ISOLATION AND CHARACTERIZATION OF BOVINE PARVOVIRUS DNA

by

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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY

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December, 1978

Blacksburg, Virginia

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Robert C. Bates, for his exellent guidance during the course of this work and for all the discussions and suggestions. He taught me that frustration is a challenge. Sincere thanks to the other members of my committee, Dr. John L. Johnson and Dr. Ernest R. Stout, who gave freely of their time and were always available with helpful hints. Thanks for giving me a gentle push, when I needed it the most. Special thanks are due to Dr. Donald R. Lightfoot, who helped me with the melting profile experiment. From him I learned a lot.

To Sirry, my wife, who had to put up with a lot of nonsense, but never gave in and was a source of constant encouragement. She typed the final copy of this thesis.

To my lab partners, Rebecca B. Young, who provided the electon micrographs, and John T. Patton who developed the virus purification procedure and worked out the hybridization technique. Thanks for all the good times and the serious and not so serious discussions.

Last but not least I want to thank the Department of Biology for providing me with the financial assistance necessary to complete my studies.

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1. Introduction.

Between the late 1950's and the early 1970's a number of viruses were isolated that shared enough characteristics to justify their classification in a separate family, the Parvoviridae. Viruses belonging to this family are nonenveloped, icosahedral, ranging in diameter from 18-26 nm. Their density, in CsCl, ranges between 1.37-1.47 g/cm^3, and they have a sedimentation coefficient of approximately 110 S. The viral capsid is composed of two to three different polypeptides, and encloses linear single-stranded DNA ranging in molecular weight between 1.35-2.0 x 10^6 daltons. Parvoviral DNA has a buoyant density, in neutral CsCl, ranging between 1.720-1.729 g/cm^3, and sedimentation coefficients ranging between 20-28 S in neutral sucrose, but between 15-18 S in alkaline sucrose. (Siegl, 1976; Salzman, 1978; Mayor and Kurstak, 1974; Tattersall and Ward, 1978; Bachmann et al., 1975). Based on differences in the mode of replication, the family Parvoviridae can be divided into three genera (Bachmann et al., 1975):

1. Viruses belonging to the genus Adeno-associated virus (AAV) or the defective parvoviruses, require the presence of a co-infecting helper virus. The helper virus is usually an adenovirus. This genus includes AAV serotypes 1, 2, 3, and 4 (Berns, 1974; Rose, 1974; Salzman, 1978).
2. Viruses in the genus Parvovirus, which are more commonly known as the nondefective or autonomous parvoviruses, do not require the presence of a helper virus, but require actively dividing

cells for their replication. This genus includes: Kilham rat virus (KRV), H-1, minute virus of mice (MVM), LuIII, and bovine parvovirus (BPV; Siegl, 1976; Salzman, 1978).

3. Viruses grouped in the genus Densovirus (DNV), or the arthropod parvoviruses, unlike the viruses mentioned above, do not infect vertebrates. Only two viruses have been described for this genus, densonucleosis virus 1 (DNV1) and densonucleosis virus 2 (DNV2; Mayor and Kurstak, 1974; Kelly et al., 1977).

The fact that members of the family Parvoviridae contain linear single-stranded DNA has posed some interesting questions as to how this DNA might be replicated. The presence of linear double-stranded replicative forms in infected cells led Tattersall et al. (1973) to propose, that such intermediates could be formed, if the 3' end of the viral DNA could function as a primer for the replication of a new strand. It is known that single-stranded DNA fragments, from denatured eucaryotic DNA, form double-stranded regions. These regions have been named palindromes, because the nucleotide sequence reads the same both backward and forward (Wilson and Thomas, 1974). It was then proposed that such palindromes at the 3' end of single-stranded DNA might act as a primer for DNA synthesis (Cavalier-Smith, 1974). Subsequent examination of several parvoviral DNA's revealed that short palindromes were present not only on the 3' end, but also on the 5' end (Kelly and Bud, 1978; Tattersall and Ward, 1978). Several models have been proposed for the replication of parvoviral DNA, based on these findings (Tattersall and Ward, 1976; Berns and Hauswirth, 1978; Strauss et al., 1976).

Although recent evidence suggests that the BPV genome contains palindromes at both termini (Chow and Ward, 1977, 1978), less is known about some of the other physico-chemical characteristics of BPV DNA. The objective of this study was to:

1. Determine the configuration of BPV DNA by hydroxyapatite chromatography and digestion with S1 endonuclease and bacterial exonuclease I.
2. Determine the sedimentation coefficient of BPV DNA in neutral and alkaline sucrose, and hence the molecular weight.
3. Determine the base composition of BPV DNA.
4. Determine the buoyant density of BPV DNA in neutral CsCl.

2. Literature Review.

2.1 Prologue.

Since the discovery of the first parvovirus, over 20 species have been isolated from various sources. Table 1 lists some of these viruses and their source. Extensive work has been carried out on many of these viruses, involving characterization of all aspects of viral replication. It is beyond the scope of this thesis to cover all the work that has accumulated in parvoviral research, instead this review will focus mainly on the characterization of the viral genomes. The interested reader is referred to any of the number of reviews, that have appeared in recent years (Kurstak, 1972; Berns, 1974; Rose, 1974; Mayor and Kurstak, 1974; Siegl, 1976; Carter, 1978; Salzman, 1978; Tattersall, 1978; Tattersall and Ward, 1978).

2.2 The Defective Parvoviruses (Genus Adeno-associated virus).

A number of viruses have been isolated from preparations of different mammalian and avian adenoviruses, that have unique morphological characteristics. A special feature of these viruses is their inability to replicate by themselves in cell cultures. They need the presence of a helper-virus, usually an adenovirus, to carry out a complete replication cycle. The best characterized of these adeno-associated viruses (AAV) are the simian and human serotypes 1, 2, 3, and 4 (Berns, 1974; Rose, 1974; Mayor and Kurstak, 1974; Salzman, 1978).

Table 1. The family Parvoviridae.

Genus	Name of virus	Source and date of isolation
Adeno-associated virus	AAV type 1	Simian adenovirus 15 stock (1965)
	AAV type 2	Human adenovirus 12 stock (1966)
	AAV type 3	Human adenovirus 7 stock (1966)
	AAV type 4	Simian adenovirus 15 stock (1967)
Parvovirus	Kilham rat virus	Rat sarcoma and leukemia (1959)
	H-1	Human Hep-1 transplantable tumor (1960)
	Minute virus of mice	Mouse adenovirus stock (1966)
	LuIII	Human cell lines (1971)
	Bovine parvovirus	Calf feces and intestinal tract (1961)
Densovirus	DNV1	Galleria mellonella larvae (1964)
	DNV2	Junonia coenia larvae (1972)

2.2.1 Adeno-associated viruses.

In 1965 Atchison et al. reported the discovery of the first adeno-associated virus, AAV-1. Within two years of this discovery three other adeno-associated viruses were isolated AAV-2, AAV-3, (Hoggan et al., 1966) and AAV-4 (Parks et al., 1967a). Because all of these viruses have very similar physico-chemical characteristics, they will be referred to collectively as adeno-associated virus (AAV) in this review.

Initial cytochemical studies on the type of nucleic acid and its strandedness, indicated that it was double-stranded DNA (Mayor et al., 1965). Later it was shown with acridine orange, which forms a green complex with double-stranded DNA and RNA, but a red complex with single-stranded DNA and RNA, that the AAV genome consisted of single-stranded DNA within the virion (Mayor and Melnick, 1966). However, when the DNA was extracted from the virions, it had characteristics of double-stranded DNA with a molecular weight of $3 - 3.6 \times 10^6$ daltons (Rose et al., 1966; Parks et al., 1967b). Crawford et al. (1969) compared AAV with other parvoviruses known to have single-stranded DNA as their genome. They speculated that AAV capsid or virion was too small to accommodate a genome of the size previously reported, and suggested that complimentary strands were encapsidated into separate virions. These complimentary strands reannealed upon release during the isolation procedure giving the impression that the DNA was double-stranded in the virions. This hypothesis was tested by Rose et al., (1969). By substituting 5-bromo-2'-deoxyuridine (BUdR) for thymidine in the growth medium, they

were able to isolate double-stranded DNA that had much higher buoyant density in CsCl than unsubstituded DNA. By mixing virions that contained substituded DNA with virions that contained unsubstituted DNA and then extracting the DNA, double-stranded DNA was formed that had buoyant density intermediate between substituted and unsubstituded DNA. This would not occur if the DNA was double-stranded in the virions. This finding was confirmed by extracting the DNA from AAV under conditions that would not cause denaturation of double-stranded DNA and minimized the reassociation of single-stranded DNA. The DNA released from the virions was indeed single-stranded, but reannealed quickly, when the conditions were altered to allow for maximum reassociation (Mayor et al., 1969; Berns and Rose, 1970). Complete reannealing of the single-strands suggested that equivalent synthesis of both strands must occur in infected cells (Carter et al., 1972). This finding and the fact that there was a considerable difference in thymine content between the strands (Rose et al., 1966; Rose and Koczot, 1971) enabled partial separation of the two virion types in CsCl after BUdR had been substituted for thymine (Berns and Adler, 1972). Recently the two strands have been separated directly on agarose-acrylamide gels (Hauswirth and Berns, 1977). It has thus been conclusively shown that AAV contains single-stranded DNA with molecular weight of 1.35×10^6 daltons (Rose, 1974) and that the plus and minus strands are encapsidated into separate virions.

Intial observations with the electron microscope indicated that double-strands AAV DNA was linear (Parks et al., 1967b). Upon closer examination of DNA released from AAV, Vernon et al. (1971) found

small numbers of double-stranded circular molecules. Further examination by other investigators also revealed the presence of double-stranded dimers and oligomers, both linear and circular (Gerry et al., 1973; Koczot et al., 1973). This led to the conclusion that AAV DNA was permuted to some extent. Not only was the AAV DNA found to be permuted, but it was also found to have inverted terminal repetition. These sequences were discovered by Carter et al. (1972). It was observed that about 50% of purified minus strands chromatographed as double-stranded DNA on hydroxyapatite (HAP), but HAP chromatography is commonly used to distinguish between single- and double-stranded DNA (Bernardi, 1971). This DNA was not completely double-stranded as it was partially susceptible to S1 endonuclease, which is specific for single-stranded DNA or RNA (Vogt, 1973). These molecules were observed with the electron microscope to be single-stranded circles. Furthermore, these circles were shown to be closed by a short duplex region, a so-called panhandle (Koczot et al., 1973). Berns and Kelly (1974) estimated by direct length measurements that the length of these duplex regions was about 1-2.5% of the AAV genome. The AAV genome has thus been found to have not only a natural terminal repetition but also an inverted terminal repetition. It was suggested, that these terminal repetitions might represent a palindrome of the type 122'1'-122'1' (Gerry et al., 1973). Such a palindrome has now been shown to be present on both the 3' and the 5' ends, representing 3-4% of the viral genome (Fife et al., 1977).

By using several restriction endonucleases, HindII and HindIII (Berns et al., 1975), EcoRI (Carter et al., 1975), HaeIII (Denhardt

et al., 1976), and SalI, PstI and HaeII (de la Maza and Carter, 1976), a physical map of the AAV genome is now available, and sequencing is under way (Berns et al., 1978).

2.3 The Autonomous Parvoviruses (Genus Parvovirus).

Although mink enteritis virus (MEV) was not grouped with the parvoviruses until 25 years after its discovery, it was the first to be isolated. A number of viruses have been discovered since then that have been grouped together within the family Parvoviridae in the genus Parvovirus, but are more commonly known as the autonomous or nondefective parvoviruses. Unlike the adeno-associated viruses, the autonomous parvoviruses do not require the presence of a helper virus, but do require actively dividing cells for their propagation (Bachmann et al., 1975; Salzman, 1978). Several of these viruses have been extensively characterized, but as before, this review will focus mainly on the characterization of the genomes of some of the better known autonomous parvoviruses.

2.3.1 Kilham rat virus.

In their search for an oncogenic papovavirus, Kilham and Oliver (1959) isolated a virus from several rat tumors, that did not share the characteristics of the papovavirus group. This new virus was appropriately, at that time, named after its discoverer and its source, Kilham rat virus (KRV).

The first indication that KRV contained DNA was drawn from the observation that intranuclear inclusions were Feulgen positive (Rabson

et al., 1961). Several years later it was shown that KRV incorporated ^{3}H-thymidine into its genome, but not ^{3}H-uridine, strongly suggesting a DNA nature of the KRV genome (Whalley, 1965). The first indications, that KRV might contain single-stranded DNA, came from studies on the then newly discovered and apparently closely related X-14 virus, isolated from the mammary tissue of X-irradiated and methylcholanthrene-treated rats (Payne et al., 1963). Jamison and Mayor (1965) showed that pure preparations of this virus were stained deeply red with acridine orange, a reaction expected, if the virions contained single-stranded DNA or RNA. Upon disruption of the viral capsid and subsequent treatment with DNase the stain was abolished, but the staining properties were not altered upon treatment with RNase. At first it was indicated that this finding might not necessarily be true where KRV was concerned. May et al., (1967) found that DNA isolated from pure preparations of KRV gave yellow-green staining with acridine orange, indicating double-stranded DNA. These investigators also showed that the DNA did not react with formaldehyde which reacts only with the free amino groups of single-stranded nucleic acid. Furthermore it was shown that the DNA had the characteristic melting profile of double-stranded DNA, and it was observed to be double-stranded and linear in the electron microscope. The molecular weight was determined to be 2×10^{6} daltons, and interestingly enough the DNA was found to be infectious. These results contradicted sharply with the later finding of Robinson and Hetrick (1969). Their studies showed that KRV DNA did indeed react with formaldehyde, lacked the characteristic melting profile of double-stranded DNA, and electron

microscopy showed pooling and sharp folds expected of completely single-stranded DNA when prepared by the aqueous technique for electron microscopy. The molecular weight was determined to be 1.2×10^6 daltons. May and May (1970) confirmed this finding and admitted that their initial characterizations of the KRV DNA might have been hampered by contaminating rat cell DNA. Further proof for the single-stranded nature of the KRV genome came from studies on base composition. It was shown that adenine was not equal to thymine and guanine was not equal to cytosine as would not be expected if the DNA was double-stranded (McGeoch et al., 1970) although exceptions are known (Thomas and MacHattie, 1967). Salzman and Jori (1970) supported this finding and determined the molecular weight to be 1.7×10^6 daltons. Further studies using a combination of electron microscopy and digestion with exonuclease I, which is specific for the 3' end of single-stranded DNA (Lehman, 1966) showed that the DNA of KRV was indeed single-stranded and linear, and the molecular weight was reported to be approximately 1.6×10^6 daltons (Salzman et al., 1971).

Gunther and May (1976), working on KRV replication intermediates, found that a portion of the viral strand was covalently linked to the complimentary strand. They also found upon denaturation of replicative intermediates that the single-stranded DNA did not behave as completely single-stranded DNA on HAP. These investigators suggested that KRV DNA might thus contain palindromes, like those that had been reported for AAV (Gunther and May, 1976). Digestion of KRV DNA with S1 endonuclease resulted in 5-6% of the DNA eluting as double-stranded from benzoyl-naphtoyl-DEAE (BND) cellulose, which is commonly employed

in the separation of single-stranded DNA from double-stranded DNA (Iyer and Rupp, 1971). By using exonuclease III which is specific for the 3' end of double-stranded DNA (Jovin et al., 1969) and then S1 endonuclease, the amount of double-stranded DNA eluting from BND-cellulose decreased by approximately one-half. This suggested that both the 3' and 5' termini of KRV were double-stranded, each piece being 115-150 base pairs (Salzman, 1977). This finding was essentially supported by Chow and Ward (1977). More recent work with KRV DNA confirmed the presence of two duplex regions and indicated that the larger region was located at the 3' end of the viral genome (Lavelle and Mitra, 1978). These investigators showed first that the KRV DNA had some double-stranded character by examining its elution profile from HAP. The DNA eluted in an intermediate position between completely single- and double-stranded DNA markers as would be expected if it contained short duplex regions (Wilson and Thomas, 1973). The viral DNA was also found to be resistant to digestion by exonuclease I, which contradicted the previous finding by Salzman et al. (1971). However, prior treatment with exonuclease III rendered the viral genome susceptible to exonuclease I. Treatment of the KRV DNA with S1 endonuclease and exonuclease I enabled the separation of two double-stranded fragments by gel electrophoresis. By comparing mobilities to those of Hind restriction endonuclease fragments of ØX174 RF I DNA their sizes were determined to be 110 and 135 base pairs. Treatment of KRV DNA with exonuclease III and then with exonuclease VII, which is specific for both 3' and 5' ends of single-stranded DNA (Chase and Richardson, 1974), resulted in the loss of the larger double-stranded

fragment. This larger fragment is thus located on the 3' end of the viral genome (Lavelle and Mitra, 1978). However, Chow and Ward (1978), determined the size of the 3' palindrome to be 110 base pairs and the 5' palindrome to be 130 base pairs. This discrepancy remains to be solved.

Recently Salzman et al. (1978), reported on the mapping of the KRV genome with the restriction endonucleases EcoRI, HpaII, and HindIII.

2.3.2 H-1.

H-1 virus was discovered in samples originating from the transplantable human tumor Hep-1, after it had been serially passed in X-irradiated and cortisone treated rats (Toolan et al., 1960).

The first indication that this new virus contained DNA was the successful staining of intranuclear inclusions with acridine orange (Hampton, 1964). These findings were supported by the fact that ^{3}H-thymidine was incorporated into the H-1 genome, but not ^{3}H-uridine, and the genome was found to be sensitive to DNase, but not RNase (Cheong et al., 1965). Using methods similar to those described for KRV, Usategui-Comez et al. (1969) showed that the DNA isolated from pure preparations of H-1 reacted with formaldehyde, did not have a sharp melting profile, and adenine and thymine were unequal as were guanine and cytosine. These investigators estimated the molecular weight of the H-1 DNA to be in the range of 2.3-3.1 x 10^{6} daltons and concluded that the genome was single-stranded. Further studies on base composition supported these findings, but the H-1 genome size of 1.7 x 10^{6} daltons was considerably smaller than previous estimates

(McGeoch et al., 1970).

Studies on viral replicative forms isolated from cells infected with H-1 indicated the presence of a palindromic sequence of about 50 base pairs at the 3' end (Rhode, 1977a). In a following study, Rhode (1977b) suggested that a palindrome was also present at the 5' end, but from results obtained with restriction endonucleases, he suggested that the palindrome at the 5' end might be more complex than a simple duplex. In a comparative study on several parvoviral DNA's Chow and Ward (1977) confirmed the presence of hairpin loops at both the 3' and the 5' termini of the H-1 genome. These were later shown to consist of 110 and 130 base pairs, respectively (Chow and Ward, 1978).

A restriction endonuclease map of the H-1 genome has been constructed using EcoRI, HaeII, HaeIII, HindII, HindIII and HpaII endonucleases (Rhode, 1977b) and DNA sequencing has begun (Chow and Ward, 1978).

2.3.3 Minute virus of mice.

The discovery of the adeno-associated viruses prompted investigators to look for these small viruses in their adenovirus stocks. Crawford (1966) working with mouse adenovirus detected a small virus that had completely different morphological characteristics than the mouse adenovirus. Because of the small size of this newly discovered virus, it was named minute virus of mice (MVM). At first it was thought that MVM, because of its source, might be related to the adeno-associated viruses. However it was soon discovered, that MVM was capable of replication in cell cultures without the presence of a helper virus, and MVM has thus been placed with the autonomous

parvoviruses.

In his original paper, Crawford (1966) had already provided proof for the single-stranded nature of the MVM genome. He showed that the nucleic acid isolated from pure preparations of MVM reacted with formaldehyde and was sensitive to DNase but not RNase. Further studies on base composition, sedimentation behaviour, and with the electron microscope supported the previous finding that the MVM genome was single-stranded DNA, and in addition it was shown to be linear with a molecular weight of 1.5×10^6 daltons (Crawford et al., 1969; Mcgeoch et al., 1970).

Detailed studies on the MVM DNA revealed that the molecule contained a stable hairpin loop of approximately 130 base pairs located at the 5' end (Bourguignon et al., 1976). About 6.5% of the MVM DNA was found to be resistant to digestion with S1 endonuclease. The MVM DNA eluted from HAP at a buffer concentration slightly higher than that needed to elute completely single-stranded DNA. Final evidence for the presence of a duplex region was obtained by ethidium bromide fluorimetry. At pH 12 stable duplex DNA retains its double-stranded structure, whereas nonspecific base paired regions do not. The dye can become intercalated into a double-helical region and can thus increase the fluoresence of the DNA sample. The results indicated that approximately 3% of the MVM DNA existed in a duplex conformation at pH 12. The size of the double-stranded region as measured by velocity sedimentation and gel electrophoresis was an average of 130 base pairs. This represents about 6% of the total number of bases. It was thus concluded that all the double-stranded

region must be present in one segment of the MVM genome, since the percentage of MVM DNA resistant to S1 endonuclease digestion was also about 6%. This was further confirmed by digestion with exonuclease I. About 4-5% of the DNA remained trichloroacetic acid (TCA) precipitable after treatment with exonuclease I, hence the duplex region was located primarily at the 5' end. In this same study, it was shown that the MVM DNA could act as a template for a number of DNA polymerases. It was thus assumed that the 3' end could fold back on itself to form an unstable palindrome that could then act as a primer for DNA synthesis (Bourguignon et al., 1976; Chow and Ward, 1977). More recent studies indicate that the 3' terminus is more stable than previously thought and that the MVM genome actually contains two palindromic sequences, one on each end. The size of these palindromes were determined by gel electrophoresis, the one at the 3' end was estimated to be 110 base pairs, and the one on the 5' end was 130 base pairs (Chow and Ward, 1978).

A simple map of the MVM genome has been worked out using the restriction endonuclease EcoRI (Ward and Dadachanji,1978), and the sequence of several nucleotides at the ends has been determined (Chow and Ward, 1978).

2.3.4 LuIII.

In the early 1960's a small hemagglutinating agent was discovered in extracts from permanent human cell lines, that had enough distinct biological and morphological characteristics to justify its classification into a separate group (Zwillenberg and Hallauer, 1962).

Closer examination of a number of human cell lines finally resulted in the isolation of three distinct serotypes: KBSH, TVX and LuIII (Hallauer et al., 1972). Of these three, LuIII has received the most attention.

As with other parvoviruses, the first indication that LuIII contained single-stranded DNA came from studies with acridine orange. Highly concentrated and pure preparations of LuIII were stained flame-red with acridine orange. Prior treatment with DNase abolished the stain, but treatment with RNase did not alter the staining properties. Moreover, it was shown that ^{3}H-thymidine was incorporated into the LuIII genome, and replication was inhibited with the antimetabolite 5-iodo-2'deoxyuridine (IUdR). It was thus concluded that the LuIII genome was single-stranded DNA (Siegl et al., 1971). Further studies employing BND cellulose chromatography, electron microscopy, and exonuclease (from the fungus Verongia aerophoba) digestion confirmed that the LuIII genome was linear single-stranded DNA, and the molecular weight was determined to be 1.59×10^{6} daltons (Siegl, 1973).

Further experiments are necessary to determine, whether LuIII DNA contains some of the unique features described for KRV, H-1 and MVM, and final characterization of the LuIII genome would have to include restriction endonuclease mapping.

2.3.5 Bovine parvovirus.

This virus was first isolated from the intestinal tract of normal calves. Because of the ability of this virus to agglutinate red blod cells from various sources, it was referred to as hemadsorbing enteric

virus of calves (HADEN; Abinanti and Warfield, 1961).

First attempts to characterize the nucleic acid of this virus indicated it might be RNA, and the virus was consequently classified as an enterovirus (Spahn et al., 1966). Further studies showed that intranuclear inclusions stained Feulgen positive, and replication was inhibited to some extent with actinomycin D and the antimetabolites BUdR and 5-fluoro-2'-deoxyuridine (FUdR). These results suggested that HADEN was a DNA virus and it was subsequently grouped among the parvoviruses and named bovine parvoviruses (BPV: Storz and Warren, 1970). These results have been supported by Bachmann (1971).

Little work has been done on further characterization of the BPV genome. Chow and Ward (1977) showed that BPV DNA was partially susceptible to S1 endonuclease and exonuclease I, and determined that a small duplex region, about 130 base pairs, was located at the 5' terminus. It was also shown that BPV DNA was utilized as a template by DNA polymerases, suggesting the presence of a palindrome at the 3' end. Accurate sizing of these hairpin regions was never achieved, but it was suggested that they were similar to those of KRV, H-1 and MVM (Chow and Ward, 1978).

2.4 The Arthropod Parvoviruses (Genus Densovirus).

Because of the presence of a linear single-stranded DNA inside the virion of densonucleosis viruses, they have been grouped among the parvoviruses although the viral capsid may contain a greater number of capsomeres than has been reported for the other parvoviruses. Like the adeno-associated viruses, the densonucleosis viruses contain the

complimentary plus and minus strand in separate virions in equal amounts. But unlike the adeno-associated viruses, the densonucleosis viruses are capable of autonomous replication; they do not require the presence of a helper virus (Mayor and Kurstak, 1974).

2.4.1 Densonucleosis viruses.

In 1964 a virus was isolated from diseased larvae of the moth Galleria mellonella, and was named densonucleosis virus, according to the cytopathic effects shown by infected cells (Kurstak, 1972). About 10 years later another virus was isolated from infected larvae of the butterfly Junonia coenia (Rivers and Longworth, 1972). Because these two viruses were apparently very closely related, the virus isolated from Galleria was referred to as DNV1 and the virus from Junonia was called DNV2 (Kelly et al., 1977). In this review they will be commonly referred to as DNV.

Because of the unusual situation, where the plus and minus strands are in separate virions, DNV presented a dilemma similar to AAV. As expected, the first results obtained with DNV DNA, indicated that it was double-stranded (Truffaut et al., 1967). Examination of the melting profile and reaction of DNV DNA with formaldehyde led to the conclusion that DNV DNA was single-stranded in the virion (Barwise and Walker, 1970). This observation was confirmed by Vernoux and Kurstak (1972) who in addition showed that purified virus stained red with acridine orange. Later the single-stranded DNA was extracted under low ionic strength conditions and conclusively shown to be single-stranded and linear when viewed under the electron microscope. The

molecular weight was determined to be about 2×10^6 daltons (Kurstak et al., 1973). These studies have been repeated and confirmed by Kelly et al., (1977), who in addition found the DNA's of DNV1 and DNV2 to be 87% homologous.

Because of the many similarities between AAV and DNV, it was of interest to see whether DNV DNA shared some of the peculiar characteristics of AAV DNA. As was expected, a sizable portion of double-stranded DNA preparations contained, in addition to linear unit length molecules, circles and concatameres, indicating that the DNV genome has limited permutation. Furthermore, in preparations of DNV DNA that had been denatured and allowed to reanneal under conditions which were selective for intramolecular annealing, 95% of the single-stranded molecules were circular. About 20% of the circles contained panhandles, 1-2.5% of the genome length (Kelly and Bud, 1978). These results were in an amazingly close agreement with the finding of Berns and Kelly (1974) for AAV DNA. Upon treatment of the single-stranded DNV DNA circles with S1 endonuclease, about 6% of the genome (380 base pairs) was found to be resistant (Kelly and Bud,1978). A similar discrepancy has been observed in size determinations of the panhandles of AAV (Carter et al., 1972; Berns and Kelly, 1974). From these studies on the DNV genome it can be concluded that it contains similar end structures as were proposed for AAV DNA (Gerry et al., 1973). The fact that DNV is capable of autonomous replication, while AAV is not, remains to be explained. The obvious difference in the genome sizes could contribute to this difference in requirements.

2.5 Other Candidate Parvoviruses.

Because of almost identical physico-chemical properties the well characterized bacteriophage ØX174 has served as a model in the characterization of the parvoviruses. But because ØX174 contains circular single-stranded DNA as its genome, and hence has a somewhat different replication strategy (Sinsheimer, 1968), it will probably not be grouped with the parvoviruses.

Recently a plant virus, Bean Golden Yellow Mosaic Virus (GYMV), was shown to contain single-stranded DNA (Goodman, 1977). It remains to be established whether GYMV shares sufficient characteristics with other members of the family Parvoviridae to justify its classification as parvovirus.

2.6. Summary.

Several viruses have been isolated in the past two decades which contain linear single-stranded DNA as their genome. An unique characteristic of these viruses is the presence of short, palindromes at both termini of the DNA. It has been shown that these viral DNA's can serve as templates for a number of different DNA polymerases *in vitro* indicating that the palindrome at the 3' end serves as primer for DNA synthesis. Several models have been proposed for parvoviral replication based on these findings. The function of the palindrome at the 5' end is obscure. It could be a protective device against exonucleases within the cell, or it might function as a recognition site for capsid proteins, during strand displacement of a newly synthesized progeny strand from a double-stranded replicative

intermediate.

Although the viruses within the family Parvoviridae share many characteristics, there are also many apparent differences. The autonomous parvoviruses seemingly only package the minus strand, while the defective parvoviruses and the arthropod parvoviruses package the plus and minus strands in equal proportions into separate virions. The defective parvoviruses, however, require the presence of a helper virus to complete their replication cycle, while the autonomous and arthropod parvoviruses are capable of autonomous replication. Both the defective and arthropod parvoviruses have natural as well as inverted terminal repetitions, which enable the single-stranded genomes to circularize with the formation of so-called panhandles. This property has not been observed in the autonomous parvoviruses. The fact that adenovirus DNA contains such inverted terminal repetitions and that adenoviruses are required as helper viruses for the defective parvoviruses suggests that these sequences might be important in the interaction between these viruses in infection. However, this does not explain the presence of these sequences in the arthropod parvoviruses, as they do not need a helper virus to complete their replication cycle.

Parvoviruses have proved to be an interesting problem and although the literature is full of contradictions, a clear picture is now emerging on the chemical, physical and biological properties of this amazing group of viruses. Some of the physico-chemical properties of several parvoviral genomes are summarized in table 2.

Table 2. Physico-chemical properties of parvoviral genomes.

Virus	MW (x 10^6)	S[a]	CsCl[b] (g/cm^3)		Base composition A	T	G	C	G+C	References
AAV-2	1.35	24 (15)	1.726 (1.714)	(-)	20.5	26.5	26.7	26.3	53.0	1,9
				(+)	25.2	21.7	26.6	26.5		
KRV	1.6	27	1.726		26.8	29.6	20.6	22.9	43.5	2,3
H-1	1.7	27.8	1.720		25.5	29.3	22.6	22.6	45.2	3,4
MVM	1.5	20	1.722		26.5	32.7	19.5	21.4	40.9	3,5,8
LuIII	1.59	24	1.7254		-	-	-	-	-	6
BPV	-	-	-		-	-	-	-	-	
DNV1	2.0	- (16.1)	1.711 (1.701)		-	-	-	-	42[c]	7

[a] S values in parenthesis are for double-stranded DNA

[b] Densities " " " " "

[c] Calculated from buoyant density of the double-stranded DNA

1) Rose and Koczot, 1971
2) Salzman and Jori, 1970
3) McGeoch et al., 1970
4) Usategui-Gomez et al., 1969
5) Crawford et al., 1969
6) Siegl, 1973
7) Kurstak et al., 1973
8) Bourguignon et al., 1976
9) Rose et al., 1969

3. Materials and Methods.

3.1 Viruses.

3.1.1 Bovine parvovirus.

BPV was obtained from the original isolate of Abinanti and Warfield (1961) and has been maintained in this laboratory. BPV (71-1-20W; Bates et al., 1972) was further purified by the dilution end-point method prior to use in these studies.

Viral stocks were grown in bovine fetal spleen cells that had been seeded into plastic flasks (75 cm^2; Falcon) at a density of 1-1.5 x 10^6 cells per flask in Eagle's minimum essential medium (MEM; Flow Laboratories Inc.) containing 10% lamb serum (LS; Flow Laboratories Inc.). The cells were incubated at 37 ^oC overnight. The media was removed and the cell layer washed 2X with Dulbecco's salt solution (see appendix). About 0.1 ml of BPV was added to each flask in 5 ml of MEM and incubated for 1 h at 37 ^oC. Then 20 ml of MEM containing 10% LS was added and the cells were observed for cytopathic effects (CPE). When the CPE was 100%, the cell suspensions were freeze-thawed three times, combined and the cell debris was pelleted at 6,500 rpm for 10 min in a Sorvall superspeed RC2-B automatic refrigerated centrifuge. The supernatant was collected and titer of the virus determined by plaque assay. The titer of the stock virus was usually 5 x 10^7 plaque forming units (pfu)/ml and it was stored at -20 ^oC.

3.1.2 Kilham rat virus.

KRV was a gift of Dr. G. Lavelle. This virus was not further purified and viral stocks were prepared as described above, except KRV was grown in normal rat kidney (NRK) cells in MEM containing 10% fetal bovine serum (FBS;Flow Laboratories Inc.). Titer of stock was not determined.

3.1.3 Adenovirus type 2 and Adeno-associated virus type 2.

These viruses were a gift of Dr. G. Lavelle and were not purified further. Titers of virus stocks were not determined.

3.2 Cells.

3.2.1 Bovine fetal spleen cells.

Bovine fetuses, 3 to 6 months old, were obtained from the Valleydale meatpacking plant. The spleens were removed aseptically and cut with sterile scissors into small pieces. The pieces were placed in a sterile Erlenmeyer flask (500 ml) and about 250 ml of 0.25% trypsin solution was added. A magnetic stir bar was used for mixing. When the tissue was almost fully digested the cell-trypsin suspension was poured into 50 ml centrifuge tubes and the cells pelleted at 1500 rpm in a IEC HN-S table top centrifuge for 10 min. The cell pellets were resuspended in MEM and seeded into tissue culture bottles (TC;Brockway Glass Co.) in MEM containing 10% LS. When the cells had formed a monolayer, they were dispersed with trypsin-versene solution and a portion was reseeded into TC bottles with the remainder frozen and stored in liquid nitrogen.

BFS cells were maintained in MEM containing 10% FBS. The cells formed a monolayer in 5-7 days. They were then dispersed and split at a ratio of 1:3-5. Only cells from the fifth to the twenty-fifth passage were used.

3.2.2 Buffalo lung cells.

Bu cells (CCL 40) were obtained from the American Type Culture Collection (ATCC) and were maintained in MEM containing 10% FBS. The cells had formed a monolayer in 5-7 days and were then split at a ratio of 1:3-4. Only cells from the twentieth to the thirty-fifth passage were used.

3.2.3 Bovine turbinate cells.

BT cells (CRL 1390) were obtained from ATCC and maintained in MEM containing 10% horse serum (HS;Flow Laboratories Inc.) and nonessential amino acids (NEAA). BT cells formed a monolayer in 7-12 days and were split at a ratio of 1:2-3. Only cells from the twentieth to the thirty-fifth passage were used.

3.2.4 Normal rat kidney cells.

NRK cells were a gift of Dr. G. Lavelle and were maintained in MEM containing 10% FBS. The cells had formed a monolayer after 4-5 days and were split at a ratio of 1:5. Cells from the twentieth to the twenty-fifth passage were used in these studies.

3.2.5 KB cells.

KB cells adapted for spinner culture were a gift of Dr. G. Lavelle. These cells were maintained in MEM for suspension cultures (Grand Island Biological Co.) containing 10% dialyzed HS (see section 3.3.1) and NEAA.

3.3 Radioactive labelling of DNA.

3.3.1 BPV DNA.

For labelling with ^{3}H-thymidine (^{3}H-TdR) or ^{14}C-thymidine (^{14}C-TdR), glass roller bottles (RB;Wheaton Scientific) were used. About 15 x 10^{6} cells were seeded per RB in 50 ml MEM containing 10% of the appropriate serum and incubated until the cells were 70% confluent. The medium was then removed and the cell monolayer washed 2X with Dulbecco's salt solution. BPV was added at a multiplicity of infection (m.o.i.) of 0.1-0.5 pfu/cell in 5 ml of MEM and virus was absorbed for 1-2 h. The unabsorbed virus was removed, and the cells were washed with Dulbecco's salt solution. When BFS cells or Bu cells were used, 20 ml of MEM containing 10% dialayzed LS (dLS) was added. The dLS was prepared by dialyzing LS against an excess of 0.15 M NaCl for 5 h at 4 ^{o}C. If BT cells were used, 20 ml of MEM containing 10% HS and NEAA was added. Label was added 24 h post infection (p.i.), 2.5 uCi ^{3}H-TdR/ml (ICN; 81.2 Ci/mmole) or 0.1 uCi ^{14}C-TdR/ml (ICN; 55.7 mCi/mmole). Cells were scraped into the medium when CPE was 80-90% and collected by centrifugation at 10,000 rpm for 10 min at 4 ^{o}C. The supernatant was discarded and the cell pellets were resuspended in 5-10 ml of 0.01 M Tris-HCl pH 8.0 and stored at 20 ^{o}C, until the virus was purified.

For labelling with carrier free ^{32}P-orthophosphate the cells were seeded into plastic RB (850 cm^2; Corning) at about 20 million cells per RB. The cells were prepared as described above, but infected with 1-2 pfu/ml. After the adsorption period, the cells were washed 2X with Saline A (see appendix) and 20 ml of phosphate-free MEM supplemented with 3% MEM (3×10^{-5} M PO_4^{-3}) and 10% of the appropriate serum. About 12-24 h p.i. 2 uCi/ml of carrier free ^{32}P-orthophosphate (ICN; 82.1 mCi/mmole) was added and the cells were collected and stored as described above.

3.3.2 KRV DNA.

KRV DNA labeled with ^{3}H-TdR was prepared as described above for BPV, except KRV was grown in NRK cells containing 10% FBS. Since virus titer was not determined, 0.5 ml-1.0 ml of the virus stock were added per RB.

3.3.3 AAV-2 DNA.

KB cells were adjusted to a density of 5×10^5 cells /ml in a spinner flask. When the cells had reached 1×10^6 cells /ml they were infected with 2 ml of adenovirus type 2 stock and 2 ml of AAV-2 stock. About 10 h p.i. ^{3}H-TdR was added to a final concentration of 10 uCi/ml. When majority of cells showed the characteristics CPE they were collected as described in section 3.3.1.

3.3.4 Cell DNA.

BFS cells were seeded into petri plates (100 x 15 mm; Lux), 2.5 x 10^6 cells per plate. Plates were incubated at 37 ^oC in a CO_2 incubator. After 20 h of incubation 10 uCi of ^3H-TdR or 1 uCi of ^{14}C-TdR was added per plate. This was followed by further incubation and after about 60 h the media was removed and the cell monolayer was washed 2X with Dulbecco's salt solution without phenol red and antibiotics. The cells were now ready for DNA extraction (see section 3.5).

3.4 Isolation of virus and viral DNA.

3.4.1 BPV, KRV, AAV-2.

The method used for purification of virus from the infected cells was developed by John T. Patton in this laboratory.

The cell pellet in 0.01 M tris-HCl pH 8.0 was made 5 mM in EDTA and pH adjusted back to 8.0 by adding 7.5% $NaHCO_3$. The cells were then homogenized with a Brinkman PCU-2 homogenizer at a setting of 4-5 for four 15-sec bursts. This was followed by sonication with the large probe of Virtis Virsonic model No. 150 at an output of 150 watts for four 15-sec bursts. The suspension was made 5 mM in $CaCl_2$ and 5 mM in $MgCl_2$ and DNase I (Sigma Chemical Co.; Cat. no. D-4763) was added to a final concentration of 100 ug/ml. Incubation was carried out for 30 min at 37 ^oC. Then 1 mg/ml of RNase (Sigma Chemical Co.; Cat. no. R-4875) and 1 unit (U)/ml of micrococcal nuclease (Sigma Chemical Co.; Cat. no. N-3755) were added and incubation continued for another 30 min at 37 ^oC. After nuclease digestion was completed, 1 mg/ml of papain (Sigma Chemical Co.; Cat. no. P-3125), 50 ug/ml trypsin (Sigma

Chemical Co.; Cat. no. T 8003), and 50 ug/ml chymotrypsin (Sigma Chemical Co.; Cat. no. C-4129) were added and incubation extended to 1 h at 37 °C. Finally the suspension was made 1% in sodium dodecyl sulfate (SDS), and was now ready to be layered on a step-gradient.

A step-gradient was prepared essentially as described by Tattersall et al. (1976). Sucrose (3 ml; 1.0 M) in TE1 pH 8.7 (50 mM tris-HCl + 5 mM EDTA) was layered on top of 5 ml of CsCl in TE1 pH 8.7 (1.40 g/cm^3) in a 17 ml nitrocellulose tube, and the sample was layered on top of the sucrose. The gradient was centrifuged in an SW 27.1 rotor in a Beckman model L5-50 ultracentrifuge at 26,000 rpm for 20-24 h at 4 °C.

After centrifugation distinct virus bands were observed in the CsCl, and the band corresponding to the density of the complete virus was collected. Virus prepared by a similar procedure has been shown to be free of cell debris by gel electrophoresis, is strongly hemagglutinating, and highly infectious (Pritchard et al., 1978). The virus band was dialyzed overnight against TE2 pH 7.5 (10 mM tris-HCl + 1 mM EDTA). All dialysis bags were prepared by boiling them in 5% $NaHCO_3$ containing 1 mM EDTA, pH 8.5, for 10 min and they were then stored in 50% EtOH at 4 °C

The sample was then made 0.3 M in NaOH and incubated for 30 min at room temperature. The pH was adjusted to neutral by adding 0.2 volumes of 1 M tris-HCl pH 8.6, 0.3 volumes of 1 N HCl, and 0.5 volumes of H_2O, and the sample was made 100 ug/ml in proteinase K (E. Merk Biochemical; Cat. no. 24568) and incubated for 37 °C for 24 h. This procedure yielded DNA with A_{260}/A_{280} of 1.5-1.7.

3.4.2 M13 DNA.

M13 RF I DNA was a generous gift of Dr. S. Mitra.

3.5 Isolation of cell DNA.

Cell DNA that had been labeled with ^{3}H-TdR (Section 3.3.2) was isolated by a modification of the method of Marmur (1961).

To the cell monolayer in the plates, 1 ml of 5 M EDTA and 0.1 ml of 25% SDS were added, and the plates incubated at 37 ^{o}C, with rotation every 10 min, until all the cells had lysed. The cells were then scraped into a clean sterile 50 ml centrifuge tube and made 1 M with $NaClO_4$. An equal volume of chloroform-isoamylalcohol (24:1) was added and the tube shaken gently for 10 min. The sample was centrifuged at 2,500 rpm for 5 min. The upper phase was removed with an inverted pipette and reextracted. Again the upper phase was removed and was dialyzed against 1X SSC-E (0.15 M NaCl + 0.015 M sodium citrate + 1 mM EDTA; pH 7.3) overnight. The volume of buffer in the bag was reduced by hydroextraction with polyethylene glycol 6000, and the sample was redialyzed against 1X SSC-E. The DNA sample was made 0.3 M with sodium acetate and precipitated with ice cold 95% EtOH overnight at -20 ^{o}C. The DNA was then pelleted by centrifugation at 10,000 rpm for 30 min at -2 ^{o}C, and the DNA was resuspended in TE2 buffer, pH 7.5.

3.6 Hydroxyapatite Chromatography.

DNA grade hydroxyapatite (HAP) was purchased from Bio-Rad Laboratories (Control No. 16225). The HAP was prepared by washing about 30 g with 300 ml of 0.01 M sodium phospate (NaP) buffer, pH 7.2

by gentle swirling. The crystals were allowed to settle for 10 min and the fines were decanted. This washing was repeated once more. The slurry was finally resuspended in 100-150 ml of 0.01 M NaP and stored at 4 ^{o}C.

Plastic (10cc) syringes were used as columns. Glass wool was used as gel support. Columns were prepared by pipetting 8-10 ml of slurry into the column. This gave about 1-2 ml of packed HAP. The column was then washed with small volume of 0.02 M NaP pH 7.2 and the sample added. A 50 ml NaP gradient, either 0.05 M-0.4 M or 0.1 M-0.3 M, was run through the column. Flow rate was adjusted to about 1 ml/min with a Buchler peristaltic pump. Fractions of 2 ml were collected. Usually 0.2 ml were drawn from each fraction and added to 4 ml of ACS scintillation fluid (Amersham) with 0.4 ml of H_2O, and counted in a Beckman LS-230 liquid scintillation system. Peaks of radioactivity were collected and pooled and dialyzed against TE2 pH 7.5. The DNA was then precipitated as described in section 3.5 and resuspended in TE2 buffer. The concentration of DNA was determined by measuring the absorbance in Gilford 240 spectrophotometer at 260 nm. The molarity of the fractions was determined by determining the refractive index of each fraction with a Bausch & Lomb refractometer and the use of a standard curve. Recovery of DNA from the column was 80-85%.

Marker DNA (section 3.5) was sheared by sonication for 2 min with a small probe size at an output of 90 watts. Single-stranded marker DNA was obtained by denaturing cell DNA in a boiling waterbath and then cooling quickly on ice, or by adding 0.1 volume of 1 N NaOH, incubating for 10 min at room temperature, and then bringing the pH to neutral

with 0.1 volume of 1.5 M NaH_2PO_4.

3.7 Melting profile.

DNA samples were dialyzed against 0.1X SSC (15 mM NaCl + 1.5 mM sodium citrate) and were then brought to a final volume of about 2.5 ml. All samples contained a final concentration of about 10 ug DNA/ml. Increases in optical densities were measured by a Beckman spectrophotometer Acta III and recorded with a Hewlett-Packard 7044A X-Y recorder. A Lauda waterbath was used to increase the temperature of the sample chamber, and the temperature was recorded by a Yellow Springs Instruments (YSI) thermometer model 43 with the aid of a YSI thermistor model 427.

3.8. Gradients.

3.8.1 CsCl gradients.

Buoyant density of BPV DNA was determined by mixing the DNA sample with CsCl dissolved in TE2 buffer pH 8.0 (1.71 g/cm^3) in 5 ml nitrocellulose tubes. The sample was then centrifuged in a SW 50.1 rotor at 30,000-33,000 rpm 40-48 h at 18 ^{o}C. Fractions of five to ten drops (0.1-0.2 ml) were collected by bottom puncture directly into minivials and the density of the CsCl was determined from refractive indices. Gradient-Solv (4 ml; Beckman) along with 0.2 ml of H_2O were then added to the vials and the fractions counted by liquid scintillation spectrophotometry.

Alkaline CsCl was prepared by making neutral CsCl 0.2 N in NaOH. Centrifugation was carried out as described above, except polyallomer tubes were used.

3.8.2 Sucrose gradients.

Alkaline sucrose was prepared according to Koczot et al., (1973), containing 0.3 M NaOH, 0.7 M NaCl, 1 mM EDTA, and 0.15% sarkosyl (pH 12.1). The sample, 0.1 ml, was layered on top of a 5-20% gradient in 5 ml nitrocellulose tube, and centrifuge in a SW 50.1 rotor at 42,000 rpm for 4 h at 20°C. Fractions were either counted directly in 4 ml of ACS containing 0.4 ml of H_2O, or 50 ul of each fraction were transferred to mini-vials containing 3 ml of ACS and 0.2 ml of H_2O, counted and the fractions containing peak radioactivity were collected and dialyzed.

Two types of neutral sucrose gradients were prepared containing either no salt or 1.0 M NaCl (high salt). These gradients were centrifuged for 3 h at 39,000 rpm at 20 °C, and fractions collected and counted as described above.

3.9 Enzyme Assays.

All viral DNA was purified on alkaline sucrose gradients and only the peak of radioactivity, corresponding to "unit length" DNA, was used in these enzyme studies.

3.9.1 Exonuclease I.

This enzyme was a generous gift of Dr. S. Mitra, and assay conditions were as described previously (Mitra and Stallions, 1976).

Exonuclease I reaction mixture contained 0.67 M sodium glycinate pH 9.5, 0.067 M $MgCl_2$, and 0.01 M 2-mercaptoethanol. Exonuclease I diluent, used to stabilize the enzyme, contained 0.05 M tris-HCl,

0.25 M $(NH_4)_2SO_4$, and 1 mg/ml bovine serum albumin (Sigma Chemical Co.). Nicked and denatured M13 RF I DNA was prepared by mixing 20 ul of the DNA sample with 10 ul of 1 M tris-HCl pH 8.6, 2 ul of 0.1 M DTE, 6 ul of 0.1 M $MgCl_2$, 10 ul of DNase (100 ng/ml), and H_2O to 200 ul. Incubation was carried out in a 37 ^oC waterbath for 15 min. The reaction was stopped by adding 5 ul of 0.2 M EDTA, and the DNA was denatured in a boiling waterbath for 3 min and then chilled on ice.

About 0.1 ug of viral ^3H-DNA (specific activity ca. 8 x 10^4 cpm/ug) was mixed with 30 ul of exonuclease I cocktail, 5 ul of exonuclease I diluent and 5 ul of exonuclease I. The mixture was incubated for 30 min in a 37 ^oC waterbath, and the reaction was then stopped by adding 500 ul of 10% TCA containing 0.2 M sodium pyrophosphate (PP_i). Calf thymus DNA (Calbiochem; 10 ul; 2.5 mg/ml in TE2) was added as carrier and 50 ul of 0.1 M PP_i was also added. The samples were then kept on ice for 10 min. Precipitated DNA was collected on Whatman GF/C filters that had been presoaked in 0.1 M PP_i. The filters were first placed on a Millipore 12 place manifold. The sample was then diluted with 5 ml of ice cold 5% TCA and poured onto the filters. The tubes were washed once with 5% TCA, and the filters were washed a third time with 5% TCA. This was followed by three 5 ml washes of ice cold 95% EtOH. The filters were dried under a heat lamp, and then placed in vials and counted in 3 ml of scintillation fluid (toluene-PPO-POPOP).

3.9.2 S1 Endonuclease.

S1 endonuclease was purchased from Sigma Chemical Co. (Cat. no. N-5255) and assay conditions were determined by digesting denatured

cell DNA with different concentrations of the enzyme. Values reported here are obtained by digestion with 20 U of S1 endonuclease for 1 h at 37 °C.

Assay conditions were essentially the same as those described by Bourguignon et al. (1976). The sample to be digested (0.1-0.2 ug DNA) was diluted to 100 ul with TNE buffer pH 7.4 (10 mM tris-HCl; 1 mM EDTA; 0.1 M NaCl). Denatured calf thymus DNA was added to a final concentration of 5 ug/ml. Then 20 U of S1 endonuclease were added and the sample diluted to 1 ml with S1 endonuclease reaction mixture (0.03 M sodium acetate; 0.3 M NaCl; 2 mM $ZnCl_2$; pH 4.4) and incubation carried out for 1 h at 37 °C.

The DNA was precipitated onto filters and the amount of acid-insoluble radioactivity was determined as described in section 3.9.1, except 1 ml of 10% TCA containing 0.2 M PP_i was used initially.

3.10 Base Composition.

3.10.1 3'-mononucleotides.

A modification of the technique described by Shatkin (1969) was used. Purified ^{32}P-labelled BPV DNA with a specific activity of 5×10^6 cpm/ml or 2×10^4 cpm/ug was suspended in 0.01 M tris-HCl pH 8.6. Thirty ul of this solution was mixed with 5 ml of $CaCl_2$-tris buffer (0.4 M tris-HCl pH 8.6; 0.2 M $CaCl_2$) and 5 ul of micrococcal nuclease (30 U/ml in 0.1 M potassium phosphate buffer (KP) pH 7.3). This was followed by a 2 h incubation in a 37 °C waterbath. Then 20 ul of 0.5 M KP pH 7.0 was added to decrease the pH to 7.0, along with 10 ul of spleen phosphodiesterase (19 U/ml in H_2O; Worthington

Biochemical Corporation; Cat. no. 3603). This was followed by a 1 h incubation and then an additional 10 ul of phosophodiesterase was added and the sample reincubated. This was repeated once more, for a total of 30 ul of phosphodiesterase and 3 h of incubation. At the end of the incubation period 50 ul of each of the four 3'-mononucleotides (Sigma Chemical Co.; 2 mg/ml H_2O) were added and 75-150 ul were put in a small spot on a Whatman no. 1 filter paper strip (4 x 40 cm). Paper chromatography was carried out in an ascending fashion in a NH_4OH-isobutyric acid buffer (33 ml 2.1 N NH_4OH + 67 ml isobutyric acid) overnight at room temperature. The spots were located under UV light (UVS-12), circled, cut out and counted in 4 ml of toluene-PPO-POPOP. As a control for recovery of radioactivity, the same sample size as was used for spotting was put on filter paper and counted directly.

3.10.2 5'-mononucleotides.

A modification of the method described by Crawford et al. (1969) was used. ^{32}P-labelled BPV DNA was mixed with denatured calf thymus DNA in a total volume of 0.5 ml, containing a total of 100 ug of DNA and 4 x 10^4 cpm, in $MgSO_4$-tris buffer (5 mM $MgSO_4$ + 5 mM tris-HCl; pH 8.5). Aliquots (0.1 ml) of this solution were treated with DNase (in 0.01 M tris-HCl pH 8.0) at a final concentration of 100 ug/ml for 2 h at 37 ^oC. Snake venom phosphodiesterase (2.5 mg/ml H_2O Worhington Biochemical Corporation; Cat. no. 3926) was then added to a final concentration of 200 ug/ml and incubation continued for additional 2 h. At the end of the incubation period 25 ul of each of the four 5'-mononucleotides (Sigma Chemical Co.; 2 mg/ml H_2O) were

added. The DNA digest was then spotted, chromatographed, located and counted as described above.

3.11 Hybridization.

All the DNA samples used in these studies were dialyzed against an excess of 0.1X SSC and chemically denatured as described in section 3.6. ^{14}C-DNA was attached to nitrocellulose filters (S&S; 25 mm) as described by Gillespie and Spiegelman (1965). The filters were presoaked in 6X SSC for 20 min and then placed on a Millipore 12 place manifold, where they were washed 1X with 10 ml of 6X SSC. The samples were made 6X SSC and the volume was brought to 10 ml. The DNA was then adsorbed to the filters, each filter containing about 2 ug of DNA. The filters were washed 5X with 10 ml of 2X SSC, and finally placed into clean glass scintillation vials and incubated overnight at 60 ^{o}C.

Hybridization was carried out essentially as described by Green et al. (1969). ^{3}H-DNA probes were denatured as described above. The samples were made 6X SSC and the volume brought to 1 ml. The samples, containing about 0.1 ug of DNA, were then made 0.1% in SDS and incubated with the filters at 66 ^{o}C for 24 h. After incubation, the filters were removed from the vials and washed 2X with 300 ml of 3 mM tris-HCl pH 9.2. The filters were then placed on the manifold and washed 10X with 10 ml of the same buffer. Filters were finally dryed under a heat lamp and counted in 3 ml of toluene-PPO-POPOP.

3.12 Electron Microscopy.

Electron microscopy of nucleic acids is being done routinely in our

laboratory by Rebecca B. Young. The method she uses has been described in detail elsewhere (Inman and Schnos, 1970) and will not be described further.

4. Results.

4.1 Hydroxyapatite chromatography.

Hydroxyapatite chromatography (HAP) has been shown to be an effective method for the separation of single- and double-stranded DNA (Bernardi, 1971). Furthermore, it has been shown that single-stranded polynucleotide chains with short duplex regions, about 30-1000 base pairs, will elute from HAP in an intermediate position between single- and double-stranded DNA (Wilson and Thomas, 1973). Because of the palindromes present at both termini of the parvoviral genomes, they would be expected to behave in this manner, when chromatographed on HAP.

When purified BPV DNA was chromatographed on HAP, two peaks of radioactivity were observed (Fig. 1). Peak I DNA eluted at a sodium phosphate (NaP) concentration of 0.17 M, intermediate between single- and double stranded DNA markers, which eluted at 0.12 M and 0.24 M NaP respectively. Peak II DNA eluted in the same position as double-stranded DNA.

Bernardi (1965) observed that native DNA sometimes eluted from HAP at two different concentrations, although there was no apparent difference between the DNA's in the two peaks. He referred to the peak eluting at the higher buffer concentration as a "false peak". The possibility that peak II represented such a false peak could not be overlooked. However, when peak II DNA was rechromatographed on HAP, it still eluted at 0.24 M NaP. Furthermore, heat denaturation and quick cooling of BPV DNA preparations caused almost all the DNA to elute in

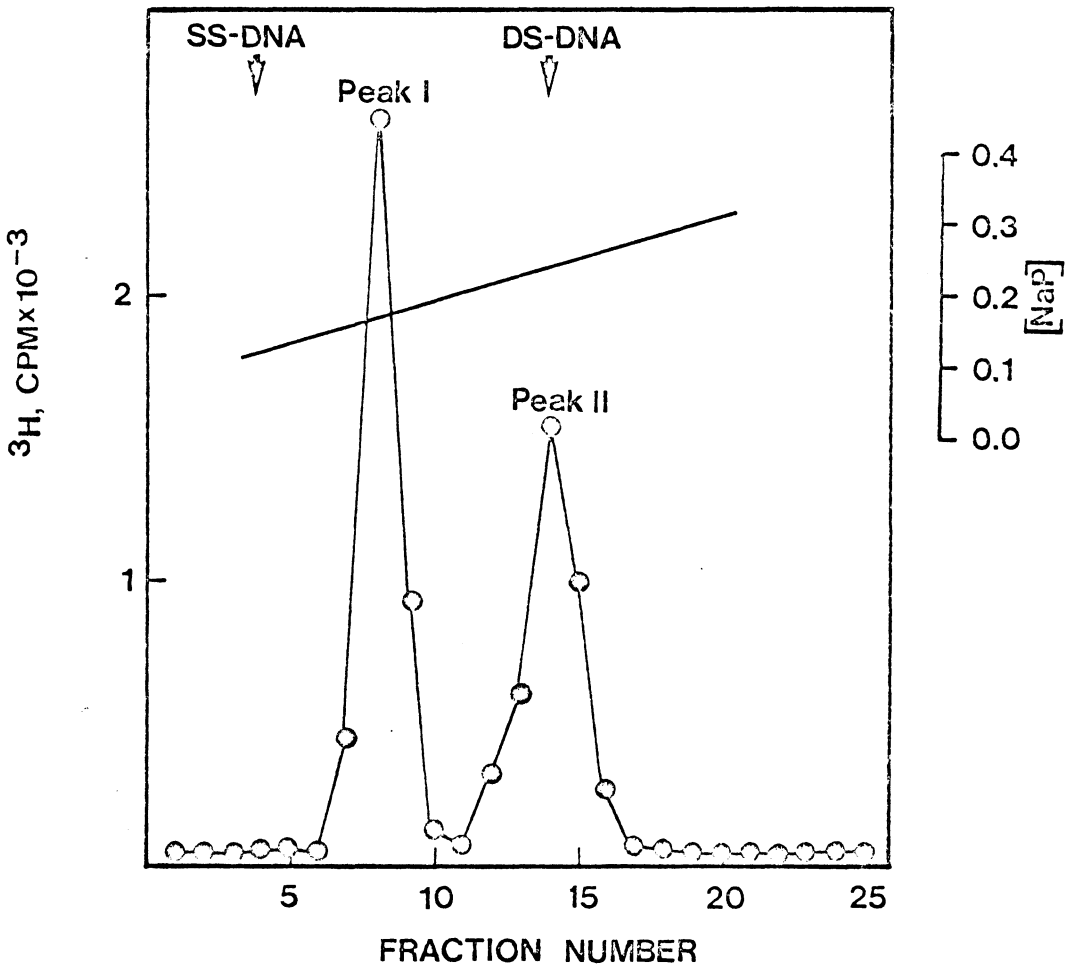

Figure 1. Hydroxyapatite profile of BPV DNA. The virus was isolated from infected Bu cells. Arrows indicate the position of single- and double-stranded DNA markers.

peak I (Fig. 2), but heat denaturation and slow cooling resulted in the reappearance of peak II (not shown). The ratios between the two peaks were the same as observed before denaturation.

An interesting observation was that the ratio between the amount of peak I and peak II DNA seemed to vary depending on the cell type used for propagation of the virus. In the case of BFS and BT cells, which are both of bovine origin, the ratio was about 2/3 peak I to 1/3 peak II. However, if Bu cells were used the ratio between the two peaks was just about equal (Table 3).

It was of interest to see how DNA's isolated from other parvoviruses would behave on HAP. I chose to examine KRV DNA and AAV-2 DNA. KRV is an autonomous parvovirus of rodent origin that presumably only packages the minus strand. AAV-2, however, is a defective parvovirus that packages both plus and minus strands into separate virions in equal proportions. As expected, the bulk of KRV DNA eluted at 0.17 M NaP, but a minor fraction (about 10%) eluted at 0.24 M NaP. Moreover, no AAV-2 DNA eluted at 0.17 M NaP, but eluted instead in two distinct peaks at 0.20 M and 0.24 M NaP respectively (Fig. 3). The possible nature of the peak eluting at 0.20 M will be discussed later.

Because of the characteristic elution profile of peak I DNA, and the fact that it eluted in the same position as KRV DNA, this peak was believed to represent the single-stranded BPV genome. Furthermore, the elution of the main portion of BPV DNA, intermediate between single- and double-stranded markers, clearly indicated that some secondary structure was present in the BPV genome. The origin of BPV peak II DNA was obscure, but the possibility that it was of viral origin could not

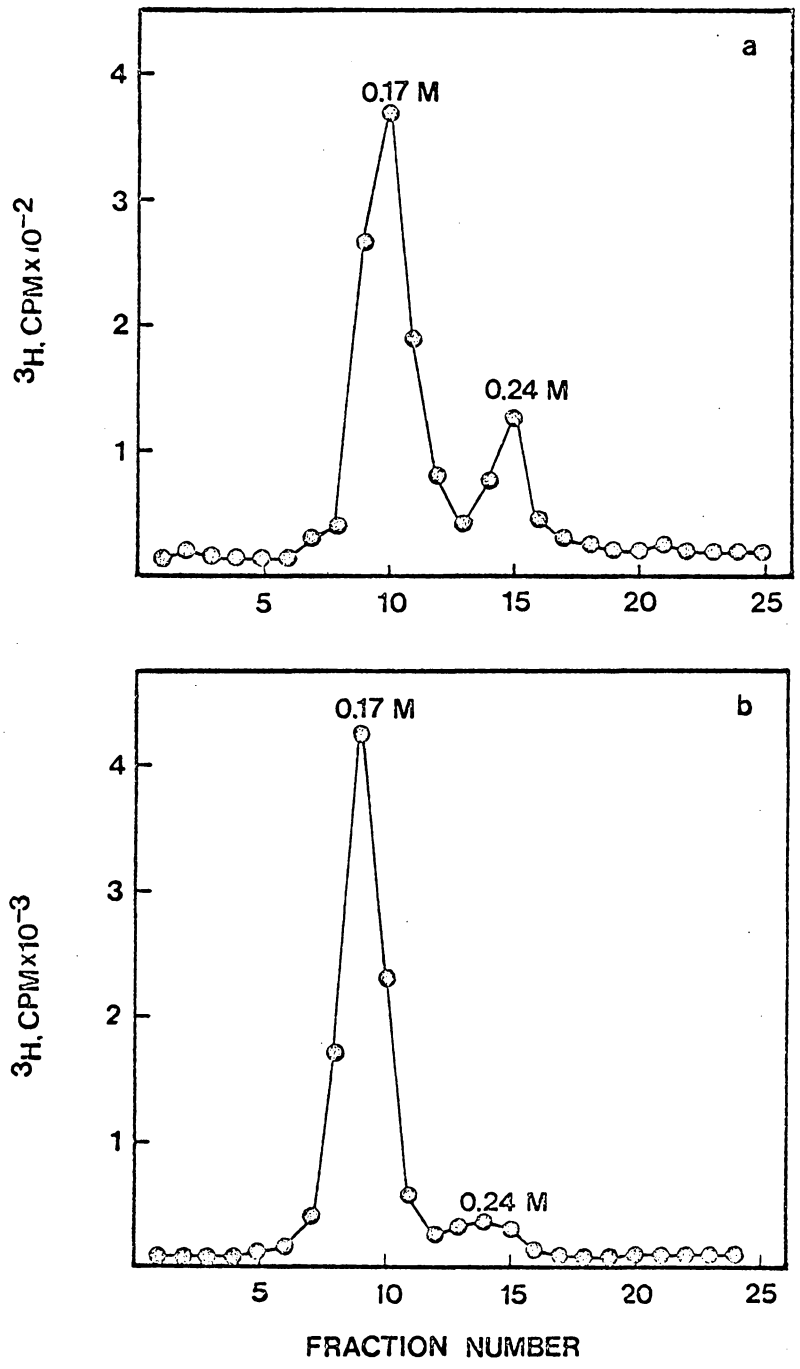

Figure 2. Hydroxyapatite profile of BPV DNA. a) Before denaturation; b) After heat denaturation and quick cooling.

Table 3. Percent distribution of BPV DNA in peaks I and II after chromatography on hydroxyapatite.[a]

cell type	% peak I	% peak II	No. trials
BFS	74	26	3
BT	68	32	3
Bu	48	52	3

[a]All cells were low passage. Percent values were determined by comparing the total radioactivity in the two peaks and/or by measuring the area under the peaks. These values were directly comparable to the microgram amounts of DNA in each peak, as measured by spectrophotometry at 260 nm.

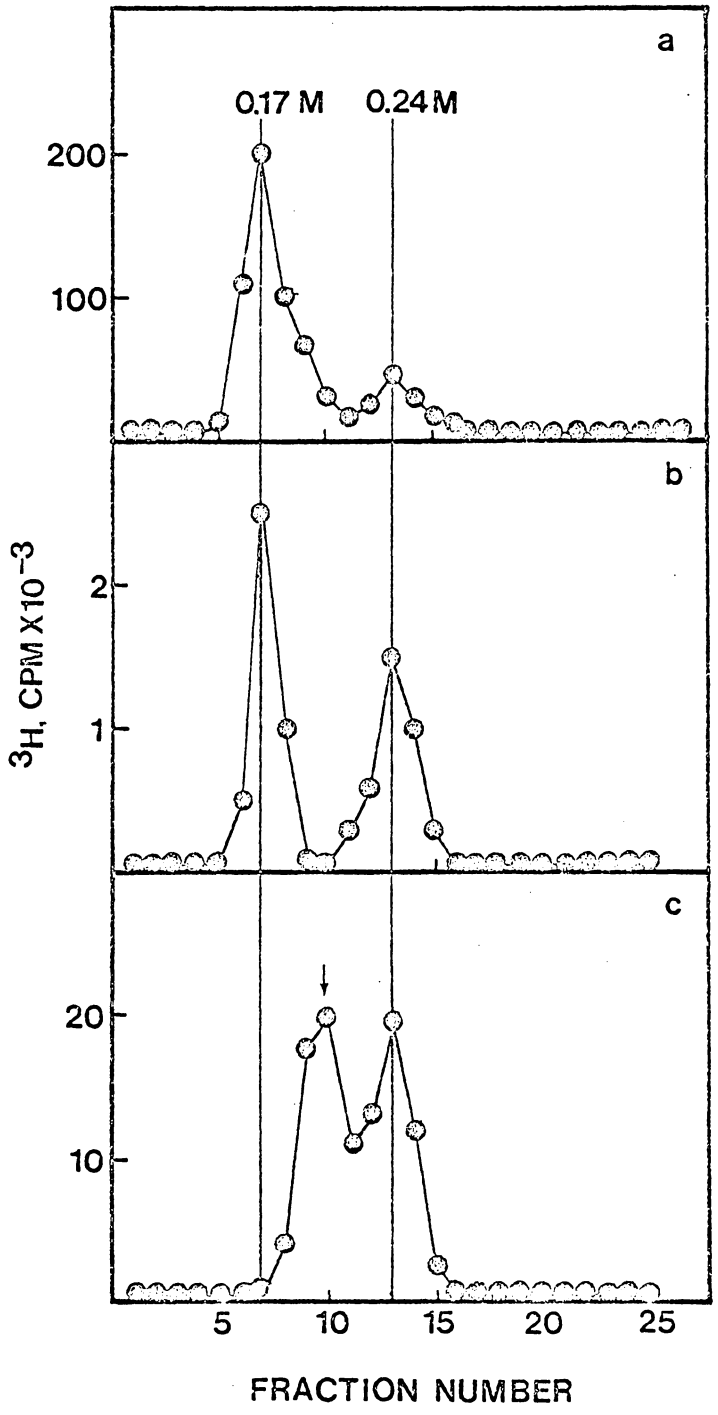

Figure 3. Hydroxyapatite profile of three parvoviral DNA's. a) KRV DNA; b) BPV DNA; c) AAV-2 DNA. The arrow indicates the position of the peak eluting at 0.20 M NaP.

be ignored.

4.2 Exonuclease I.

Bacterial exonuclease I has been shown to be specific for the 3' end of completely single-stranded DNA (Lehman, 1966). If a palindrome is present at the 3' end of single-stranded DNA, that DNA would be resistant to digestion with exonuclease I.

About 85% of the BPV DNA, from peak I, was found to be resistant to digestion with exonuclease I. RF I of bacteriophage M13, which is circular double-stranded DNA, was completely resistant, but became almost completely susceptible, when nicked and denatured (Table 4). These results indicated that some secondary structure is present at or close to the 3' end of the BPV genome. Peak II DNA was also found to be about 85% resistant to digestion with exonuclease I after denaturation, indicating the possibility of some secondary structure on the 3' ends of the denatured DNA (Table 4).

4.3 S1 Endonuclease.

The presence of secondary structure, in the BPV genome, could be further demonstrated by treating the DNA with S1 endonuclease. This enzyme has been found to be specific for single-stranded DNA and RNA (Vogt, 1973).

Peak I DNA was found to be about 7% resistant to digestion with S1 endonuclease. This figure was an average of three separate determinations with a range from 5.4%-8.3%. Under similar conditions, KRV DNA was found to be about 6% resistant to digestion with S1

Table 4. Digestion with exonuclease I.

Sample	Without enzyme (cpm)	With enzyme (cpm)	Resistance (%)
BPV DNA, peak I	8341	7097	85.1
BPV DNA, peak II (denatured)	6367	5389	84.6
M13 RF I	4542	4767	105.0
M13 RF I (nicked & denatured)	5199	136	2.6

endonuclease. Peak II DNA was about 70% resistant to S1 endonuclease digestion, indicating that it had considerable double-stranded character. Double-stranded cell DNA was more than 95% resistant to digestion with S1 endonuclease, while denatured cell DNA was more than 99% susceptible (Table 5).

4.4 Melting profile.

Studies on the melting profile can give valuable information about the configuration of DNA, as well as the base composition of double-stranded DNA (Marmur and Doty, 1962). As double-stranded DNA is heated gradually from 20 ^{o}C to 100 ^{o}C the two strands will be separated and the absorbance at 260 nm increases. The temperature at which half the strands are separated is the transition temperature (T_m), which is directly related to the GC content of the double-stranded DNA (Marmur and Doty, 1962).

Figure 4 shows that, when peak I DNA was melted, it did not show the characteristic sharp melting curve, as did the double-stranded calf thymus DNA and BPV peak II DNA. However, BPV peak I DNA did show a slight transition at about 57 ^{o}C that could indicate that a short AT rich duplex was being melted. T_m values for calf thymus DNA and BPV peak II DNA were determined to be 68.5 ^{o}C and 73.5 ^{o}C, respectively. Assuming that there is a direct relationship between the T_m and the GC content and taking the percent GC of calf thymus DNA to be 42% (Marmur and Doty, 1962) yielded GC ratio of 45% for BPV peak II DNA. Frank-Kamenetskii (1971) has derived at a formula for calculating the %GC of double-stranded DNA based on T_m and the ionic strength,

Table 5. Digestion with S1 endonuclease.

Sample	Without enzyme (cpm)	With enzyme (cpm)	Resistance (%)
BPV DNA, peak I	2569	174	6.8
BPV DNA, peak II	2104	1426	67.8
KRV DNA	55234	2002	5.7
Cell DNA	52805	50640	95.9
Cell DNA (denatured)	123022	873	0.7

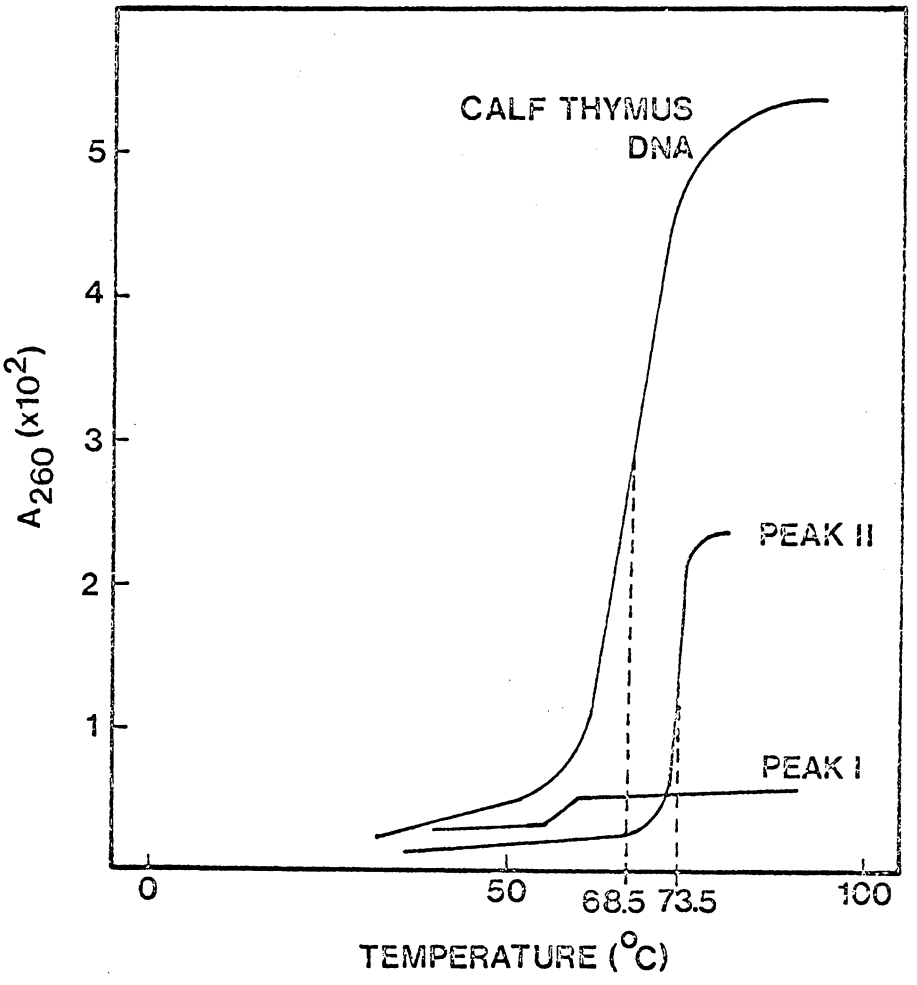

Figure 4. DNA melting profiles. All samples were dissolved in 0.1X SSC. T_m values for calf thymus DNA and BPV peak II DNA are shown.

% GC = [tan(70.077 + 3.32(log $[Na^+]$)] (T_m - 175.95) + 260.34.

Since the DNA samples were in 0.1X SSC, instead of the more classical 1X SSC, this formula can be used to obtain direct estimates of the GC content. However, the use of this formula yields a %GC of 39% for calf thymus DNA and 49% for BPV peak II DNA. These values are obviously somewhat different from the values obtained above.

4.5 Base composition.

In double-stranded DNA, adenine (A) equals thymine (T) and guanine (G) equals cytosine (C). But where single-stranded DNA is concerned, this does not necessarily have to be the case. Thus if a DNA does not contain equimolar amounts of A and T and of G and C, it can usually be concluded that the DNA in question is not double-stranded (Crawford et al., 1969).

In the case of parvovirus DNA, direct chemical analysis is difficult, because of the difficulty in obtaining sufficient amounts of DNA. Uniform labelling of the DNA with ^{32}P-orthophosphate and subsequent enzyme digestion into 3' or 5' mononucleotides has been commonly employed in elucidating the base composition of several parvoviral DNA's (Table 2).

In this study, the base composition of BPV DNA was determined, not so much to show that the BPV genome is single-stranded, but rather to compare the base composition of BPV DNA to that of other parvovirus DNA's. Table 6 shows the average of five individual determinations for each mononucleotide. Only those values that corresponded to a recovery of more than 90% of the nucleotides were accepted. As was expected,

Table 6. Base composition of bovine parvovirus DNA[a]

	3'-Mononucleotides.			
	A	T	G	C
	20.6	30.7	22.0	24.2
	22.6	31.5	22.4	23.9
	25.5	29.7	25.0	24.0
	22.7	29.0	21.6	22.6
	24.8	28.4	26.2	22.7
	5'-Mononucleotides.			
	A	T	G	C
	20.3	33.1	23.1	22.9
	25.7	35.7	21.1	22.9
	25.4	29.2	21.2	22.1
	20.6	34.1	21.2	24.1
	25.5	33.4	19.9	20.6
$(\bar{x})$	23.2	31.3	22.2	22.9
(S.E.)	$\pm$0.7	$\pm$0.7	$\pm$0.5	$\pm$0.4

[a]Highest and lowest values for each base determination were discarded.

thymine was the most common base, representing 31.3% of the total number of bases. Adenine represented 23.3%, while guanine and cytosine represented 22.2% and 22.9% respectively. These determinations corresponded to a GC ratio of 45.1%.

4.6 Buoyant density.

Measurement of the buoyant densities in CsCl gradients has become a standard physical parameter of viruses and nucleic acids. In this study BPV peak I DNA was found to have a buoyant density of 1.721 g/cm^3 in neutral CsCl (Fig. 5; Table 7), and BPV peak II DNA was determined to have a buoyant density of 1.706 g/cm^3 (Table 7). The buoyant density of BPV peak I and II DNA in alkaline CsCl was determined to be 1.763 g/cm^3 and 1.755 g/cm^3 respectively (Table 7).

Schildkraut *et al.* (1962) have derived at a formula ($\underline{p}$ = 1.660 + 0.098(GC)) for calculating the GC ratio of double-stranded DNA from its buoyant density in neutral CsCl. By substituting the buoyant density of BPV peak II DNA into this formula, a value of 47% GC is obtained, which correlates closely with the GC ratio obtained from the T_m.

4.7 Velocity sedimentation in sucrose.

The sedimentation rate of DNA depends upon the effective mass of the molecule as well as upon shape and size. The relationship between the sedimentation coefficient (S) and the molecular weight (M) is depicted in the Svedberg equation; $M = RTS/(1 - \bar{v}p)D$, where R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume

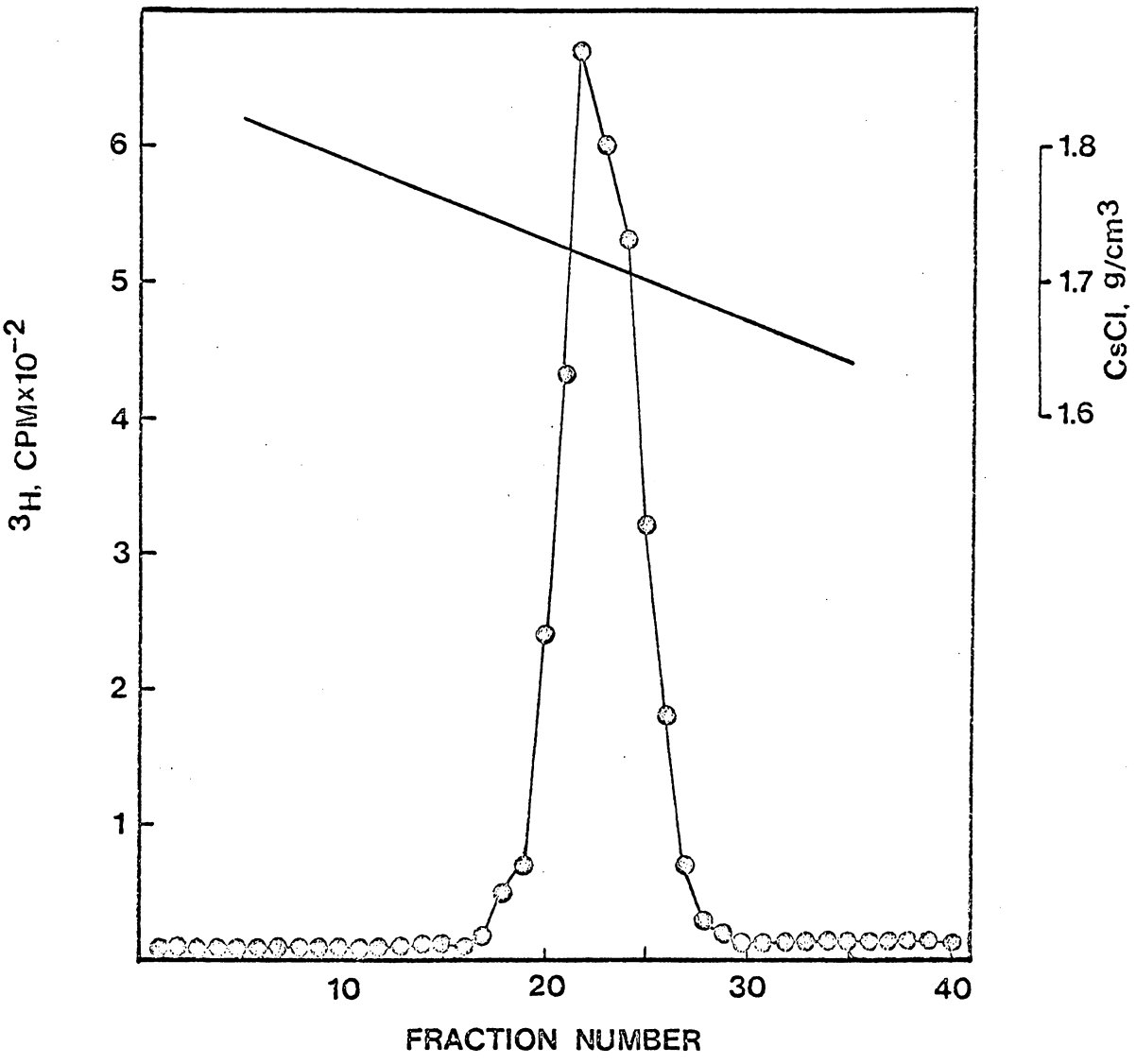

Figure 5. Density of the BPV genome in neutral CsCl. Sedimentation is from right to left.

Table 7. Buoyant densities of BPV DNA in CsCl.

	Peak I	Peak II
Neutral CsCl	1.721 ± 0.001 (2)[a]	1.706 ± 0.001 (2)
Alkaline CsCl	1.763 ± 0.003 (2)	1.755 ± 0.003 (2)

[a]Buoyant densities are in g/cm^3; Numbers in parenthesis represent number of determinations.

of the DNA, $\underline{p}$ the density of the solution, and D the diffusion coefficient (Mazzone, 1967). This equation can be somewhat simplified by determining the relationship for S and M for a number of molecules with known molecular weights, and assuming they all have the same hydrodynamic properties under the same standard conditions. The most direct approach is the co-sedimentation of labeled DNA (e.g. ^{3}H), of a known molecular weight, with DNA of an unknown molecular weight containing an alternate label (e.g. ^{14}C or ^{32}P). The ratio between the distance sedimented can be related to the ratio between the molecular weights (Studier, 1965; Burgi and Hersey, 1963; Thomas and MacHattie, 1967).

BPV DNA from peak I was found repeatedly to sediment one fraction faster than KRV DNA and two fractions faster than AAV-2 DNA in alkaline (pH 12.1) sucrose (Fig. 6). In an alkaline gradient all secondary structure is completely denatured, and the DNA is completely stretched out, hence the sedimentation properties will depend primarily on the molecular weight of the DNA. KRV DNA has been determined to have a sedimentation coefficient of 16 S in an alkaline gradient (Salzman and Jori, 1970), while AAV-2 DNA has a sedimentation coefficient of 15 S (Gerry $\underline{et\ al.}$, 1973). Assuming that the gradient is linear, the sedimentation coefficient of BPV DNA was determined to be approximately 16.5 S. Studier (1965) has derived an equation ($S^{0}_{20,w} = 0.0528\ M^{0.400}$) for calculating the molecular weight of DNA in alkaline sucrose. If the sedimentation coefficient obtained for BPV in this study is substituted for $S^{0}_{20,w}$ in Studier's equation, it yields a molecular weight of about 1.7×10^{6} for BPV DNA.

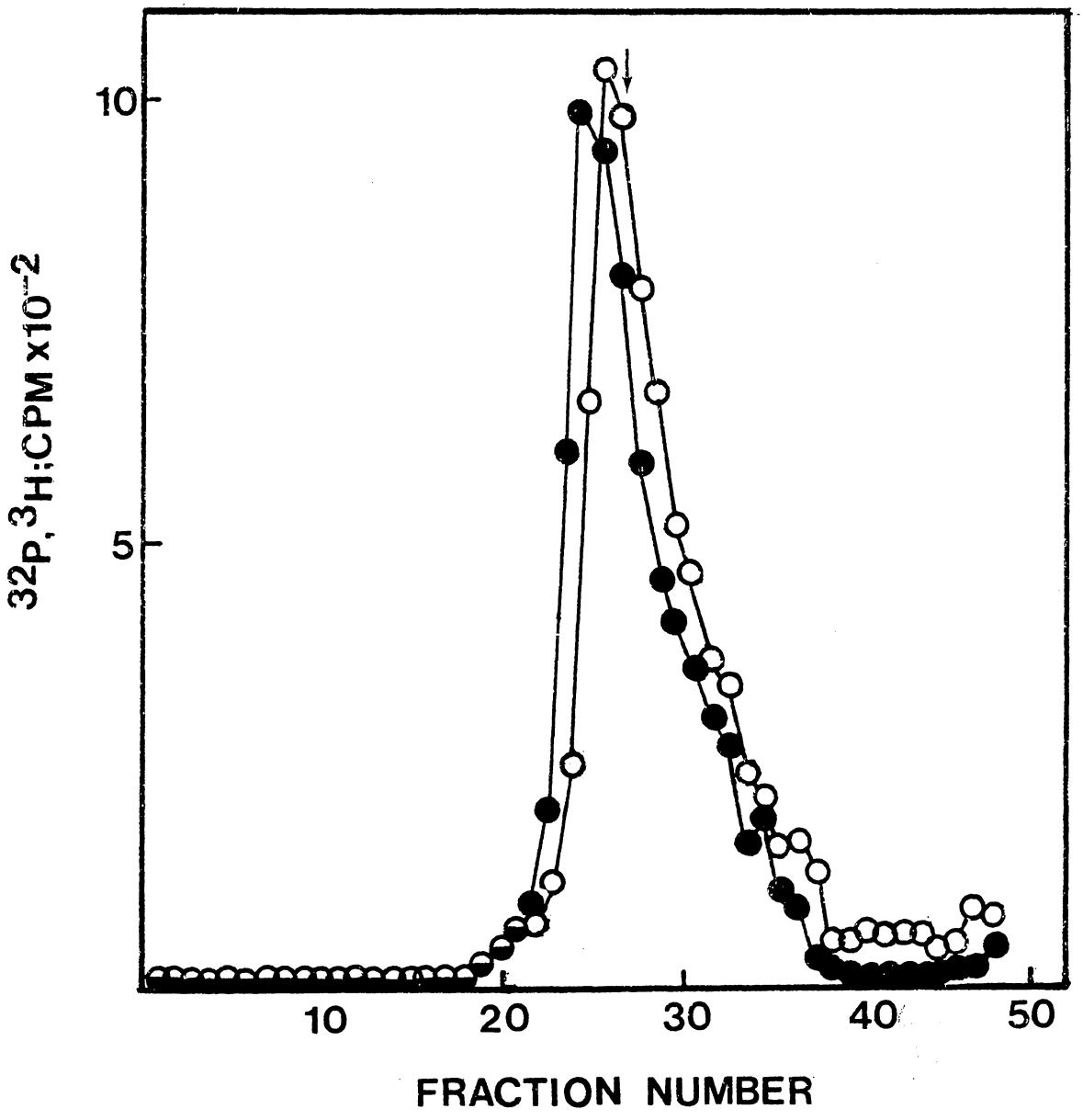

Figure 6. Co-sedimentation of BPV ^{32}P-DNA (●—●) and KRV ^{3}H-DNA (○—○) in alkaline sucrose. Sedimentation is from right to left. Arrow indicates the position of AAV-2 DNA on a similar gradient.

It was of interest to examine the sedimentation properties of BPV peak II DNA on alkaline sucrose. When BPV peak II DNA was centrifuged along with BPV peak I DNA through alkaline sucrose, both DNA's sedimented to the same position in the gradient (Fig. 7). This clearly indicated that peak II DNA was of the same size as peak I DNA. However, BPV peak II DNA sedimented farther in a neutral (pH 8.0) sucrose containing no salt, confirming its double-stranded nature (Fig. 8).

Although sedimentation coefficients derived from alkaline gradients have proven more useful in calculating molecular weights, sedimentation coefficients in neutral sucrose are more commonly reported in the literature. It was thus decided to determine the sedimentation coefficient of BPV DNA in neutral sucrose containing 1.0 M NaCl (high salt). In a gradient of this type, single-stranded DNA sediments faster than double-stranded DNA because of the high ionic strength. KRV DNA has been determined to have a sedimentation coefficient of 25 S in a gradient of this type (Lavelle, personal communication). BPV peak I DNA sedimented one fraction faster than KRV DNA on parallel gradients, which would correspond to a sedimentation coefficient of about 27 S for the BPV genome in neutral sucrose (Fig. 9). Single-stranded AAV-2 has a sedimentation coefficient of 24 S in neutral high salt sucrose, while double-stranded AAV-2 has a sedimentation coefficient of 15 S in such a gradient (Rose, 1974). Sedimentation of AAV-2 DNA and BPV peak II DNA on parallel gradients yielded a sedimentation coefficient of about 17 S for the double-stranded peak II DNA (Fig. 10).

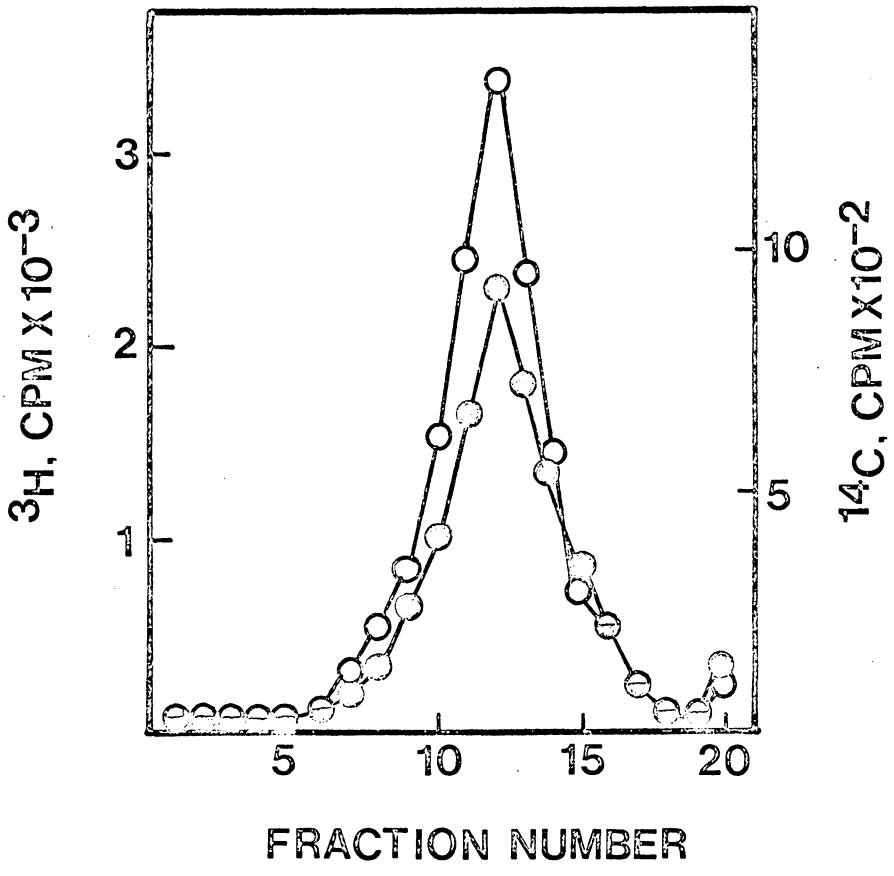

Figure 7. Co-sedimentation of BPV peak I ^{14}C-DNA (○——○) and BPV peak II ^{3}H-DNA (○——◎) in alkaline sucrose. Sedimentation is from right to left.

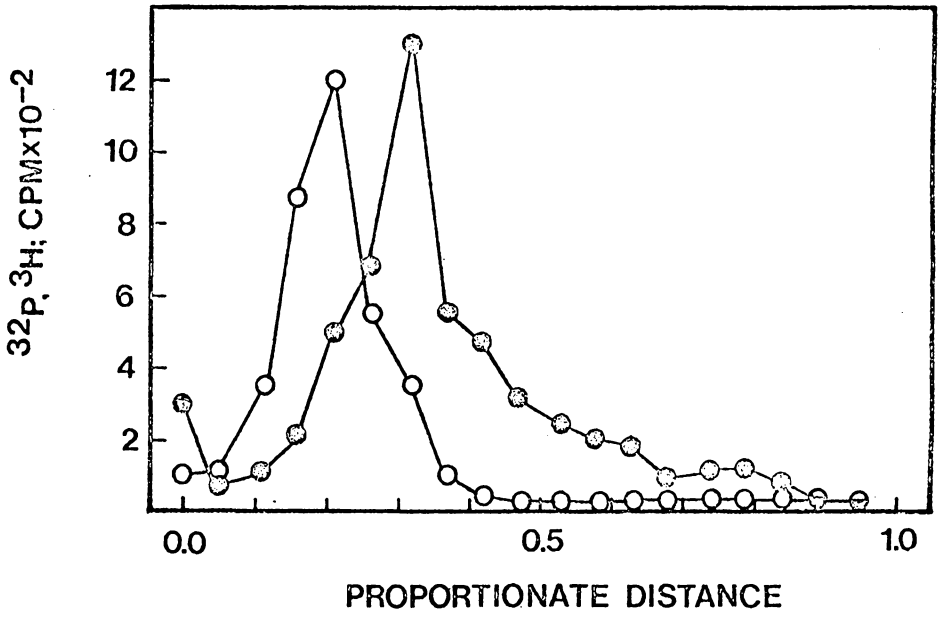

Figure 8. Co-sedimentation of BPV peak I ^{32}P-DNA (○—○) and BPV peak II ^{3}H-DNA (○——○) in neutral sucrose containing no salt. Sedimentation is from left to right.

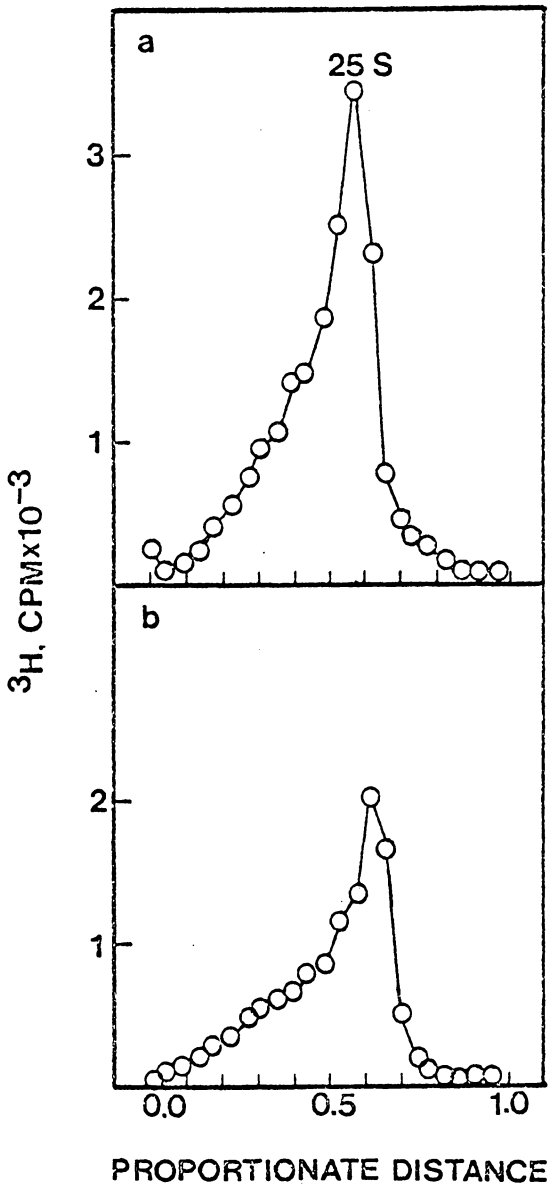

Figure 9. Sedimentation of parvoviral DNA's in parallel neutral sucrose containing 1.0 M NaCl (high salt). a) KRV DNA; b) BPV DNA from peak I. Sedimentation is from left to right.

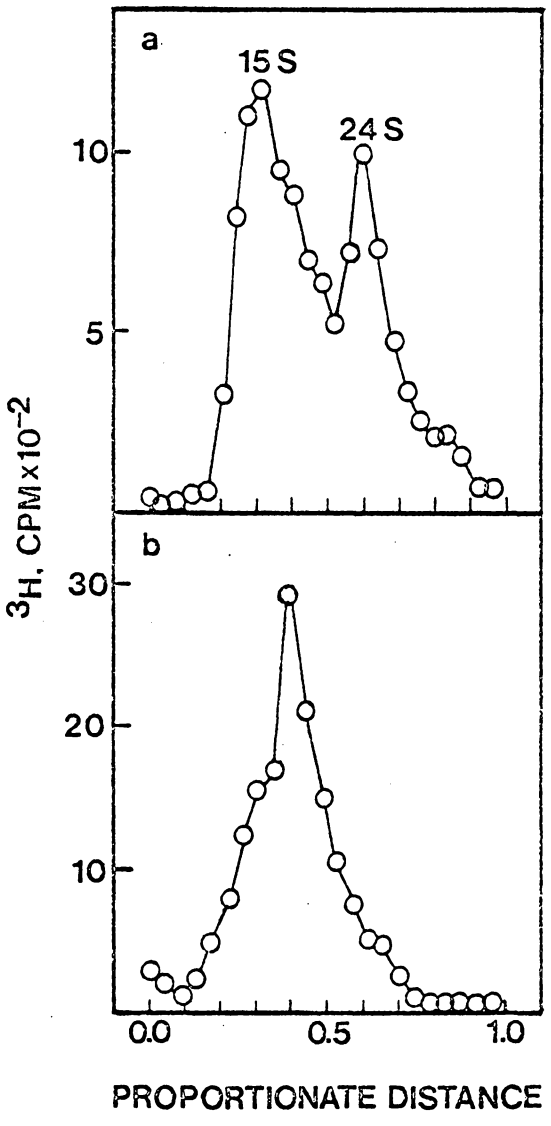

Figure 10. Sedimentation of parvoviral DNA's in parallel neutral sucrose gradients containing 1.0 M NaCl (high salt). a) single- (24 S) and double-stranded (15 S) AAV-2 DNA; b) BPV DNA from peak II. Sedimentation is from left to right.

4.8 Hybridization.

Hybridization between DNA and DNA or DNA and RNA has become a useful tool in analyzing the homology between nucleic acids of different viruses, in the isolation of viral specific mRNA from infected cells, and in the identification of viral replicative forms and transcription complexes (Green et al., 1969). Since the results have indicated, so far, that the peak II DNA is truly of viral origin, it was decided to see whether BPV peak I DNA would hybridize to peak II DNA in significant amounts.

Denaturation of peak II ^{32}P-DNA in the presence of ^{3}H-DNA from peak I and subsequent renaturation at high ionic strength and 60 ^{o}C causes some of the ^{32}P counts to be shifted into peak I and some of the ^{3}H counts to shifted into peak II (Fig. 11). Since the amount of DNA used in this experiment, and hence the DNA-DNA ratio, was not known, it was decided to conduct a more carefully planned experiment using the filter method. Furthermore, it was of interest to see if either peak I or peak II DNA would hybridize in significant amounts to cell DNA. Table 8 shows that over 70% of peak I DNA hybridized with peak II DNA under the conditions employed in this study, while radioactivity associated with cellular DNA was not significantly above background. These results clearly indicated that the peak II DNA was indeed of viral origin.

4.9 Electron microscopy.

The visualization of nucleic acids was very difficult until the surface spreading technique was developed (Kleinschmidt, 1968). Since

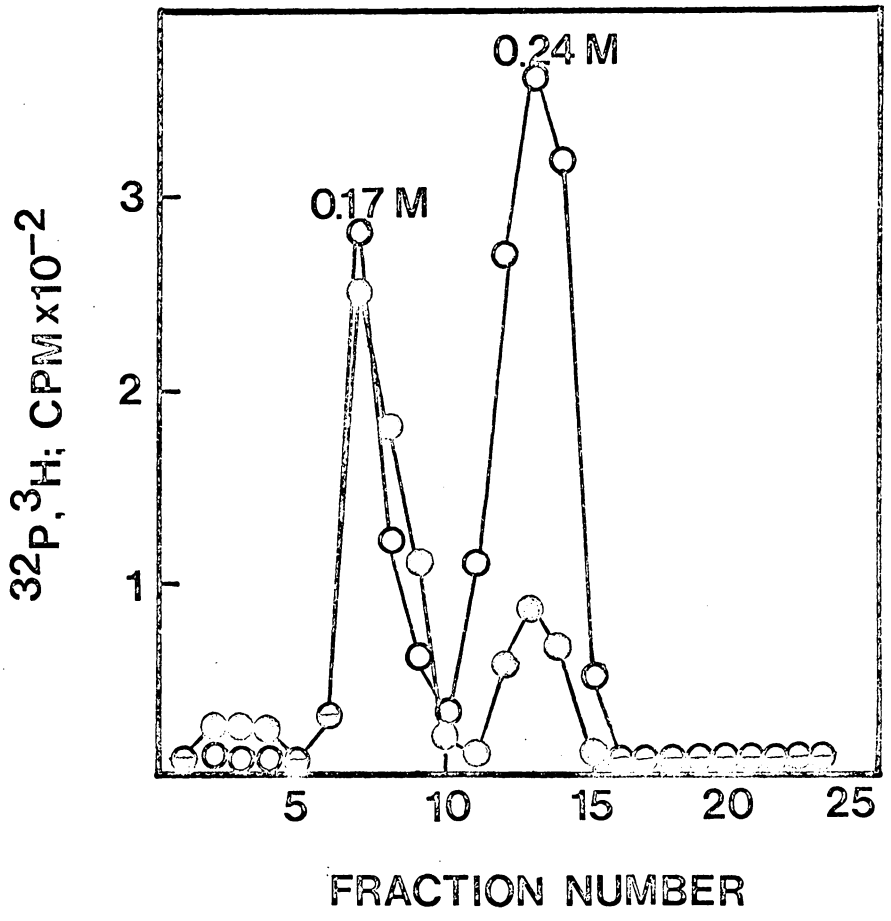

Figure 11. Hydroxyapatite chromatography of BPV peak II ^{32}P-DNA (O—O) denatured and then renatured in the presence of BPV peak I ^{3}H-DNA (O—O).

Table 8. DNA-DNA hybridization.

Input ^{3}H-DNA	Immobilized ^{14}C-DNA	Bound DNA (% of input)
BPV DNA, peak I (0.1 ug)	BPV DNA, peak II (2 ug)	71
	BFS cell DNA (2 ug)	0
BPV DNA, peak II (0.1 ug)	BPV DNA, peak II (2 ug)	67
	BFS cell DNA (2 ug)	0

then a great number of modifications of the original technique have appeared, making it possible to examine a number of different types of nucleic acids. A very important aspect of electron microscopy is, that it has made possible the estimation of the molecular weight of nucleic acids independent of other methods, by calculation of the linear density (daltons/um) of the nucleic acid in question (Thomas and MacHattie, 1967).

Time did not allow for the measurement of a sufficient number of molecules from electron micrographs to make a statistically accurate estimate of the molecular weight. However, electron micrographs were obtained of BPV peak I and II DNA that did make generalizations possible, about the configuration of these molecules. Figure 12 shows the linear single-stranded BPV genome (peak I). The length of this one molecule is about 1.5 um which corresponds well to the estimated molecular weight obtained from velocity sedimentation studies (section 4.7). Peak II DNA turned out to be, not surprisingly, a linear double-stranded molecule (Fig. 13). ØX174 RF II (New England BioLabs), which is circular double-stranded DNA, was used as an internal standard. The ØX174 RF II DNA has been shown to have a molecular weight 3.49×10^6 (Sanger et al., 1977) and close examination of figure 13 shows that the contour lengths of the two molecules are quite similar, indicating that the two molecules have similar molecular weights. If this holds true, the molecular weight of the peak II DNA is twice that of the BPV genome.

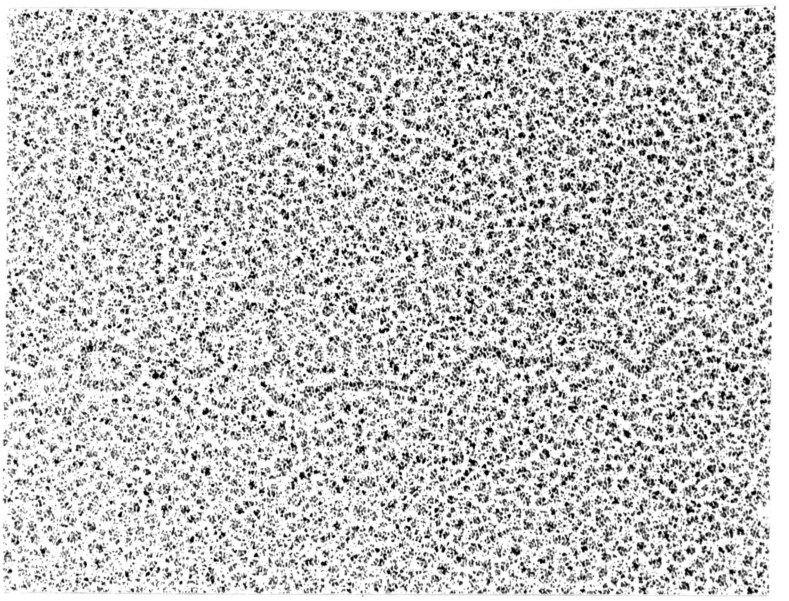

Figure 12. Electron micrograph of linear single-stranded BPV DNA from peak I. The DNA was mounted by the formamide technique and rotary shadowed with platinum-palladium. Magnification 95,000 x.

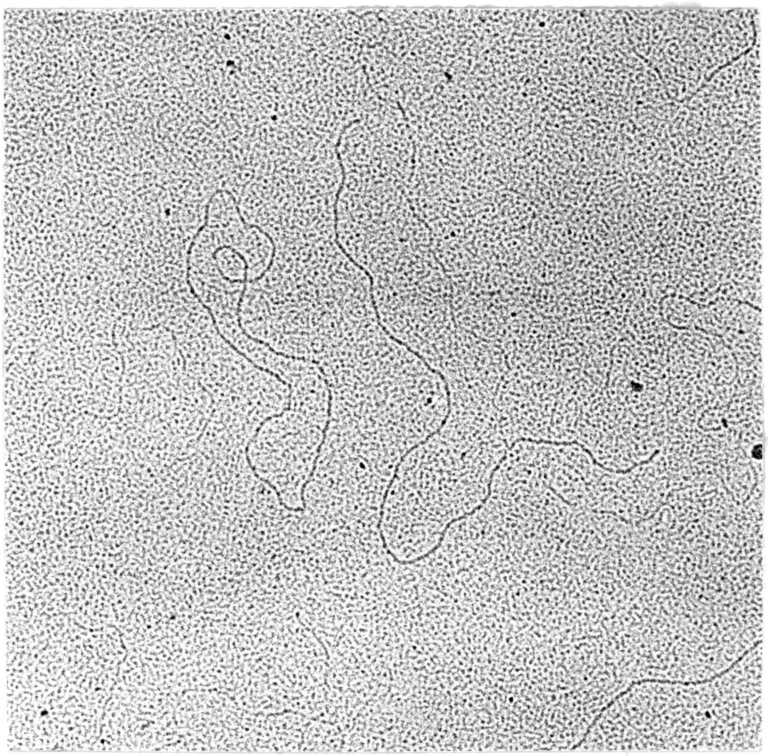

Figure 13. Electron micrograph of BPV peak II DNA. Circular double-stranded ØX174 RF II DNA (New England BioLabs) was added as an internal standard. The molecular weight of ØX174 RF II DNA is 3.49×10^6 daltons. Magnification 75,000 x.

5. Discussion.

5.1 Hydroxyapatite chromatography of BPV DNA.

Because of the palindromes, parvovirus DNA would be expected to elute from HAP columns in an intermediate position between single- and double-stranded DNA markers. This has been shown to be the case, where the DNA's of MVM (Bourguignon et al., 1976) and KRV (Gunther and May, 1976; Lavelle and Mitra, 1978) are concerned.

BPV DNA was found to behave in a similar manner, when chromatographed on HAP, except two peaks of radioactivity were consistently observed. Peak I eluted from HAP at 0.17 M NaP, intermediate between single- and double-stranded DNA. Peak II, however, eluted in the position of double-stranded DNA at 0.24 M NaP. This peak was shown to be a true peak, and furthermore, was shown to elute in the position of peak I after denaturation. This indicated that the DNA in peak II might be of viral origin. Because of this unexpected behavior of BPV DNA on HAP, it was decided to carry out HAP chromatography on other well characterized parvoviral DNA's.

When DNA isolated from KRV was chromatographed on HAP, most of the radioactivity eluted in a position characteristic of parvoviral genomes. However, a small portion (about 10%) of the KRV DNA eluted as double-stranded. Since AAV-2 has been found to encapsidate both the plus and the minus strands into separate virions (Rose, 1974), it was of interest to see how DNA isolated from AAV-2 would behave on HAP. If the isolation procedure used in this study allows for the renaturation of

single-stranded complimentary strands most of the AAV-2 DNA would be expected to elute in the position of double-stranded DNA. This was indeed the case. No AAV-2 DNA eluted at 0.17 M NaP, however, a significant portion of the DNA eluted at 0.20 M NaP. Whether the DNA eluting at 0.20 M NaP represents panhandles as described by Carter et al. (1972) remains to be established.

These results clearly indicated that BPV peak I DNA represented the linear single-stranded viral genome. The DNA in peak II, eluting in the position of double-stranded DNA, posed some interesting questions as to its origin. The nature of this DNA will be discussed in section 5.3.

5.2 Physico-chemical characterization of the BPV genome (peak I).

5.2.1 Exonuclease I digestion.

The BPV genome was found to be about 85% resistant to digestion with exonuclease I. This suggested that there is some secondary structure located at or close to the 3' end. The DNA used in these studies was "unit length" DNA from alkaline sucrose, hence the DNA preparations should have been rather homogeneous. The fact that BPV DNA was only 85% resistant, but not completely resistant as was shown for KRV DNA (Lavelle and Mitra, 1978), could be the result of a palindrome located close to the 3' end, but not directly on it. This secondary structure can still serve as a primer for DNA synthesis by several polymerases (Chow and Ward, 1977). Another possibility is, that the 3' end palindrome is unstable, flopping back and forth. This is supported by the observation, that when salt was added to the enzyme assay, the resist-

ance increased from 60-70% to the value reported here. The possibility that the 3' end palindrome might represent a loosely hydrogen-bonded secondary structure has been pointed out previously (Bourguignon et al., 1976; Chow and Ward, 1977). This is further supported by the fact that during the initial characterization of several parvoviral DNA's, exonucleases specific for the 3' end of single-stranded DNA, were used to show that the genomes were linear and single-stranded (Siegl, 1973; Chow and Ward, 1977; Bourguignon et al., 1976; Salzman et al., 1971). It has also been suggested that the presence of a phosphate group at the 3' end could result in the resistance of otherwise single-stranded DNA to the action of exonuclease I (Lavelle and Mitra, 1978). These same investigators showed, however, that after pretreatment of KRV DNA with bacterial alkaline phosphatase, the DNA was still resistant to exonuclease I. A similar experiment was not done on BPV DNA, but since a phosphate group at the 3' end is rather unusual, and since such a phosphate group has not been demonstrated for the 3' end of other parvoviral genomes, I feel safe to assume that such a phosphate group is not present on the BPV genome.

5.2.2 S1 endonuclease digestion.

BPV DNA from peak I was found to be about 7% resistant to digestion with S1 endonuclease. KRV DNA used as a control for the assay conditions was found to be about 6% resistant. These values agree closely with the 5-6% reported for KRV DNA (Salzman, 1977; Lavelle and Mitra, 1978), the 6.5 % reported for MVM DNA (Bourguignon et al., 1976),and the 3-4% reported for AAV DNA (Fife et al., 1977).

It has been reported that a detectable and significant amount of double-stranded DNA is digested from the 5' end by S1 endonuclease (Fife et al., 1977). This extra nuclease activity could account for the variation in values observed in this study and by others (Bourguignon et al., 1976). But by carefully controlling the assay conditions and by the use of proper controls, the error that this would contribute should be minimized.

5.2.3 Melting profile of BPV DNA.

Single-stranded DNA does not show a characteristic melting transition when heated gradually from room temperature to about 100 ^{o}C. Because of this fact, this method has been used by several investigators to provide proof for the single-stranded nature of the parvoviral genomes (Robinson and Hetrick, 1969; Salzman et al., 1971; Usategui-Gomez et al., 1969).

As was expected, the BPV genome did not exhibit a melting profile, characteristic of double-stranded DNA, as compared to native calf thymus DNA. However, a small transition was observed at 57 ^{o}C. This T_m value would correspond to a GC content of about 16%, when substituted for T_m in the equation derived at by Frank-Kamentskii (1971) for the relationship between T_m and GC. However, this T_m value would correspond to a GC ratio of 35%, if a linear relationship is assumed between T_m and GC and if calf thymus DNA is assumed to contain 42% GC (Marmur and Doty, 1962). The reason for this discrepancy is not clear. One could speculate that this transition corresponds to the melting of a short AT rich secondary structure. S1 endonuclease digestion of the BPV genome

and subsequent separation of the palindromes could provide material for studying the base composition and possibly configuration of the palindromes by melting (Lightfoot, personal communication).

5.2.4 Base composition.

BPV DNA was high in thymine (31.3%) and the GC ratio was determined to be 45%, a value very comparable to the values previously reported for the other nondefective parvoviruses (McGeoch et al., 1970). Although some variation was observed between individual determinations, the standard error was well below 1% for all four bases. Some discrepancy can be observed in the literature between base determinations performed by different investigators on the DNA of the same virus. For example Usategui-Gomez et al. (1969)determined the GC content of H-1 to be 41.7% from base analysis of the viral DNA. Later McGeoch et al. (1970) repeated this experiment and determined the GC content to be 45.2%. Similarily Salzman and Jori (1970) determined the thymine content of KRV DNA to be 30.8%, but McGeoch et al. (1970) obtained 29.6%. It has even been suggested that the thymine content of KRV DNA may be as high as 34-35% (Mitra, personal communication). This discrepancy could probably be minimized by standardizing the conditions for an assay of this type.

No unusual bases have been reported in parvovirus DNA, and the method employed here did not allow for the detection of such bases, if present.

5.2.5 Buoyant density.

Buoyant density of BPV DNA in neutral CsCl was determined to be 1.721 g/cm^3, in close agreement with the value of 1.720 g/cm^3 found by Parris (Ph.D. dissertation, 1975). This value is also in close agreement with the buoyant densities of other parvoviral DNA's (Table 2; Salzman 1978).

Riva et al. (1969) have derived an equation ($p_i = 1.6267A_i + 1.7580G_i + 1.7681C_i + 1.7424T_i$) for calculating the buoyant density of single-stranded DNA from the base composition. By substituting the values obtained in this study into this equation, it yields a buoyant density for BPV DNA of 1.720 g/cm^3. I thus feel confident that the values on base composition and buoyant density reported here are correct.

5.2.6 Sedimentation coefficients.

The BPV genome was determined to have a sedimentation coefficient of 16.5 S in alkaline sucrose and about 27 S in neutral sucrose. By substituting these values into corresponding formulas derived by Studier (1965), the BPV DNA is estimated to have a molecular weight of about 1.7×10^6 daltons.

In these calculations it was assumed that $S = S^0_{20,w}$, which for all purpose holds true in this case. Other investigators have made similar allowance in their calculation of molecular weights, (Salzman, 1977; Siegl, 1972; Salzman and Jori, 1970; Bourguignon et al., 1976). I realize that this molecular weight determination of the BPV DNA is based only on its sedimentation coefficient. However, preliminary observations with the electron microscope and by gel electrophoresis indicate that the

value reported here will not change significantly.

It might be mentioned, that considerable discrepancy exists in the literature between molecular weight determinations by individual investigators for the same viral DNA (Siegl, 1976). Most of this discrepancy could be contributed to the use of a wide variety of standards, whose molecular weights have been subject to change. This discrepancy has for the most part been solved and the molecular weights of the parvoviral genomes have been fixed (Salzman, 1978).

5.3 BPV peak II DNA.

5.3.1 The possible origin of peak II DNA.

There were several possible explanations for the presence of peak II DNA eluting at NaP concentration of 0.24 M, in the position of double-stranded DNA.

1. Contaminating cellular DNA or viral replicative forms could have been co-purified with the virus. This DNA would, however, most likely have been removed by nuclease treatment during purification.
2. Some double-stranded cell DNA might have been packaged by mistake, as has been shown to be the case, where some bacterial viruses are concerned (Thomas and MacHattie, 1967).
3. Viral mRNA, co-purified with the virus, could have reannealed with the DNA strand, upon its release from the capsid, providing the minus strand is being encapsidated.
4. Double-stranded viral replicative forms could be encapsidated by mistake. This has to be considered unlikly, since it has

been calculated that the parvoviral capsids can not enclose double-stranded DNA of that size (Crawford et al., 1969).

5. The presence of palindromic sequences on the ends of the viral genome could possibly result in two strands being connected together end to end to form a dimer (concatamer). The amount of secondary structure, thus obtained, could be sufficient to cause the DNA to elute as double-stranded.

6. So-called panhandles have been observed in preparations of single-stranded AAV DNA (Koczot et al., 1973) and DNV DNA (Kelly and Bud, 1978). These have been shown to elute from HAP in the position of double-stranded DNA (Carter et al., 1972). The possibility that peak II might represent such panhandles, could not be overlooked.

7. Although it is commonly believed that the autonomous parvoviruses package only the minus strand (Salzman, 1978), there was a possibility that BPV might be packaging the plus strand also, to some extent. The two strands could then reanneal upon release from the virions, as is the case where AAV and DNV are concerned (Mayor and Kurstak, 1974).

5.3.2 The viral nature of peak II DNA.

It was considered unlike by that peak II represented a DNA-RNA hybrid, since extensive treatment with RNase during the isolation of the virus and of purified virions, did not abolish peak II. Furthermore, after double labelling of infected cells with ^{3}H-TdR and ^{14}C-uridine, no ^{14}C counts were observed in either peak (results not shown).

Several results were consistent with double-stranded nature of the peak II DNA. Sedimentation of peak II DNA in neutral sucrose clearly indicated that the DNA was double-stranded, and the sedimentation coefficient was determined to be approximately 17 S. The sharpness of the bands in sucrose indicated that the DNA population was rather homogeneous, which would not be expected if the DNA in peak II was of cellular origin. The melting profile of peak II was characteristic of double-stranded DNA, and this DNA was found to be about 70% resistant to S1 endonuclease. The most conclusive evidence for the double-stranded nature of peak II came from electron microscopy. Visualization by electron microscopy clearly showed that peak II DNA was double-stranded and furthermore, that it was linear.

Schildkraut et al. (1962) have pointed out that single-stranded DNA is 0.015-0.017 g/cm^3 more dense in neutral CsCl, than its double-stranded counterpart. If 0.015 is subtracted from the buoyant density of the BPV genome (1.721 g/cm^3), it yields a value of 1.706 g/cm^3, exactly the buoyant density of peak II DNA in neutral CsCl. Furthermore, Riva et al. (1969) have derived at an equation for calculating the buoyant density of single-stranded or denatured DNA from the known density of double-stranded DNA ($\rho_{den} = 0.8\rho_{nat} + 0.3566$ g/cm^3). Substitution of the buoyant density of the BPV genome into this equation yields the same results as described above. These buoyant density studies thus strongly indicated that the double-stranded DNA in peak II was of viral origin. The GC ratio of peak II DNA, as determined from T_m and buoyant density, was found to quite comparable to that of the BPV genome, as determined by base composition analysis. Peak II DNA sedimented to the same

position as the BPV genome in alkaline sucrose, indicating that the two DNA's were of the same size. About 70% of the BPV genome was found to hybridize with peak II DNA. This value is quite comparable to the value of 80% reported for adenovirus DNA-DNA hybridization under similar experimental conditions (Green et al., 1969). Peak II DNA was shown to hybridize almost 70% with itself, a much higher value than would be expected, if the DNA in peak II was of cellular origin (Green et al., 1969). Furthermore, attachment of BPV DNA and peak II DNA to filters containing cell DNA was not significantly above background levels. Extraction of AAV-2 DNA under the same conditions used to extract BPV DNA in this study, resulted in all of the AAV-2 DNA eluting as double-stranded, hence indicating that reassociation of complimentary strands does occur under those conditions. Finally, extraction of the DNA from BPV under conditions of low ionic strength (Berns and Rose, 1970), resulted in no DNA eluting from HAP in the position of double-stranded DNA. All of the BPV DNA eluted in peak I. Subsequent incubation of the DNA in peak I at high ionic strengths and elevated temperatures and rechromatography on HAP, resulted in the reappearance of peak II (results not shown). The evidence thus clearly indicates that not only is peak II DNA of viral origin, but that it represents plus and minus strands that have reannealed during the isolation of the DNA.

5.3.3 Encapsidation of the plus strand in other autonomous parvoviruses.

The encapsidation of both the plus and the minus strands into

separate capsid is well documented where AAV and DNV are concerned (Mayor and Kurstak, 1974). The autonomous parvoviruses, however, have hitherto been reported to package only the minus strand (Salzman, 1978; Tattersall and Ward, 1978). Several investigators have, nevertheless, reported on apparent packaging errors in several of the autonomous parvoviruses.

Bourguignon et al. (1976) reported that about 1% of MVM DNA preparations appeared to be unit length double-stranded molecules in the electron microscope. These investigators concluded that these double-stranded molecules might be formed, if the plus strand was being packaged in minute amounts. Rhode (1977b) has suggested that about 1 out of every 400 virions (0.25%) of H-1 contains a plus strand. DNA isolated from LuIII was found to band as two distinct peaks in neutral CsCl, but as a single peak in alkaline CsCl (Siegl, 1973). However, LuIII DNA behaved as completely single-stranded on BND-cellulose. The fact that a portion of the viral DNA behaved as double-stranded on gradients was thus attributed to the formation of intra-molecular bonds in the single-stranded DNA. But the idea that this double-stranded DNA represented reannealed plus and minus strands was discarded (Siegl, 1973). Although encapsidation of the plus strand has not been reported for KRV, one should bear in mind that KRV DNA was initially characterized as being completely double-stranded (May et al., 1967). In the present study, a minor portion of KRV DNA was found to elute from HAP as double-stranded, a finding supported by others (Lavelle, personal communication). Furthermore, preliminary studies on this double-stranded DNA on agarose gels show that it migrates as a single

homogeneous band, slightly faster than BPV DNA from peak II. KRV might thus also package the plus strand in greater amount than previously thought.

5.4 Concluding remarks.

The following conclusion can be drawn from the results presented above:

1. BPV DNA is linear and single-stranded with a sedimentation coefficient of 16.5 S in alkaline sucrose, hence a molecular weight of 1.7×10^6 daltons.
2. BPV DNA contains duplex regions that represent about 7% of the genome length.
3. BPV DNA contains 23.2% adenine, 31.3% thymine, 22.2% guanine, and 22.9% cytosine. Therefore, BPV DNA has a GC content of 45.1%.
4. BPV DNA has a buoyant density of 1.721 g/cm^3 in neutral CsCl.
5. BPV encapsidates complimentary single-strands into separate virions, allowing for the formation of double-stranded unit length DNA molecules during release from the virion.

BPV DNA has been found to share many of the physico-chemical characteristics of the other parvoviral genomes (Table 2.). A significant difference between BPV and the other autonomous parvoviruses is the extent of an apparent packaging error. Base sequences of the ends of the BPV genome have also been shown to be different from those of other autonomous parvoviruses (Ward, personal communication).

The best characterized of the autonomous parvoviruses, KRV, H-1, and MVM are all of rodent origin. Nevertheless, all of the autonomous

parvoviruses require similar conditions for their propagation in cell culture in vitro. It has been shown that the viral DNA of the autonomous parvoviruses is replicated along with the cellular DNA in late S phase (Salzman, 1978; Tattersall and Ward, 1978). It has also been postulated that all the enzymes that the autonomous parvoviruses require for their replication are of cellular origin (Berns and Hauswirth, 1978; Pritchard et al., 1978; Patton et al., in review). The observation that the extent of this packaging error in BPV infected cells seems to depend on the cell type is not surprising.

It has been postulated that after formation of a double-stranded replicative intermediate in infected cells, displacement synthesis produces both plus and minus strands. It has also been postulated that the 5' end palindrome might serve as a recognition site for capsid proteins. Because of the inverted terminal repetition, the 5' end of the plus and minus strands of AAV will look exactly alike, hence the strands are encapsidated in equal amounts (Berns and Hauswirth, 1978). Such a terminal repetition is apparently not present in the genomes of the rodent parvoviruses (Bourguignon et al., 1976), so only the minus strand is recognized and encapsidated (Berns and Hauswirth, 1978). When the plus strand is encapsidated in significant amounts, it might be because of a rapid accumulation of BPV capsid protein, with assembly occuring even if no minus strands are available. This might also explain the release of empty capsids or capsids containing only a fraction of the genome. The difference in cell types might thus reflect differences in the rates of transcription and/or translation of viral specific components.

The fact that the other autonomous parvoviruses package only the minus strand has caused some problems. Restriction endonuclease mapping has only been possible after initial synthesis of the complimentary plus strand *in vitro* or by use of isolated replicative forms. This has proved to be time consuming and possibly inaccurate. The finding here that BPV packages the plus strand into 15-25% of the virions (depending on cell type) and the subsequent formation of double-stranded molecules during isolation of the DNA, could provide the proper material for restriction endonuclease mapping of the BPV genome, without *in vitro* manipulations. The possibility that the extent of this packaging error of BPV is dependent on cell physiology could provide suitable basis for future investigation of the requirements for parvovirus replication.

6. Literature cited.

Abinanti, F.R., and M.S. Warfield. 1961. Recovery of hemadsorbing virus (HADEN) from gastrointestinal tract of calves. Virology 14:288-289.

Atchison, R.W., B.C. Casto, and W.McD. Hammon. 1965. Adenovirus-associated defective virus particles. Science 149:754-756.

Bachmann, P.A. 1971. Properties of a bovine parvovirus (Brief report). Zbl. Vet. Med. B. 18:80-85.

Bachmann, P.A., M.D. Hoggan, J.L Melnick, H.G. Pereira, and C. Vago. 1975. Parvoviridae. Intervirology 5:83-92.

Barwise, P.A., and I.O. Walker. 1970. Studies on the DNA of a virus from Galleria mellonella L. FEBS Lett. 6:13-16.

Bates, R.C., J. Storz, and D.E. Reed. 1972. Isolation and comparison of bovine parvoviruses. J. Infect. Dis. 126:531-536.

Bernardi, G. 1965. Chromatography of nucleic acids on hydroxyapatite. Nature 206:779-783.

Bernardi, G. 1971. Chromatography of nucleic acids on hydroxyapatite columns, p. 95-147. In L. Grossman and K Moldave (ed.), Methods in enzymology, vol. 21. Academic Press Inc., New York.

Berns, K.I. 1974. Molecular biology of the adeno-associated viruses. Curr. Top. Microbiol. Immunol. 65:1-20.

Berns, K.I., and S. Adler. 1972. Separation of two types of adeno-associated virus particles containing complimentary polynucleotide chains. J. Virol. 9:394-396.

Berns, K.I., and W.W. Hauswirth. 1978. Parvovirus DNA structure and replication, p. 13-32. In D.C. Ward and P. Tattersall (ed.), Replication of the mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Berns, K.I., and T.J. Kelly, Jr. 1974. Visualization of the inverted terminal repetition in adeno-associated virus DNA. J. Mol. Biol. 82:267-271.

Berns, K.I., and J.A. Rose. 1970. Evidence for a single-stranded adeno-virus-associated virus genome: Isolation and separation of

complimentary single-strands. J. Virol. 5:693-699.

Berns, K.I., W.W. Hauswirth, K.H. Fife, and J.S. Spear. 1978. Terminal structure of adeno-associated-virus DNA, p. 179-192. In D.C. Ward and P. Tattersall (ed.), Replication of the mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Berns, K.I., J. Kort, K.H. Fife, W.E. Grogan, and J. Spear. 1975. Study of the fine structure of adeno-assocated virus DNA with bacterial endonucleases. J. Virol. 16:712-719.

Bourguignon, G.J., P.J. Tattersall, and D.C. Ward. 1976. DNA of minute virus of mice: Self-priming, nonpermuted, single-stranded genome with a 5'-terminal hairpin duplex. J. Virol. 20:290-306.

Burgi, E., and A.D. Hersey. 1963. Sedimentation rate as a measure of molecular weight of DNA. Biophys. J. 3:309-321.

Carter, B.J. 1978. Parvovirus transcription, p. 34-52. In D.C. Ward and P. Tattersall (ed.), Replication of the mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Carter, B.J., G. Khoury, and D.T. Denhardt. 1975. Physical map and strand polarity of specific fragments of adenovirus-associated virus DNA produced by endonuclease R.EcoRI. J. Virol. 16:559-568.

Carter, B.J., G. Khoury, and J.A. Rose. 1972. Adenovirus-associated virus multiplication. IX. Extent of transcription of the viral genome in vitro. J. Virol. 10:1118-1125.

Cavalier-Smith, T. 1974. Palindromic base sequences and replication of eukaryote chromosome ends. Nature 250:467-470.

Chase, J.W., and C.C. Richardson. 1974. Exonuclease VII of Escherichia coli. J. Biol. Chem. 249:4553-4561.

Cheong, L., J. Fogh, and R.K. Borday. 1965. Some properties of the H-1 virus and nucleic acid. Federation Proc. 24:596.

Chow, M.B., and D.C. Ward. 1977. Terminal hairpin structures in non-defective parvovirus DNA's. Federation Proc. 36:740.

Chow, M.B., and D.C. Ward. 1978. Comparison of the terminal nucleotide structures in the DNA of nondefective parvoviruses, p. 205-217. In D.C. Ward and P. Tattersall (ed.), Replication of the mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Crawford, L.V. 1966. A minute virus of mice. Virology 29:605-612.

Crawford, L.V., E.A.C. Follett, M.G. Burdon, and D.J. McGeoch. 1969.

The DNA of a minute virus of mice. J. Gen. Virol. 4:37-46.

de la Maza, C.M., and B.J. Carter. 1976. Cleavage of adeno-associated virus DNA with SalI, PstI and HaeII restriction endonucleases. Nucl. Acids Res. 3:2605-2616.

Denhardt, D.T., G. Eisenberg, K. Bartok, and B.J. Carter. 1976. Multiple structures of adeno-associated virus DNA: Analysis of terminally labelled molecules with endonuclease R.HaeIII. J. Virol. 18:672-684.

Fife, K.H., K.I. Berns, and K. Murray. 1977. Structure and nucleotide sequence of the terminal regions of adeno-associated virus DNA. Virology 78:475-487.

Frank-Kamenetskii, M.D. 1971. Simplification of the empirical relationship between melting temperature of DNA, its GC content and concentration of sodium ions in solution. Biopolymers 10:2623-2624.

Gerry, H.W., T.J. Kelly, Jr., and K.I. Berns. 1973. Arrangement of nucleotide sequences in adeno-associated virus DNA. J. Mol. Biol. 79:207-225.

Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12: 829-842.

Goodman, R.M. 1977. Single-stranded DNA genome in a whitefly-transmitted plant virus. Virology 83:171-179.

Green, M., K. Fujinaga, and M. Pina. 1969. The use of DNA-RNA and DNA-DNA hybridization on nitrocellulose membranes in virus research, p. 467-480. In K. Habel and N.P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.

Gunther, M., and P. May. 1976. Isolation and structural characterization of monomeric and dimeric forms of replicative intermediates of Kilham rat virus DNA. J. Virol. 20:86-95.

Hallauer, C., G. Kronauer, and G. Siegl. 1972. Parvoviruses as contaminants of permanent human cell lines. I. Virus isolation from 1960-1970. Arch. Ges. Virusforsch. 38:366-382.

Hampton, E.G. 1964. Viral antigen in rat embryo in culture infected with H-1 virus isolated from transplantable human tumors: Cytochemical studies. Cancer Res. 24:1534-1543.

Hauswirth, W.W., and K.I. Berns. 1977. Origin and termination of adeno-associated virus DNA replication. Virology 78:488-499.

Hoggan, M.D., N.R. Blacklow, and W.P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: Physical, biological, and immunological characteristics. Proc. Nat. Acad. Sci. U.S.A. 55:1467-1474.

Inman, R.B., and M. Schnos. 1970. Partial denaturation of thymine- and 5-bromouracil-containing DNA in alkali. J. Mol. Biol. 49:93-98.

Iyer, V.N., and W.D. Rupp. 1971. Usefulness of benzoylated naphtoylated DEAE-cellulose to distinguish and fractionate double-stranded DNA bearing different extents of single-stranded regions. Biochim. Biophys. Acta 228:117-126.

Jamison, R.M., and H.D. Mayor. 1965. Acridine orange staining of purified rat virus strain X14. J. Bacteriol. 90:1486-1488.

Jovin, T.M., P.T. Englund, and L.L. Bertsch. 1969. Enzymatic synthesis of deoxyribonucleic acid. XXVI. Physical and chemical studies of homogeneous deoxyribonucleic acid polymerase. J. Biol. Chem. 244: 2996-3008.

Kelly, D.C., and H.M. Bud. 1978. Densonucleosis virus DNA: Analysis of fine structure by electron microscopy and agarose gel electrophoresis. J. Gen. Virol. 40:33-43.

Kelly, D.C., A.H. Barwise, and I.O. Walker. 1977. DNA contained by two densonucleosis viruses. J. Virol. 21:396-407.

Kilham, L., and L.J. Oliver. 1959. A latent virus of rats isolated in tissue culture. Virology 7:428-437.

Kleinschmidt, A.K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules, p. 361-377. In L. Grossman and K. Moldave (ed.), Methods in enzymology. Academic Press Inc., New York.

Koczot, F.J., B.J. Carter, C.F. Garon, and J.A. Rose. 1973. Self-complimentary of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. Proc. Nat. Acad. Sci. U.S.A. 70: 215-219.

Kurstak, E. 1972. Small DNA densonucleosis virus (DNV). Adv. Virus Res. 17:207-241.

Kurstak, E., J.P. Vernoux, and L. Brakier-Gingras. 1973. Etude biophysique de l'acide desoxyribonucleique du virus de la densonucleose (VDN). II. Extraction du DNA viral et mise en evidence de la presence de chaines polynucleotidiques complementaires encapsidees seperement dans les virions VDN. Arch. Ges. Virusforsch. 40:274-284.

Lavelle, G., and S. Mitra. 1978. Double-helical regions in Kilham rat virus DNA, p. 219-229. In D.C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Lehman, J.R. 1966. Exonuclease I (phosphodiesterase from Escherichia coli), p. 203. In G.L. Cantoni and D.R. Davies (ed.), Procedures in nucleic acid research. Harper and Rowe, New York.

Marmur, J. 1961. A procedure for isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.

May, P., and E. May. 1970. The DNA of Kilham rat virus. J. Gen. Virol. 6:437-439.

May, P., A. Niveleau, G. Berger, and C. Brailovsky. 1967. Recherches sur le DNA du virus K du rat (Parvovirus ratti). J. Mol. Biol. 27: 603-614.

Mayor, H.D., and E. Kurstak. 1974. Viruses with separately encapsidated complimentary DNA strands, p. 55-78. In E. Kurstak and K. Maramorosch, Viruses, evolution, and cancer. Academic Press Inc., New York.

Mayor, H.D., and J.L. Melnick. 1966. Small deoxyribonucleic acid-containing viruses (picodnavirus group). Nature 210:331-332.

Mayor, H.D., R.M. Jamison, L.E. Jordan, and J.L. Melnick. 1965. Structure and composition of a small particle prepared from a simian adenovirus. J. Bacteriol. 90:235-242.

Mayor, H.D., K. Torkai, and J.L. Melnick. 1969. Plus and minus single-stranded DNA separately encapsidated in adeno-associated satellite virions. Science 166:1280-1282.

Mazzone, H.M. 1967. Equilibrium ultracentrifugation, p. 41-92. In K. Maramorosch and H. Koprowski, Methods in virology, vol. 2. Academic Press Inc., New York.

McGeoch, D.J., L.V. Crawford, and E.A.C. Follett. 1970. The DNA's of three parvoviruses. J. Gen. Virol. 6:33-40.

Mitra, S., and D.R. Stallions. 1976. The role of Escherichia coli dnaA gene and its integrative suppression in M13 coliphage DNA synthesis. Eur. J. Biochem. 67:37-45.

Parks, W.P., J.L. Melnick, R. Rongey, and H.D. Mayor. 1967a. Physical assay and growth cycle studies of a defective adeno-satellite virus. J. Virol. 1:171-180.

Parks, W.P., M. Green, M. Pina, and J.L. Melnick. 1967b. Physicochemical characterization of adeno-associated satellite virus type 4 and its nucleic acid. J. Virol. 1:980-987.

Parris, D.S. 1975. Replication of bovine parvovirus. Ph.D. Dissertation at Virginia Polytechnic Institute and State University.

Payne, F.E., C.J. Shellaberger, and R.W. Schmidt. 1963. A virus from mammary tissue of rats treated with X-rays or methylcholanthrene. Proc. Amer. Ass. Cancer Res. 4:51.

Pritchard, C., J.T. Patton, R.C. Bates, and E.R. Stout. 1978. Replication of nondefective parvoviruses: Lack of a virion-associated DNA polymerase. J. Virol. 28:20-27.

Rabson, A.S., L. Kilham, and R.L. Kirschstein. 1961. Intranuclear inclusions in Rattus (mastomys) natalensis infected with rat virus. J. Natl. Cancer Inst. 27:1217-1221.

Rhode III, S.L. 1977a. Replication process of the parvovirus H-1. VI. Characterization of a replication terminus of H-1 replicative-form DNA. J. Virol. 21:694-712.

Rhode III, S.L. 1977b. Replication process of the parvovirus H-1. IX. Physical mapping studies of the H-1 genome. J. Virol. 22:446-458.

Rivers, C.F., and J.F. Longworth. 1972. A non-occluded virus of Junonia coenia (Nymphalidae: Lepidoptera). J. Invert. Pathol. 20: 369-370.

Robinson, D.M., and F.M. Hetrick. 1969. Single-stranded DNA from Kilham rat virus. J. Gen. Virol. 4:269-281.

Rose, J.A. 1974. Parvovirus reproduction, p. 1-61. In H. Fraenkel-Conrat and R.R. Wagner (ed.), Comprehensive virology, vol. 3. Plenum Press, New York.

Rose, J.A., and F.J. Koczot. 1971. Adenovirus-associated virus multiplication. VI. Base composition of the deoxyribonucleic acid strand species and strand specific in vitro transcription. J. Virol. 8:771-777.

Rose, J.A., K.I. Berns, M.D. Hoggan, and F.J. Koczot. 1969. Evidence for a single-stranded adenovirus-associated virus genome: Formation of a DNA density hybrid on release of viral DNA. Proc. Nat. Acad. Sci. U.S.A. 64:863-869.

Rose, J.A., M.D. Hoggan, and A.J. Shatkin. 1966. Nucleic acid from an adeno-associated virus: Chemical and physical studies. Proc. Nat. Acad. Sci. U.S.A. 56:86-92.

Salzman, L.A. 1977. Evidence for terminal S1-nuclease-resistant regions on single-stranded linear DNA. Vriology 76:454-458.

Salzman, L.A. 1978. The parvoviruses, p. 539-587. In D.P. Nayak (ed.), The molecular biology of animal viruses, vol. 2. Marcel Dekker Inc., New York.

Salzman, L.A., and L.A. Jori. 1970. Characterization of the Kilham rat virus. J. Virol. 5:114-122.

Salzman, L.A., P. Fabisch, R. Parr, C. Garon, and T. Wali. 1978. In vitro synthesis of double-stranded DNA from the Kilham rat virus single-stranded DNA genome. J. Virol. 27:784-790.

Salzman, L.A., W.L. White, and T. Kakefuda. 1971. Linear, single-stranded deoxyribonucleic acid isolated from Kilham rat virus. J. Virol. 7:830-835.

Sanger, F., G.M. Air, B.G. Barrell, N.L. Brown, A.R. Coulson, J.C. Fiddes, C.A. Hutchinson III, P.M. Slocombe, and M. Smith. 1977. Nucleotide sequence of bacteriophage ØX174 DNA. Nature 265:687-695.

Schilkraut, C.L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430-443.

Shatkin, A.J. 1969. Base composition analysis of DNA, p. 496 - 501. In K. Habel and N.P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.

Siegl, G. 1972. Parvoviruses as contaminants of permanent human cell lines. V. The nucleic acid of KBSH-virus. Arch. Ges. Virusforsch. 37:267-274.

Siegl, G. 1973. Physicochemical characteristics of the DNA of parvovirus LuIII. Arch. Ges. Virusforsch. 43:334-344.

Siegl, G. 1976. The parvoviruses. Springer-Verlag, Wien.

Siegl, G., C. Hallauer, A. Novak, and G. Kronauer. 1971. Parvoviruses as contaminants of permanent human cell lines. II. Physicochemical properties of the isolated viruses. Arch. Ges. Virusforsch. 35: 91-103.

Sinsheimer, R.L. 1968. Bacteriophage ØX174 and related viruses. Progr. Nucleic Acid Res. Mol. Biol. 8:115-169.

Spahn, G.J., S.B. Mohanty, and F.M. Hetrick. 1966. Characteristics of hemadsorbing enteric virus (HADEN). Canad. J. Microbiol. 12:653-661.

Storz, J., and G.S. Warren. 1970. Effect of antimetabolites and actinomycin D on the replication of HADEN, a bovine parvovirus. Arch. Ges. Virusforsch. 30:271-274.

Straus, S.F., E.D. Sebring, and J.A. Rose. 1976. Concatamers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. Proc. Nat. Acad. Sci. U.S.A. 73: 742-746.

Studier, F.W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390.

Tattersall, P. 1978. Parvovirus protein structure and virion maturation, p. 53-72. In D.C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Tattersall, P., and D.C. Ward. 1976. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature 263:106-109.

Tattersall, P., and D.C. Ward. 1978. The parvoviruses-an introduction, p. 3-12. In D.C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Tattersall, P., P.J. Cawte, A.J. Shatkin, and D.C. Ward. 1976. Three structural polypeptides coded for by minute virus of mice, a parvovirus. J. Virol. 20:273-289.

Tattersall, P., L.V. Crawford, and A.J. Shatkin. 1973. Replication of the parvovirus MVM. II. Isolation and characterization of intermediates in the replication of the viral deoxyribonucleic acid. J. Virol. 12:1446-1456.

Thomas, Jr., C.A., and L.A. MacHattie. 1967. The anatomy of viral DNA molecules. Ann. Rev. Biochem. 36:485-518.

Toolan, H.W., G. Dallendorf, M. Barclay, S. Chandra, and A.M. Moore. 1960. An uidentified filtrable agent isolated from transplanted human tumors. Proc. Nat. Acad. Sci. U.S.A. 46:1256-1258.

Truffaut, N., G. Berger, A. Niveleau, P. May, M. Bergoin, and C. Vago. 1967. Recherche sur l'acide nucleique du virus de la densonucleose du lepidoptore Galleria mellonella L. Arch. Ges. Virusforsch. 21: 467-474.

Usategui-Gomez, M., H.W. Toolan, N. Ledenko, F. Al-Lami, and M.S. Hopkins. 1969. Single-stranded DNA from parvovirus, H-1. Virology 39:617-621.

Vernon, S.K., J.T. Stasny, A.R. Neurath, and B.A. Rubin. 1971. Electron microscopy of DNA from adeno-associated virus type 1. J. Gen. Virol. 10:267-271.

Vernoux, J.P., and E. Kurstak. 1972. Etude biophysique de l'acide desoxyribonucleique du virus de la densonucleose (VDN). I. Purification du VDN et ses proprietes en relation directe avec la structure de l'acide nucleique encapside. Arch. Ges. Virusforsch. 39:190-195.

Vogt, V.M. 1973. Purification and further properties of single-stranded nuclease from Aspergillus oryzae. Eur. J. Biochem. 33:192-200.

Ward, D.C., and D.K. Dadachanji. 1978. Replication of minute-virus-of-mice DNA, p. 297-313. In D.C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Press, Cold Spring Harbor.

Whalley, J.M. 1965. Rat-virus nucleic acid. Biochem. J. 94:10P.

Wilson, D.A., and C.A. Thomas, Jr. 1973. Hydroxyapatite chromatography of short double-helical DNA. Biochim. Biophys. Acta 331:333-340.

Wilson, D.A., and C.A. Thomas, Jr. 1974. Palindromes in chromosomes. J. Mol. Biol. 84:115-138.

Zwillenberg, L.O., and C. Hallauer. 1962. An unidentified hemagglutinin from human tumor tissue cultures. Arch. Ges. Virusforsch. 38:366-382.

7. Appendix.

Dulbeeco's Salt Solution

Ingredients per liter:

Sodium Chloride	8	g
Disodium Phosphate·H_2O	0.12	g
Potassium Chloride	0.2	g
Magnesium Chloride·H_2O	0.1	g
Monopotassium Phosphate	0.2	g
Calcium Chloride	0.1	g
Phenol Red	0.005	g
Streptomycin	0.1	g
Penicillin	100,000	units

Saline A

Ingredients per liter:

Sodium Chloride	8	g
Potassium Chloride	0.4	g
Sodium Bicarbonate	0.35	g
Glucose	1	g
Phenol Red	0.02	g
Streptomycin	0.1	g
Penicillin	100,000	units

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ISOLATION AND CHARACTERIZATION OF BOVINE PARVOVIRUS DNA

by

Ari Kristjan Saemundsen

(ABSTRACT)

The best characterized of the autonomous parvoviruses are those of rodent origin. The purpose of this study was to examine the physico-chemical characteristics of the genome of an autonomous parvovirus of non-rodent origin, bovine parvovirus (BPV).

BPV was isolated from infected cells by centrifugation through a sucrose-CsCl step-gradient. The virion DNA was released from the capsids by alkali treatment. Upon chromatography on hydroxyapatite, two peaks of radioactivity were consistantly observed. Peak I eluted at a sodium phosphate concentration of 0.17 M, intermediate between single- and double-stranded DNA markers. Peak II eluted in the same position as double-stranded DNA.

The DNA eluting in peak I represented the viral genome. About 7% of the genome length was found to exist as a duplex. The BPV DNA was found to have sedimentation coefficients of 16.5 S and 27 S in alkaline and neutral sucrose, respectively. These S values corresponded to a molecular weight of approximately 1.7×10^6 daltons. The BPV DNA was rich in thymine (31.3%) and had a GC content of 45.1%. The buoyant

density of the single-stranded BPV genome was determined to be 1.721 g/cm^3.

The DNA eluting in peak II was shown by hybridization to be of BPV origin. Furthermore, the peak II DNA was shown to be of the same size as the single-stranded BPV genome, by sedimentation in alkaline sucrose. Electron microscopy revealed that the peak II DNA was linear and double-stranded. It is concluded that BPV packages the plus strand into separate virions in significant amounts, resulting in the reannealing of complimentary plus and minus strands upon release from the virions during isolation of the DNA.