated and probed with the left BPV terminus (Hind III - Xba I) fragment from pVT501. The markers in lanes 1 and 6 are the same as in panel A and B. The markers in lanes 3 and 4 were Nde I digests of the same fragments. The exposure time was adjusted between 24 and 72 hrs so that all possible fragments were visible.

The observed replicated fragments could have originated from deletions of the full length replicated BPV. However, upon digestion of the Hirt DNA with Nde I (Fig. 10, panel C, lanes 4 and 5) the replicated fragments migrate as 2 fragments of the same size as the Nde I digested viral DNA fragment of pVT2 x 3 (678 nt and 315 nt). There are no similar sized fragments in the Nde I digested DNA from cells transfected with pVT501 (lane 2).

Any fragment that had recombined with a right end from pVT501 would have hybridized to the right terminus probe, and any deletion that arose during the replication of pVT501 would have hybridized with the left terminus probe and would likely have Nde I restriction fragments of novel size. Thus, the observed replicating fragment is actually two left termini originating from pVT2 x 3.

Replication of a Clone with Duplicate Right Termini

To determine if the *cis* signals for replication are also present in the right terminus, BFL cells were cotransfected with pVT501 and the pVT2 x 5 construct with duplicate right termini. The rescue and replication of the 682 bp viral DNA insert of pVT2 x 5 (Fig. 11, panel B, lane 1) demonstrates that duplicate right termini can replicate. While these termini are replicated to only a limited extent, they hybridize only to BPV probes specific for the right end (panel B compared to panel A). These results suggests that these fragments are not natural deletions or recombination products of the replicating pVT501.

The possibility exists that pVT2 x 5 is less efficiently replicated because of the small size of the viral DNA fragment compared to that of dl 13-89. It should be noted that each assay varies a great deal because of the nature of each individual transfection and subsequent infection. Therefore, the exact quantification of the degree of replication of an individual chimera was impossible. However, several repetitions of the transient replication assay using the duplicate end chimeras demonstrated that they are less efficiently replicated than dl 6-95 and dl 13-89.

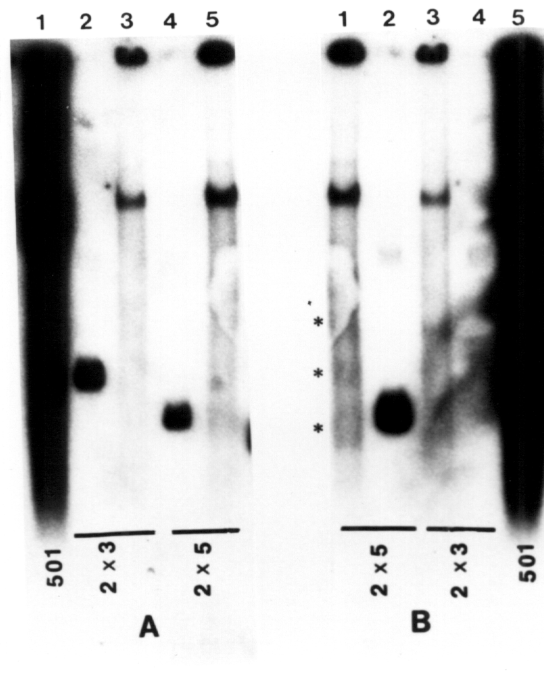


Figure 11. Replication of a clone with duplicate right termini: Panel A and B are the same Southern blot. In panel A the DNA was hybridized with the ^{32}P-labeled full length BPV fragment from pVT501; and in panel B, the probe was the Aat II fragment (mu 85 to 95) of BPV from pVT501. Lanes 2 and 4 contain the Sal I digested plasmid DNA indicated as a viral ds DNA marker. Lanes 1, 3, and 5 of each panel contain Dpn I digested DNA from cells transfected with the plasmids indicated. In lanes 3 and 5 in panel A and 1 and 3 in panel B, the cells were cotransfected with pVT501 as well. The asterisks denote the monomer length and larger replicated ds duplicate right termini fragments. The membrane in both panels was was exposed for equivalent periods of time.

Interestingly, there are two apparent higher molecular weight bands of the replicated pVT2 x 5 fragment (Fig. 11, see asterisks). Similar bands were observed for pVT2 x 3 suggesting that the viral DNA fragments were amplified and processed differently than chimeras containing both termini.

Replication of Genomic Clones with Altered Termini

Evidence that altered BPV termini are efficiently replicated is presented in Figure 12. BPV is clearly replicated in pVT503 transfected cells (lane 9). The clone pVTAB65 (lane 8) that contains elements of the AAV termini surrounding the BPV genome replicates as efficiently as pVT501. It should be noted that this clone also transfects BFL cells very efficiently and produces infectious virion (data not shown). This clone was constructed by inserting the Klenow repaired, Sal I bounded, BPV genome of pVT501 into a pUC8 vector containing the AAV ends represented in the AAV clone dl 3-94 (McLaughlin et al., 1988). Sequence analysis of the clone indicated that the BPV genome including the termini are intact but the AAV ends are deleted to some extent. No BPV RF is present in cells transfected with pVT502Δ52 (Shull et al., 1987) which is missing nt 1-52 in the left terminus. This suggests that there is a minimum number of nucleotides in the left terminus which are required for replication. There also might be different *cis* signals required for efficient replication to yield the normal BPV distribution of ss DNA versus ds DNA. Cells transfected with pVT504 which contains the 65 bp deletion of its left terminus do not appear to produce any ss DNA (Fig. 12, lane 7).

Replication of BPV / AAV Chimeras in BFL Cells

The secondary conformation of the termini has been demonstrated to be important for the replication of AAV (Lefebvre et al., 1983). The intact BPV left terminus has a secondary

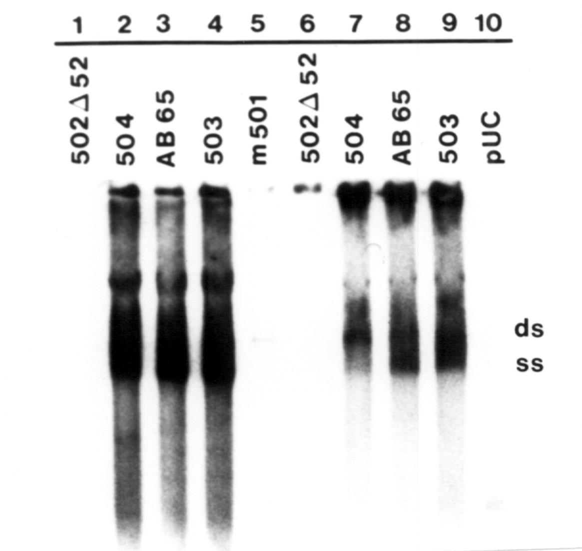


Figure 12. Replication of genomic clones with altered termini: DNA was probed with the ³²P-labeled full length BPV fragment of pVT501, Lanes 1-4 and 6-9 contain DNA from cells transfected with the plasmids indicated. The DNA in lanes 6-9 was digested with Dpn I. The Sal I fragment of pVT501 was used as the full length BPV (5517 bp) genome marker (lane 5). The ds and ss replicated DNA molecules are indicated.

conformation similar to that of the AAV termini (Fig. 5). Beyond the T-shape conformation, the termini are quite different. They share only 32% sequence homology and the BPV terminus is AT rich and the cross arms are shorter compared to those of AAV. It was of interest to determine if an AAV terminus could be exchanged for a BPV terminus and still allow replication of the chimeric molecule. If this molecule replicated, the necessity of a specific conformation at the BPV *ori* instead of a specific sequence would be confirmed. The Southern blot in Figure 13 shows DNA isolated from BFL cells transfected with the BPV-AAV chimeras and pVT501. Unlike the other transient replication assays, no obvious rescue or replication of the expected ds monomer length viral DNA fragment (no band at the position of the arrowhead) was apparent even after extended radioautographic exposure. No replication was observed either when pAB35 was cotransfected into BFL cells with both Bovine Adenovirus and BPV (data not shown). While different combinations of this type of experiment could be tested using both BFL and HeLa cells, it is apparent from these results that the AAV terminus cannot simply replace a BPV terminus nor can a single BPV terminus function alone.

DISCUSSION

The Termini of BPV Act Alone as a Replicon

It is apparent from other DNA replication studies on SV40, adenovirus, as well as eukaryotic and prokaryotic chromosomes (Bramhill and Kornberg, 1988; Stillman, 1989) that specific DNA sequences are required for initiation of DNA synthesis and define the origin of replication. The origin of BPV replication is probably similar and work with AAV (Lefebvre et al., 1983; Bohensky et al., 1989) suggests that the secondary structure of the termini will also be an important component of the parvovirus origin.

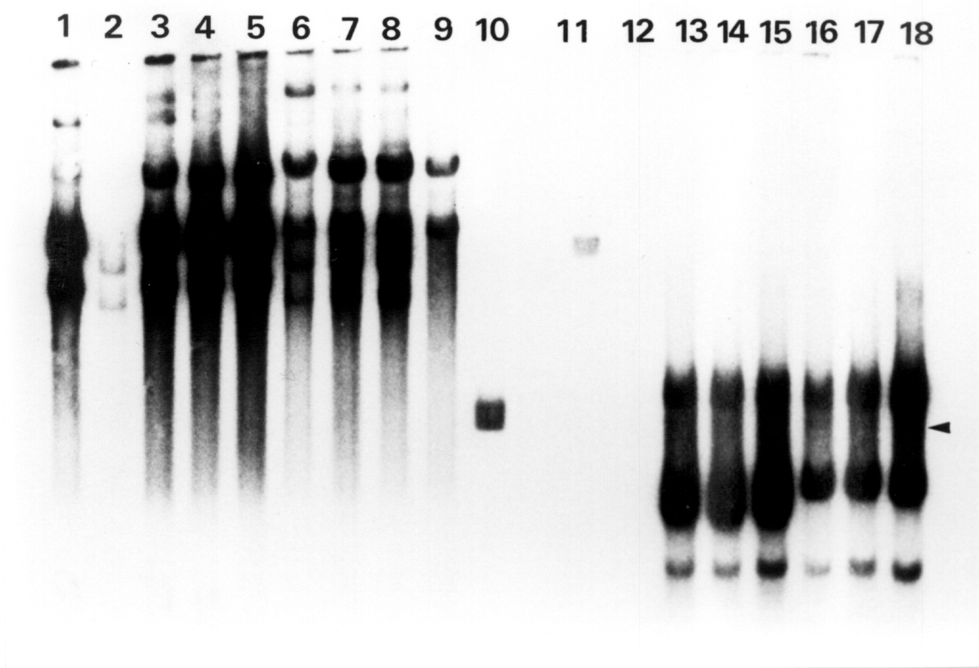


Figure 13. Replication of pAB53 and pAB33 by cotransfection with pVT501: DNA and BFL cells were treated as described in figure 8. Lane 1 contains DNA from cells transfected with 10ug of pAB53. Lane 2 contains DNA from cells transfected with 6ug of pAB33. Lane 9 is DNA from cells transfected with 10ug of pVT501. Lanes 3-8 contain undigested DNA from cells cotransfected with pVT501 and pAB53 (lanes 3-5) or pAB33 (lanes 6-8) at three different molar ratios of pVT501 to chimeric plasmid: 1:1.5 (lanes 3 and 6), 1:2 (lanes 4 and 7), and 1:3 (lanes 5 and 8). Lanes 13-18 contain the same group of DNA digested with Dpn I. The markers are; lane 10, Sal I digested pAB53 to excise the 1,414 bp AAV-BPV chimeric fragment ; lane 11, Sal I digested pVT501; lane 12, Eco RI digested pUC8. Arrowhead, see text.

The results of the replication of the chimeras shows that BPV replication depends on a sequence specific *ori* located in the termini. The replication of dl 13-89 and dl 6-95 shows that the BPV termini act as a replicon. The natural BPV *ori* then must be located within nt 1-348, and nt 5,268 to 5,517. These transfection results are similar to those found for AAV in which internal deletion mutants were replicated in the host cell (Mclaughlin et al., 1988), and the data showing that parvovirus genomes devoid of all but the terminal 250 nt are replicated and packaged (Faust and Ward, 1979; De la Maza and Carter, 1980).

The results are also consistent with the proposed models for parvovirus replication which require that replication initiate at both termini to yield replicative intermediates necessary for generation of ss DNA progeny. The lack of replicated multimer ds mini-genomes of dl 13-89 suggests that BPV may not replicate through an obligatory dimer intermediate as does MVM. It is likely that the replicated termini are being processed by a mechanism consistent with the unified model of Chen et al. (1989) for parvovirus replication.

The Minimal Sequences Sufficient For BPV Replication.

The replication of pVT2 x 3 and pVT2 x 5 in the transient replication assay demonstrates that all of the DNA sequences necessary for the initiation and of replication are present in either terminus and lie within the first 291 nt of the left end and the last 249 nt of the right end. Recent work using the LuIII infectious genomic clone (Diffoot et al., 1989), in which various chimeric genomes were constructed and tested (Rhode, 1989) for their ability to replicate, demonstrated that a clone with two left termini was capable of replication. Rhodes' clones were slightly different than these mini-genomes in that elements of both the left and right termini were present at the ends of the chimeras. The apparent relatively inefficient replication of both BPV duplicate end clones compared to dl 13-89 could be due to the absence of one of the native termini or to the small size of the viral DNA fragment. To test the efficiency

of replication of a genome with duplicate ends, a full length BPV genome with either two left or two right ends is being used to transfect BFL cells (K. Chen, unpublished results).

The higher and lower than monomer length forms of the replicated mini-genomes, pVT2 x 3, pVT2 x 5, and dl 6-95, suggest that an alternate mechanism of processing is occurring during replication of these genomes. This could be due to the 65 nt deletion, which alters the normal secondary structure of the palindrome, in the left terminus of dl 6-95, pVT2 x 3, and pVT504. More generally, it could be due to the absence of one of the wild type termini. This suggests that the BPV *ori* is defined by more than one domain within the termini; a sequence specific domain and a conformation specific domain.

When the conformation of the BPV termini included in pVT2 x 3 and pVT2 x 5 are compared, the right end palindrome can form a dramatically different cruciform than that formed by the left end. So while these secondary structures may be important in determining the efficiency of replication from one virus terminus, they are not the sole determining factors in the origin of replication. A comparison of the sequences of the termini included in pVT2 x 3 and pVT2 x 5 shows only 50% homology. However, there are 2 regions of significant similarity. The first region (Fig. 14, domain 1), as discussed previously (Shull, 1987), is between nt 115 and 148 of the left terminus and nt 5341 and 5381 of the right terminus and shows 75% homology. It is important to note that when the left terminus is in the hairpin conformation (Fig. 5) nt 1 - 58 and nt 88 - 150 are basepaired. The conserved TA_5T region (Shull, 1987) is within this domain as is the palindromic sequence ATTGGC-N_7-GCCAAT. Perhaps coincidentally, the region between nt 30 and 55 shares some homology with the NFI binding site in adenovirus 2 (Jones et al., 1987). There is little homology between these sequences and the G-rich sequence near the right terminal palindrome of H-1 proposed to be the origin of RF replication (Rhode and Klaasen, 1982). The results of the replication of the full length BPV genomes with altered termini suggest that the removal of the first 52 nucleotides from BPV inhibits replication. Therefore, domain 1 in the left and right terminus and their complementary sequences in their respective terminus likely define the BPV core origin of replication.

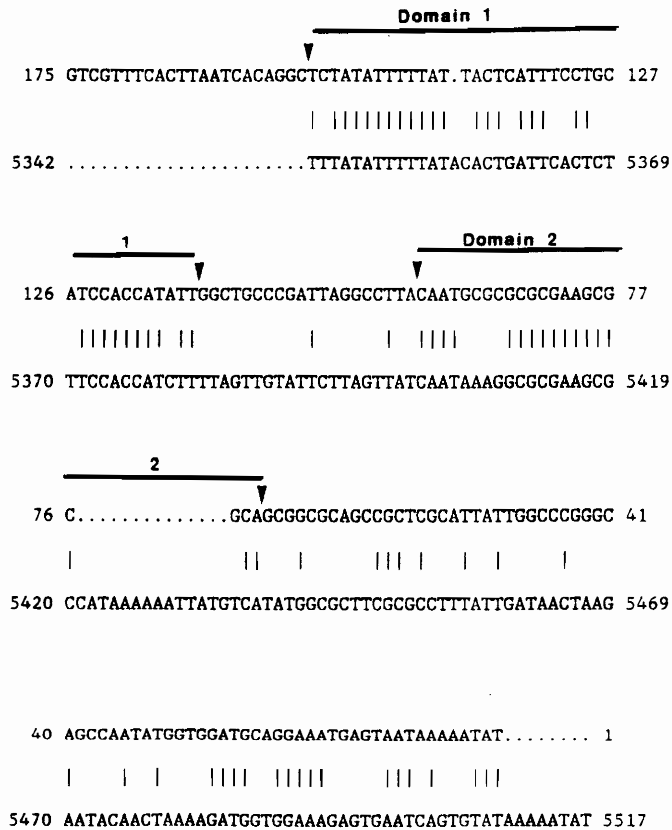


Figure 14. Two homologous regions of the left and right termini of BPV: The sequence depicted is that of the plus strand left terminus of BPV in the flip conformation (top) and the right terminus of the plus strand in the flip conformation (bottom). The sequences were aligned using the GAP program from UWGCG.

The second region, between nt 30 and 98 of the left end and nt 5398 and 5460 of the right end, shows only 44% homology. However, this region includes a smaller domain (Fig. 14, domain 2) of significant base complementarity. Interestingly included within this region are the nucleotides which comprise the "loop" at the right terminus and the cross arms of the left terminus. This GC rich domain 2 has a role in BPV replication. It may direct the efficiency of amplification by influencing the initiation of DNA synthesis from the termini, because when nucleotides 54-118 are deleted,as in pVT504, BPV replication is similar to wild type except that only monomer ds BPV DNA was detected. Whether the actual sequence or the secondary conformation is most important in this domain remains undetermined.

In AAV, the primary sequence of the termini is relatively unimportant. As long as the secondary conformation of the cross arms of the terminal palindrome are maintained, AAV will replicate (Lefebvre et al., 1984;Bohenzky et al., 1988). The fact that an AAV terminus cannot replace those of BPV appears to support the conclusion that specific sequences in the termini are required for BPV replication. The demonstration of two domains in the BPV *ori* which are not completely identical in both termini, may partially account for the differential replication kinetics proposed by Chen et al (1989 and 1990).

Termini Interact with Replication Proteins

The replicated dl 6-95 and dl 13-89 appear to act as defective interfering molecules to the replication of BPV in the same manner as naturally occurring DI particles might (Carter et al., 1984a). Since the rescue and replication of the chimeric molecules requires that the cell be cotransfected with pVT501, it can be inferred that proteins coded for by BPV necessary for replication and perhaps rescue are provided in *trans*. These proteins are likely to be the *rep* proteins i.e., the BPV large nonstructural proteins. Rhode (1989) has demonstrated that the NS-1 protein of LuIII is required for the rescue and replication of the viral genome in HeLa cells transfected with chimeric LuIII clones. The observed replication interference of the mini-

genomes on BPV is then probably due to their acting as competitors for the available BPV *rep* proteins. The competition is probably effective because the small size (< 1500 bp) of the chimeras allows them to be replicated by the cell DNA synthesis enzymes more frequently than the full length BPV molecule. Therefore, they act as a sink for all the replication factors.

Recent evidence from DNA-protein binding studies with AAV termini show that the AAV *rep* proteins bind specifically to the termini (Im and Muzyczka, 1989; Ashktorab and Srivastava, 1989). The *in vivo* data presented here suggest that viral protein-DNA interactions are occurring at the BPV *ori* to initiate replication. Therefore, specific *in vitro* DNA-protein binding assays should be able to identify the specific *trans* factors involved in the initiation of BPV replication and the DNA sequences to which they bind.

Chapter III

PROTEIN RECOGNITION OF BOVINE PARVOVIRUS TERMINAL DNA SEQUENCES

Introduction

Bovine Parvovirus (BPV) is an autonomously replicating virus with a linear single strand (ss) DNA genome of 5517 bases (Chen et al. 1986, 1988). It has three major open reading frames (ORF) that divide the genome into left, middle, and right sections. Except for the presence of the middle ORF, this same genomic organization is found in all parvoviruses (for a review see Cotmore and Tattersall, 1987). The left ORF in adeno-associated virus (AAV), a parvovirus that generally requires a helper virus to complete its replication cycle (Carter and Laughlin, 1984), and in minute virus of mice (MVM), an autonomously replicating virus, codes for nonstructural (NS) proteins (Mendelson et al.,1986; Cotmore and Tattersall 1986). The four nonstructural proteins of AAV have been determined to be important in both replication and transcription of the genome (Hermonant et al., 1984; Tratschin et al., 1986). At least one of

these NS proteins (68 kDa) is capable of binding to the terminus of AAV (Im and Muzyczka, 1989) and is involved in the resolution of the terminal hairpin (Snyder et al., 1990). Similarly, the large NS protein (NS-1) of MVM has been shown to be covalently bound to the 5′ terminus of the genome (Cotmore and Tattersall, 1988), and the NS-1 of another rodent virus, H-1, has trans-activating functions (Rhode, 1985). The structural and functional similarities between the NS proteins of these viruses likewise suggests that the NS proteins of BPV, which are immunologically related to the NS-1 protein of MVM and B19 (Lederman et al., 1987), would have comparable functions.

Infectious genomic AAV2 and BPV clone mutants containing deletions in the termini are unable to replicate (Samulski et al.,1983; Shull et al., 1988). Alternatively, a deletion of the infectious BPV genomic clone, pVT501, containing 348 nt of the left (3′ OH) and 249 nt of the right (5′ P) terminus, can be replicated when all of the *rep* functions are provided in *trans* by an intact BPV genome (see Chapter I). These data and the parvovirus replication models (Chen et al., 1989; Hauswirth and Berns, 1979; Cotmore and Tattersall, 1987) suggest that, as with other mammalian DNA viruses, the initiation of BPV DNA replication requires specific recognition of the termini by viral proteins. Also, BPV and other autonomous parvoviruses have a strict requirement for an S-phase host cell factor (Wolter et al., 1980) to be able to replicate their genomes. This factor could cooperatively bind with the viral protein to recognize the termini.

The resolution of the gel retardation assay and the specific amplification of the polymerase chain reaction (PCR) was used to identify proteins in crude nuclear extracts of BPV-infected and uninfected primary bovine fetal lung (BFL) cells that interact with the first 173 nt of the left end of BPV (3′ OH terminus of the viral minus strand). Three specific DNA-protein complexes are formed with this terminus when in its cruciform conformation. By specific competition with anti-BPV capsid antibody, one of the complexes was shown to involve a capsid protein. By specific competition with unlabeled competitor DNA and anti-BPV antibodies, a second complex was shown to be due to a BPV NS protein that likely recognizes a sequence with similarities to the consensus CTF/NFI site proposed by Jones et al. (1987).

The identity of the cellular factor is undetermined but it may recognize the GC rich cross-arm of the left end hairpin and is in a 5 fold higher concentration in actively dividing cells than in contact inhibited cells.

MATERIALS AND METHODS

Cell Cultures

Bovine fetal lung (BFL) cells were cultured as previously described by Parris and Bates (1976) in minimal essential medium (MEM) containing 10% fetal bovine serum. Contact-inhibited cells were obtained from cultures at least seven days post-seeding when the monolayer had been confluent for two days. Uninfected, actively dividing S-phase cells were obtained from 50-75% confluent monolayer cell cultures two days post-seeding. BPV-infected cells were obtained by infecting parasynchronous BFL cells 24 hours post-seeding and harvesting the infected cells 24 to 36 hr post-inoculation. Hydroxyurea (HU) synchronized cells were obtained for infection by seeding BFL cells in MEM containing 10% FBS and 2 mM HU (Parris et al., 1975). These cells were infected with BPV 24 hours post-seeding and released from the HU block as described by Pritchard et al. (1981).

Nuclear Extracts

Nuclei were prepared essentially as described by Challberg and Kelly (1979) with some modifications. Cells from 7-10 roller bottles ($\simeq 6 \times 10^7$ cells) were harvested and combined give 2.5×10^7 cells per tube. Cells were washed twice with 20 ml of hypotonic buffer (20 mM

Hepes (N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid) - KOH [pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 M sucrose) (Challberg and Kelly, 1979) and the cells resuspend in 6 ml cold hypotonic buffer without sucrose. Cells were allowed to swell on ice for two hours and lysed by 150 strokes in a Dounce homogenizer using pestle A. Nuclei were pelleted by centrifugation at 1000 x g for 15 min and washed once in 10 ml Buffer A (25mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA [pH 7.5], 1mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride) (Im and Muzyczka, 1989). The nuclear pellet was resuspended in 1 ml Buffer A and brought to 1M NaC1. The nuclei were incubated on ice for 1 hr and then pelleted at 65,000 rpm for 30 min.. Typically 1.8 ml of supernatant was recovered and dialyzed overnight against cold 0.1 x Buffer A. The dialysate was cleared by centrifugation and concentrated by vacuum 10 fold. The protein concentration was determined using the BCA protein assay (Pierce Chemical Co.) and glycerol was added to a final concentration of 15%. Typical yields were 2-7 μg protein/μl. The lysate was stored at -20 °C.

To increase cell lysis and recovery of nuclei, an alternative method of nuclear isolation (Mirkovitch et al., 1984) was also used. Approximately 6 x 10^7 cells were collected into a large volume to give 1 x 10^7 cells per tube. Each cell pellet was washed twice with 20 ml of wash buffer (20 mM Tris hydrochloride [pH 7.4], 0.05 mM spermine, 0.125 mM spermidine, 1% dithiodiglycol, 20 mM KCl) with 100 TIU aprotinin and 10 μl of 1 mM phenylmethylsulfonylfluoride added per microliter. The cell pellet was suspended in 12 ml of homogenization buffer (wash buffer with 0.1% digitonin (Fluka), and the cells were lysed by 50 strokes in a Dounce homogenizer with pestle A. The nuclei were washed three times in wash buffer and finally resuspended in 1 ml Buffer A. NaCl (5M) was added to a final concentration of 1M NaCl. The nuclei were incubated on ice for 1 hr and dialyzed and concentrated as described above.

Preparation of Radiolabeled DNA

Radiolabeled homogeneous BPV left terminus was obtained either by conventional methods (Maniatis et al., 1982) or by an arithmetic polymerase chain reaction (PCR). The left terminus was obtained using the clone pVTC350. This clone is a deletion of the BPV genomic clone, pVT501 (Shull et al., 1988). The Nhe I fragment of BPV from map unit 6 to 92 was removed to leave a 809 bp BPV DNA insert in pUC 8. The Cla I site was introduced at nt 170 of the plus strand of the left terminus. This was accomplished using PCR mutagenesis (Higuchi et al., 1988) and a mutated oligonucleotide,
5′ GTGATTAAGTGAATCGATATAGGCGAGGAC 3′ (Applied Biosystems 381A DNA synthesizer), representing nt 158 to 187. Bases 14 and 18 within the oligonucleotide are mutated from the wild type sequence. The clone also contains a Cla I site at nt 5329 near the right terminus of the BPV genome. This site was introduced using the oligonucleotide
5′ AAAAAGTCAATCGATACACCGCTTTATA 3′ with bases 10 and 13 mutated from the wild type sequence. The left terminus of BPV was electroeluted from an agarose gel as the 171 basepair Hind III to Cla I fragment from pVTC350. The ends of the purified fragment were filled in with α^{32}P-dCTP (3000 Ci/mM) (New England Nuclear) and a mix of cold nucleotides using the Klenow fragment. After organic extraction, unincorporated nucleotides were removed using a spin column containing BioRad P-30 or P-100 matrix. The specific activity of the fragment typically was 1 x 10^7 cpm/μg.

Alternatively, a labeled left terminus was obtained by PCR amplification (Saiki et al., 1988) using a single primer. The template for the reaction was an isolated left terminus of pVT501 or pVTC350. A synthetic primer,
5′ TGTCGTTTCACTTAATCACAGGCTCTATAT 3′ , which is complementary to nucleotides 151 through 176 of the plus strand of the BPV left terminus, was used to synthesize the terminal 150 nucleotides of the minus strand of BPV. The reaction mixture was that suggested for basic PCR by the manufacturer (Perkin-Elmer Cetus), except that 3μM α^{32}P-dCTP (3000 Ci/mM) was also incorporated into the reaction. The parameters of the reaction cycle were 94° C for 3

min followed by 40 cycles of 94° C for 50 sec, 55° C for 1 min. 80° C for 2 min, and final extension for 7 minutes at 80° C. Disassociation of the amplified single strands was at 94° C for 5 minutes. Two reaction products were usually produced. The larger one was recovered by electrophoreses on a 12% native polyacrylamide gel and electro-elution of the fragment into a Centricon 30 filter cartridge (Amicon) followed by subsequent concentration. This larger fragment corresponds to the full length (176 nt) single strand intrastrand basepaired left terminus in the flop conformation. The conformation was confirmed by Hha I restriction enzyme digestion (Fig. 15). Typical yields of gel purified fragment were 5 x 10^6 cpm/ug.

Mobility Shift Assay

The mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981) used to detect DNA binding proteins was based on the assay described by Im and Muzyczka (1989). Crude 1M NaCl nuclear extract (7 ug protein) was mixed in a 20μl reaction mixture (25 mM Hepes - KOH [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 2% glycerol, 2.6 μg bovine serum albumin, 50 mM NaCl, 0.01% Nonidet P-40) containing 2 μg heat-denatured, sonicated, salmon sperm DNA as non-specific competitor DNA and the specific competitor DNA or antiserum described in the Results. The reactions were allowed to incubate at room temperature (RT) for 20 min before adding the labeled fragment which had been boiled for 5 min and immediately chilled on ice for 5 min. The extract and labeled fragment were further incubated for 20 min at RT. The specific labeled left terminus was added to give $\simeq$ 5 x 10^3 cpm in each reaction (0.5 ng - 0.8 ng DNA). The reactions were applied to a 4% polyacrylamide gel (39:1) and electrophoresed at RT for 3 hr in 1 x TBE (0 .089 M Tris, 0.089 M Boric acid, 0.2 mM EDTA [pH 7.6]) at 28 mA.

Competitor DNA Preparation

Unlabeled viral termini (AAV or BPV) were obtained by electroeluting the appropriate restriction fragments from agarose gels. The AAV termini were isolated as Pvu II and Xba I fragments from the SSV16 isolate of psub201(+) plasmid DNA (Samulski et al.,1987; a gift from N. Muzyczka). The termini were either used as double stranded DNA fragments or boiled for 5 min and immediately chilled on ice for 5 min and used as single stranded hairpined fragments. The fragment containing the sequence 5' CATATTGGCTGCCCGGGCCAATAATG 3' between nt 31 and 56 of the BPV genome, which is similar to the CTF/NF-1 consensus sequence, was created by annealing 2 μg each of complementary oligonucletides. The oligonucleotides were mixed, heated to 94° C for 3 min cooled to 55° C over a period of 10 min and maintained at 55° C for 10 min and then allowed to cool for 30 min to 37° C. Approximately, 20 to 40 percent of the oligonucleotides reannealed using this procedure.

RESULTS

Terminal Fragment Conformation

The fragment used in the mobility shift assay was the left terminus of BPV in its hairpin conformation. The fragment was obtained using either asymmetric PCR or by boiling an isolated left terminus restriction fragment. In both cases the final labeled product was homogenous (Fig. 15). Two PCR fragments were consistently produced and found to be 30 nt different in length and were purified from each other by isolation from a polyacrylamide gel.

The conformation and identity of each fragment was determined by digestion with HhaI and observation of the size of the restriction fragments (Fig. 15). In lane 1 the undigested left

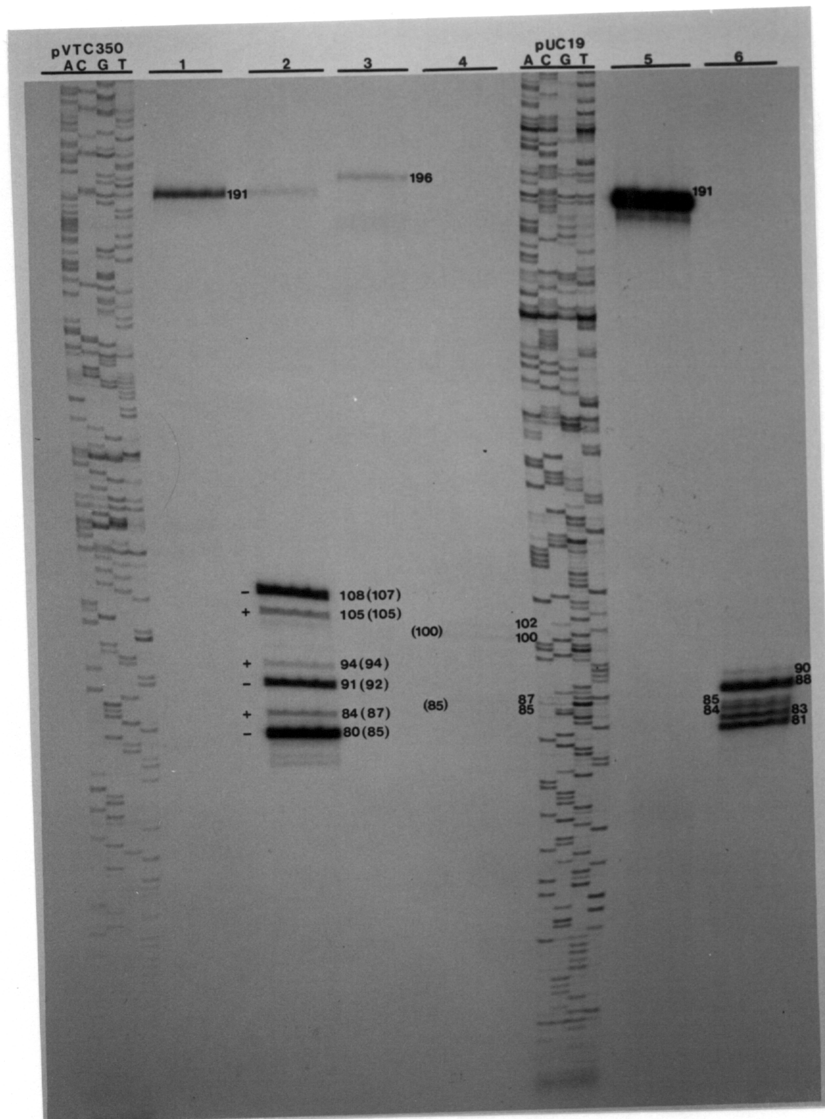


Figure 15. Conformation of terminal fragment: The size of each labeled fragment was determined based on a comparison of the migration distance with the dideoxy sequence of the left end of pVTC350 or pUC19. The 3′ end-labeled Hind III-Cla I fragment of pVTC350 was either boiled for 5 min and immediately chilled prior to loading and restriction enzyme digestion (lanes 1 and 2) or used in its native ds DNA form (lanes 5 and 6). The same Hind III-Cla I fragment was used as a template to obtain uniformly labeled PCR amplified single strand left terminus (lanes 3 and 4) which was also boiled prior to digestion and loading. Full length fragments are in lanes 1, 3, and 5. Fragments treated with Hha I for 1 hr are in lanes 2, 4, and 6. The observed fragment lengths are indicated. The expected fragment lengths are indicated in parentheses.

terminus restriction fragment migrates as a fragment 191 nt long which corresponds well with the expected length of 193 nt. This fragment includes 173 nt of BPV sequence from the Klenow repaired Cla I site through 20 nt of pUC8 sequence to the Hind III site. The same fragment in lane 5 which was not heat-treated, migrates to the same position. As expected, the uniformly labeled PCR product (lane 3) migrates as a fragment 195 nt long. Digestion of the left terminus of BPV with Hha I gives unique size fragments which allow the determination of the terminal sequence orientation; flip versus flop (Chen et al., 1988). Knowing the sequence orientation of the left terminus restriction fragment, digestion with Hha I was used to confirm its size and conformation. In lane 2, the fragment which was pretreated by boiling and quick chilling yields restriction fragments whose sizes agree almost completely with the expected size (see parenthesis). Also, due to the end labeling method used the plus and minus strands of the fragment are differentially labeled. The majority of the radioactivity is incorporated into the minus strand. Similarly, upon Hha I digestion, the PCR fragment yields fragments close to the expected size fragments for the minus strand DNA in its cruciform conformation (lane 4). The greater variation can be explained if the enzyme recognizes similar but alternative sites. The restriction fragments obtained for both the PCR and restriction enzyme product (Fig. 15, lanes 2 and 6) agree completely with the expected fragment sizes (see parentheses) of a single strand hairpin terminus and not with a basepaired ds DNA fragment. The fragments obtained from the left terminus when it was not pretreated by boiling and digested with Hha I (lane 6) are similar to those expected from a ds DNA terminus. The actual fragment size is 20 nt greater than the expected size due to the vector DNA contained in the fragment. This confirms that the terminus used in the mobility shift assay is in the hairpin confirguration and is full length with no deletions.

Identification of Viral DNA-Protein Interaction

To visualize the interaction of proteins that specifically bind to the left terminus of BPV, the mobility shift assay was used. Proteins from nuclear extracts of BPV-infected BFL cells were mixed with radiolabeled DNA of the BPV left terminus and non-specific unlabled competitor DNA. The results of this assay using varying concentration of protein are shown in Figure 16.

The optimum assay conditions were those containing 7 ug of protein and 2 ug of salmon sperm DNA. It is clear that there are three different DNA protein complexes formed as represented by the altered mobility of the DNA in the bands marked C1, C2, and C3 (lane 6) as compared to the unbound fragment in lane 1. By virtue of the restriction site used to isolate the left end fragment, the plus and minus strands were differentially labeled. The majority of the radioactivity was incorporated into the minus strand. Therefore, the observed complexes involve mostly the minus strand of the left hairpin.

It is important to note that the nuclear extracts used in the assay shown in Figure 16 were from BPV-infected BFL cells which had been synchronized. The intent of the synchronization was to achieve a more uniform viral infection (Parris and Bates, 1976) and thus a more consistent yield of viral proteins. The results of this assay indicate that the altered mobility of the labeled DNA fragment is due to protein. As expected, the mobility shift is sensitive to both treatment with proteinase K and phenol-chloroform extraction (Fig. 17).

In this assay, the restriction fragment (Fig. 17, panel A, lane 1) and the PCR product (Fig. 17, panel B, lane 1) migrate freely in the gel in absence of any nuclear extract. Both nuclear extract from infected (Panel A, lane 2) and uninfected cells (Panel B, lane 2) retard the mobility of the fragment. However, if the extracts are treated so as to remove or degrade the protein, the fragment migrates freely (Panel A and B, lane 3). The slight altered mobility of the unbound fragment in lane 3 (Panel B) is perhaps the result of interaction with protein. The extract may contain some nuclease or topoisomerase activity which alters the conformation or size of the fragment. Optimization of the DNA-protein interaction was attempted by altering the

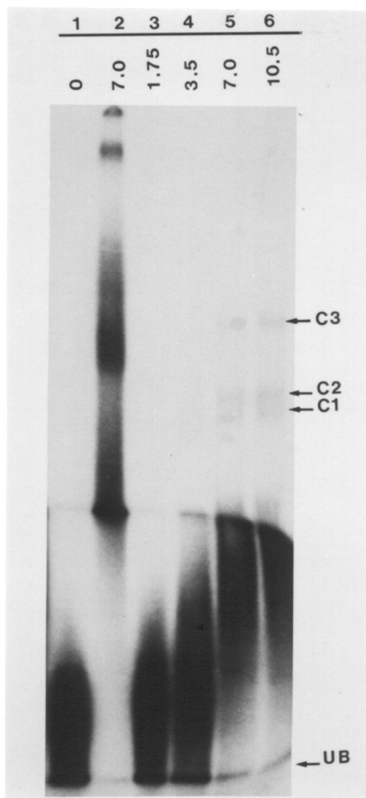


Figure 16. Binding of BPV infected-cell proteins to the BPV left terminus: The 3′ end labeled HindII-Cla I fragment of pVTC350 (0.5 ng) was incubated with 2 μg nonspecific competitor DNA in all cases, except in lane 2, and either no protein (lane 1) or increasing concentrations of protein from nuclear extracts of BPV-infected BFL cells as indicated. The 3 major DNA-protein complexes are indicated by arrows as is the unbound fragment (u).

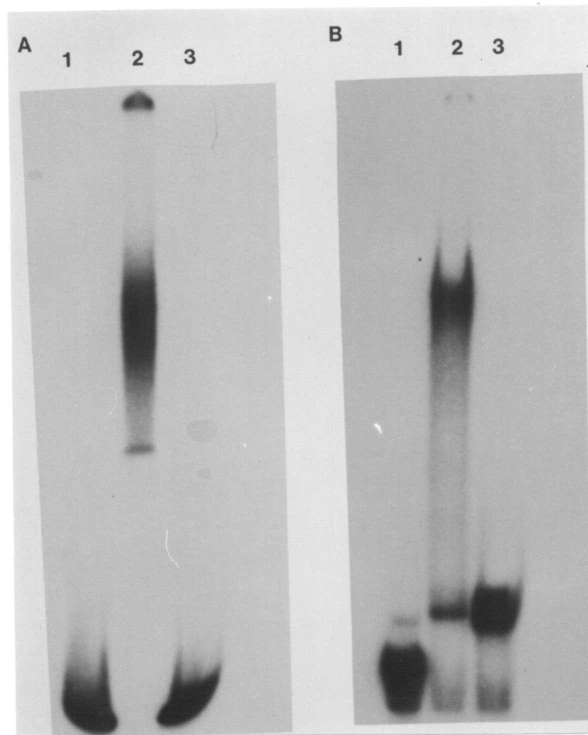


Figure 17. Altered mobility of the left terminus due to protein binding: Panel A, the labeled left terminus, Hind III-Cla I fragment, was incubated with no protein (lane 1), 7 μg of protein from infected cell nuclear extracts, or nuclear extracts pretreated by phenol-chloroform extraction (lane 3). Panel B, the labeled PCR fragment of the left terminus was incubated with no protein (lane 1), 7 μg of protein from uninfected cell nuclear extracts lane 2), or 7 μg of protein pretreated with 1 μl of proteinase K (10 mg/ml) for 30 min. at room temperature.

nonspecific competitor DNA used in the standard reaction. Each competitor dIC, dAT, and calf thymus DNA appeared to inhibit the formation of one or more of the specific protein-DNA complexes observed using salmon sperm DNA. Alteration of the assay buffer by lowering the ionic strength or altering the pH generally decreased the number or intensity of the DNA-protein complexes.

Cellular factor binds BPV terminus

The assay in Figure 18 again shows the DNA-protein complexes formed by proteins from infected nuclear extracts (lane 5). The C1 and C2 complexes are running as a broad band with a slightly slower mobility in this assay as compared to the ones in Figure 16. The intensity of the C3 complex is slightly diminished because the cells used to obtain the extracts were not synchronized prior to infection. The C1 complex also can be identified in nuclear extracts from uninfected cells (lane 3 and 4). However, in spite of using equivalent concentrations of protein in the assay, significantly less C1 complex is formed by the extract from contact-inhibited BFL cells (lane 3). The C1 complex formation is approximately 5 fold greater in extracts from uninfected, actively dividing S-phase BFL cells (lane 4). This dramatic increase in specific DNA binding activity in these extracts suggest that the protein involved is regulated during the cell cycle. The exact identity of the protein will need to be investigated further.

Identification of Viral Proteins Binding to the BPV terminus

The absence of the C2 and C3 complexes in the reactions containing nuclear extracts from uninfected cells suggests that they are caused by viral proteins. To start to identify virus specific proteins which bind to the BPV terminus, a modification of the basic gel shift was

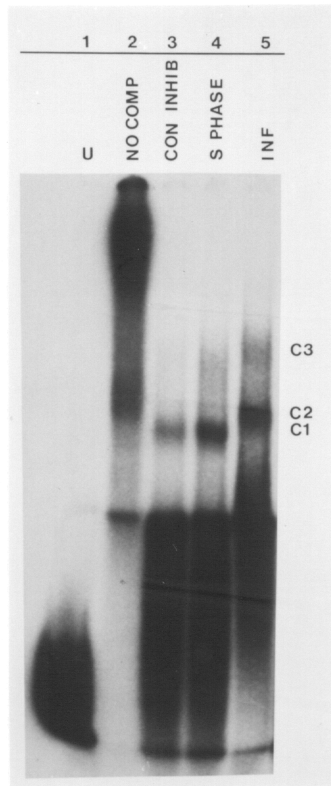


Figure 18. Cellular protein binds the BPV left terminus: The labeled left terminus was incubated with 7 μg of protein from nuclear extracts of contact-inhibited BFL cells (lane 3), actively dividing BFL cells (lane 4), BPV-infected BFL cells (lane 5). Lane 1 has no nuclear extract added. In lane 2, the reaction had 7 μg of infected cell nuclear extract added but no nonspecific salmon sperm competitor DNA.

performed. This assay involved the addition of polyclonal antibodies to the basic reaction mixture prior to the addition of the radiolabeled probe.

The antisera used was either calf 86 serum or rabbit antiserum 0118. Calf 86 serum is a convalescent serum from a BPV-infected calf that reacts with both the nonstructural and structural and nonstructural proteins of BPV (Lederman et al.,1987). The 0118 serum is from a rabbit experimentally inoculated with purified BPV capsids. It is clear from the inhibition of the C2 complex (Fig. 19, lane 3 and 4) and the decrease in the C3 complex that these shifts are due to viral proteins. Since the C3 complex is completely inhibited by the 0118 serum (lane 6), this complex is due to interaction with the capsid. The decrease in the C3 complex caused by calf 86 is, thus, also due to anti-capsid antibodies in the serum. Therefore, the inhibition of the C2 complex by calf 86 and the lack of its inhibition by the 0118 serum (compare lane 4 and 6) suggest that the complex involves the BPV NS proteins. Which of the NS proteins or capsid proteins cause the shifts is as yet undetermined. By analogy to AAV, though, it is likely the 83kDa or 75kDa protein is involved; and based on earlier protein-DNA studies by Lederman et al. (1987), VP2 could also be involved. Further since the C1 complex is unaffected, except for a slight altered mobility due to the mass effect of the serum protein (see negative controls, lane 5 and 7), it is clear that this shift is due to a cellular protein.

Specific Inhibition of DNA-Protein Complex

The sequence specificity of the DNA-protein interaction was determined by the addition of non-radioactive single strand hairpin DNA as a competitor to the DNA binding reaction (Fig. 20). Using a 50 fold excess concentration of DNA containing the BPV left terminus as competitor there is almost no DNA-protein complex formation (Fig. 20, lane 3). A slightly greater concentration completely inhibits any complex formation. In contrast, a 50 fold excess of unlabeled nonspecific competitor DNA such as ds pUC19 DNA or ss M13mp19 DNA does not inhibit the formation of the three complexes (Fig. 20, lanes 9 and 10).

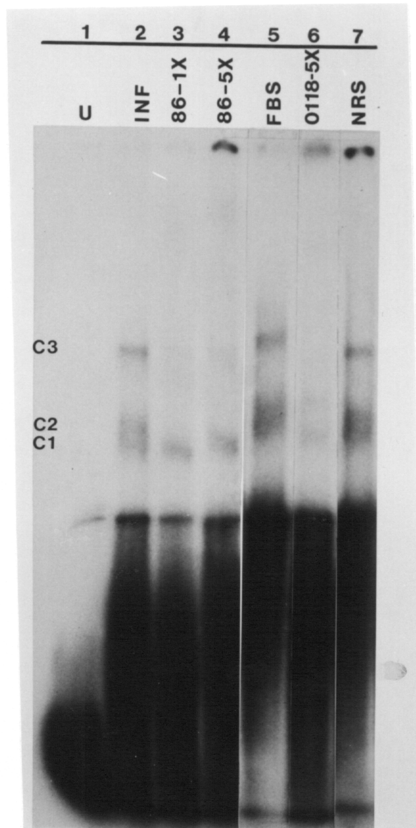


Figure 19. Antiserum inhibition of C2 and C3 complexes: The basic mobility shift reaction containing 7 μg of protein from synchronized infected-cell nuclear extracts which were preincubated with 1 μl of calf 86 convalescent serum (lane 3) containing 100 μg of protein or 5 μl of Calf 86 serum (lane 4). In lane 6, the basic reaction was preincubated with 5 μl of anti-BPV capsid antiserum, 0118. Lanes 1 and 2 contained no extract and no antiserum respectively. Lane 5 contained normal fetal bovine serum and lane 7 contained normal rabbit serum with a final protein concentration of 500 μg in each reaction.

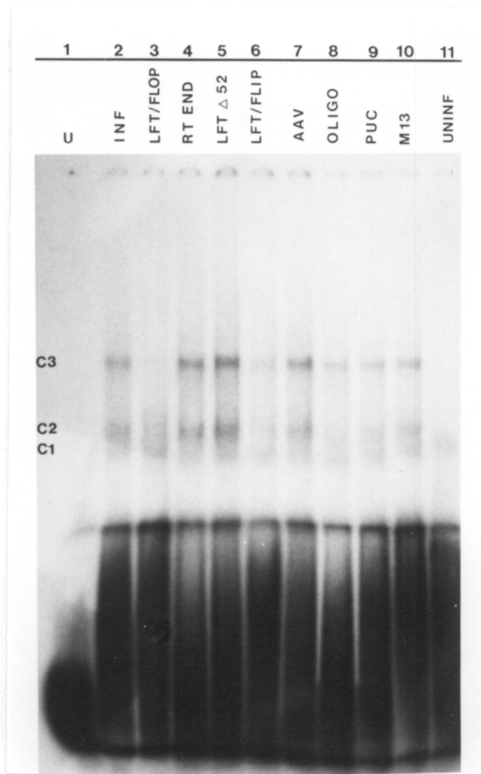


Figure 20. Specific competition using hairpinned termini: The basic mobility shift reaction was performed containing 0.8 ng of labeled left terminus and either no protein (lane u) or 7 μg of protein from nuclear extracts from BPV-infected HU synchronized BFL cells (lanes 2-10) or uninfected contact-inhibited BFL cells (lane 11). The specific competitor DNA was boiled and immediately chilled and included in a 50 fold excess compared to the amount of labeled terminus: Hind III-Cla I fragment of pVTC350 (lane 3), BamHI-Cla I fragment of pVTC350 (lane 4), the Hind III-Pst I fragment of pVT502 Δ52 (lane 5), the HInd III-XbaI fragment of pVT502 (lane 6), and the AAV termini (lane 7). In addition, the following competitor DNAs which were not boiled were included: the oligonucleotide with the sequence of nt 32 - 56 of BPV (lane 8), pUC19 (lane 9), M13mp19 (lane 10).

Sequences Important in DNA-Protein Complex Formation

To define those sequences important in the observed DNA-protein complex formation the mobility shift assay was performed using specific cold competitor DNA (Fig. 20). It is clear when the shifts in lane 2, 3,and 4 are compared, the terminal 171 nt of the BPV right end is an inefficient competitor compared to the left end for the viral protein but an efficient competitor for the cellular proteins. The ss hairpin of the left terminus from pVT502Δ52 (Fig. 20, lane 5) inhibits the DNA-protein complex to the same extent as the right end. This fragment is missing nt 1 through 52 of the BPV left end and is known to be unable to replicate (Shull et al., 1988). The deletion prevents the possibility of stem formation at the left end (Fig. 22). However, the fragment could still form the GC crossarms of the hairpin which may account for the ability of the fragment to inhibit the cellular protein complex. Interestingly, a partially palindromic sequence similar to the CTF/NF-1 binding site
5′ TTGGCT-N_3-AGCCAA 3′ (Jones et al., 1987)) exists between nt 31 and 56 of the BPV left end (Fig. 22). When an oligonucleotide containing this sequence was annealed to its complement and used as a competitor (lane 8), it inhibited the formation of the viral protein complexes, although not to the same degree as the intact left end. However, this may only reflect the efficiency of duplex formation between the oligonucleotides and not the inefficiency of protein binding. The NF-1- like binding site is differentially represented in the flip and flop orientations of the hairpin form of the left terminus due to the imperfect palindromic nature of the BPV terminus. Therefore, a fragment of the left terminus of pVT502 (Shull et al., 1988), which is in the flip orientation, was used as a competitor (lane 6). This fragment competed efficiently for both the viral and cellular proteins which recognize the labeled fragment (compare to lanes 2 and 3), which is in the flop orientation.

As Im and Muzyczka (1989) demonstrated, the AAV terminus and BPV terminus do not compete for the same viral proteins. This is evident from the continued presence of the C2 and C3 complexes when the AAV terminus is used as competitor (Fig. 20 lane 7). However, as with pVT502Δ52 and the BPV right end, the AAV terminus inhibits the formation of the cel-

lular protein complexes. This inhibition suggests that there is some secondary structure or DNA sequence in common between these termini which is recognized by the cellular protein. Lastly, neither the left nor the right end of BPV was able to inhibit any of the DNA-protein complexes in their native double strand form (Fig. 21).

DISCUSSION

Recent studies on BPV (see Chapter I) and AAV (Mclaughlin et al., 1988) have shown that the viral origin of replication (*ori*) lies completely within the terminal palindromes of these viruses. Further, our work with BPV (Shull et al., 1988) and studies with AAV (Samulski et al., 1983; Lefebvre et al., 1984; Hermonant et al., 1984; Bohenzky et al.,1988) have demonstrated that deletions within these palindromes can be lethal to replication. These studies also suggest that both the primary sequence and secondary structure of the terminus define domains within the *ori*. Recent studies with AAV (Im and Muzyczka, 1989; Snyder et al., 1990) provide evidence that the nonstructural proteins of the virus specifically bind to the terminus and likely are involved with replication.

The results presented here provide clear evidence that the BPV left terminus can form the proposed hairpin structure (Chen et al., 1986) *in vitro* (Fig. 15) and this hairpin is recognized by proteins in crude nuclear extracts of BFL cells. More than one protein binds to the terminus. The proteins involved in the C2 and C3 DNA-protein complex formation are virally coded. They are not formed using uninfected cell nuclear extracts and are inhibited by BPV-specific antibodies. The protein involved in C1 protein-DNA complex formation is a cellular protein which is present in uninfected cell nuclear extracts.

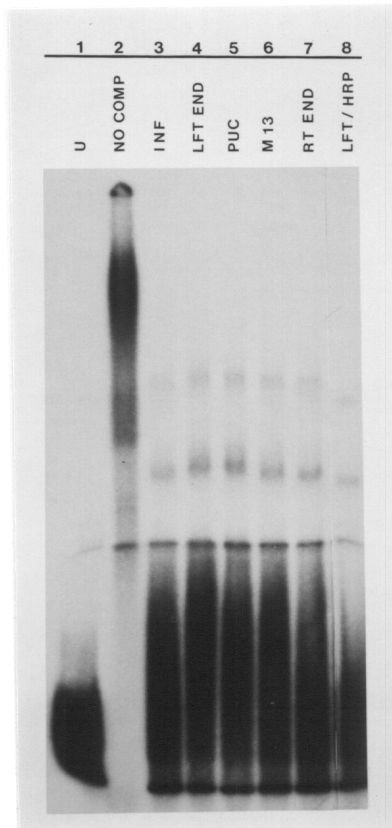


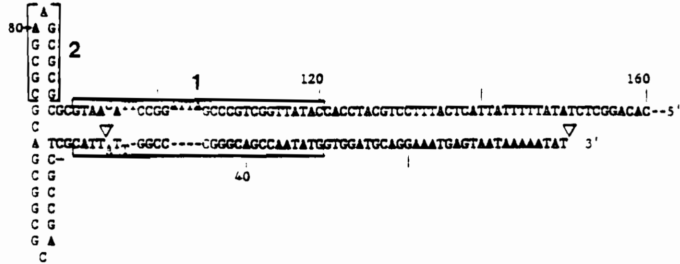
Figure 21. Specific competition using double strand termini: The basic mobility shift reaction was performed using 0.5 ng of labeled left terminus and either no protein (lane U) or 7 μg of protein of nuclear extracts from uninfected BFL cells. Lane 1 contained no salmon sperm DNA. Lane 8 contained 50 fold excess of the single strand hairpin of the Hind III-Cla I fragment of pVTC350. The following competitor DNAs in double strand form were included in a 50 fold excess concentration: the Hind III-Cla I fragment of pVTC350 (lane 4), pUC19 (lane 5), single strand M13mp19 DNA (lane 6), the Bam HI-Cla I fragment of pVTC350 (lane 7).

Viral Protein Recognizes Termini

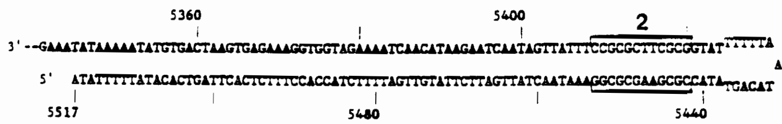
The antibody inhibition of the formation of the C3 complex by the capsid specific antiserum, 0118, suggest that the protein binding to the DNA is one of the BPV capsid proteins. The lack of inhibition of the C2 complex by 0118 and the inhibition of the same complex by calf 86 serum suggests that a BPV NS protein is binding to the left end. Interestingly, the antibody inhibition of the formation of the DNA-protein complex as opposed to the alteration of the shift indicates that the recognized epitope could possibly be contiguous with the DNA binding domain. While the inhibition of the C2 and the C3 complex by anti-BPV serum confirms that both complexes involve viral proteins, it does not answer whether the one or more proteins are involved in each complex. The answer to this question requires further purification of the proteins in the nuclear extract and antibodies against specific BPV coded proteins.

Still, the inhibition of protein binding by some of the non-radioactive competitor DNA fragments suggest two possible BPV proteins that may be involved in the DNA-protein complex formation. First, pVT502 and pVT502Δ52 differ only by the absence of the first 52 nt in pVT502Δ52. Yet, pVT502 inhibits all protein binding to the labeled fragment and pVT502Δ52 only inhibits cellular protein binding. This suggests the viral proteins recognize a sequence within the first 52 nt of the left end. Importantly, the synthetic NF-1 like oligonucleotide (nt 32 - 56 of the BPV left end) also appears to inhibit the binding of the viral proteins. The proteins involved likely recognize the partial palindrome, CATATTGGC-N_7-GCCAATA, present within the oligonucleotide (Fig. 22). Reasonably, the protein may have properties similar to those of the CTF/NF1 protein. The binding domain of this protein (Mermod et al., 1989) consists of a large number of positively charged amino acids (30% of the 131 amino acids in the binding domain are basic). The deduced protein of the BPV mid ORF (Chen et al., 1987), which is likely transcribed and translated based on recent RNA studies (N.Diffoot, unpublished data), also contains predominantly basic amino acids at its amino terminus (33% of the first 131 amino acids). The only other BPV protein containing many basic amino acids is coded for by the BPV right ORF. This protein is VP2 and 25% of its first 130 amino acids are basic. Pre-

(A) BPV minus strand, left (3') terminus, flip conformation



(B) BPV minus strand, right (5') terminus, flip conformation



(C) AAV minus strand, left (3') or right (5') terminus, flip conformation

Figure 22. Sequence of the left termini of BPV and AAV and the right termini of BPV: The sequences are that of the minus strand of each virus. The boxed regions are those important in DNA-protein binding. Box 1 is the proposed viral protein binding region. Box 2 is the proposed cellular binding region. The Δ indicates the deletion present in pVT502Δ52.

vious studies by Lederman et al. (1987) have demonstrated that VP2 does bind to the left 290 nt of BPV. If both of these proteins are binding to the left end, the left end may play a role in both packaging and replication.

The indication that the proteins which recognize the stem of the left hairpin are important in replication is suggested by the lack of inhibition of viral protein binding by the replication-deficient left terminus of pVT502Δ52. Further, the nonidentical ends of BPV appear to inhibit protein binding differently. Both the left and right ends inhibit the cellular proteins from binding the labeled fragment but only the left end inhibits the viral proteins from binding. This suggests that perhaps different viral proteins are required for replication from either end or are different for the initiation of viral replication versus RF amplification. While this was not necessarily predicted in the rolling hairpin model of parvovirus replication (Astell et al., 1985; Cotmore and Tattersall, 1987), it does lend support to the need for differential replication kinetics from the different ends to account for the asymmetric virion DNA strand production of parvoviruses as suggested by Chen (1989).

Whether the viral protein identified in this study behaves as a site specific "nickase" and helicase as does rep 68 for AAV (Snyder et al., 1990) remains to be determined. A BPV NS protein is likely binding to the terminus as demonstrated by the specific antibody competition (Fig 8). By comparison of proposed amino acid homology (Chen et al., 1987) and immunological similarities (Lederman et al., 1988) between the NS proteins of AAV, MVM, and BPV, the large NS proteins of BPV (73kDa, 83kDa) may have a function similar to that of rep 68. Alternatively, NP-1 may be functioning as terminal recognition protein.

Cellular Protein Recognizes Termini

A protein present in nuclear extracts from uninfected BFL cells binds to the left terminus of BPV. Interestingly, in the specific competition assays, it appears that the cellular protein-DNA complex can be inhibited without the inhibition of the viral protein-DNA com-

plexes. Similarly, the results of the anti-BPV serum inhibition show that the viral protein-DNA complexes can be inhibited without affecting the cellular protein complex. These results suggest that binding by either a viral or cellular protein can occur independently. However, these same results do not rule out the possibility that the C2 or C3 complex consists of both viral and cellular proteins.

The binding of cellular proteins is inhibited by using the AAV termini, pVT502Δ52, or the right end of BPV as non-radioactive competitor. There is little overall sequence homology between these termini. A computer search for small regions of homology showed that the only region of significant homology between pVT502Δ52 and the right end of BPV lies between bases 5410 - 5420 of the right end and bases 75 - 85 of the left end. All of the other regions of homology identified would be expected to be ss DNA when pVT502Δ52 is in a hairpin conformation, and therefore, they would not affect the DNA-protein interaction in this assay. The region of greatest homology between the AAV termini and pVT502Δ52 encompasses this same region. There is 81% homology between nt 72 - 94 of BPV and nt 16 - 37 of AAV with only 2 nucleotide differences between 75 - 86 of BPV and 16 - 29 of AAV. The only significant homology between the AAV termini and the right end of BPV, although minimal at 50%, is between nt 5402 - 5423 of BPV and nt 16 - 37 of AAV. These significant regions of homology between the competitors suggest that the cellular protein is likely recognizing the partial palindrome 5′ GCGCGAAGCGC 3′ in the short arm of the T-shaped hairpin of BPV (Fig. 22). This region is also the region of greatest homology between the left terminus of BPV and MVM (nt 43 - 63). In looking for homology in the DNA sequences of the rodent-like parvoviruses, Bodnar (1989) has identified this region as a potential Z DNA element. While DNase I footprinting is needed to identify the binding region specifically, protein binding to the T-shaped hairpin appears reasonable because the left terminus as native ds DNA did not inhibit the protein binding reaction. Also the results of assays on protein binding to the AAV terminus (Im and Muzyczka 1989, Ashktorab and Srivastava, 1989) demonstrate the importance of the secondary DNA structure for protein binding.

The requirements of actively dividing cells for autonomous parvovirus replication has been recognized for at least 20 years (Tennant et al., 1969). Studies indicate that this actively dividing cell requirement is a factor which occurs in the S-phase of the cell cycle allowing conversion of the ss DNA genome to a ds DNA RF(Wolter et al., 1980). Recent work demonstrating the replication of AAV without adenovirus coinfection in actively dividing, hydroxyurea synchronized cells (Yakobson et al., 1987) establishes the requirement as a basic property for all parvoviruses. While the nature of this requirement remains unclear, the cellular protein identified in this study is a good candidate for the S-phase factor.

It will be of interest to determine if uninfected cell lines such as HeLa or NBE cells contain a cellular protein which binds to the termini of the parvoviruses for which they are permissive, or perhaps even to BPV. The studies of Im and Muzyczka (1989) and Ashktorab and Srivastava (1989) did not demonstrate proteins from uninfected cells which bind to the AAV terminus. Therefore, synchronized cell lines might more appropriately be used to identify a common terminal binding S-phase factor.

Role of DNA Binding by Viral and Cellular Protein in Replication

Two likely functions of the viral proteins binding to the left terminus can be imagined. The essential first step in replication of parvoviruses is conversion of virion ss DNA to ds DNA and its conversion to an extended open end RF DNA. The left terminus must be able to initiate these processes, because in parvoviruses, 50% - 90% of the infecting virion DNA genome is of the minus polarity. Viral proteins will likely direct the reactions necessary for this conversion. Evidence from studies with AAV (Snyder et al., 1990) and MVM (Cotmore and Tattersall 1988) suggest viral NS proteins bind to the terminus and resolve the hairpin into an extended ds DNA form. As mentioned, the NS protein of BPV probably has a similar function. In addition viral proteins could be interacting with cellular proteins to initiate DNA replication from the hairpin.

Assessing the function of the cellular binding protein is more speculative because there are only two other reports of cellular proteins interacting with a parvovirus terminus. One study (Avalosse et al., 1989) demonstrated that a 102 kDa protein from transformed human cells bound to the MVM left hairpin. If recognition of the BPV terminus is specific to BFL cells and other BPV permissive cells, then the protein may be important in the host cell restriction of BPV. In this case, the protein may function in manner similar to that suggested by Labow and Berns(1988) for the NS proteins of AAV. When the cell protein is present in sufficient concentration it allows the NS proteins of BPV (*rep* proteins) to activate replication. Further, the binding of the cellular protein to the terminus, thereby sequestering the protein, may serve as the basis of the well documented onco-suppressive function of parvoviruses in some cells (Winocour et al., 1988). An alternative possibility is that the identified protein is the required S-phase factor for replication. If so, it could be functioning in the conversion of ss DNA to ds DNA. Here it could have two roles, either in resolution of the terminal hairpin or elongation of the nascent DNA strand with the 3'OH of the hairpin serving as a primer for a DNA polymerase (Bates et al., 1980; Prithchard et al., 1981). An intriguing possibility is the S-phase factor could be PCNA, an auxillary protien for DNA polymerase δ (Bravo et al., 1985). Polymerase δ could be the enzyme that replicates parvoviruses. Studies showing polymerase α as the replicating enzyme for BPV (Pritchard et al., 1981; Robertson et al., 1984) and for RV (Gunther et al., 1984) and H-1 (Kollek et al., 1982) were based on the replication of the viruses being sensitive to aphidicolin and resistant to the effects of ddTTP. Both of these properties are the same for polymerase α and δ. Further, the absence of Okazaki fragments in H-1 replication (Tseng et al., 1979) and the ability of the hairpins to serve as primers obviates the need for a DNA primase which is associated with polymerase α and not δ. Thus, parvovirus replication appears more similar to leading strand DNA synthesis which appears to be carried out by polymerase δ (Perlich et al., 1988, Stillman, 1989). However, since the concentration of the S-phase factor is increased in actively dividing cells, it is probably a positive regulator of replication. Except for the origin unwinding protein (Roberts and D'Urso, 1988) and PCNA, few

positive regulators of eukaryotic DNA replication have been identified: therefore, the cellular binding protein may be a unique regulator.

Chapter IV

GENOME HOMOLOGIES BETWEEN BPV AND LPV

Introduction

The family of small single strand (ss) DNA viruses known as Parvorididae has two genera which infect vertebrates; Parvovirus and Dependovirus (Siegl et al., 1985). The Dependoviruses, various adeno-associated viruses (AAV), require a helper virus (Siegl et al., 1985) for a productive infection. The members of the genus Parvovirus replicate autonomously to generate infectious virus. Lapine parvovirus (LPV) is an autonomously replicating parvovirus (Matsunaga et al., 1977; Seigl et al., 1985). Beyond its basic description as a parvovirus and the identification of its structural and some of its nonstructural proteins (Matsunaga and Matsuno, 1983) LPV is not well characterized, especially with respect to the characteristics of its genome.

As a parvovirus infecting a rodent, LPV is unique because its icosahedral capsid is made up of three proteins each individually coded for by the viral genome (Matsunaga and Matsuno, 1983). The other parvoviruses which infect rodents have only two such capsid proteins with the

third being a proteolytic product of a larger capsid protein (Cotmore and Tattersall, 1987). The only other autonomous parvovirus with three capsid proteins is bovine parvovirus (BPV) (Lederman et al., 1983). BPV and LPV also code for small nonstructural (NS) proteins with molecular weights of 20,000-30,000 daltons (Matsunaga and Matsuno, 1983; Lederman et al., 1984). This gross similarity between BPV and LPV proteins is striking, particularly since BPV seems to represent a distinct subgroup of parvoviruses (Chen et al., 1986; Cotmore and Tattersall, 1987).

In the present study, a direct comparison was made between the proteins and DNA genomes of BPV and LPV. The protein relationship both in terms of size and number was confirmed and further investigated by an immunological assay. The relationship between the genomes was compared by direct comparison of genome size, DNA/DNA hybridization, and sequencing of cloned LPV DNA fragments. LPV was not found to be as closely related to BPV as their similar capsid structure suggested. It appears that LPV may have an intermediate evolutionary relationship to BPV and other rodent parvoviruses.

Methods

Cell Culture and Virus Infection

Bovine fetal lung cell (BFL) cultures were grown and infected with plaque-purified BPV as previously described (Parris and Bates, 1976). Low passage primary rabbit kidney (RK) cells or a RK cell line (a gift from G. Siegl) were grown in monolayer cultures in Eagles Minimal Essential Medium supplemented with 10% fetal bovine serum and infected with a cell culture adapted LPV isolate, LPV-F79, obtained from Y. Matsunaga (Matsunaga et al., 1977).

Polyacrylamide Gel Electrophosesis and Immunoblotting

Proteins in cell lysates of mock-infected or virus-infected BFL or RK cells were electrophoretically separated in 10% SDS-polyacrylamide gels. Cell lysates were prepared one day post infection as previously described (Lederman et al., 1987). The gels were stained with 0.2% Coomassie Brilliant blue R (Sigma) in 50% methanol - 10% acetic acid and de-stained in 30% methanol - 10% acetic acid. Immunoblotting of LPV or BPV specific proteins was also carried out according to the procedure described by Lederman et al., (1987). Proteins from cell lysates were separated on SDS-polyacrylamide gels and electrophoretically transfered to nitrocellulose membranes. The proteins were detected using a 1:100 dilution of a convalescent calf serum (Calf 86) from a BPV-infected calf containing antibody specific for BPV capsids and NS proteins or a convalescent serum from a rabbit naturally infected with LPV as the first antibody. The second antibody was a 1:500 dilution of protein A coupled to horseradish peroxidase (Bio-Rad).

Isolation and Cloning of LPV

Both ss viral DNA and reannealed plus and minus strand DNA were isolated from CsCl-purified LPV virions as previously described for BPV DNA by Chen et al. (1986). To obtain double-stranded (ds) LPV DNA for cloning, a pool of both ss DNA and ds DNA was treated with *E. coli* DNA polymerase I (Klenow fragment), as previously described (Burd et al., 1983). The 3′ palindromic end of LPV was efficiently used as a self-primer, and all of the ss DNA replicated to ds DNA. This DNA was digested with either EcoRI or PstI and cloned into pUC8 digested with EcoRI or PstI and SmaI. The largest LPV-containing recombinants resulted from the cloning of EcoR1 fragments. Two of these clones, LPV 3 and LPV 23, were used for analyzing the LPV genome. Transformation of *E. coli* JM107 with the recombinant plasmids was performed as described by Hanahan (1983).

DNA/DNA Hybridization

The EcoR1 fragment of LPV3 was hybridized with genomic length fragments from two parvovirus genomic clones. The BPV fragment (mu 0 - 100) was excised by Sal I digestion from pVT501 (Shull et al., 1988). The LuIII fragment (mu 0 - 100) was excised by digestion with Bam H1 from a genomic clone of the virus (Diffoot et al., 1989). All fragments were separated by electrophoresis in 1% agarose and phenol extracted from excised gel chunks (Maniatis et al., 1982). DNA in TE (10mM Tris, pH 8.0 and 1mM EDTA) was spotted onto a Zeta Probe (BioRad) membrane using a dot blot apparatus and washed three times with 75 ul of 0.4 M NaOH. Hybridization of the DNA was performed according to instructions for the use of Zeta Probe in 1.5 x SSPE ($[Na+]=0.27$ M). Detection of varying degrees of homology between the parvoviral DNAs was performed by hybridizing the DNA at different temperatures. To detect 100% homology, DNA was hybridized at 68°C. For detection of 75% and 50% homology, DNA hybridization was performed at 32°C and 22°C, respectively. Typically, DNA fragments were radiolabeled using ^{35}S-dCTP (1000 ci/mmol) or ^{32}P-dCTP (3000 ci/mmol, New England Nuclear) by the random priming method of Feinberg and Vogelstein (1983).

PCR Amplification of LPV Termini

Clones containing regions of the left terminus of LPV were obtained using the polymerase chain reaction (PCR) (Saiki et al., 1988). The template DNA was obtained by extracting LPV-infected cells by a modified Hirt procedure (Robertson et al., 1984). Approximately 1 x 10^7 RK cells were infected with LPV one day post-seeding. Three days post-infection, with CPE readily apparent and 50% of the cells rounding, the cells were collected and treated with proteinase K, SDS, and NaCl as described in Chapter 1. The precipitated DNA was centrifuged through a 4 ml neutral high salt sucrose gradient (Chen et al., 1986) to separate the LPV DNA fractions from higher and lower molecular weight DNA.

To amplify the left terminus, LPV DNA from the sucrose gradient was treated with terminal transferase and dATP. The DNA was then asymmetrically amplified using a single primer and the basic reaction conditions prescribed by Perkin-Elmer Cetus. After dissassociating the DNA at 94°C for 3 min, the parameters for amplification were 40 cycles of 94°C for 50 sec, 50°C for 1 min, and 72°C for 3 min. A final extension period of 7 min at 72°C was carried out after the last amplification cycle. In the first asymmetric amplification, a primer, 5′ TTCCCGTCCGTCGAACCTCCCGCAAGA 3′ (LPVLFT), specific for LPV sequence in LPV 23 was used to synthesize ss DNA from the end of the cloned region toward the terminus. In a second amplification, a heterogeneous oligo dT primer 12 to 18 nucleotides in length was used to synthesize ss DNA from any terminus that a had acquired a polyadenylate tail. The discrete reaction products from each of these reactions were isolated from agarose gels and heated to 94°C for 5 min and slowly allowed to cool. This reannealed DNA was amplified in a basic PCR reaction for 55 cycles using the LPVLFT primer and a poly-T adapter primer (5′ ATCTAGTCGACATCGATATC-T_{17}-3′) containing a Sal I restriction site. The discrete reaction products of this second amplification were also isolated from an agarose gel and reamplified. These products were digested with Sal I and Eco RI (an Eco R1 site is present 180 nucleotides toward the left terminus from the LPVLFT primer). Several Sal I - Eco RI clones were recovered in pUC8 vectors. One clone, LPV 62-14, with a 700 bp insert, was used to identify terminal fragments of the LPV genome.

Results

Comparison of Proteins by PAGE and Immunoblotting

When cell lysates of LPV and BPV infected cells were compared in Coomassie-stained SDS - polyacrylamide gels (Fig. 23), the three capsid proteins of each virus were readily apparent. As expected, the capsid proteins of LPV, VP1 (96kDa), VP2 (85kDa), VP3 (75kDa) (Matsunaga and Matsuno, 1983) are each larger than the corresponding capsid proteins of BPV of 80kDa, 72kDa, and 62kDa (Lederman et al., 1983). The Coomassie stained gel demonstrates the presence of NP-1 (Lederman et al., 1984), a small NS protein, in BPV infected cells and a 25 kDa nonstructural protein present in LPV infected cells.

In the immunoblots, the difference in the size of the capsid proteins is again apparent. The anti-LPV convalescent rabbit serum clearly detected a small LPV NS protein of approximately 33,000 daltons (Fig. 24, panel A, lane 3) while the calf 86 serum detected the small (28 kDa) NS protein, NP-1, of BPV (Fig. 24, panel B, lane 1). Interestingly, neither the anti-LPV or anti-BPV serum show immunological cross reactivity with the heterologus virus (Fig. 24, compare panel A and B).

Comparison of Virion DNA

While it may be possible to subgroup parvoviruses according to their capsid subunits, it has been also suggested that they could be grouped according to the proportion of plus and minus strand DNA packaged in the virion (Bachmann et al., 1979). In this classification, AAV, the human parvovirus B-19, and the rodent virus LuIII would form a group which packages equal amounts of plus and minus strand DNA. The parvoviruses such as minute virus of mice

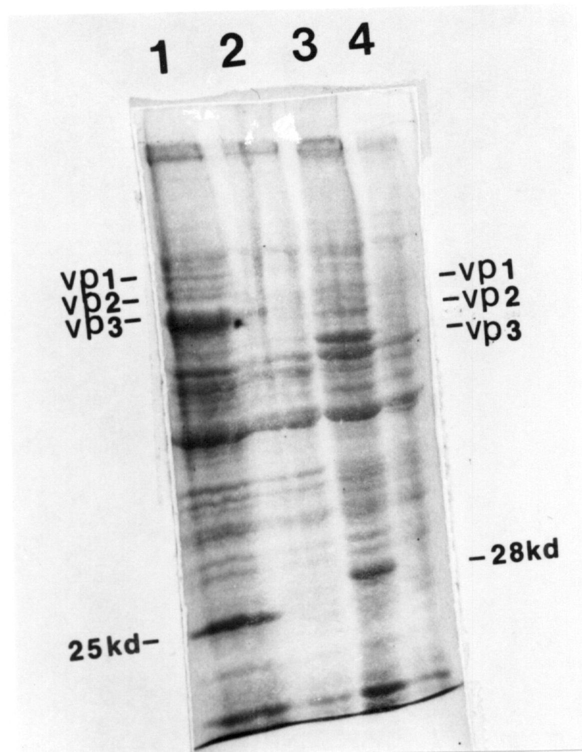


Figure 23. Comparison of proteins from mock and infected cell lysates: Cell lysates from LPV-F79-infected (lane 1) and uninfected (lane 2) RK cells and BPV-infected (lane 3) and uninfected (4) BFL cells were electrophoresed on a 10% SDS-polacrylamide gel and stained with Coomassie brilliant blue.

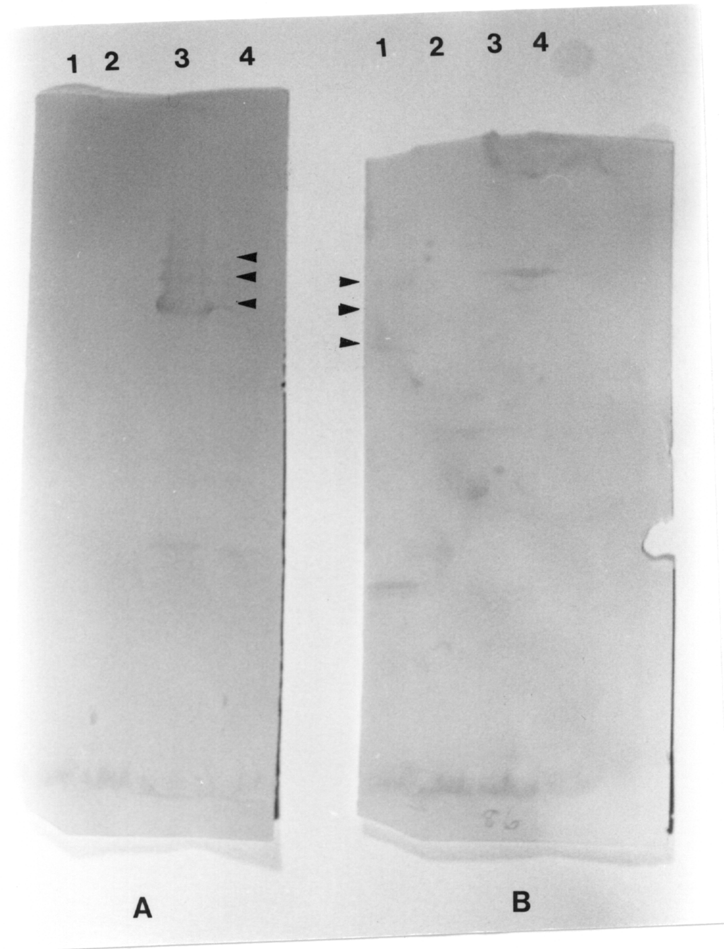


Figure 24. Detection of protein homology by immunoblotting: Proteins from cell lysates were separated as in Figure 1: lane 1, BPV-infected BFL cells; lane 2, uninfected BFL cells; lane 3, LPV-F79-infected RK cells; lane 4, uninfected RK cells. Proteins were reacted with anti-LPV 225 serum (panel A) or anti-BPV calf 86 serum (panel B).

(MVM), which package >95% minus strand DNA, would form another group. In this classification, BPV represents another distinct group, as it packages 10% plus strand and 90% minus strand DNA (Siegl et al., 1985).

It was of interest to examine how LPV was related to BPV in terms of this classification. Since BPV encapsidates mostly ss DNA of the minus polarity, the amount of plus strand encapsidated is reflected by the quantity of monomer ds DNA (reannealed plus and minus strands) obtained from isolated virions. When LPV virion DNA was compared to that of BPV (Fig. 25), it was clear that the proportion of plus to minus strands encapsidated by LPV was similar to that encapsidated by BPV. The intensity of the ethidium bromide stain of monomer ds DNA is similar for both viruses (Fig. 25, compare lanes 2 and 3). The slightly different migration distance for both the ds and ss DNA of LPV from that of BPV suggested also that the LPV genome is larger than the BPV genome. A more specific comparison of the genome size is presented in Figure 26. The migration distances of LPV RF DNA (lanes 2 and 3) and BPV RF DNA (lane 5) were compared to a BPV ds and ss DNA marker (lane 4) as well as to a 1 kilobase (Kb) DNA ladder (lane 1). While the mobility of both RF DNAs is slightly retarded due to the presence of the high salt used in the Hirt isolation procedure, the LPV genome appears to be approximately 5,700 bases in length compared to the 5,517 base genome of BPV (Chen et al., 1988).

DNA/DNA Hybridization

To determine if any similarity exists between the DNA genomes of LPV and other parvoviruses, DNA/DNA hybridization experiments were performed using cloned DNA from LPV, BPV and LuIII. The results of the hybridization (Fig. 27) demonstrated that under the most stringent conditions, LPV does not share a high degree of similarity with LuIII or BPV. However, at a stringency (32°C) where genomes sharing 75% homology should hybridize, LPV shows some similarity to BPV. Under the least stringent conditions, LPV again shows some

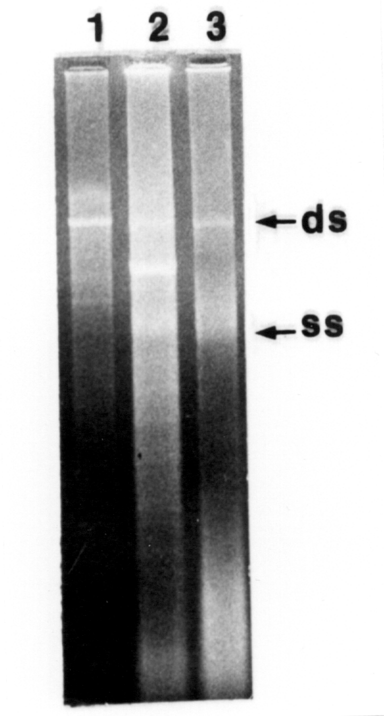


Figure 25. Comparison of virion DNA by agarose gel electrophoresis: DNA isolated from virions purified by CsCl gradient centirfugation was separated on a 1% agarose gel and stained with ethidium bromide. The DNA was loaded on the gel as follows: lane 1, ds BPV DNA marker; lane 2, LPV virion DNA; lane 3, BPV virion DNA.

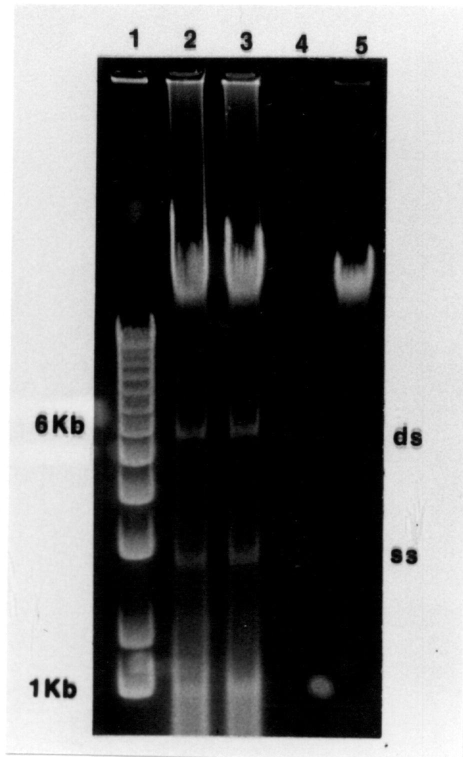


Figure 26. Size comparison of LPV and BPV viral DNA: DNA isolated from virus-infected cells by a modified Hirt procedure was electrophoresed on a 1% agarose gel and stained with ethidium bromide. The markers in lane 1 and 4 are a 1 kb ds DNA ladder and ds plus ss BPV DNA respectively. Lanes 2 and 3 contain DNA from LPV infected RK cells. Lane 5 contains DNA from BPV infected BFL cells.

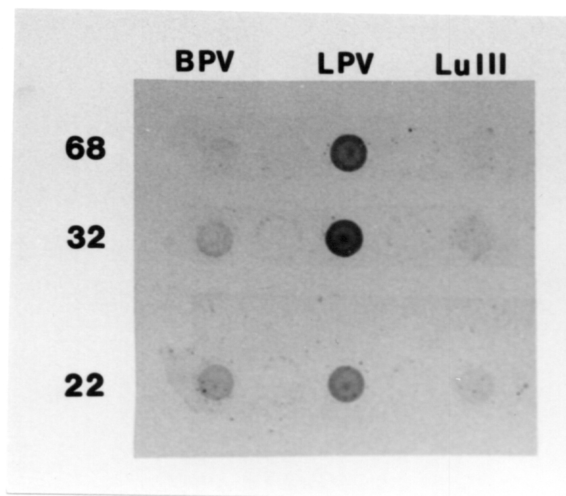


Figure 27. **DNA/DNA cross hybridization:** Full length genomic DNA of BPV and LuIII and the large LPV 3 clone fragment were applied in a concentration of 30 ng to each well of a dot blot apparatus. Membrane strips of each DNA were hybridized with the ^{35}S-labeled large viral DNA fragment of the LPV 3 clone at the temperatures indicated.

similarity to BPV as well as LuIII, although the hybridization signal between BPV and LPV is more intense than that between LPV and LuIII. Comparable results were also observed when radiolabeled genomic length BPV was hybridized with the Eco R1 fragment of LPV3 (data not shown).

Amino Acid Sequence Similarities

To be able to sequence the LPV genome and further compare it to the BPV genome, several strategies were undertaken to clone a significant proportion of the LPV genome. Two large Eco R1 clones, LPV 3 and LPV 23, containing LPV DNA inserts of 4,500 bp and 800 bp respectively were initially recovered. The sequence data on these two clones was used to identify restriction sites in LPV which were used to isolate several smaller clones. Assuming a genome size of 5,700 bp, approximately half of the genome has been sequenced. However, there are numerous gaps between the sequenced regions. The majority of gaps are located in the left half of the genome. Therefore it was not reasonable to make comparisons between the left half of the BPV and LPV genomes.

As suggested above, there is little identity between the known LPV sequence and BPV or LuIII. However, when some of the LPV sequence is translated by computer, the amino acid sequence of one of the possible reading frames is similar (~40%) to the deduced amino acid sequence of the BPV right open reading frame (ORF) (Fig. 28). A similar comparison of the possible LPV right ORF amino acid sequence to that of LuIII and MVM showed only ~28% similarity. It is important to note that in this comparison conservative amino acid changes such as arginine to lysine and alanine to valine or leucine to isoleucine were scored as matches. Also some of the regions of amino acid homology between LPV and BPV include amino acid sequences that are present in most parvoviruses: the glycine rich region in sequence A; the TPW, DWQ, and YNNDLTA sequences in sequence B; the PIW sequence in se-

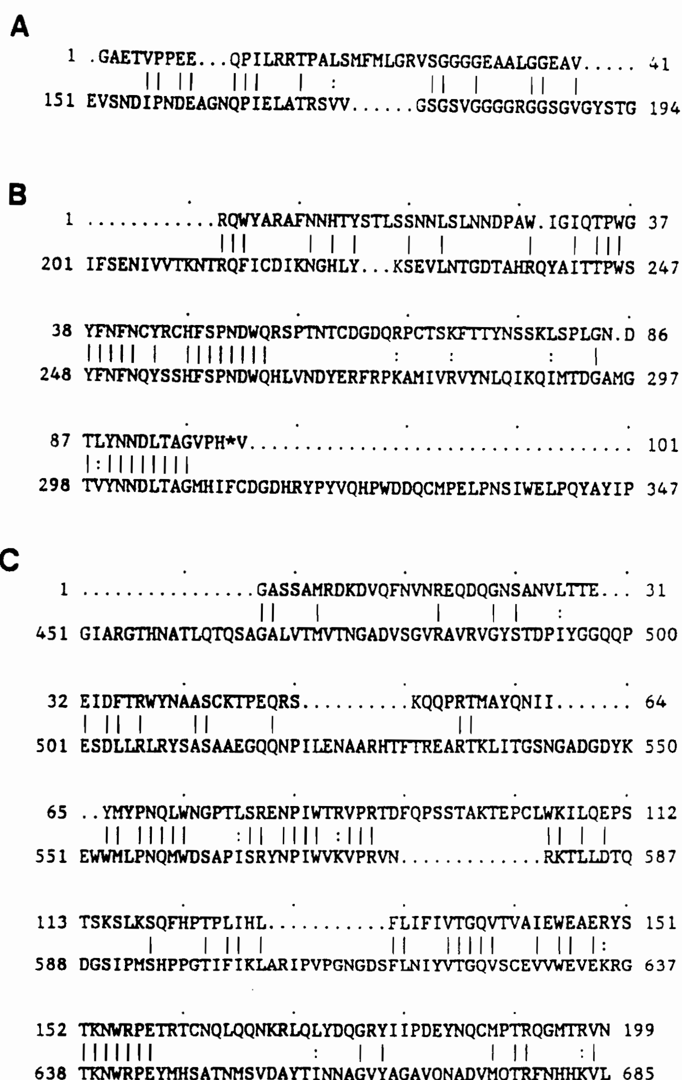


Figure 28. Amino acid sequence similarities between LPV and BPV: Three discontinuous amino acid sequences (A, B, C) of the probable right ORF of LPV as deduced from the DNA sequence are displayed on the bottom line. They are aligned with the deduced amino acid sequence of the BPV right ORF. The amino acids are numbered from the amino to the carboxyl end of the BPV protein and from the 5′ to 3′ end of the known LPV DNA sequence. The sequences were aligned using the GAP file from the UWGCG DNA sequence program.

quence C. However, the other regions of significant homology (Fig. 28, see brackets) are only similar between LPV and BPV and not between LPV and either LuIII or MVM.

Restriction Enzyme Analysis

Several attempts to clone the left terminus of LPV by conventional methods were tried and proved unsuccessful. Instead, left end clones were generated using PCR. These clones contained fragments ranging in size from 200 bp to 700 bp but did not contain the palindromic left terminus sequence. The largest of these clones, LPV 62-14, has sequence with limited homology (46%) to the region just internal to the BPV hairpin (nucleotide (nt) 190 through 421). The greatest similarities are centered around the promoter sequence, TATATAAA, at nt 249 of BPV. The LPV 62-14 clone was used as a probe of Hirt DNA from LPV-F79-infected RK cells (Fig. 29, panel B). It is clear from this Southern blot that this clone actually contains an LPV insert that hybridizes only to LPV DNA sequences. Similarly, when full length ds LPV DNA is used as a probe (Panel A), it hybridized to LPV 62-14. The clone LPV 62-1 (lane 5), which is also a PCR product clone, and LPV 3 (lane 6) contain LPV DNA inserts. There are also several significant bands which are present in lanes 1 and 2 of panel B that are absent in panel A. This is most likely because the fragments identified in panel B predominantly contain the left terminus specific sequences represented in LPV 62-14. As expected, neither the clone or the LPV viral DNA hybridized to a left terminus clone of BPV (data not shown).

Based on the different Pst I and Eco RI fragments of LPV which hybridized with LPV 62-14 (Panel B, lanes 2 and 3) and the full length LPV probe (Panel A, lanes 2 and 3), the left terminus is probably represented within the smallest (780 bp) Pst I fragment of the genome. Since the LPV 62-14 probe also hybridizes to the Pst A fragment and not to the Pst B fragment, the Pst I fragments of LPV can be arranged in their linear order from the left to the right end of the genome as follows: C (780 bp), A (3,800 bp), B (1,500). The sizes of each fragment were determined based on a comparison of the migration distance of a 1 Kb ladder that does not

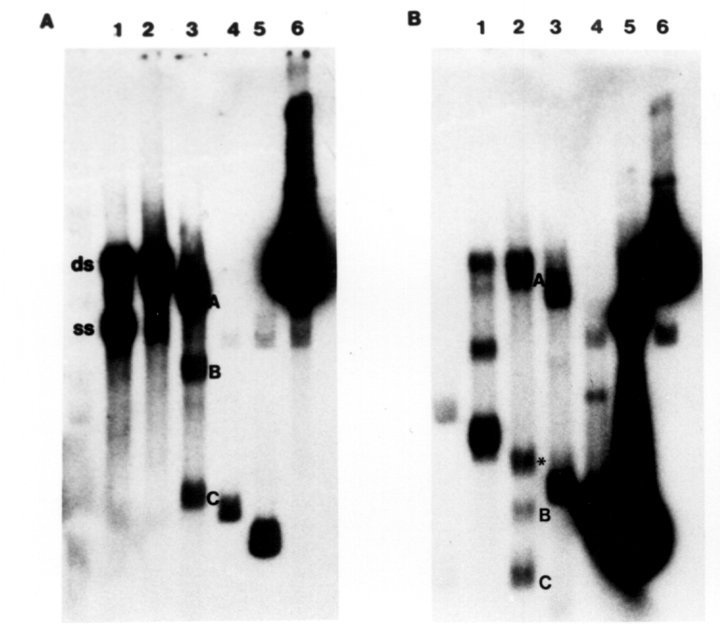


Figure 29. Southern blot of LPV DNA and PCR product clones: The Zeta Probe membrane in panel A was hybridized to gel purified, full length ds LPV DNA labeled with ^{32}P-dCTP. The same membrane was stripped of all significant radioactivity and re-hybridized with a ^{32}P-labeled gel purified insert from LPV 62-14 (panel B). The DNA was separated on a 1% agarose gel loaded as follows: lane 1, Hirt isolated sucrose gradient purified DNA from LPV-F79-infected RK cells; lanes 2 and 3, Eco RI and Pst I digests of the DNA in lane 1; lane 4 and 5, Sal I digested LPV clones 62-14 and 62-1 respectively; lane 6, Eco RI digested LPV 3 clone.

appear in the Southern blot to any significant extent. The sizes are likely larger than the actual size because together they would constitute a genome of ~ 6,000 bp. As mentioned though, estimates of the ds LPV DNA genome size are ~5,700 bp. There are only two Eco RI sites. The largest fragment (A) is approximately 4,500 bp. Fragments B and C are 700 bp and 450 bp each. The fragment marked with an asterisk is approximately 1,100 bp and likely is comprised of both the B and C fragments. The two smaller fragments also comprise the left terminus of the genome based on their intense hybridization with the LPV 62-14 clone.

Discussion

The comparisons made in this study confirm that there are similarities between LPV and BPV. The lack of immunogenic cross-reactivity between LPV and BPV suggests that the two viruses share few conserved regions in these proteins. This finding is in agreement with previous studies showing no immunological cross-reactivity between BPV and other autonomous parvoviruses (Siegl, 1984). This study does not address whether there is immunological cross-activity between LPV and other rodent parvoviruses. It seems unlikely that any cross-reactivity exists though, because a previous study (Matsunaga et al., 1977) showed that anti-LPV serum did not inhibit hemagglutination by rat virus. Still, the deduced amino acid sequence of the putative right ORF of LPV does show several regions similar to that of BPV.

Conserved antigenic regions in the small nonstructural proteins, common to LPV and BPV, probably exist. The nonstructural proteins of MVM and AAV have been shown to be necessary for viral replication (Hermonant et al., 1984; Tratschin etal., 1984). Immunological cross-reactivity between BPV, B-19, and MVM to a conserved region of the NS-1 protein was recently described (Lederman, 1987) and it is likely that there are functional similarities between these proteins. There are also conserved antigenic regions among the small nonstruc-

tural proteins of MVM, LuII, H-1, and BPV (Cotmore and Tattersall, 1986). The finding that LPV and BPV encapsidate similar porportions of plus and minus strand DNA suggests that the LPV mechanism of replication is similar to that of BPV. It seems reasonable then that the function of those nonstructural proteins which are involved in the replication of LPV would be similar to those of BPV. These proteins will likely share some conserved antigenic regions.

The DNA hybridization assay demonstrated only limited DNA identity (50% homology) between the LPV and BPV genomes and less similarity (40% homology) with LuIII. Although LuIII was isolated from a human cell line (Siegl, 1984), it has a high degree of homology with rodent parvoviruses. According to previous heteroduplex mapping by electron microscopy (Banerjee et al., 1983) and studies by N. Diffoot (manuscript in preparation), LuIII shares 80% homology with MVM, H-1, and rat virus. Therefore, based on DNA hybridization LPV appears to be more related to BPV than to the other rodent parvoviruses. Further, the sequence data of LPV has shown that there is limited homology between LPV and either LuIII or MVM or BPV. The amino acid sequence similarities between these parvoviruses suggest that LPV is more similar to BPV than the other rodent parvoviruses. Significantly, besides containing several regions of amino acids that are present in most parvoviruses, LPV shares regions of amino acid homology only with BPV.

In conclusion, LPV appears not to be highly related to BPV. According to two proposed subgroupings of parvoviruses (Cotmore and Tattersall, 1987; Bachmann et al., 1979), it appears that such a relationship should exist. LPV probably occupies an evolutionarily intermediate position between the rodent parvoviruses and BPV. The DNA sequence is similar to both BPV and rodent parvoviruses, but the functional organization of the sequence more closely resembles BPV. A more definitive picture will be known with the determination of the LPV DNA sequence.

Chapter V

SUMMARY

The research for this dissertation has clearly defined two basic domains within the BPV origin of replication of the left terminus of the genome. The demonstration of the interaction of specific viral proteins with a viral terminus was also achieved. Most interestingly, a novel cellular protein that specifically binds to the BPV *ori* was demonstrated. Further, some of the basic ground work was set for ultimately investigating similar phenomenon with LPV.

The use of deleted recombinant mini-genomes in a transient replication assay was a novel approach for identifying the *cis* sequences important in BPV replication. It was a method that defined the minimum size of the termini sufficient for replication. The replication of the duplicate end chimeras showed that either terminus can function as an *ori* and allowed the identification of domain 1 (nt 115 - 148 of the left end and nt 5341 - 5381 of the right end) and domain 2 (nt 73 - 98 of the left end and nt 5402 - 5437 of the right end) as important in replication.

The limitation of these experiments was that the quantification of replication was limited by the small number of initially transfected cells. Also, the BPV *rep* proteins were provided in *trans* by a separate genome and the absolute efficiency of replication of the mini-genomes could not be determined. Both of these limitations can be addressed by con-

structing full length genomes with chimeric termini. With these genomes, the transfection efficiency should be greater than with the mini-genomes. They should also form infectious virions so that transfection procedures could be avoided entirely. The fortuitous 65 bp internal palindrome deletion in pVT504, which could not have been easily constructed, will be useful in further assays on BPV replication. Since the mini-genomes also do not replicate when transfected alone, they might serve as useful vectors in an assay for the integration of BPV termini.

My research resulted in the adaptation of the gel retardation assay for use in identifying proteins that bind to the BPV termini. This assay demonstrated that the two nonidentical BPV termini bind proteins differently. It also demonstrated that a BPV NS protein and a capsid protein interact the left termini and likely recognize the sequence 5′ CATATTGGC-N_7-GCCAATA 3′. As well, a host cell S-phase protein was identified with a putative recognition sequence of 5′ GCGCGAAGCGC 3′. These two different recognition sequences again define two different domains for the BPV *ori*. The domains correspond to those identified by the replication of the mini-genomes. These observations suggest that the cellular protein binds to the GC rich region of domain 2 to increase the efficiency of DNA synthesis; and viral proteins bind to domain 1 to initiate DNA synthesis.

These findings invite many more questions. First, DNase I protection experiments are required to confirm the protein binding sites. These experiments could use the deleted termini from clones, pVT502Δ52 and pVT504, which have known replication phenotypes, as DNase I substrates to determine exactly how the deletions within the terminus affect protein binding. Competition experiments, similar to those described in chapter 2, using synthetic oligonucleotides could be performed to assess the affinity of the protein for different binding sites. Second, similar experiments to those described in chapter 2 could be conducted using the right terminus. All of the protein binding studies could be refined by generating site specific mutations within the terminus. Since the protein binds to the single stranded hairpin terminus , all of the mutations will have to be duplicate mutations. Unless, the desired outcome of the mutation is to introduce a bubble in the hairpin, complementary sequences will

have to be introduced at two different sites along the terminus when it is in its double stranded form in the plasmid. One possible method of accomplishing this would be to use PCR mutagenesis.

The results suggest that left and the right terminus bind the identified proteins differently. This differential protein binding suggests they may each function with varying efficiency as an *ori* at different stages of replication. It is possible to imagine that since the left end must (at least 90% of the time) function as an *ori* for the conversion of virion ss DNA to ds DNA, then, perhaps, the right end functions poorly at the same replication step. If this were true one could test whether the plus and minus strand of BPV were equally infectious. In contrast, the proteins which bind to the right end might allow it to function better as an *ori* for the amplification of ds RF molecules and virion minus polarity ss DNA, as the kinetic hairpin transfer model suggests. The proposed experiments with chimeric viruses can address this question.

The demonstration of the S-phase requirement for replication will begin to answer questions concerning the role of the host cell during parvovirus replication. The identification of the S-phase protein could be accomplished using several approaches. A conventional purification scheme could yield the specific protein. Alternatively, the terminus could be used as a radioactive probe to screen a bovine cell cDNA expression library. The third method would be a judicious shot-gun approach. The specific protein - DNA binding could be inhibited using various competitors. For example, anti-PCNA antibodies or other anti-replisome protein antibodies could be used to try to inhibit the protein - DNA interaction. Alternatively, our lab has observed that BPV replicates poorly in primary bovine cells that have been transformed by SV40. This suggests that the large T antigen is down regulating some factor required for BPV replication. Therefore, SV40 T antigen, which binds several cellular proteins, could be pre-incubated with the nuclear extracts to determine the effect on protein - DNA binding. These experiments might identify the cellular protein involved in BPV replication.

A more generalized function of the S-phase factor may be in determining cellular permissiveness for BPV replication. The gel retardation assay could be used to detect the S-phase protein in uninfected cell cultures of various bovine cell types. This assay could be

extended to look for its presence in Bovine adenovirus infected bovine cells or tumor cell lines. The adenovirus infection may induce the S-phase factor in a greater number of cells or to a higher concentration as it appears to do for cells infected with AAV. Similarly, if the S-phase factor is an integral cellular replication protein then some tumor cell lines might be expected to have an increased concentration of the protein.

Another approach to investigating the influence the host cell has on BPV replication is to investigate a similar virus replicating in a different host cell. To this end, the work on identifying the similarities between LPV and BPV was carried out. The easiest way to compare the two viruses would be to sequence the entire genome of LPV. However lacking this information, the results obtained show that LPV is probably more similar to BPV than to other parvoviruses that infect rodents. Based on the recovery and isolation of enough LPV RF DNA for restriction enzyme analysis, it is now possible to grow LPV efficiently enough to isolate sufficient amounts of RF DNA to clone the viral genome. Based on the mapping of the Pst 1 fragments with the PCR products, the termini can be cloned as Pst I-Sma I fragments (as suspected all along) and the major coding region as a Pst I fragment. These would be significant steps to obtaining the sequence of LPV.

The results of my research provide answers to all of the objectives set forth at the beginning of my dissertation. Perhaps more importantly, they begin to define the specific proteins and DNA sequences required for the accurate replication of BPV.

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EMPLOYMENT:

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