THE CHARACTERIZATION OF AN EQUINE VIRUS ISOLATE

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A Thesis Presented to the Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

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In Partial Fulfillment of the Requirements for the

Degree of Master of Science

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by

Keith Peter Nielsen

February 1985

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KEITH PETER NIELSEN

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

An uncharacterized viral agent isolated from an aborted equine fetus was cultured in a fallopian tube cell line of porcine fallopian tube (PFT) derivation. The viral replicative cycle was compared in two systems. In cells synchronized by a double thymidine block, the infectious virus titre peaked at 24 hours post infection (hpi), while growth in asynchronous culture plateaued at 36 hpi. The synchronization protocol was of greatest value in achieving a higher degree of cell cycle G1/S boundary alignment, for use in biological and molecular studies. The multiplication of equine virus infected PFT cells was inhibited by 68% at 24 hpi, relative to an uninfected control, thereby indicating a mitolytic property.

Viral stocks retained full infectivity after heating at 56°C for 30 minutes. Incubation at 70°C resulted in a 1-2 $\log_{10}$ decline in titre, with total inactivation from 80°C and upwards. Infectivity was not diminished by ether extraction, or exposure to 1% concentrations of: sodium dodecyl sulfate, Nonidet P40, deoxycholate, or n-butanol.

Morphologically, viral particles having a cesium chloride buoyant density of 1.416 g/cm^3, were 20 - 23 nm in diameter, icosahedral and excluded phosphotungstic acid stain.

Two classes of full particles were identified, a heavy full (HF) and light full (LF) with respective CsCl buoyant densities of 1.435 and 1.395 g/cm^3. Both were infectious. There were three viral capsid proteins visualized, VP1, VP2, and VP3, with respective molecular weights of 87,000, 70,500, and 68,000 daltons. VP2, the most abundant polypeptide, was cleaved *in vitro* by trypsin to VP3, with no effect on infectivity. A

nonstructural protein having a nuclear location and molecular weight of 17,000 daltons, was observed by SDS-PAGE analysis of the total lysates from EV infected cells.

The viral genome is single-stranded DNA, being resistant to ribonuclease A, and digested by both deoxyribonuclease I and nuclease S1. Sensitivity to nuclease S1 was further quantitated relative to a control single-stranded DNA substrate. The average S1 resistance of native genome was 6.9% more than the control material. When the genome was heat denatured, a core of 3.2% remained resistant and was concluded to represent sequences with the capacity for spontaneous reannealing by virtue of a hairpin arrangement.

The intracellular replicative forms (RF) consisted of double-stranded, dimeric (di) and monomeric (m) RF molecules, plus single-stranded DNA species. Denaturation demonstrated a relationship between the diRF and mRF. The mRF had a length of 5050 base pairs and was composed of two molecular types, a denaturable extended form, and a hairpin conformer which migrated like mRF after heat quenching. Nine restriction endonuclease recognition sites were mapped on the mRF. Similarities were detected between certain of these points and the physical map of a porcine parvovirus.

In conclusion, the equine virus isolated has features entirely consistent for its classification as a member of the genus Parvovirus.

INTRODUCTION

Recently, a high incidence of equine abortion was observed in Manitoba. No known etiological agent (eg. equine herpes virus) was established. A number of these fetuses were submitted to the Manitoba Agriculture Services, Winnipeg, for autopsy. No apparent pathological manifestation was observed. Homogenates were prepared from the liver tissue of two fetuses. Both of these induced a weak cytopathological effect (CPE) against the porcine fallopian tube (PFT) cell line (Bouillant, et al, 1973) following three days incubation. Infectious virus was suggested because bacteria or fungi were not detected in either the innoculum or the infected PFT culture supernatant by electron microscopy or culture growth.

This thesis deals with the identification, classification, and genome structure elucidation of the equine virus isolate derived from one of the fetal liver homogenates. The results may provide an insight into the origin of the equine virus in the horse stock of Manitoba.

LITERATURE REVIEW

3.1. Classification

The family Parvoviridae was established by the International Committee on the Nomenclature of Viruses and is subdivided into 3 genera (Siegl, 1976). Members of the genus Densovirus (DNV): infect arthropod hosts, require no helper virus, and package equal amounts of complementary single stranded DNA, in separate particles. The genus Adeno-associated virus (AAV; also known as Dependovirus) has members which infect vertebrate hosts and like the DNV, encapsidate equal amounts of both sense strands in separate virions (Rose et al, 1969; Berns and Rose, 1970). AAV require coinfection with adenovirus (Ad) for efficient growth. Early Ad genes are necessary for full AAV helper function. The report of Myers et al (1980) suggests that the Ad E72 protein may be involved in the modulation of AAV mRNAs. The third genus is Parvovirus.

Vertebrates are also the hosts for the genus Parvovirus. They differ from AAV in that they require no helper virus coinfection and therefore are also referred to as autonomous or nondefective parvoviruses. Parvoviruses had been defined by their encapsidation of a minus sense single stranded molecule of DNA (Rose, 1974). However, Bourguignon et al (1975) found that preparations of minute virus of mice (MVM) contained 1% of particles with the complimentary sense strand. Recently, several other autonomous parvovirus systems have also been found to package variable amounts of the second strand (to be discussed later) and thus it would appear that the criterion of strand selection should be re-examined. These viruses also rely on elements expressed during the late S or early G2 phases of the cell cycle (Hampton, 1970; Rhode, 1973; Siegl and Gautschi, 1974). As it will be shown by the following results, the equine virus isolate belongs to the

autonomous genus of Parvoviridiae. The following discussion of parvovirus refers to the autonomous parvoviruses, unless otherwise specified.

3.2. Physical and Biological Properties of Parvovirus Virions

Parvovirus capsids have icosahedral symmetry and are 20 - 25 nm in diameter (Siegl, 1976). The particles are very stable to heating. The infectivity of porcine PV did not drop after heating at 56°C for 30 minutes (Cartwright and Huck, 1967; Mayr et al, 1968). Siegl (1976) found that LuIII resisted heat inactivation at 56°C for 1 hour. Biological actvity was abolished at 80°C for both Kilham rat virus (Brailovsky, 1966) and PPV (Cartwright et al, 1969).

The proteolytic enzymes: chymotrypsin, trypsin, and papain, as well as the nucleases RNase and DNase, did not diminish the infectivity of H-1, H-3, or Kilham rat virus (KRV) (Green, 1964; Kongsvik and Toolan, 1972).

Feline panleukopenia virus infectivity was unchanged by exposure to acid (pH 3), chloroform, or ether (Johson, 1967,1969; Kilham and Margolis, 1967; Studdert and Peterson, 1973).

The hamster viruses, H-1 and H-3, retained complete infectivity even after 6 years storage (Toolan et al, 1960; Green, 1964).

The parvoviral capsids formed during infection are resolved by their buoyant densities in CsCl gradients. Generally, particles banding at density 1.38 - 1.43 g/cm^3 possess the highest infectivity. Heavier populations, from density 1.46 - 1.48 g/cm^3, have been observed in many systems and are usually less infectious. Empty capsids band at 1.30 - 1.32 g/cm^3 (Tattersall and Ward, 1978).

Johnson et al (1974) have identified four distinct types of particles (densities 1.44, 1.41, 1.36, and 1.31 g/cm^3) in both the feline panleukopenia virus and mink enteritis virus systems. The species at

1.36 g/cm^3 packaged only a partial genome. In the analysis of the LuIII particle spectrum, Muller et al (1978) identified at least four defective types with densities of 1.33 - 1.37 g/cm^3. These possessed between 9 and 43% of the nucleic acid content of the infectious form.

3.3. Structural Proteins

The autonomous parvoviruses studied to date have between 2 - 4 structural proteins, designated A through D (or V1 to VP4). Molecular weights have been reported in the following ranges: A) 93,000 - 73,000; B) 80,000 - 64,000; C) 67,000 - 56,000; and D) 56,000 - 40,000 daltons (Tattersall, 1978). The A protein is usually the least abundant, representing about 10% of the capsid makeup. The B and C proteins are related in that C appears to be formed as a result of proteolytic cleavage of B (note: B is often designated VP2' and C as VP2). A functional role for this modification has been pursued with regard to maturation. In the composition analysis of the nondefective parvoviruses: H-3, H-1, KRV, and minute virus of mice (MVM), Peterson et al (1978) demonstrated that empty virions contained only A and B polypeptides, whereas A, B, and C were found in full particles. A recent examination of the autonomous porcine PV again confirmed that the C polypeptide predominated over B in full virions (Molitor et al, 1983). In the rabbit PV system, four structural proteins have been identified: A, B, C, and C'. The C' polypeptide was generated by a cleavage of C, late in infection. However, unlike the previously discussed systems, the C to C' conversion was not nearly as extensive (Matsunaga and Matsuno, 1983).

3.4. Nonstructural Proteins

Astell et al (1983a), in sequencing the MVM genome, identified possible coding regions in the 3' half of molecule. Pintel et al (1983)

identified a 4.8 kilobase transcript which mapped to the 3' end. However, no MVM nonstructural protein has been reported.

Recently, Matsunaga and Matsuno (1983) have immunoprecipitated two nonstructural proteins (25,000 and 22,000 daltons) predominantly nuclear in origin, from rabbit PV infected cells.

A new H-1 parvovirus gene product was found when mRNA from H-1 infected cells was translated in vitro and immunoprecipitated with antiserum from infected animals (Rhode and Paradiso, 1983). Like the speculated noncapsid MVM product, the H-1 transcript mapped to the 3' half of the genome. Paradiso (1984) has since identified two related forms of this protein, NCVP1 and NCVP1', having molecular weights of 84,000 and 92,000 daltons, respectively. NCVP1' was phosphorylated to a higher degree than NCVP1. The appearance of both proteins was coincident with the capsid proteins at 9 hpi.

It is believed that nonstructural proteins such as these, are involved in parvoviral DNA synthesis and therefore have been termed RF "rep" proteins (Rhode and Paradiso, 1983).

3.5. The Autonomous Parvovirus Genome

a) Sizes

The parvoviruses encapsidate a single-stranded, linear molecule of DNA, having molecular weight 1.2×10^6 to 2.2×10^6 daltons (Berns and Hauswirth, 1978). Bates et al (1984) have reported a length of 5,000 nucleotides for both LuIII and KRV. The genome of rodent virus, H-1, has been sequenced and found to be 5,176 bases (Rhode and Paradiso, 1983). Again, by complete DNA sequence analysis of the MVM genome, a length of

5,081 nucleotides was determined (Astell et al, 1983a). Synder et al (1982) reported that the bovine parvovirus genome (BPV) was 5,550 bases long, making it one of the largest known to date.

b) Genome Polarity and Packaging

The nature of the parvoviral genome has been the subject of extensive investigation over the past 20 years. The DNA strand serving as the template for messenger RNA transcription is referred to as having negative (minus) polarity or sense. It was originally believed that AAV possessed a duplex genome (Rose et al, 1966), but when isolated, its apparent size of 3.6×10^6 daltons was thought too large for the virion's capacity (Crawford et al, 1969). The existance of plus and minus single-strands packaged in separate particles was proven by Rose et al (1969). Virus was propagated in a system supplemented with the thymidine analog, bromodeoxyuridine (BUdR). The substitution of the heavier BUdR for thymidine in replicated progeny genomes, followed by CsCl gradient centrifugation, lead to the resolution of positive and negative strand-containing particles (Rose et al, 1969; Berns and Rose, 1970).

Rose and Koczot (1971) demonstrated that the success of the earlier experiments was made possible due to a thymidine content differential between positive (21%) and negative (27%) strands.

Whereas the helper-dependent viruses showed no selection in the encapsidation process, the autonomous parvoviruses were classically defined by their discrimination in packaging unique negative sense molecules (Rose, 1974). The rodent virus, MVM, has been shown to package a unique single-stranded molecule (Crawford et al, 1969). Canine parvovirus, as a representative of higher mammalian systems, also packages a unique strand (Paradiso et al, 1982).

However, the specificity of strand selection for the autonomous parvoviruses has recently come under closer scrutiny. Saemundsen (1980) demonstrated that between 20 - 30% of extracted bovine parvovirus virion DNA displayed features indicative of double-strandedness. Parvovirus LuIII has been reported to package strands of both senses (Muller and Siegl, 1983). These findings were corroborated by Bates et al (1984) who showed that DNA from LuIII virions annealed under the condition of 1 M NaCl at room temperature. Omission of this procedure yielded preparations having higher amounts of single-stranded material. Annealed material migrated at a position similar to KRV replicative form (RF) DNA in neutral agarose gels and demonstrated a resistance to the single-strand (ss) specific nuclease S1, whereas control ss KRV DNA was digested.

Bates et al (1984) further examined their experimental system to determine whether packaging was a cell modulated or virus-coded feature. When the autonomous parvovirus H-1 was progagated in the same culture system employed for LuIII, virion DNA was almost exclusively negative sense. Therefore, it appeared that encapsidation was a viral directed function. It remains uncertain as to whether other factors such as input multiplicity of infection (MOI) or the cell's differentiated state play a role. Thus, for at least two autonomous parvoviruses encapsidation specificity deviates from the classical axiom and parallels that of members of the adeno-associated genus.

3.6. The Duplex Nature of Genomic Termini

Evidence for regions of double-strandedness in the genome of autonomous parvoviruses came from studies of secondary structure with MVM (Bourguignon et al, 1976). The single-strand (ss) specific nuclease S1

(Ando, 1966) was used to digest viral genome labelled uniformly with [^{3}H]-thymidine and at the 5' terminus, with [^{32}P]-phosphate. Whereas tritium counts were almost completely solubilized, up to 80% of the [^{32}P] label remained resistant, indicating that a duplex existed at the 5' end. Gel analysis, following an S1 reaction, permitted the visualization of a 130 base pair (bp) fragment.

Self-annealing was also believed to be a property of the 3' end of the viral molecule. Various DNA polymerases were able to utilize the 3' terminus of MVM as a template-primer from which double-strand DNA was made (Bouguignon et al, 1976). Lavelle and Mitra (1978) proved that the 3' end of KRV genome existed in a snapback form when treatment with exonuclease I, which hydrolyzes ssDNA in the 3' to 5' direction, had no effect.

The palindromic sequences in the 5' end of the MVM genome permit the formation of a linear hairpin in which 200 of the 206 nucleotides are capable of base pairing (Astell et al, 1983a). Rhode and Klassen (1982) have found the 5' snapback of H-1 virus to consist of 242 nucleotides. Sequencing studies have revealed that the 5' termini of MVM and H-1 may each exist in two orientations, coined "flip" and "flop", which are the inverted complements of each other. In a less direct manner, this same heterogeneity has been inferred from results of mapping studies with both bovine parvovirus (Burd et al, 1983) and LuIII (Bates et al, 1984). The restriction enzyme EcoRI was known to cleave the monomeric replicative form of LuIII at one site, near the 5' terminus. When digests were analyzed on neutral agarose gels, one large fragment from the 3' end was seen, and two small, closely migrating bands were resolved, representing a heterogenous 5' end.

Astell et al (1979) have sequenced the genomic 3' termini of : H-1, H-3, KRV, and MVM autonomous parvoviruses. The hairpins were very homologous with each containing either 115 or 116 nucleotides. The unique sequence of MVM had the potential for snapback arrangement in which all but 11 bases were paired. The double-stranded stem region of this foldback possessed a 5 base mismatch referred to as the "bubble". This feature was common among the rodent viruses and was suggested to be a recognition site for a nicking enzyme during the replication process (Astell et al, 1983).

3.7. Intracellular Parvovirus Nucleic Acid Forms and a Model of Replication

Extracts of parvovirus infected cells contained three intracellular replicative forms for KRV (Hayward et al, 1978), H-1 (Rhode, 1978), CPV (Paradiso et al, 1982), and four from MVM (Ward and Dadachangi, 1978). The smallest was a double-stranded DNA molecule having the same length as the viral genomes. It existed in two configurations: an extended (open linear) and snapback (having one end covalently closed). These species are referred to as the monomeric replicative forms (mRF). The other type was a dimer of the smaller mRF, designated diRF. The unique molecule in the MVM preparation was a tetramer of the mRF.

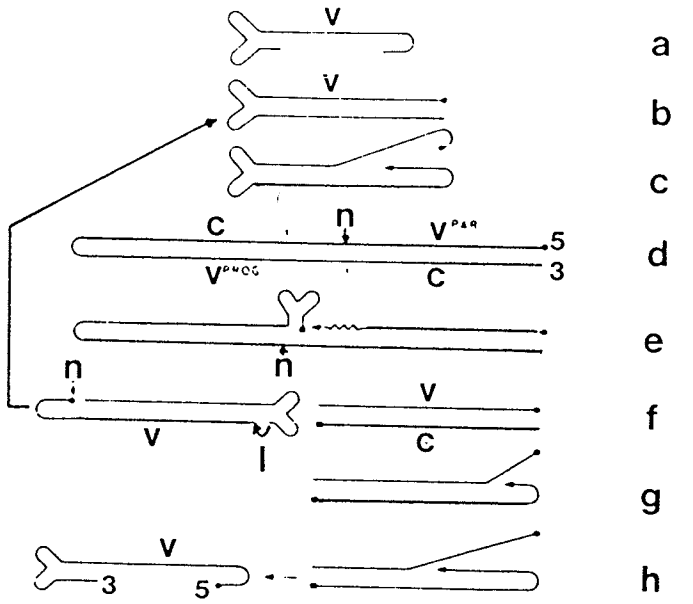
Recently, Astell et al (1983b), have presented a detailed model for parvoviral replication (Figure 1) which incorporates the above intermediates together with the observations made on 5' and 3' terminal sequences (Astell et al, 1979; Astell et al, 1983a). It has been well established that the autonomous parvoviral genome is converted to a double-stranded species (Figure 1a,b), called the monomeric replicative form (mRF) as the initial step in replication. The palindromic nature of the genomic 3'-OH serves as the primer for certain cellular DNA polymerases

(Kollek and Goulian, 1981; Pritchard et al, 1981). Polymerization proceeds to the 5' end of the virus genome and the 5' snapback is extended allowing the completion of the copy strand (Figure 1b). After this, the viral 5' hairpin is believed to reform. The complementary sequences on the copy strand also anneal, thereby creating a new 3'-OH primer. Thus, replication of the mRF begins (Figure 1c). The nascent copy strand is used as the template from which a second viral sequence is produced. Replication proceeds back to the original viral and copy junction, through this hairpin, and to the end of the molecule. The product is a dimeric RF, which consists of alternating copy and viral strands (2 each), which are actually the two mRF species arranged in a tail-to-tail fashion, with the viral sense 3' termini overlapped (Figure 1d).

A nicking enzyme is postulated to recognize a specific sequence within this overlap region. Its cutting, allows the self-complementary sequences of the 3' terminus to form a hairpin. In nicking, a 3'-OH is produced, from which polymerization proceeds, displacing the hairpin structure (Figure 1e). A second nick is introduced on the opposing strand at a similar site and the foldback is then ligated. These enzymatic functions are believed to be directed by the same protein (Astell et al, 1983a,b). This process creates the two mRF species and in doing so, maintains the unique orientation of the 3' terminus (Figure 1f).

The snapback mRF is nicked and recycled through the dimer RF. Progeny viral stands are generated from the extended mRF (Figure 1g,h) by repeated cycles of displacement-synthesis. Alternation of the 5' hairpin by nicking and transfer accounts for the inverted complementary sequence orientations, reported by Rhode and Klassen (1982) and Astell et al (1983a).

Figure 1. Hypothetical model of autonomous parvovirus genome replication.



Letters on right refer to description in text, also v, viral sense strand; c, copy sense; n, nickase; and l, ligase.

[a] From Astell, C.R., Thomson, M., Chow, M.B., and Ward, D.C. (1983b). Structure and replication of minute virus of mice DNA. Cold Spring Harbor Symp. Quant. Biol. 47:751-762.

3.8. Transcription

The minus strand of parvoviral DNA serves as the only template from which RNA transcription proceeds (Green et al, 1979; Tal et al, 1979). In analyzing the MVM genome, Pintel et al (1983) found that all four virus-specific transcripts isolated were complementary to the virion DNA. The genome possessed functional promoters for RNA polymerase II binding, this enzyme being responsible for the synthesis of heterogenous nuclear RNA (hnRNA), the precursor to mRNA. Canonical TATA sequences (or Hogness box) of "TATATAA" and "TATAAAT" were identified for the two transcriptional units of MVM, at 4 and 39 map units, respectively. These consensus sequences signal the start of messages about 30 nucleotides downstream. The second gene was also preceeded by the "CAAT" box, a sequence of "CCAATCT", lying 87 bases upstream of the TATA site, which is a feature of some promoters (Lewin, 1983).

Analysis of the three reading frames of the H-1 parvovirus, by Rhode and Paradiso (1983), identified two major (1 and 2) and two minor (3 and 4) open regions, which covered essentially all of the coding capacity of the genome. Both 1 and 2 were headed by promoter sequences, at map positions 4 and 38, respectively.

The mapping of gene products to their origin within the genome, was facilitated by hydrid-arrested translation (HART), as described by Paterson (1977). Briefly, total RNA from infected cell extracts was hybridized to cloned restriction fragments of H-1 DNA. The RNA which remained unbound was translated in a rabbit reticulocyte cell lysate, in vitro translation system, with [^{35}S] methionine. Labelled products were immunoprecipiated with antisera from either animals which had survived neonatal infection, or serum prepared against purified capsid proteins. The HART experiments of Rhode and Paradiso (1983) permitted the identification of a new viral coded

protein which mapped to the 3' end of the genome. This protein was only precipitated by the serum obtained from survivors of neonatal disease and therefore designated as noncapsid viral protein 1 (NCVP1). A functional role for nonstructural proteins such as this has been discussed in the sections on proteins and replication models.

Both structural proteins VP1 and VP2' arise from sequences on the 5' half of the genome. The mRNA for VP1 is believed to utilize the same promoter as NCVP1, and then is spliced at about map unit 40. The third transcript is initiated from the second promoter at map unit 38. All three transcripts are thought to have a common short internal splice junction and do possess polyadenylation signals at map unit 95.

3.9. Pathogenesis

The predilection of autonomous parvoviruses for actively growing cell populations has been described *in vivo* (Hampton, 1970; Rhode, 1973; Siegl and Gautschi, 1973). Parvoviruses are able to cross both the blood-brain and placental barriers, and lead to both fetal and neonatal death (Tattersall and Ward, 1978). The following is a short list of diseases caused by parvovirus infection. The hamster osteolytic viruses: H-1, KRV, H-3, and others, have been shown to cause mongolism, craniofacial lesions, and microcephaly in rodents. The diseases are believed to stem from the susceptibility of dividing cells in skeletal and dental tissues (Siegl, references therein, 1976; Tattersall and Ward, 1978).

Canine PV causes gastric enteritis in dogs of all ages and myocarditis in puppies (Carmichael and Binn, 1981). Disease, as a consequence of procine (PV) infection, is manifested by fetal death, mummification, and often by infertility and stillbirth (Joo *et al*, 1976; Mengeling and Cutlip, 1975; Mengeling *et al*, 1979).

Adult animals are usually resistant to disease, however, feline panleukopenia virus (FPV), in addition to causing spontaneous ataxia in kittens, also produces infectious panleukopenia and enteritis in adult cats (Siegl, 1976).

3.10. Parvovirus Infection

a) Host Restriction

In surveying the factors which determine the ability of a parvovirus to carry out a productive infection, the most frequently cited observation has been the reliance on elements expressed during the S phase of the cell cycle (Hampton, 1970; Rhode, 1973; Siegl and Gautschi, 1973; Tattersall, 1972; and Tennant et al, 1969). Secondly, in vivo pathogenesis has been correlated with proliferating cell populations (Siegl, 1976). Thirdly, virus-host interactions are generally defined by restricted species range. For instance, although the feline panleukemia virus (FPV) and canine parvovirus (CPV) are closely related antigenically (Johnson and Spradbrow, 1979; Lenghaus and Studdert, 1980) and share extensive DNA homology (McMaster et al, 1981), the simian virus transformed dog kidney (DKSV) cell line used to culture CPV (Paradiso et al, 1982) was a nonproductive host system for FPV (P.R. Paradiso, S.L. Rhode, III, and I.I. Singer, unpublished data).

Studies with MVM suggested that the differentiated phenotype of the host cell may play a role in determining if replication will be permitted. The MVM prototype strain, MVM(p), was plaque purified by Tattersall (1972). Bonnard et al (1976) described the isolation of an immunosuppressive MVM strain, MVM(i), as a contaminant of in vivo passaged EL4 lymphoma cells. These strain variants were indistinguishable by heterologous antisera (Tattersall and Bratton, 1983). A comparison of

virions of MVM(i) and the prototype strains by McMaster et al (1981) found no detectable differences in buoyant densities, sedimentation properties, or structural proteins. Restriction endonuclease mapping of the genomes showed that 80% of known sites were shared. A 60 nucleotide deletion was detected at map unit 92 (5' end of the viral strand) in MVM(i) and may be of possible significance.

Each virus has demonstrated a target cell specificity for productive replication, with MVM(p) multiplying in murine cells of fibroblast origin and MVM(i) in mouse T lymphocytes. The growth of each strain was restricted in the opposing host system, with no effects on the rate of infected cell growth or viability (Tattersall and Bratton, 1983). Both strains bind to the same surface receptor on each cell line, regardless of whether or not the line is restrictive (Spalholz and Tattersall, 1983). The loss of the MVM(p) receptor by the A9-8E cell line culminated in no binding of MVM(i). The viral adsorption step was not the point of restriction. By monitoring the conversion of isotopically-labelled input viral genome to the replicative form in each cell type, these authors observed a lag period of about 3 hours in the restrictive as opposed to the productive system, but by 15 hours post infection, the same levels had been reached. The suggestion has been put forward that the replication of viral DNA in the restrictive system proceeds at a reduced level relative to the productive counterpart.

Since hybrids of the fibroblast and lymphocyte cell lines supported lytic infection by either virus, it was ventured that intracellular host factors facilitated successful multiplication (Spalholz and Tattersall, 1983).

b) Binding and Uptake

The necessary first step in the replication process of any virus must be attachment or adsorption. The cytotropism displayed by MVM for the

mouse cell line A-9 has been described. In their initial work, Linser and Armentrout (1978) examined the binding kinetics of labelled full viral particles to the surface of this cell line. Optimum binding took place at pH 7.2, was 75% complete after 15 minutes of exposure, and appeared to be independent of temperature in the 4 - 37°C span studied. Attachment to cell receptors followed biphasic kinetics: a linear portion of saturable binding at 5-7 x 10^5 particles per cell, and a nonsaturable phase attributed to nonspecific binding.

The nature of the interactions at the adsorption level were explored. It was possible to elute virus which had been adsorbed to cells (at 4°C) by brief treatment with 1 mM EDTA, whereas washing failed to disrupt the association. After a two hour adsorption period, considered sufficient to attain steady-state saturation of available receptors, it was still possible to remove radiolabelled bound virion when an excess of unlabelled virus was added. Competition was also shown with empty capsids.

An infection resistant clone of A-9 was isolated and its capacity for MVM attachment was demonstrated to be nonsaturable, binding far less virus than the permissive host.

In summary, permissive cells appear to have a limited number of surface receptors. The binding of MVM to cell surface receptors was both saturable and reversible, and loss of viral specific receptors resulted in the resistance to MVM infection .

Electron microscopy of cells after an adsorption period found virus to be: randomly bound, in patches on filopodia, and also clustered in endocytotic depressions, termed coated pits. This receptor distribution reflected the internalization process, in which, a number of initially diffuse receptors were brought together by the cytoskeletal apparatus, and clustered at coated pits prior to virus uptake (Linser and Armentrout, 1978).

c) Kinetics of Virus Growth

Siegl and Gautschi (1973) monitored growth parameters of parvovirus LuIII in Hela cells by immunofluorescent staining. Cytoplasmic fluorescence was observed beginning at four hpi in both randomly growing and synchronized cultures. It was concluded that early steps in viral infection, adsorption, uptake, and transcription, were independent of the cell's position within the cell cycle. The onset of intranuclear staining was from 10 to 12 hpi in both types of cultures and peaked at 16 hpi. However, synchronous cultures displayed a much sharper rise in intranuclear labelling than did the asynchronous counterparts, having a substantially greater number of nuclei stained. The difference in competence reflected the cyclic distribution of cells in the two cultures.

Actively dividing cell populations are known to be the optimal targets for parvoviral replication, in vivo, and the sites of disease in animal systems (Siegl, 1976). Hampton (1970) demonstrated the preference of parvovirus H-1 for a cellular function(s) expressed during the late S or early G2 phases of the cell cycle. Proliferating eukaryotic cells migrate through the cell cycle prior to division. This repetitive cycle has been broken down into four distinct parts. The replication of DNA occurs during the "S" or synthesis phase. This copying period is flanked by pre- and post-DNA synthetic "gap" periods, termed G_1 and G_2, respectively. The second gap preceeds mitosis and division, "M". The intervals of the cell cycle have approximate durations of: G_1, 6 - 9 hours; S, 6 - 8 hours; G_2, 2 - 5 hours; and M, 0.5 - 2 hours. This cycle is repeated approximately every 24 hours by cultured animal cells (Flickinger, 1979).

Siegl and Gautschi (1973) observed that the time of intranuclear antigen appearance in synchronized Hela cells did not change when LuIII infection took place as late as 3.5 hours into the DNA replication period.

Cells infected at the end of the S or early G_2 phase, demonstrated kinetics paralleling that of the asynchronous culture.

These authors also reported on the effects of replicating virus on the growth of Hela cells. Infection in early S phase resulted in the complete cessation of cellular division. When virus was added to cultures during the latter half of DNA replication, the mitotic burst was both suppressed and delayed by approximately 3 hours, relative to a mock infected control. Cells were found to divide at the same time as the control, when infected during the G_2 period (ie., when DNA replication had been completed). It was concluded that the critical events in LuIII replication were associated with late S phase.

Paradiso et al (1982) described the growth cycle of canine parvovirus by immunofluorescent staining. Like LuIII, intranuclear antigen was initially detected at 12 hpi, rose markedly over the following 12 hours, and plateaued thereafter.

d) Replication and Host Cytopathology

Parvoviral replication is known to take place in the nucleus, beginning in and around the nucleolus (Singer, 1975). Immunoelectron microscopy identified newly synthesized H-1 antigen on both nucleolar chromatin and extranucleolar heterochromatin between 8 and 12 hours post-infection (Singer, 1976). The reason for viral specific staining in these regions is believed to stem from the affinity of the net acidic amino acid content of H-1 proteins (Kongsvik and Toolan, 1972) for the basic proteins within the nucleolar complex (Singer, 1975).

The first structural alteration was detected at 12 hpi, with the contraction of the nucleolar chromatin in the fibrous regions. Extranucleolar chromatin also condensed between 12 and 24 hpi. Retraction of chromatin in these regions created vacuoles, initially in the nucleolus

and later (18 - 36 hpi) in the nucleus. Unassembled viral protein was situated within the condensed chromatin, whereas assembled virions were attached to extranucleolar fibers. The dissocation of full particles from chromatin coincided with cellular breakdown at around 36 hpi (Singer and Toolan, 1975).

In conclusion, vacuolation and margination were the prominent cytopathological changes observed in H-1 infected cells. These "processes" are postulated to arise as a direct consequence of H-1 antigen binding.

Similar observations were made in canine parvovirus infected DKSV cells (Paradiso et al, 1982). Comparable alterations have also been reported in the myocardial cell nuclei of CPV infected young dogs (Hayes et al, 1979; and Carpenter et al, 1980).

e) Virion Maturation

It is now evident that both the helper dependent adeno-associated viruses (AAV) and several autonomous parvoviruses have two forms of the full particle, designated "heavy" full (HF) and "light" full (LF) which differ in buoyant density by about 0.04 g/cm^3. In the work of Kongsvik et al (1978), the basis for this phenomenon was pursued with the rodent virus, H-1. The authors labelled H-1 infected cells and harvested the virus at 24 and 96 hours post-infection. At the 24 hpi point, there was a predominance of the HF particles (60%) over the LF species. At 26 hpi, the radioactivity associated with VP2' (relative to the whole virion) was 76% for HF and 67% for LF. By 96 hpi, VP2' radioactivity had declined to 44% in the LF. A new polypeptide, designated VP2, replaced VP2' as the major component and had a molecular weight of 3,800 daltons less than VP2'. The generation of VP2 was mimicked in vitro, by trypsin or chymotrypsin. However, only conditioned medium from cultures at late infection could modify the particle from HF to LF.

A density differential and maturation scheme similar to the above has also been described for MVM (Richards et al, 1978). The authors concluded that the HF to LF processing took place primarily in the nucleus.

MATERIALS AND METHODS

4.1. Virus Origin and Stock Preparation

Two culture supernatants, #21-1A and #22-1A, deriving from equine fetus liver extract infected PFT cultures which demonstrated mild cytopathological effect (CPE), were submitted to this laboratory by Dr. Frank Wong (Manitoba Agriculture Services, Winnipeg) for virus identification and classification. Both stocks, #21-1A and #22-1A, were expanded in PFT cells, at 0.25 ml/2.5 x 10^6 cells in the 25 cm^2 flasks. A very mild CPE was demonstrated by the cultures. However, studies by electron microscopy demonstrated the presence of naked icosahedral viral capsid-like structures of homogeneous size (about 22 nm) in the secondary culture supernatants of both stocks #21-1A and #22-1A. This suggested that the viruses derived from the two fetuses were probably similar.

This thesis deals with the identification, classification and genomic structure of the equine virus isolate derived from #21-1A. Early parallel studies with #22-1A to confirm that the isolates from the two fetuses to be similar viruses are not discussed here.

Generally, a PFT monolayer, at near 70% confluency, was infected with a multiplicity of infection (MOI) of 0.01 - 0.001 $TCID_{50}$/cell (subsequently determined) in a reduced volume of serum free medium. Adsorption was conducted for one hour at room temperature (while occasionally rocking the flask) and then the culture was placed at 37°C for an additional one hour. At 2 hours post infection (hpi) the inoculum was removed and replaced with MEM supplemented with 2% FCS. The infectious culture medium was harvested at 72 hpi and clarified at 1000x g (IEC).

The preparation of a high titre equine virus stock (EPV; as it will be shown later, the equine virus isolate is a parvovirus and is thus referred

to as EPV for equine parvovirus here) will be described here since it was used to generate much of the data of this report. Briefly, a 150 cm^2 flask was seeded with $4x10^6$ cells and allowed to grow for 2 days. The growth medium was removed, the cell sheet was washed once with warm PBS, and a synchronization medium, consisting of MEM with 2% FCS and 2 mM thymidine, was added. The flask was incubated for a period of 16 hours at 37°C. After 8 hours, the cultures were synchronized for the second time by the same procedure as in Section 4.3.

Two hours before the removal of the second block, the medium was changed to serum free conditions, and the culture was infected at an MOI of about 0.5 TCID/cell, with 2 ml of EPV stock 10-8-83 ($1.6x10^6$ $TCID_{50}$/ml). After 1 hr adsorption at room temperature and a further 1 hr incubation at 37°C, the inoculum was removed, the cells were washed twice with warm PBS, and MEM with 2% FCS was added. At 72 hpi, the culture which displayed greater than 90% cytopathological effect (CPE) was harvested by freeze/thaw (in toto) and designated EPV stock 13-11-83 (1×10^8 $TCID_{50}$/ml).

4.2. Cell Culture

The porcine fallopian tube (PFT) cell line was characterized by Bouillant et al (1973). Its utility in supporting the growth of a number of RNA and DNA viruses has been reported (Bouillant et al, 1975). The cellular morphology was observed to change from an epithelial to a more fibroblastic state at around the fiftieth passage.

The cell line was obtained at passage 68 (P68) from Dr. Frank Wong (Manitoba Agriculture Services, Winnipeg). Stock cultures were prepared at P68 and P69, and stored at -70°C.

PFT cells were maintained in minimal essential medium (MEM, Gibco Cat. No. 410-1100) supplemented with 7% FCS, 1% (v/v) L-glutamine (0.292

g/l), and a 1% (v/v) antibiotic solution containing: 5 mg penicillin, 5 mg streptomycin, and 10 mg neomycin/ml (Gibco Cat. No. 600-5640). Confluent monolayers were trypsinized with an enzyme preparation (Gibco Cat. No. 610-5300) and passaged in 2 flasks at 1:7 and 1:13 splits (ie., 14 and 8% confluency, respectively). When seeded at 1:7, the cells became confluent in 3 days. All cultures were incubated at 37°C, in a water saturated atmosphere having a 2% carbon dioxide level.

4.3. Synchronization Protocol

A method of achieving a higher degree of cell synchrony was employed with the PFT cell line. Briefly, when randomly growing cells receive an initial thymidine block (in mM excess) only those cells in S phase become arrested from proceeding through the generation cycle. The remainder, distributed throughout the pre- and post-DNA sythetic periods (in G2, M, and G1) will continue to proceed to the next S phase, and accumulate at the G1/S boundary. Following a blocked interval, the cells become partially synchronized at the G1/S and S phase of the generation cycle. Removal of the thymidine from the culture releases the block and the cells migrate through the S phase. A substantially higher percentage of cells may be arrested at the G1/S boundary by applying a second thymidine block after a period of release from the first block. This release permits those cells at G1/S to completely transverse S phase but is sufficiently short that those cells at the end of S could not yet reach the next S phase. Thus at the time of application of the second block, no cells (theoretically) are in S phase and, therefore, most of the population can proceed through the cell cycle to the G1/S boundary (Cleaver, 1967).

The method employed for the synchronization of PFT cells was based on the divisions defined by Cleaver (1967). Generally, subconfluent PFT

cultures were trypsinized and reseeded at 25% confluency (depending on the culture vessel) in the maintenance medium described above (Section 4.2). Twenty-four hours later, the culture was washed once with warm PBS and synchronization medium consisting of MEM with 2% FCS, 1% L-glutamine, and 1% antibiotics, supplemented with 2 mM thymidine, was added. After 16 hours incubation at 37°C, this medium was removed and the cells were washed twice with warm PBS. Fresh growth medium was added for a release period of 8 hours. Following this incubation, the medium was replaced as in the first thymidine block for a second 16 hour period.

4.4. The Tissue Culture Infectious Dose Assay (TCID)

a) Primary Infection

The assay for infectivity in an untitred sample was determined utilizing an infinite serial dilution. PFT cultures were trypsinized and the cell number was adjusted to 1.5×10^5 cells/ml in MEM with 2% FCS. Microtitre plates (96 wells) were seeded with 1.5×10^4 cells/well, delivered in 0.1 ml, using the Finnpipette multiwell dispenser. Cells were allowed to attach for 3 hours. The sample to be assayed was usually added to the first well with additional medium such that the total volume was 0.2 ml. Mixing was accomplished by drawing and expelling medium from the pipette, prior to transfer of 0.1 ml to the next well. The volume serially transferred was always 0.100 ml (unless otherwise specified) and resulted in a net 2-fold dilution between wells. Plates were incubated at 37°C and cultures were examined for CPE at 48 and 72 hpi.

b) Secondary Infection

Since CPE may not have been detectable in wells which received a low MOI (ie., those near the endpoint), it was believed that a second plate should be infected with the output of each well from the primary assay.

Beyond 72 hpi, the cell monolayer began to degrade due to overcrowding. It was not desirable to seed fewer cells in the primary assay, because the cells would grow in patches and would fail to reach confluency. Consequently, the CPE discernment would not be possible. Thus, a secondary infection would amplify the CPE, due to the increased MOI and improve the reproducibility and sensitivity of the infectivity assay.

The procedure was essentially as described for the primary infection except that the cell number was increased to 2×10^4 cells/well. Infection involved the direct transfer of up to 0.05 ml of the medium from the primary to the corresponding well in the second plate. Both the increased cell seeding number and virus input, made it possible to obtain the final CPE results at 48 hpi, by reading microscopically.

4.5. Observations on the Growth of Synchronized PFT Cells Infected with Equine Parvovirus Isolate

Two 25 cm^2 flasks of PFT cells were synchronized by the double thymidine block. Two hours before the scheduled removal of the second block, the medium in both flasks was changed to synchronization medium lacking FCS, and one was infected at an MOI of 0.8 TCID/cell. Both were maintained at room temperature, 1 hour, and then incubated for a further hour at 37°C. Each flask was trypsinized, cells were counted and reseeded at 5×10^5 cells/flask (equivalent to a 1:4 passage) in MEM with 2% FCS. At 24 hpi, the passaged cultures were trypsinized and cell number quantitated.

4.6. Determination of EPV Growth Cycle

a) Asynchronous

A subconfluent 80 cm^2 flask of PFT cells was infected at an MOI of 0.2 TCID/cell with EPV stock 13-11-83 (1x108 TCID/ml) in 5 ml of serum free

medium. The culture was rocked occasionally for one hour while at room temperature and then incubated for a further one hour period at 37°C. Cells were trypsinized, resuspended in growth medium with 2% FCS, and aliquotted at $4x10^5$ cells/35 mm tissue culture dish. Single dishes were freeze/thawed (2x) and collected in sterile 5 ml Falcon tubes at selected time points after infection. The suspensions were sonicated 2x15 seconds with intermittent cooling, using the microtip (Fisher Dismembranetor) at 40 watts. Aliquots were assayed for infectivity by the TCID method (Section 4.4).

b) Synchronous

Pig fallopian tube cells in an 80 cm^2 flask were synchronized by the double thymidine blockade method (Section 4.2). Two hours prior to the second release, the monolayer was infected with EPV (13-11-83) at a multiplicity of 1.4 $TCID_{50}$/cell, in 5 ml of synchronization medium lacking FCS. The infected cells were incubated in the same manner as the asynchronous culture. Cells were released by trypsinization, resuspended in normal MEM with 2% FCS, and dispensed at $4x10^5$ cells/35 mm dish, as above. The harvest procedure was similar to that described for the asynchronous cultures, with the exception that the scheduled points were slightly different (to be discussed in Results Section 5.3).

4.7. Temperature Stability

Aliquots of equine virus stock 9-2-83 ($2x10^4$ TCID/ml) were diluted 8 fold in PBS to a final volume of 0.200 ml. Individual samples were incubated at 0, 37, 40, 50, 55, 60, and 70°C, for 15 minutes, permitted to cool to room temperature and then maintained on ice prior to the infectivity assay (Section 4.4). On the basis of the initial results, the experiment was repeated at temperatures: 0, 50, 60, 70, 80, 90, and 100°C.

4.8. Treatment of EPV with Nonidet P40 and Sodium Dodecylsulfate

The detergent, Nonidet P40 (NP-40), was obtained from Particle Data Laboratories Ltd. (Elmhurst, Ill.) and prepared as a 10% (v/v) stock solution in sterile water. Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Laboratories (Richmond, Calif.) and made to a 10% (w/v) aqueous solution.

Equivalent amounts of undiluted EPV stock were incubated with a final concentration of either 1% (v/v) NP-40 or 1% (w/v) SDS, for 30 minutes on ice. Prior to titration, virus was diluted 500 fold, in order to prevent the lysis of PFT cells by the detergents used.

Changes in infectivity were measured relative to a virus stock control, diluted initially with a volume of sterile PBS equivalent to that of the detergent's, and then processed identically as the test specimens. The experiment was repeated twice.

4.9. Organic Solvent Extraction

A CsCl gradient purified preparation of EPV (from density 1.416 g/cm^3) was dialyzed against TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA] and extracted three times with an equal volume of ether. The aqueous phase was placed under a stream of nitrogen gas to remove residual ether prior to the TCID assay. The effects of ether extraction were measured against control untreated material from the same density which was similarly treated but without the test reagent. The experiment was conducted on a single sample and assayed once by the TCID method.

4.10. Tryptic Digestion of EPV

Into each of three microcentrifuge tubes was placed 0.090 ml of EPV stock 13-11-83 ($1x10^8$ TCID/ml). To two of these was added 0.010 ml of a commercial trypsin preparation (Gibco, Cat. No. 610-5300) for a net enzyme

concentration of 50 ug/ml. As a control, 0.010 ml of MEM was added to the third virus aliquot. All samples were incubated for 1 hour at 37°C and then assayed for infectivity.

4.11. Selection and Preparation of EPV for Electron Microscopy

A CsCl gradient preparation of EPV from a fraction having density 1.416 g/cm^3 was selected for electron microscopy. EM grids (400 mesh) were obtained precoated with 0.3% Formvar from the Electron Microscopy Unit (Department of Medical Microbiology). To prepare the virus for visualization, the grid was floated Formvar-side-down on a 10 ul droplet of the above fraction for 5 minutes. Excess liquid was carefully removed using Whatman filter paper placed at the grid's edge followed by 2 - 3 washings on drops of filtered, sterile PBS. Material on the grid was then negatively stained by floatation on a drop of 0.0025 M phosphotungstic acid (PTA; pH 7.0) for 4 minutes and excess PTA removed as above. The grid was dried and viewed with the Philips (Model 201) transmission electron microscope, at 60 kilovolts.

4.12. Preparation of EPV for Homogeneous Gradient Ultracentrifugation and Infectivity Assay

Two 80 cm^2 flasks seeded with $4.4x10^6$ PFT cells were infected as described above at an MOI of 10 $TCID_{50}$/cell or mock infected. AT 48 hpi, the infected culture displayed extensive CPE while the parallel mock infected flask demonstrated no apparent CPE. Cells were disrupted, without removing medium, by freeze/thaw (twice), collected and sonicated 5 times for 10 second durations with intermittent cooling on ice/water to dissipate heat. To aid in the release of particles not liberated through such treatment, n-butanol was added to 1% (v/v) and kept on ice for one hour, as

described by Myers and Carter (1980). Medium was clarified by centrifugation at 8,000 rpm for 15 minutes (SS-34 rotor, Sorval).

Cesium chloride salt and TE buffer were added to the infectious lysate to a final volume of 13 ml, of density 1.408 g/cm^3. Samples were spun at 32,000 rpm for 48 hours, 20°C (Beckman SW40). Fractions were collected after bottom puncture, and refractive indices were determined with a Fisher refractometer. Selected fractions were assayed for infectivity utilizing the TCID method (Section 4.4).

4.13. Preparation and Infectivity Assay of [^{3}H] Thymidine Labelled EPV

A subconfluent culture of PFT cells in 150 cm^2 flask infected with EPV at an MOI of 0.01-0.001 $TCID_{50}$/cell, was labelled for 48 hours, beginning at 24 hpi, with 500 uCi of [methyl-^{3}H] thymidine (aqueous; sp. act. 2 Ci/mmole, Amersham) in 15 ml of MEM with 2% FCS. Extracellular virus (only) was harvested at 72 hpi, as described in Section 4.15. Samples were separated on preformed linear CsCl gradients (1.2-1.45 g/cm^3) at 35,000 rpm for 16 hours, with the SW60 rotor. Four drop fractions were collected. Radioactivity was determined by liquid scintillation (LKB Rackbeta). Refractive indices were determined using the Fisher refractometer and then samples were dialyzed against 2 changes of TE buffer (pH 8.0) overnight for infectivity assay by the TCID method (Section 4.4).

4.14. Preparation of [^{35}S] Methionine Labelled Mock and EPV Infected PFT Total Cell Lysates

Two cultures of PFT cells were mock or EPV infected (10 $TCID_{50}$/cell) as described in Section 4.1, in 60 mm dishes at 50 to 60% confluency ($2x10^6$ cells). At 20 hpi, the cultures were washed once with warm PBS, and

labelled with 50 μCi/ml of [^{35}S] methionine (1000 Ci/mmol; New England Nuclear, Cat. No. NEG-009H, Boston, Mass.) in 76% methionine-free MEM (Gibco, Cat. No. 78-0114), 20% normal MEM, 2% FCS, 1% L-glutamine, and 1% antibiotics. After 24 hours growth, the labelling medium was removed and monolayers were washed with cold PBS. Total cell lysates were prepared by the addition of SDS-PAGE sample buffer containing: 2% SDS and 5% 2-mercaptoethanol directly to the dish. These were collected, denatured by boiling at 100°C for 2 minutes (Jay et al, 1983) and analyzed by SDS-PAGE on a 12.5% polyacrylamide gel. The gel was fixed in 20% trichloracetic acid, washed, and dried. Autoradiography and fluorography was carried out as described by Laskey and Mills (1975).

4.15. Preparation and Analysis of [^{35}S] Methionine Labelled EPV Virion

A 75 cm^2 tissue culture flask was seeded with 4x10^6 PFT cells and infected 3 hours later at an MOI of roughly 0.01 - 0.001 TCID/cell with EPV stock 9-2-82 (2x10^4 TCID/ml). At 29 hpi, the infected culture was washed once with warm PBS, and labelled under the conditions described in the previous Section 4.14, for 24 hours.

Extracellular virus was harvested by collecting the culture medium at 48 hpi. This was freeze/thawed (2x), clarified of debris by spinning at 2,000 rpm for 15 minutes, and the virus pelleted by centrifugation at 45,000 rpm for 2 hours in the SW60 rotor (Beckman). The pellet was redissolved in 0.2 ml of TE buffer (pH 8.0), loaded onto a preformed 1.20 to 1.45 g/cm^3 CsCl gradient, and spun at 35,000 rpm for 16 hours in the SW60 rotor. Four drop fractions (approximately 0.2 ml) were collected from the tube bottom and refractive indices recorded. Portions of fractions covering densities from 1.45 to 1.22 g/cm^3 were diluted with 3 volumes of

TE buffer and pelleted at 100,000 x g for 2 hours with the Beckman Airfuge. The pellets were redissolved directly in PAGE sample buffer, denatured by boiling for 2 minutes, and analyzed on a 10% polyacrylamide gel (Laemmli, 1970).

4.16. Comparison of Extracellular EPV (Prepared Without) and Cell Associated Virion (Isolated With) Trypsin

PFT cells were infected, labelled with [^{35}S] methionine (Section 4.14) and extracellular virion harvested at 48 hpi as described (Section 4.15). The intracellular virion was harvested separately. Briefly, the culture was washed once with cold PBS and the cells were removed by scraping. The cells were combined with the floating cells in the culture supernatant (low speed pelleted during clarification of the extracellular fraction) and pelleted at 1000x g for 15 minutes (IEC). After resuspending in TE buffer (pH 7.5), the cells were sonicated at 40W, 3 times for 5 seconds with intermittent cooling. Deoxycholate was added to 1% w/v final. The suspension was digested with trypsin (50 ug/ml) for 1 hour at 37°C. Cesium chloride gradient (1.20-1.55 g/cm^3) analysis was performed as detailed in Section 4.15.

Both extracellular and intracellular fractions were TCA precipitated, dissolved in 1X SDS-PAGE sample buffer, and analyzed on a 10% polyacrylamide gel.

4.17. Growth of EPV as a Function of Medium Phosphate Concentration

The optimum condition for *in vivo* growth of EPV in low inorganic phosphate medium was studied. Six 60 mm tissue culture dishes were seeded at 25% confluency and passed through the double thymidine blockage (Section 4.2). Two hours prior to the second release, 5 dishes were infected (0.05

- 0.1 TCID/cell) in synchronization medium lacking FCS. The sixth dish was similarly treated but without virus in the inoculum and served as the mock infected control. After 2 hours incubation, the cells were released by washing twice with phosphate-free Hepes buffered Earl's salts, followed by a 15 minute incubation with the same at room temperature (to reduce internal diffusible phosphate levels).

Labelling medium consisted of: 92% phosphate free MEM (Gibco, Cat. No. 157), 5% dialyzed FCS, 1% L-glutamine and 1% antibiotics (Gibco, Cat. No. 600-5640), supplemented with 10 mM Hepes (pH 7.4). At the time of labelling, normal growth medium was added to give final phosphate levels of 0, 1, 2, and 5% relative to the concentration of normal medium. Culture medium was maintained at near neutral pH by occasional titration with 0.2 M NaOH. One infected culture was maintained in normal medium (with 2% FCS), as a control for growth under routine stock preparation conditions. Dish contents were harvested at 72 hpi and assayed for infectivity by the TCID method (Section 4.4).

4.18. Preparation of [^{32}P] Labelled Equine Parvovirus

Two cultures of PFT cells seeded at 3-4 x 10^6 cells per 150 cm^2 flasks, were allowed to grow 18 - 24 hours prior to synchronization by the double thymidine block method (Section 4.2). Infection with EPV at 10 $TCID_{50}$/cell was carried out as described above (Section 4.1).

a) Labelling Procedure

At two hours post infection (ie., 16 hours after the second thymidine block), the inoculum was removed and each monolayer was washed twice, for a total of 10 minutes, with phosphate-free, Hepes buffered Earl's salts (to reduce inorganic phosphate levels). Following trypsinization, cells were resuspended directly in 24 ml of labelling medium: 91.5% phosphate free

MEM, 0.5% normal MEM, 5% dialyzed FCS, 10 mM Hepes (pH 7.4), 1% L-glutamine, 1% antibiotics, and 8 mCi of carrier free, [^{32}P] orthophosphate (aqueous; New England Nuclear, Cat. No. NEX-053). The cell suspension was dispensed into 150 cm^2 flasks at 12 ml/flask (ie., at 1:2 split). Due to the limited buffering capacity of Hepes, it was necessary to visually monitor and titrate medium pH with 0.2 M NaOH.

b) Harvest

At 48 hpi when extensive CPE had developed, the virus was harvested in toto by freeze/thaw (2x). After standing upright at 4°C for 5 minutes to drain, the suspension was collected and the flasks were washed twice with cold PBS which was pooled with the suspension. The cellular debris was clarified by two successive spins; low speed (2,000 rpm, IEC) and high speed (12,000 rpm, Sorval SS-34), both at 4°C for 15 minutes.

The supernatant was kept on ice until ultracentrifugation. The pellet from the clarifying spins was resuspended in 9 ml of TE buffer (pH 8.0) and sonicated twice for 15 seconds with cooling, using the Microtip at 40W (Fisher Dismembranetor). One ml of 10% (w/v) deoxycholate (in TE buffer, pH 8.0) was added and incubated on ice for 1 hour. The debris was removed by centrifugation at 12,000 rpm for 15 minutes (Sorval, SS-34). Virus originating from the supernatant and cellular debris were designated extracellular and intracellular, respectively; and pelleted separately by ultracentrifugation at 35,000 rpm for 3 hours, 4°C (Beckman Type 42.1 rotor). The pellets were resuspended in 1.2 ml of TE buffer (pH 8.0).

c) Primary Equilibrium Density Gradients

Linear cesium chloride gradients in TNE buffer (25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM Na_2 EDTA) were preformed from density 1.20 to 1.50 g/cm^3. Extra- and intracellular virus were loaded in parallel and spun at 32,000 rpm for 40 hours, 20°C (Beckman, SW40 rotor).

Five drop fractions (approximately 0.2 ml) were collected from the bottom into siliconized Eppendorf tubes. Refractive indices of alternate fractions were measured with a Fisher refractometer and converted to the corresponding densities. Total radioactivity in each fraction was estimated by Cerenkov counting in the tritium window of the LKB Rackbeta, and plotted with the fraction densities versus fraction number.

d) Infectivity Profiles

Aliquots of selected fractions were diluted in medium having 2% FCS and assayed by the TCID method (Section 4.4) to determine the densities with infectious virus.

e) Secondary Equilibrum Density Gradients

On the basis of the radioactivity and density profiles of the primary extracellular gradient (Figure 13), the following fractions were pooled: A (1.415 - 1.445 g/cm^3) and B (1.387 - 1.415 g/cm^3). The intracellular gradient possessed a single distinct peak (Figure 14) from which fractions with densities 1.381 - 1.416 g/cm^3 were combined. The pooled material was diluted with an equal volume of TE buffer (pH 8.0) and loaded on a second set of preformed, 1.30 - 1.50 g/cm^3 gradients. These were centrifuged at 40,000 rpm for 20 hours at 20°C (Beckman, SW60) and were processed in the same way as the primary gradients.

4.19. Phenol Extraction

The radioactive material of the extracellular fraction which rebanded at 1.435 g/cm^3 in two successive CsCl equilibrium gradients, was dialyzed against a buffer containing: 1 M NaCl, 0.010 M Tris-HCl (pH 8.0), and 0.001 M Na_2EDTA, to remove CsCl. Following dialysis, the material was placed in a siliconized Eppendorf tube and 2 ug of sRNA was added as carrier.

To a portion of this material was added SDS to 0.5% (v/v) from a 10% w/v stock. It was then extracted three times with a buffer saturated (TE, pH 8.0), phenol/chloroform mixture. (The buffer/phenol/$CHCl_3$ ratio was 1:1:1. Chloroform contained isoamyl alcohol at 1 part per 50). After extracting once with $CHCl_3$/isoamyl alcohol, the aqueous phase was adjusted to 0.3 M NaCl and 2.5 volumes of 95% ethanol was then added, inverted to mix, and froze at -70°C.

The precipitated nucleic acid was pelleted by centrifugation at 10,000 rpm (Microfuge) for 15 minutes, 4°C. Ethanol was removed and the pellet was dried briefly, in vacuo. Sterile TE buffer (pH 8.0) was added to redissolve the pellet and the nucleic acid was stored at 4°C.

4.20. Enzymatic Susceptibility

The nature of the equine viral genome was probed with 3 enzymes: bovine pancreatic ribonuclease A (RNase A), deoxyribonulcease I (DNase I), and nuclease S1 (S1). RNase A digests RNA, DNase I digests both single and double stranded DNA, and nuclease S1 cleaves single stranded nucleic acids, both RNA and DNA (Maniatus et al, 1982).

RNase A (Sigma, Cat. No. R-4875) was prepared as a 10 mg/ml stock solution, boiled for 5 minutes (100°C) and aliquotted for storage at -20°C. RNase A was used at a concentration of 0.1 - 1.0 µg/ml for 1 hour (37°C) in reactions containing 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, and 20 µg/ml of unlabelled sRNA carrier. Control reactions were conducted using [^{3}H] uridine cytoplasmic RNA.

DNase I (sp. act. 2200 U/mg protein; Worthington Laboratories) was used at 50 U/ml in reactions with 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 20 µg/ml unlabelled, sonicated herring sperm DNA. Incubations were conducted at 37°C for 1 hour. To monitor activity [^{3}H] thymidine labelled DNA was used.

Nuclease S1 (sp. act. 189,000 U/mg protein; Sigma) was prepared as a labroatory stock of 2,000 U/ml in a storage buffer containing: 30 mM NaOAc (pH 4.5), 150 mM NaCl, 0.5 mM $ZnSO_4$, and 1 µg/ul bovine serum albumin (BSA, Fraction V; Sigma) as carrier. An optimum amount of stock S1 for use in the digestion of single-stranded DNA was determined to be 50 U/ml, in a calibration experiment (data not shown). Cellular [^{3}H] thymidine labelled DNA (sp. act. approx. 10,000 cpm/µg) was prepared and used as a tracer for enzyme activity. All reactions contained 20 µg/ml unlabelled sonicated herring sperm DNA. Nucleic acid preparations were denatured to single-strand molecules by heating at 100°C for 10 minutes, followed by quick chilling in an ice-water bath (heat-quench). Reactions were conducted in the above buffer, with the exception that BSA was added to 0.1 µg/ml. Nuclease S1 was added, mixed, and incubated at 50°C for 1 hour. The ability of the enzyme preparation to hydrolyze native [^{3}H] cellular DNA (as trace) under this condition was also determined.

The incubations for all 3 enzymes were carried out in siliconized glassware. After the reaction, the tubes were cooled on ice and BSA was added to 100 µg/ml and mixed. Cold TCA solution was then added to 10% final concentration and vortexed. After standing for 30 minutes on ice, TCA-precipitable material was collected on Whatman GF/A glass filters using the Millipore Sampling Manifold. Filters were washed twice with cold 10% TCA, once with ethanol, dried and counted in a toluene based, liquid scintillation cocktail. To adjust for background radioactivity, the counts from a blank filter, processed identically, were subtracted from each sample.

4.21. Velocity Sedimentation of the [^{32}P] Labelled Virion Peak Fraction

The homogeneity of the twice CsCl gradient purified virion fraction was examined by its sedimentation in neutral sucrose, similar to the method of Myers and Carter (1980). Briefly, the virion material was diluted 10 fold in TE buffer (pH 8.0), and loaded on a linear preformed, 15 - 30% (w/v) sucrose gradient, in: 0.010 M Tris-HCl (pH 8.0), 1 M NaCl, and 0.001 M Na_2 EDTA. Tubes were centrifuged at 40,000 rpm for 2 hours, 4°C (Beckman SW60). Four drop fractions were collected by bottom puncture into siliconized microcentrifuge tubes and the total radioactivity in each fraction was estimated by Cerenkov counting in the tritium window of the LKB Rackbeta.

4.22. Isolation and Enzymatic Analysis of Resolved Nucleic Acids

When the radioactive profile was plotted versus the fraction number (Figure 15), a peak was detected at fraction 10, together with material which did not enter the gradient, fraction 24. Both of these were selected for analysis. Sucrose was removed by dialysis against: 0.010 M Tris-HCl (pH 8.0), 1 M NaCl, and 0.001 M Na_2 EDTA for 4 hours at 4°C. The nucleic acids were phenol extracted and ethanol precipitated, as before.

Because a few autonomous parvoviruses have now been found to package variable amounts of the complementary strand, a portion of extracted fraction 10 was denatured at 100°C for 5 minutes and then incubated under high salt conditions [15 mM NaOAc (pH 7.2), 150 mM NaCl] overnight, at 65°C to allow reassociation. After the incubation, the nucleic acid was cooled gradually to room temperature and ethanol precipitated.

Portions of fractions 10, 24, and the annealed 10, were incubated under the conditions described previously for RNase A, DNase I, and

nuclease S1 (Section 4.20). Once the incubations were completed, they were phenol extracted and ethanol precipitated. The products were analyzed by electrophoresis in a 1.4% agarose gel and visualized by autoradiography (Section 4.25).

4.23. Preparation and Isolation of Intracellular Replicative Forms of EPV

PFT cultures in two 150 cm^2 flasks were synchronized and mock infected or infected with EPV at an MOI of 5-10 $TCID_{50}$/cell as described above (Sections 4.1, 4.3).

Intracellular replicative forms were prepared by a modified Hirt method (Hirt, 1967; Jay et al., 1979). Between 20 - 24 hpi, the growth medium was decanted into sterile 50 ml Corning centrifuge tubes. The cell sheets were washed once with 5 ml cold PBS and pooled with the medium. The tubes were centrifuged at 2,000 rpm (IEC, model centrifuge) for 15 minutes, 4°C, to pellet floating cells. These were resuspended in 0.75 ml (per 150 cm^2 flask to be harvested) with 50 mM Tris-HCl (pH 7.9). The suspension was pipetted into the original flasks and 4 volumes (ie., 3 ml) of lysing buffer containing 0.75% SDS, and 10 mM Na_2 EDTA (pH 7.9) was added to yield a final 10 mM Tris-HCl (pH 7.9), 0.15% SDS, and 2 mM Na_2 EDTA. The flasks were rocked to wet the entire cell surface.

After 20 minutes at room temperature, the flask contents were gently swirled and poured into preweighed Nalgene polycarbonate centrifuge tubes. The weight differences between filled and empty tubes were used to approximate the lysate volumes (with the assumption that density was roughly 1 g/ml). One-quarter volume of 5 M NaCl (1 M NaCl final) was added, the tubes were gently inverted 10 times, and then placed on ice for 8 hours.

The tubes were spun at 12,000 rpm for 30 minutes, 4°C (Sorval, SS-34). The supernatant was collected in siliconized tubes and digested with 100 µg/ml Proteinase K (Merck, cat. no. 24568) for 1 hour at 56°C. After the incubation, the contents were phenol/chloroform extracted and ethanol precipitated (Section 4.19). The pellet was redissolved directly in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl, and treated with 50 µg/ml of RNase A for 1 hour (37°C). The reaction was terminated by the addition of SDS and phenol extracted, as above. The DNA pellet was redissolved in TE (pH 8.0) and stored at 4°C.

4.24. Preparation of [^{32}P] Labelled Intracellular Replicative Forms of EPV

In the preparation of the [^{32}P] labelled material, the cell numbers, synchronization, MOI, and harvest procedures were as described in the preceeding section. The labelling protocol was as described for the production of [^{32}P] labelled EPV virion (Section 4.18), with the exception that the net radioisotope was 1 mCi/150 cm^2 flask.

4.25. Analysis of Intracellular Replicative Forms

a) Horizontal Agarose Gel Electrophoresis

To a 250 ml Erlenmyer flask was added, an amount of agarose-ME (Marine Colloid) depending on the concentration required for 100 ml gel, 10 ml of a 10X agarose gel buffer containing 0.4 M Tris-HCl (pH 7.8), 0.005 M NaOAc, and 0.010 M Na_2 EDTA, and distilled water to 100 ml. Volume of the gel solution was re-adjusted to 100 ml after the agarose was completely melted and then cooled to 56°C. Ethidium bromide (EtBr, Bio-Rad) was added to the gel solution to a concentration of 1 µg/ml prior to casting on the horizontal gel apparatus. Running buffer was made by diluting the stock buffer to 1X concentration with water plus EtBr to 1 µg/ml.

To nucleic acid samples was added one-tenth volume of a loading buffer [10 mM Tris-HCl (pH 7.5), 10 mM Na_2 EDTA, 0.1% SDS, 0.02% bromophenol blue, 0.02% xylene cyanol, and 50% glycerol]. These were loaded into the preformed slots of the submerged gel. DNA molecular weight standards were the Hind III and EcoRI/Hind III digests of bacteriophage λ DNA (Boehringer Mannheim) 0.25 µg/slot. The Hae III digest of bacteriophage ϕX_{174} RF DNA (Bethesda Research Laboratories, BRL) was used at 0.5 µg/slot. Electrophoresis was conducted at 125 V for 2 hours or until the bromophenol blue dye had migrated 10 cm from the origin.

DNA was visualized by short wave UV irradition with a transilluminator and photographed with Polaroid Type 57 film.

Radioactive specimens were fixed in the gel matrix by shaking in a solution of 9% acetic acid for 30 minutes. After washing for 5 minutes, the gels were dried in vacuo with heating, onto Whatman filter paper. Radioactive bands were visualized by exposure to Kodak X-OMAT film at room temperature (autoradiography) or with the Dupont Cronex intensifying screen at -70°C (fluorography).

b) Preparative Agarose Gel Electrophoresis

When it was necessary to obtain specific viral replicative forms from the crude intracellular extracts, 1% agarose gels were routinely used. Up to 50 µg of infected preparation was loaded in either 2.5 or 5 cm wide slots and electrophoresed as described. Unlabelled bands were UV-visualized with EtBr and the desired bands were rapidly excised. Gels with radioactive specimens were exposed to Kodak X-OMAT film coupled with the Cronex intensifying screen.

Samples were recovered from the excised slices according to the method described by Maniatus et al (1982). Briefly, slices were placed in dialysis tubing with 0.5X agarose gel buffer together with 2 µg sRNA as

carrier. The dialysis bags were aligned perpendicular to the current direction and electrophoresis was conducted for 3 hours at 100 V. After electroelution, the contents were centrifuged into siliconized polypropylene tubes at 2,000 rpm for 10 minutes (IEC). The tubing was washed once with the same buffer, pooled, and SDS was added to 0.5%. The recovered material was phenol extracted and ethanol precipitated as before. The recovery of radioactive samples was monitored throughout with a Geiger counter.

4.26. Identification of Monomeric Replicative Form (mRF) 5' Terminus

a) Preparation and Purification of Unlabelled Hairpin mRF

Crude, unlabelled, infected extract was heated at 100°C for 5 minutes, quick cooled in an ice-water bath, and electrophoresed on a 1% agarose gel. The hairpin mRF band was UV visualized with EtBr, rapidly excised, and electroeluted as described (Section 4.25).

b) Estimation of mRF Recovery

An aliquot of the recovered hairpin molecule was rerun with the EcoRI/Hind III digest of bacteriophage λ DNA. The amount of mRF was estimated by comparing its UV intensity to the various marker bands. The percentage of the fragment, having roughly the same intensity as mRF, to the total λ molecule was calculated:

$$\frac{\text{fragment base pair}}{\text{base pair}} \times \mu g\ \lambda\ \text{marker loaded}$$

Micrograms were converted to picograms:

$$\mu g \times \frac{10^6\ pg}{\mu g} = pg\ mRF$$

Using the approximate molecular weight of 640 pg/pmole for a base pair, the pmole base pair was ascertained:

$$\frac{\text{pg mRF}}{6.40 \times 10^2 \text{ pg/pmole bp}} = \text{pmole bp mRF}$$

Since the length of the mRF molecule was around 5,000 bp, the pmole mRF was determined:

$$\frac{\text{pmole bp mRF}}{5 \times 10^3 \text{ bp}} = \text{pmole mRF loaded on gel}$$

c) Dephosphorylation of the 5' Terminus

In labelling the 5' end of mRF hairpin DNA with polynucleotide kinase and [γ -^{32}P] ATP, direct phosphorylation is more efficient than phosphate exchange. It is therefore necessary to remove the 5' terminal phsophate group prior to phosphylation with [^{32}P] by the kinase. The amount of mRF has been estimated above (Section 4.26b). Bacterial alkaline phosphatase (BRL) was obtained at 208 U/µl and diluted to a laboratory stock of 20 U/µl. All reaction conditions were described as by the supplier. Briefly, the reaction mixture contained: 3 pmole mRF and 60 U B.A.P., in 10 mM Tris-HCl (pH 8.0) in a final volume of 40 µl. After incubation at 65°C for 1 hour, the reaction was terminated by adjusting the volume to 100 µl with TE and adding SDS to 0.5%. The mixture was phenol/$CHCl_3$ extracted 3x and ethanol precipitated (Section 4.19).

d) Labelling the 5' End

T_4 polynucleotide kinase was purchased from P-L Biochemicals (Milwaukee, Wisconsin). The kinase reaction was conducted as detailed by Maniatus et al (1982). Three pmole of dephosphorylated mRF 5' ends was incubated with 15 U T_4 polynucleotide kinase and 15 pmole (43 uCi) of [γ -^{32}P] ATP (sp. act. 2900 Ci/mmole, NEN) in kinase buffer: 50 mM

Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreotol (DTT), 0.1 mM spermidine, and 0.1 mM Na_2 EDTA. After 1 hour incubation at 37°C, the reaction contents were phenol extracted and ethanol precipitated.

As a control, the marker fragments of the Hind III digest of bacteriophage λ DNA were dephosphorylated and kinased in parallel.

4.27. Orientation of Restriction Enzyme Sites

Purified monomeric replicative form EPV DNA, either uniformly (in vivo) or 5' end (polynucleotide kinase) labelled with [^{32}P], was digested with 2 U of the restriction endonucleases BglII, EcoRI, HaeIII, and HindIII, for 1 hour at 37°C in buffers as recommended by the manufacturer. DNA digests were analyzed on an agarose gel and visualized by fluorography on x-ray film (Section 4.25a).

4.28. Mapping the mRF by Single and Double Restriction Enzyme Digests

Aliquots of uniformly labelled, electrophoretically purified total mRF DNA (about 300 cpm) were incubated with 2 U of restriction endonuclease in a reaction volume of 10 µl, buffered according to the supplier's specifications. Reactions were conducted 1 - 1.5 hours at 37°C. When double enzyme digests were performed, enzymes having identical salt conditions were added and incubated simultaneously. Otherwise, pairings of low and high salt requiring endonucleases were conducted with the low salt enzyme initially. After this, salt concentration was adjusted and the second enzyme was added.

Digests were analyzed on 1 or 1.4% agarose gels as required. As a rule, unlabelled or 5' end labelled DNA molecular weight markers, covering a range from 23,000 to 100 base pairs (BRL, Boehringer Mannheim) were included in all gels from which standard plots of length versus migration were made.

RESULTS

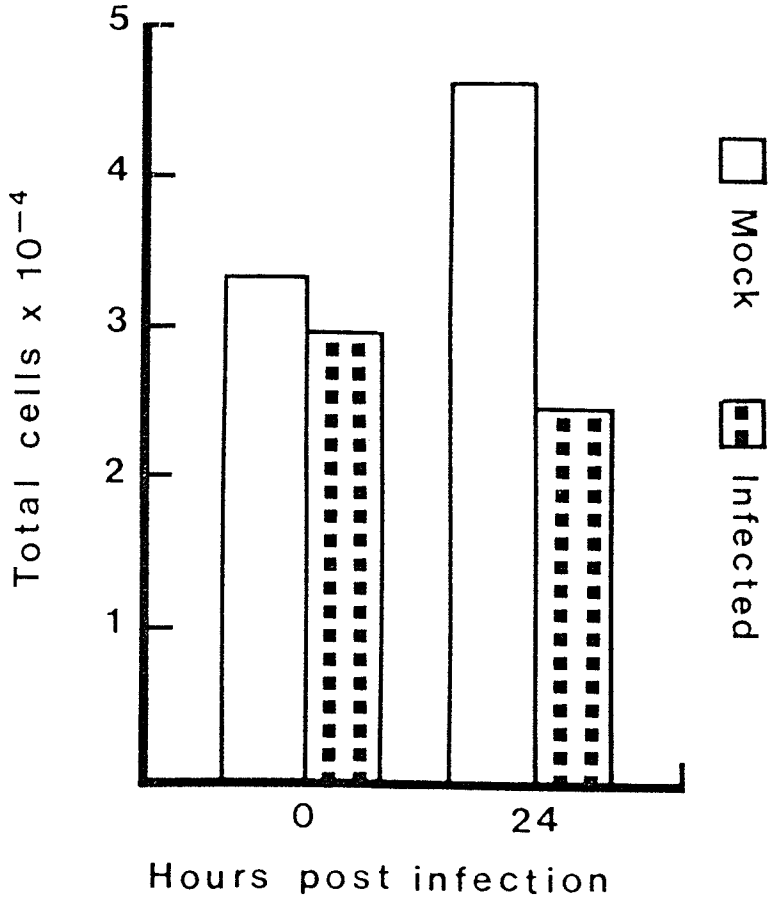
5.1. Production of High Titre Equine Parvovirus (EPV) Stock

The preparation of a high titre equine virus stock (EPV; as it will be shown later, the equine virus isolate is a parvovirus and is thus referred to as EPV, for equine parvovirus here) was desired for the characterization experiments. As there was only a small amount of the original isolate #21-1A, the virus was passaged to generate stock material. Initial culturing produced stocks having low amounts of infectivity. Generally, PFT cells were not infected with large volumes of innoculum, since this agent was an animal virus, low infectious inputs were used to avoid the generation of defective particles.

A PFT culture, synchronized by the double thymidine block (Section 4.3) was infected at MOI 0.5 $TCID_{50}$/cell and harvested by the freeze/thaw technique at 72 hpi. This material was designated as EPV stock 13-11-83 and had a titre of 1×10^8 $TCID_{50}$/ml, as determined by the TCID infectivity assay (Section 4.4). All experiments in this research project, with the exception of preliminary studies which used early low titre material, were conducted with EPV stock 13-11-83.

5.2. Observations on the Growth of Synchronized PFT Cells Infected with EPV

In this study, an attempt was made to measure the growth of PFT culture, infected at an MOI of 0.8 TCID/cell, relative to mock infected control culture. At 24 hpi, the total cell number in both flasks was measured and plotted in Figure 2. The cell number in the mock infected control increased by approximately 40% after 24 hours, from 3.30×10^5 to 4.61×10^5 cells. The infected cultures had fewer initial cells seeded


5
4
3
2
1
Total cells x 10^{-4}
Mock
Infected
0
24
Hours post infection

(2.95×10^5) and at 24 hpi, there had been a drop, to 2.46×10^5 cells. To rationalize these results, it was suggested that the drop may partially represent a loss at the point of reattachment following the initial trypsinization. However, as both the mock and EPV infected cultures were trypsinized after infection, it would be anticipated that the mock would have had the same initial decrease in reattached cells. The growth of the mock infected culture would have masked the decline. It would have been preferable to have measured the actual reattachment efficency shortly after reseeding. However, as prior experience had already shown, attachment was essentially complete after two hours incubation, thus the control was not included. When an adjustment was made for the expected infected cell number if both had grown at the same rate, the infected culture should have reached 4.13×10^5 rather than 2.46×10^5 cells total. The difference between the mock and infected cultures then becomes more prominent, as the infected flask would have been inhibited by some 68%.

Although the effect of the synchronization conditions may not be ruled out, it would be expected to be a factor common to both. Thus, it appeared from this study, that the growth of PFT cells was significantly inhibited by infection with EPV, suggesting a mitolytic property.

5.3. Determination of EPV Growth Cycle

The time course of EPV multiplication in cultured pig fallopian tube cells in synchronized and nonsynchronized cultures was studied. In each case, subconfluent PFT cultures were infected at multiplicities of 0.2 and 1.4 $TCID_{50}$/cell (determined subsequent to infection) for the nonsynchronized and synchronized cultures, respectively. After incubation, cells were trypsinized and reseeded in equal numbers with normal growth medium. Virus was harvested, *in toto*, by the freeze/thaw and

sonication methods, and infectivity was measured using the TCID assay. The data on EPV production is presented graphically in Figure 3.

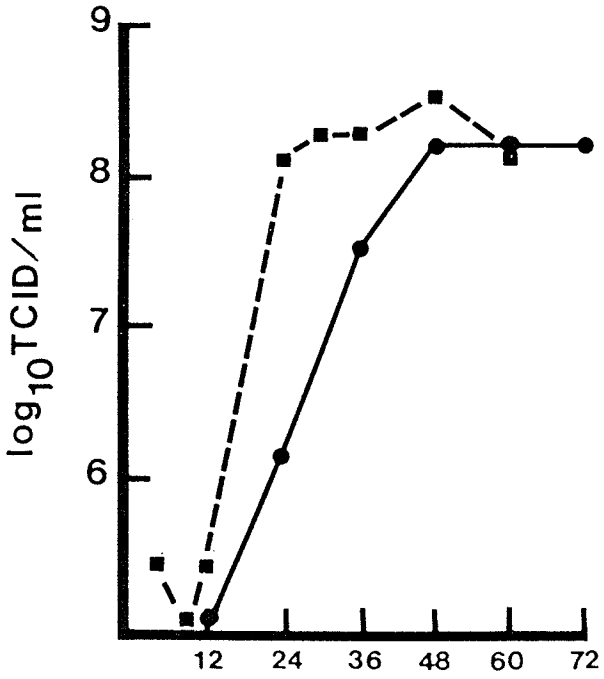
Under asynchronous conditions (Figure 3, solid line), virus was barely detectable at 12 hpi (< $2x10^4$ $TCID_{50}$/ml). The titre rose substantially in the 12 - 24 hpi interval and continued to steadily increase during the subsequent 24 hour period. Infectivity attained a maximum by 48 hpi and remained unchanged at 72 hpi.

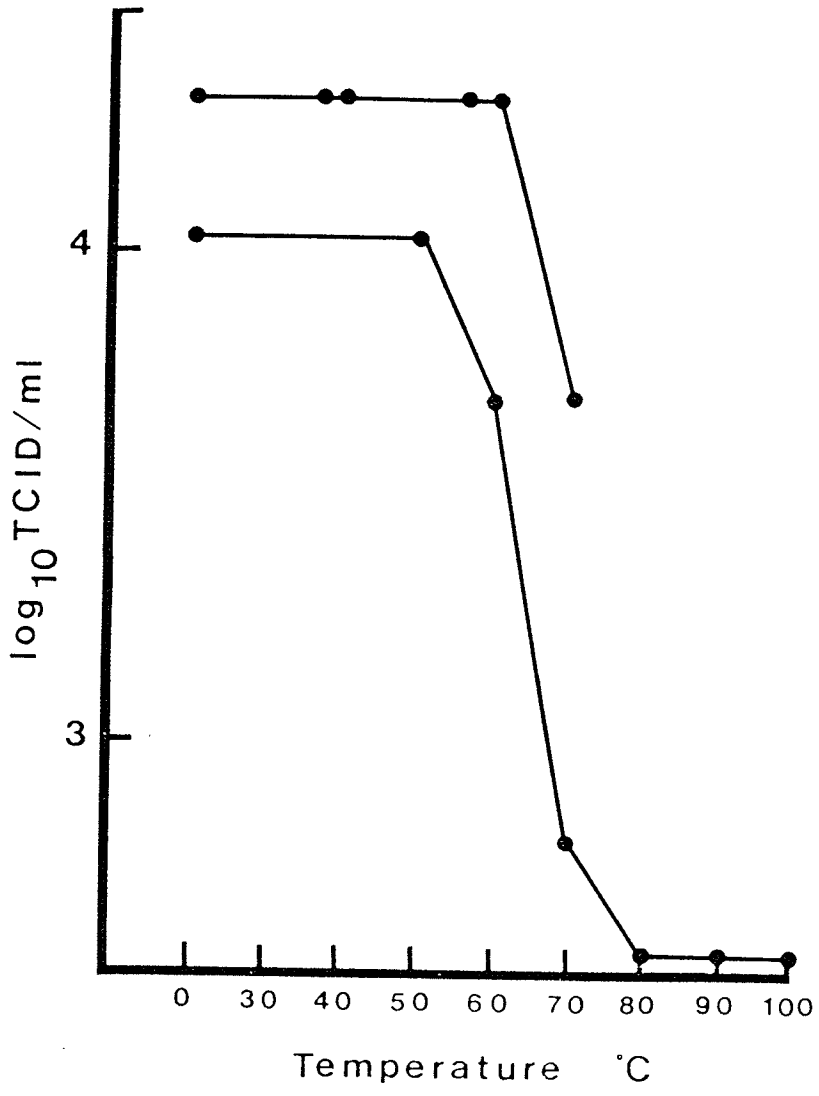
Figure 3 (broken line) indicated that infectious virus was present at 4 hpi, in the synchronized culture. It was unlikely that this represented progeny from de novo synthesis but rather residual intact input virus. The titre fell during the subsequent 4 hours, until at 8 hpi, it was not detectable at the dilution used (hence < $2x10^4$ $TCID_{50}$/ml). Infectious progeny were initially observed at 12 hpi and rose by about 3 $\log_{10}$ TCID/ml during the next 12 hour interval. Infectivity was observed to increase by a maximum 2-4 fold during the following 30-60 hpi interval. The beginnings of visually discernable cytopathological effect correlated with the plateauing of virus yields. Inspection of each dish prior to harvest established initial CPE by 30-36 hpi, with almost total monolayer disintegration by 48-60 hpi.

To conclude, the EPV growth cycle was found to have a duration of roughly 36 hours in randomly growing PFT cells. This time was shortened by at least 12 hours when the virus was grown under synchronized conditions. An analysis of EPV multiplication with respect to the effect of synchronization will be presented in the discussion.

5.4. Temperature Stability

In one study, aliquots containing 500 TCID of EPV were incubated for 15 minutes at temperatures covering a range from 0 - 70°C. Figure 4


9
8
7
6
log10TCID/ml
12
24
36
48
60
72


4
3
log10TCID/ml
0
30
40
50
60
70
80
90
100
Temperature °C

presents the effects of the various temperatures on EPV infectivity. The results indicated that no significant decrease in biological activity was found up to and including 60°C. However, when heated at 70°C, a 0.5-1 $\log_{10}$ decline in titre was observed. To ascertain if this dropped represented a true endpoint, the experiment was repeated with treatments up to 100°C. The results of the second trial (Figure 4, lower curve) again demonstrated the marked decline in infectivity at temperatures above 60°C. It was not possible to detect the presence of infectious virus at temperatures beyond 70°C. To conclude, EPV withstood heating at 60°C for 15 mintues.

5.5. The Effect of Detergent Treatment on EPV Infectivity

To test the effect of detergents on the infectivity of EPV, aliquots of stock virus were incubated with 1% aqueous solutions of the detergents, Nonidet P40 (NP-40) or sodium dodecyl sulfate (SDS) on ice for 30 minutes. The results of two separate experiments are summarized in Table 1. As can be seen, there was no decline in infectivity as a consequence of detergent treatment, in either set. In both cases, SDS exposure resulted in a slightly higher titre. However, as this actually represents a difference of 1 microtitre well in the endpoint (a net two fold increase) it was not considered significant.

Thus, EPV was found to be stable to 1% NP-40 and SDS exposure at 0°C for 30 minutes.

5.6. The Effect of Organic Sovent Extraction on EPV Infectivity

A CsCl gradient purified preparation of EPV (density 1.416 g/cm^3) was dialyzed against TE buffer (pH 8.0) and extracted 3 times with ether. The results of the infectivity assay are presented in Table 2. It was apparent

Table 1. Effect of detergents treatment on EPV infectivity.

Trial[a]	Treatment[b]	Well Positive[c]	$TCID_{50}$/ml
1	1% NP-40	4	3.2×10^5
	1% SDS	5	6.4×10^5
	Virus alone	4	3.2×10^5
	No virus[e]	-	-
2	1% NP-40	3	1.6×10^5
	1% SDS	4	3.2×10^5
	Virus alone	3	1.6×10^5
	No virus[e]	-	-

[a] Trial, experiments performed on different days.

[b] Treatment, detergent exposure as described in text.

[c] Well positive, indicates last well with CPE from the secondary infectivity assay.

[d] $TCID_{50}$/ml, infectivity converted from well positive endpoint.

[e] No virus, a negative control from cells treated with culture medium only.

Table 2. Effect of ether extraction on EPV infectivity.

Ether extraction[a]	Well positive[b]	$TCID_{50}$/ml[c]
-	9	20,480
+	9	20,480
No virus[d]	-	-

[a] Ether extraction, aliquots of CsCl purified EPV were extracted three times (+) or not (-).

[b] Well positive, indicates last well with CPE from the secondary infectivity assay.

[c] $TCID_{50}$/ml, infectivity converted from well positive endpoint.

[d] No virus, a negative control from cells treated with culture medium only.

Table 3. Effect of tryptic digestion on EPV infectivity.

Sample	Trypsin[a]	Well positive[b]	$TCID_{50}$/ml[c]
EPV	-	7	1.28×10^8
EPV	+	8	2.56×10^8
EPV	+	6	6.40×10^7
No virus	-	-	-

a Trypsin, 50 ug/ml final concentration.

b Well positive, indicates last well with CPE from the secondary infectivity assay.

c $TCID_{50}$/ml, infectivity converted from well positive endpoint.

d No virus, a negative control from cells treated with culture medium only.

that there was no reduction in EPV infectivity, relative to the control which was similarly shaken (but without diethyl ether) and dialysis.

5.7. Tryptic Digestion of EPV Stock 13-11-83

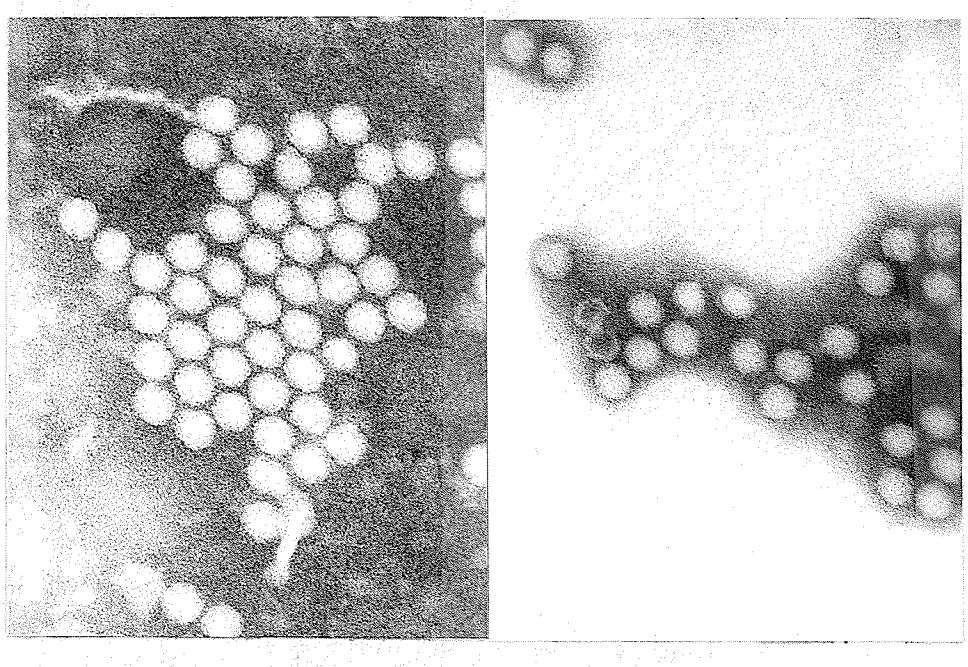
The resistance of EPV infectivity to trypsin was tested. The high titre EPV stock 13-11-83 (1 x 10^8 TCID/ml) was treated in duplicate tubes with trypsin at a concentration of 50 ug/ml, and assayed for infectivity. A control virus sample was incubated under the same conditions, 1 hour at 37°C.

The effects of trypsin exposure are summarized in Table 3. The positive virus control gave a titre of 1.28 x 10^8 $TCID_{50}$/ml, which was consistent with previous assays. After trypsin treatment, the infectivity assays showed the endpoints of the duplicate to be one well above and below that of the control. However, such single well variability was not considered significant for biological function assays in two-fold serial dilutions. The mean of the trypsin endpoints was similar to the viral control as well as the known titre of the virus stock. It was shown that EPV biological activity was not affected by trypsin.

5.8. Electron Microscopy of CsCl Gradient Purified EPV from Density 1.416 g/cm^3

Electron microscopy was conducted on an EPV preparation having a CsCl buoyant density of 1.416 g/cm^3 (Section 4.11). Figure 5 shows the electron micrographs of EPV. The particles were observed to be "full" by virtue of stain exclusion, however the occasional empty capsid was observed. They possessed icosahedral symmetry and had diameters of 20 to 23 nm. In conclusion, the EPV virion from density 1.416 g/cm^3 had both the morphology and size characteristic of typical parvoviruses.

Figure 5. Electron microscopy of CsCl gradient purified EPV from density 1.416 g/cm^3.



Micrographs were prepared with particles having a buoyant density of 1.416 g/cm^3. Virus was visualized with the Philips model 201 transmission electron microscope at 60 KV. Note the occasional empty capsids at this density.

5.9. Infectivity Profile Following Ultracentrifugation from Uniform Density

It was necessary to establish a method to resolve or characterize the biological activity (ie., infectivity) of the agent responsible for the cytopathological effect displayed in equine virus infected PFT cells. To study this, a lysate prepared from an infected culture at 48 hpi was made to 1.408 g/cm^3 with CsCl in TE buffer (pH 7.8). The gradient generated during ultracentrifugation was fractionated and assayed at particular points to produce the infectivity profile of Figure 6.

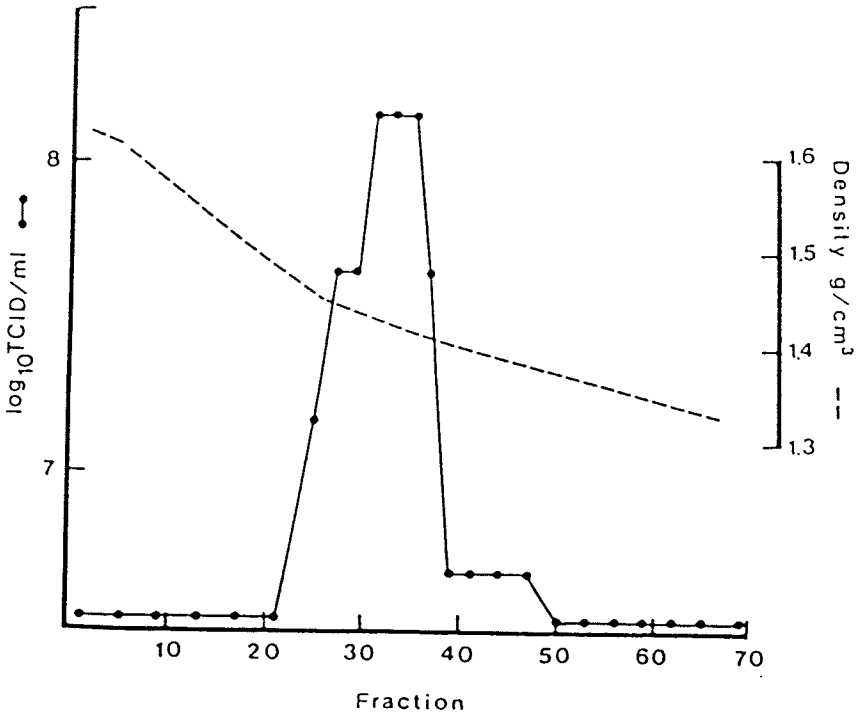
The predominant area of infectious material, possessing a maximum titre of 1.31×10^8 $TCID_{50}$/ml, peaked at density 1.423 g/cm^3. Biological activity dropped by nearly 1.5 $\log_{10}$ $TCID_{50}$/ml, immediately below density 1.39 g/cm^3, and reached only 5×10^4 $TCID_{50}$/ml at 1.31 g/cm^3. A reproducible shoulder, at density 1.44 g/cm^3, was also observed and will be discussed in view of other infectivity profiles. Thus, the peak infectivity of the equine virus was at a buoyant density of 1.41 g/cm^3.

5.10. Infectivity Profile of a [^{3}H] Thymidine Labelled EPV Preparation

The aims of this experiment were to attempt the labelling of the EPV genome using the DNA precursor, [^{3}H] thymidine, and secondly, to determine if the buoyant density of the labelled infectious material agreed with other density analyses.

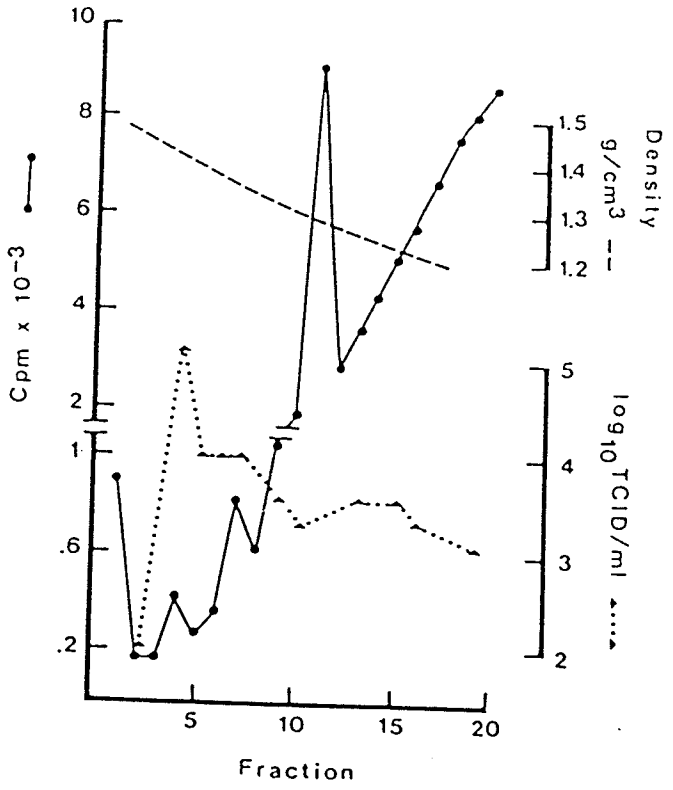
A PFT culture, infected at a low MOI, was labelled between 24 - 72 hpi with [^{3}H] thymidine. Only the progeny virus in the culture supernatant, was collected for analysis. The results presented in Figure 7, indicated that the peak infectious titre (1.6×10^5 $TCID_{50}$/ml) coincided with the fraction having density 1.416 g/cm^3. Infectivity dropped by 16 fold in the

Figure 6. The infectivity profile of uniform CsCl gradient purified EPV, prepared from <u>in toto</u> lysed PFT culture at 48 hpi.



PFT cells (4.4×10^6) were infected at MOI 10 and harvested by freeze/thaw, sonication, and 1% n-butanol, at 48 hpi. To the lysate was added TE buffer (pH 8.0) and CsCl salt to a density of 1.408 g/cm^3. The gradient was centifuged at 32,000 rpm, for 48 hours (SW 40). Infectivity was measured by the TCID method.

Figure 7. The infectivity profile of a [^{3}H] thymidine labelled EPV preparation.



A subconfluent culture of PFT cells was infected at MOI 0.01 - 0.001 TCID/cell and labelled at 24 hpi with [^{3}H] thymidine. Extracellular virus was harvested at 72 hpi, pelleted, resuspended, and loaded on preformed 1.20 to 1.45 g/cm^3 CsCl gradients. These were centrifuged at 35,000 rpm, for 16 hours (SW 60). Fractions were collected from the bottom, counted in a toluene based LSC, and a portion assayed for infectivity.

following fraction (1.392 g/cm^3) and continued to diminish throughout the upper portion of the gradient, despite the increase in tritium counts.

In summary, infectious virus (harvested at 72 hpi) had banded at density 1.416 g/cm^3. Although not further pursued, it appeared that the EPV genome had been labelled with [^{3}H] thymidine, as a distinct radioactive band was coincident with the infectivity profile.

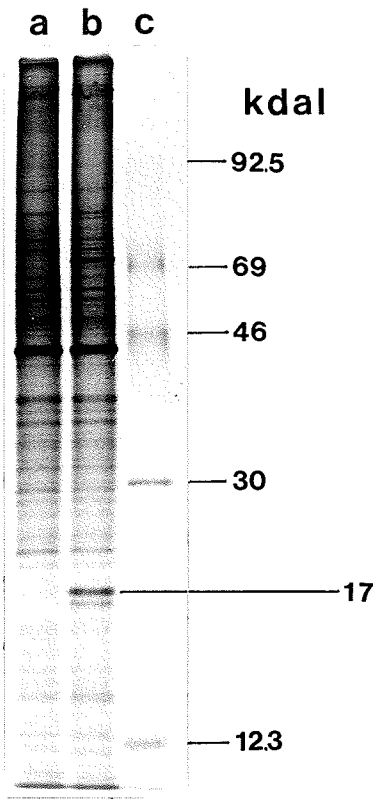
5.11. PAGE Analysis of Total Mock and EPV Infected PFT Cell Lysates

Cultures of PFT cells were infected or mock treated, labelled with [^{35}S] methionine, and the monolayers were lyzed with PAGE sample buffer, having 2% SDS and 5% 2-mercaptoethanol. Aliquots were denatured by boiling for 2 minutes. The autoradiogram of the 12.5% polyacrylamide gel is shown in Figure 8. In addition to the 87 and 70.5 Kdal proteins, the infected cell lysate contained a third protein of 17 Kdal (lane b) which was not present in the mock preparation (lane a). It was uncertain whether this protein was viral encoded, or the product of a cellular gene, induced as a consequence of viral infection. Early SDS-PAGE analysis of mock and EPV infected PFT cell lysates, prepared with a buffer which disrupted the cytoplasmic membrane while leaving the nucleus intact, had shown the presence of the 87 and 70.5 Kdal proteins (data not shown). It was therefore evident from total cell lysates (possessing both cytoplasmic and nuclear fractions) that the 17 Kdal protein was nuclear in origin.

5.12. Composition and Distribution of EPV Capsid Proteins at Various Buoyant Densities

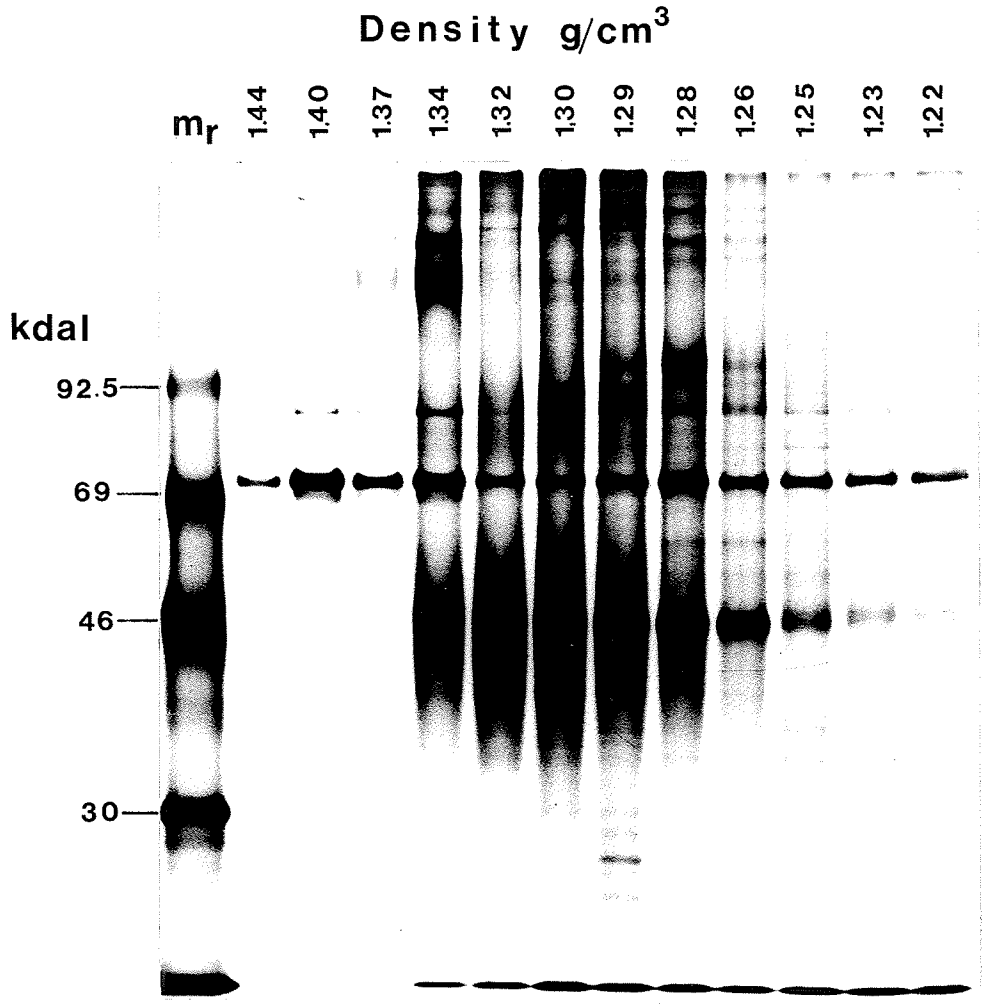
It was of interest to characterize the EPV structural and non-structural proteins and to study the synthesis of these proteins. This knowledge may be useful for the eventual development of a vaccine.

Figure 8. Analysis of total mock and EPV infected PFT cell lysates on a 12.5% polyacrylamide gel.



Asynchronous PFT cells were infected at MOI 10. At 20 hpi, the culture was labelled with 50 uCi/ml [^{35}S] methionine, in 76% methionine-free MEM, 20% normal MEM, 2% FCS, 1% L-glutamine, and 1% antibiotics. After 24 hours growth, the labelling medium was removed and total cell lysates prepared with 2% SDS and 5% 2-mercaptoethanol. The autoradiogram is: a) mock infected; b) EPV infected; and c) [^{14}C] labelled molecular weight standards.

Figure 9. A 10% polyacrylamide gel analysis of the EPV virion protein types and distribution in a linear CsCl density gradient.



PFT cells (4×10^6) were infected at MOI 0.01 - 0.001, and labelled at 29 hpi under conditions described in Section 4.14. Virus was pelleted, and analyzed on a 1.20 to 1.45 g/cm^3 CsCl gradient. Designated fractions were diluted with 3 volumes of TE buffer and pelleted at 100,000 x g for 2 hours (Airfuge, Beckman). The virion pellets were redissolved directly in SDS-PAGE sample buffer, boiled for 2 minutes and electrophoresed on a 10% polyacrylamide gel.

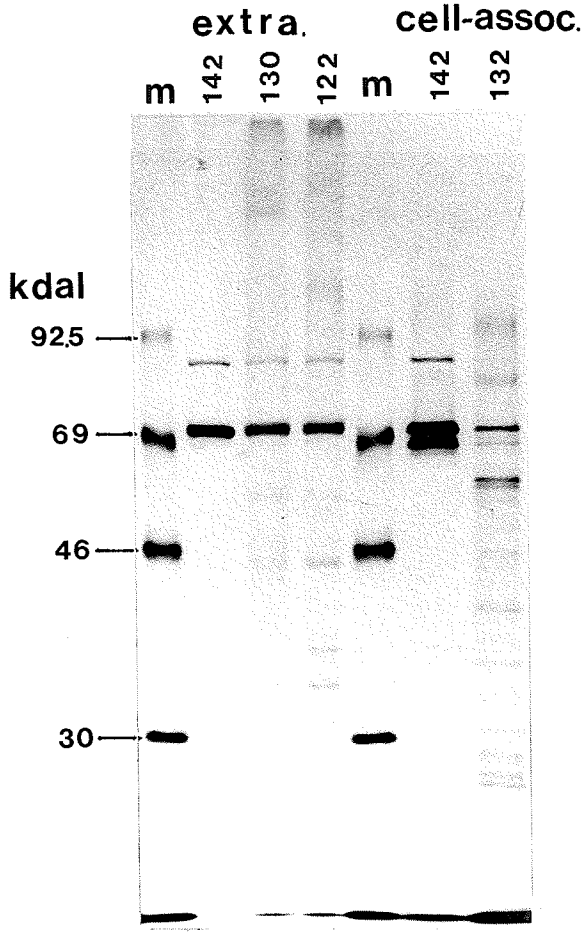
Extracellular virions labelled with [^{35}S] methionine from 12-48 hpi was separated on a CsCl gradient and the ^{35}S-labelled protein associated with particles at different densities were analyzed by SDS-PAGE. The fluorogram of the 10% PAGE analysis is presented in Figure 9. Two proteins with molecular weights of 87 Kdal (VP1) and 70.5 Kdal (VP2) were found to associate with particles at densities equal to or greater than 1.40 g/cm^3 (Figure 9). A third minor band at 68 Kdal (VP3) was observed at 1.40 g/cm^3. At density 1.37 g/cm^3, the viral bands became contaminated with cellular material. Both VP1 and VP2 were present throughout the gradient. This may suggest that the EPV particles vary from full particles with complete genomes to medium density defective particles and low density empty capsids at densities below 1.30 g/cm^3. Since the particles were pelleted after gradient fractionation, the proteins at low density must at least exist in aggregate forms such as empty capsids.

Thus, it was established that the EPV capsid prepared at 48 hpi was composed of 2 major structural components, VP1 and VP2. A possible third structural protein having a molecular weight of roughly 2 Kdal less than VP2, was also observed.

5.13. Comparison of Extracellular EPV (Prepared Without) and Cell-Associated Virion (Isolated With) Trypsin

The infectivity of EPV has been shown to be unaltered by exposure to trypsin (Table 3). However, at the structural level, trypsin does cleave the full virion of H-1 virus (Kongsvik *et al*, 1978; Paradiso, 1981) generating a VP2 protein from VP2'. The full virion of canine PV has also demonstrated a susceptibility to trypsin (Paradiso *et al*, 1982). It was therefore of interest to ascertain whether a comparable cleavage occurred with EPV.

Figure 10. A 10% polyacrylamide gel analysis of extracellular EPV (prepared without) and cell-associated virion (prepared with) trypsin.



Infection and labelling of EPV was as described in Section 4.15. Extracellular virus was pelleted from clarified medium. Cell-associated virus from the debris pellet was resuspended and digested with 50 µg/ml trypsin for 1 hour (37°C), and pelleted as for the extracellular. The two samples were loaded on separate 1.20 to 1.55 g/cm^3 CsCl gradients, spun for 16 hours at 35,000 rpm and fractionated. Fractions having the above designated densities were analyzed on a 10% polyacrylamide gel.

Table 4. Yields of infectious EPV grown under limit phosphate labelling conditions.

Sample		Well positive[d]	$TCID_{50}$/ml[e]
Mock infected[a]		-	-
Infected, normal conditions[b]		6	3.2×10^6
		5	1.6×10^6
Infected, supplemented[c] with:	0%	5	1.6×10^6
		5	1.6×10^6
	1%	6	3.2×10^6
		4	0.8×10^6
	2%	6	3.2×10^6
		5	1.6×10^6
	5%	6	3.2×10^6
		5	1.6×10^6

[a] Mock infected, culture received no virus as a negative control.

[b] Normal conditions, virus yield in cells maintained in normal medium.

[c] Supplemented, phosphate free MEM was supplemented with an amount of normal MEM.

[d] Well positive, indicates last well with CPE from secondary infectivity assay.

[e] TCID50/ml, infectivity converted from well positive endpoint.

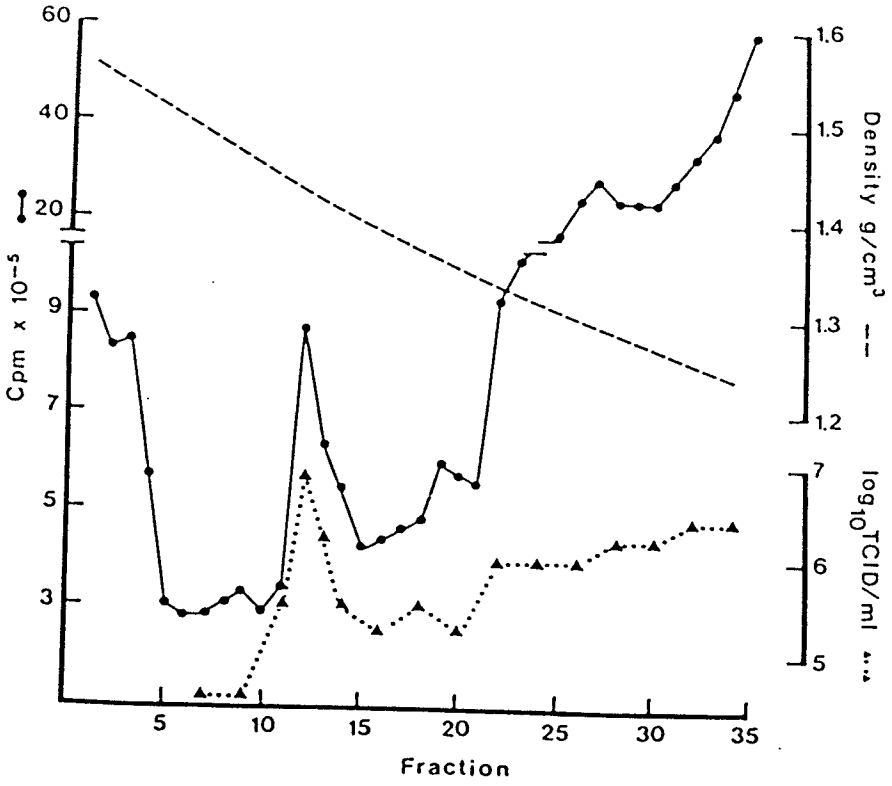
To do this, [^{35}S]-labelled virus was harvested from the culture medium (extracellular) and compared to that isolated by sonication, DOC, and tryptic digestion of the cell-associated fraction. The fluorogram of a 10% polyacrylamide gel is shown in Figure 10. The VP bands of 87 and 70.5 Kdal were observed throughout all densities in the extracellular material (Figure 10). However, the cell-associated virion had a unique third band of 68 Kdal at density 1.42 g/cm^3. Thus, it was concluded that the heavy EPV virion was susceptible to tryptic digestion.

5.14. Growth of EPV as a Function of Medium Phosphate Concentration

In order to study the nucleic acids of EPV, it was decided that ^{32}P labelling would be used to facilitate purification analysis and detection. Theoretically, the lower the concentration of phosphate in the medium, the higher the specific activity of the nucleic acid synthesized. However, the cell requires a minimum concentration of a phosphate source to support this biosynthesis, below which little or no nucleic acid can be synthesized which would affect cell viability. The minimum concentration of phosphate, defined by percent of normal medium, to allow acceptable level of virion production must be found. PFT cultures (60 mm) were infected or mock infected as described (Section 4.17). AT 72 hpi cultures were freeze/thawed, in toto (2x), and then sonicated. The viral yields in each culture were determined by the TCID assay method (Section 4.4). The results are shown in Table 4.

The mock infected control gave no CPE. Infectivity yield from cultures maintained in normal growth medium, reached a titre between 1.6 and 3.2 x 10^6 $TCID_{50}$/ml. At all phosphate concentrations tested (0,1,2, and 5%) the infectivity yields were between 1.6-3.2 x 10^6 $TCID_{50}$/ml, with the exception of a single point of 0.8 x 10^6 $TCID_{50}$/ml at 1%, and

Figure 11. The infectivity profile of CsCl gradient purified [^{32}P] labelled extracellular EPV.



Synchronized PFT cells were infected at MOI 10, and labelled at the time of trypsinization, with carrier free [^{32}P] ortrophosphate. Extracellular virus was harvested at 48 hpi by freeze/thaw, sonication, and 1% deoxycholate treatment. Virus was pelleted and loaded on preformed 1.2 to 1.50 g/cm^3 CsCl gradients, spun at 32,000 rpm for 40 hours. Five drop fractions were collected, counted by Cerenkov counting in teh tritium window of the LKB Rockbeta. Infectivity was measured by the TCID method.

therefore, had attained the output of the positive control. Thus, it was concluded, that no limiting phosphate level had been experimentally reached which would adversely affect EPV growth. These methods were applied in labelling EPV.

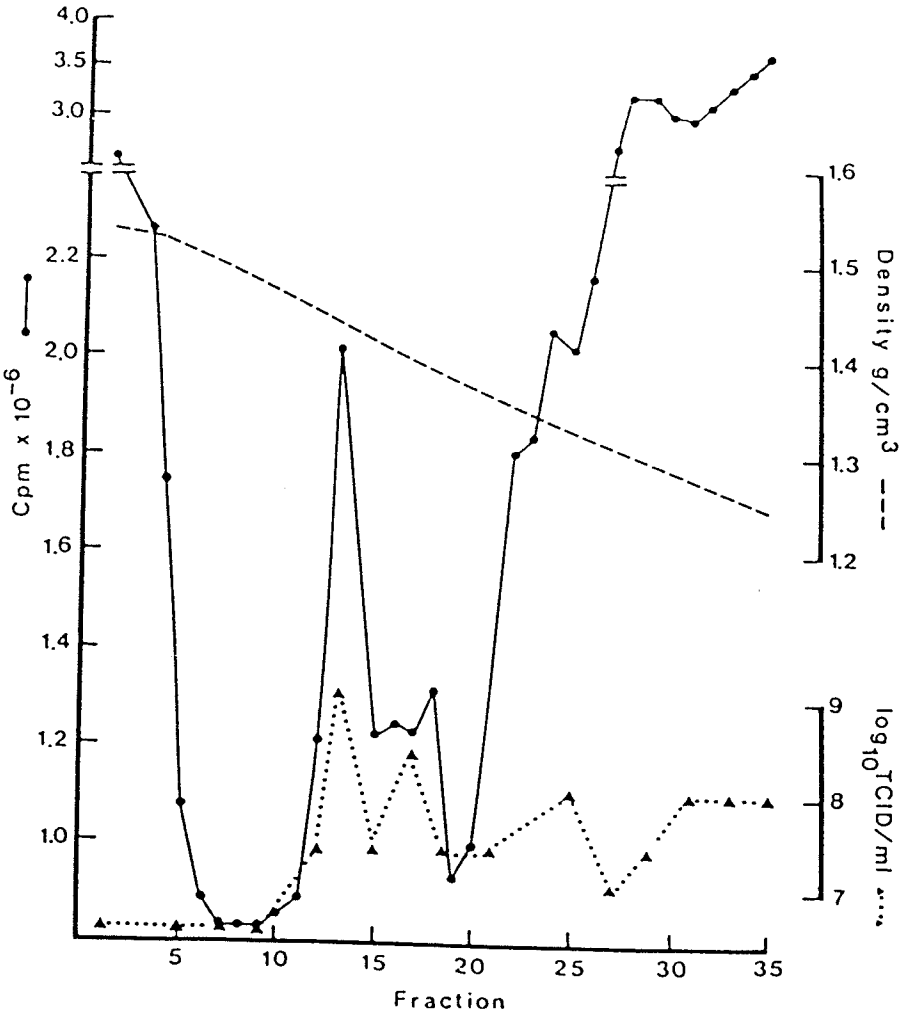
5.15. Purification and Infectivity Assay of [^{32}P] Labelled EPV on Primary CsCl Gradients

The main purpose of this experiment was to label, isolate, and characterize the equine virus genome. In addition, it was necessary to obtain an infectivity profile to corroborate previous data (Figures 6 and 7). The initial labelling trial utilized the methods described in Section 4.18. The combined radioactivity, density, and infectivity profiles of the primary extracellular gradient are presented in Figure 11. (The intracellular gradient had no radioactive peak and was not assayed for infectivity).

In the extracellular material there was a single radioactive peak at density 1.43 g/cm^3, which had a slight shoulder at lower density. The fraction having density 1.43 g/cm^3 also possessed the highest infectious virus titre, of approximately 10^7 $TCID_{50}$/ml. No infectivity was detected at higher densities. The titre dropped by about 20 fold at density 1.40 g/cm^3. A baseline level of around 10^6 $TCID_{50}$/ml was maintained from density 1.33 to 1.24 g/cm^3. This may be attributable to smearing of the infectious virus in the bottom collection procedure. To conclude, [^{32}P] based radioactivity accounted for a peak at a buoyant density of 1.43 g/cm^3 which was also coincident with the maximum infectivity. The viral genome was not isolated in this preparation.

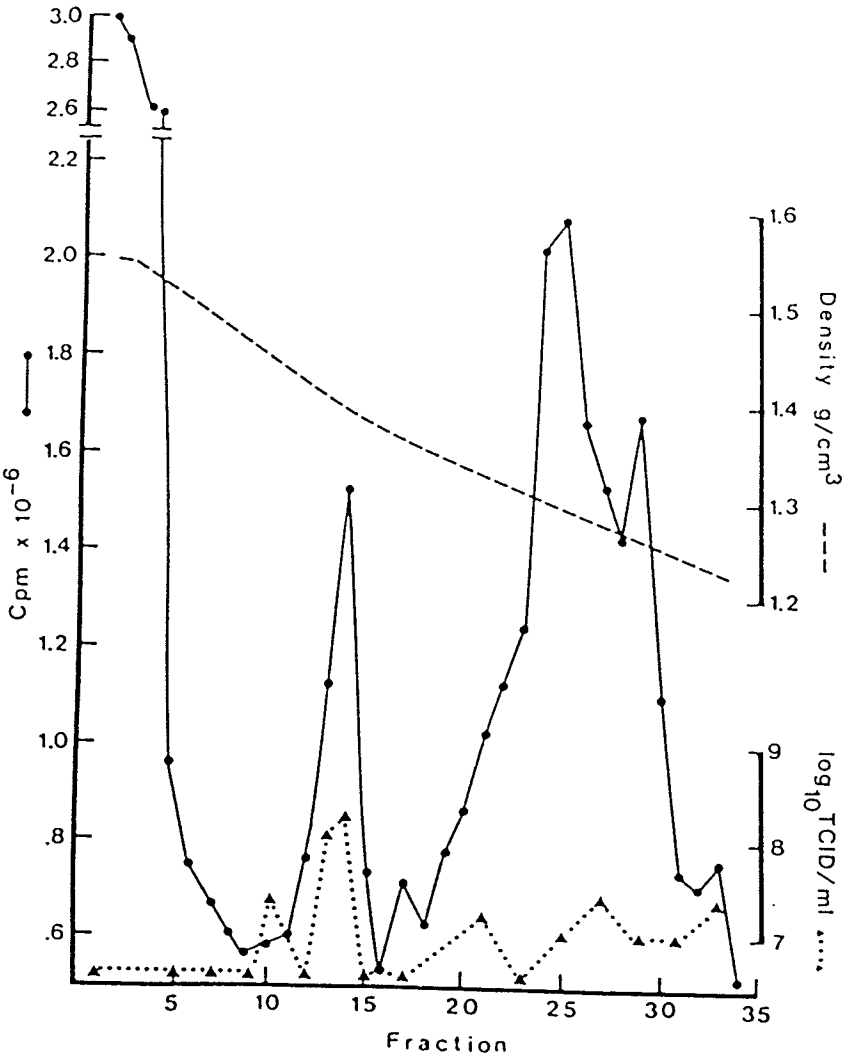
The experiment was repeated with primary extracellular and intracellular gradients as before. Both were processed as described and

Figure 12. The radioactivity, density, and infectivity profiles of CsCl gradient purified [^{32}P] labelled extracellular EPV.



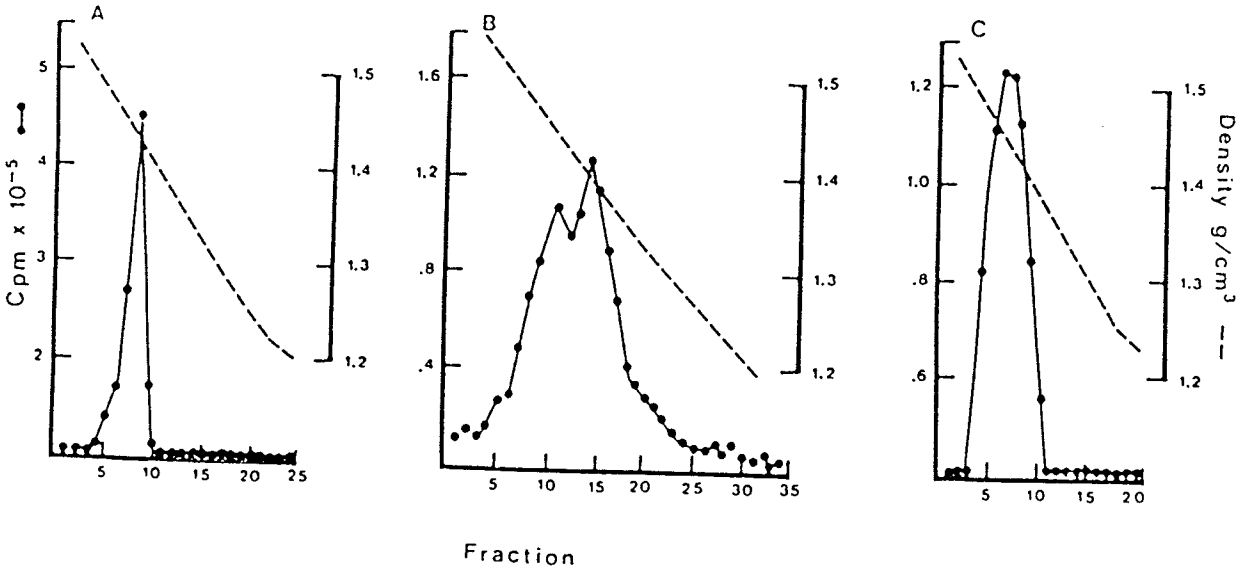
Infection, labelling, and harvest conditions were as described in Section 4.18. Extracellular virus was pelleted, resuspended in 1.2 ml TE buffer (pH 8.0) and loaded on 1.20 to 1.50 g/cm^3 CsCl gradients. Samples were centrifuged at 32,000 rpm for 40 hours (20°C). Five drop fractions were collected from the bottom.

Figure 13. The radioactivity, density, and infectivity profiles of CsCl gradient purified, [^{32}P] labelled intracellular EPV.



Infection and labelling conditions were as described in Section 4.18. Intracellular virus was extracted from cellular debris by sonication, and 1% deoxycholate treatment (1 hour, 4°C). Virus was pelleted, resuspended in 1.2 ml TE buffer (pH 8.0) and processed on CsCl gradients as the extracellular (see Figure 12).

Figure 14. The radioactivity and density profiles of secondary CsCl gradient rebanded extracellular and intracellular EPV.



Material was selected from the primary CsCl gradients (Figures 13,14), and rebanded on preformed (1.30-1.50 g/cm^3) secondary gradients. Fractions for rebanding were: A) extracellular (1.415-1.445 g/cm^3); B) extracellular (1.387-1.415 g/cm^3); and C) intracellular (1.381-1.415 g/cm^3). Radioactive and density profiles were determined as before.

assayed for infectivity. The extracellular gradient displayed a prominent radioactive peak at 1.435 g/cm^3, with a shoulder and second peak around 1.395 g/cm^3 (Figure 12). The TCID assay showed that the high density form reached a titre in excess of 1x10^9 $TCID_{50}$/ml, which then fell and rose to about 4x10^8 $TCID_{50}$/ml, at 1.395 g/cm^3. The titre then fluctuated between 10 and 100 fold less than the maximum, throughout the remainder of the gradient.

There were two major peaks defined by radioactivity, having densities 1.395 and 1.30 g/cm^3, in the intracellular gradient (Figure 13). When the infectivity data were plotted, the highest titre was found at 1.395 g/cm^3 (4x10^8 $TCID_{50}$/ml). Despite the fact that no discernable radioactive peak was present at 1.435 g/cm^3, infectious virus did exist.

The portion of the gradient flanking the 1.30 g/cm^3 reading, had variable amounts of biological activity, with most fractions having a titre approximately 40 fold less than the peak. It is speculated that the background infectivity in both gradients may be attributed, in part, to contamination of lower densities through smearing of the heavier forms during bottom collection.

To conclude, the procedure described in this experiment permitted the elucidation of 2 infectious forms of equine virus, differing in buoyant density by a significant 0.03-0.04 g/cm^3.

5.16. Secondary Gradient Rebanding

A second set of preformed (1.30-1.50 g/cm^3) CsCl gradients were used to purify the once banded material from the primary gradients. From the extracellular sample, two regions were pooled, which originated from the peaks: A (1.415-1.445 g/cm^3) and B (1.387-1.415 g/cm^3). The intracellular gradient had a single high density peak, C (1.381-1.415 g/cm^3). These

Table 5. Preliminary RNase A susceptibility analysis of the nucleic acid from twice CsCl gradient purified extracellular virion[a].

Substrate	RNase A	Acid precipitable cpm[b]	Mean	% Reduction[c]
1) [^{3}H] RNA	-	929		
	-	848	888	-
	+	8		
	+	16	12	98.6
2) [^{32}P] n.a.	-	1567		
	-	1399	1483	-
	+	606		
	+	598	602	59.4

[a] Virion, from density 1.435 g/cm^{3}.

[b] Acid precipitable cpm, counts were adjusted by subtraction of 25 or 12 cpm in the [^{3}H] and [^{32}P] window, respectively, in the LKB Rackbeta.

[c] % Reduction, calculated from:

$$\frac{\text{cpm (no enzyme)} - \text{cpm (enzyme)}}{\text{cpm (no enzyme)}} \times 100\%$$

Table 6. Preliminary DNase I susceptibility analysis of the nucleic acid from twice CsCl gradient purified extracellular virion[a].

Substrate	DNase I	Acid precipitable cpm[b]	Mean	% Reduction[c]
1) [^{3}H] DNA	-	1554		
	-	1737	1646	-
	+	8		
	+	1	5	99.7
2) [^{32}P] n.a.	-	1323		
	-	1474	1398	-
	+	585		
	+	604	594	57.5

[a] Virion, from density 1.435 g/cm^3.

[b] Acid precipitable cpm, counts were adjusted by subtraction of 25 or 12 cpm in the [^{3}H] and [^{32}P] window, respectively, in the LKB Rackbeta.

[c] % Reduction, calculated from: $\frac{\text{cpm (no enzyme)} - \text{cpm (enzyme)}}{\text{cpm (no enzyme)}} \times 100\%$

steps were taken to both purify the material and also to determine if the peaks would reband to their original densities. The radioactivity and density profiles are plotted versus the fraction, for the 3 secondary gradients (Figure 14).

The (A) pool of the extracellular rebanded at 1.435 g/cm^3 (Figure 14A). The gradient of the "shoulder and minor peak" of the extracellular (B) was purposely collected in smaller fractions. In this manner, it was possible to detect 2 peaks with densitites 1.435 and 1.395 g/cm^3 (Figure 14B). The sole inconsistency in the secondary set, was the (C) material of the intracellular gradient. This had a much broader radioactive profile (Figure 14C) and a peak density of around 1.45 g/cm^3. The reason for such a result is unknown.

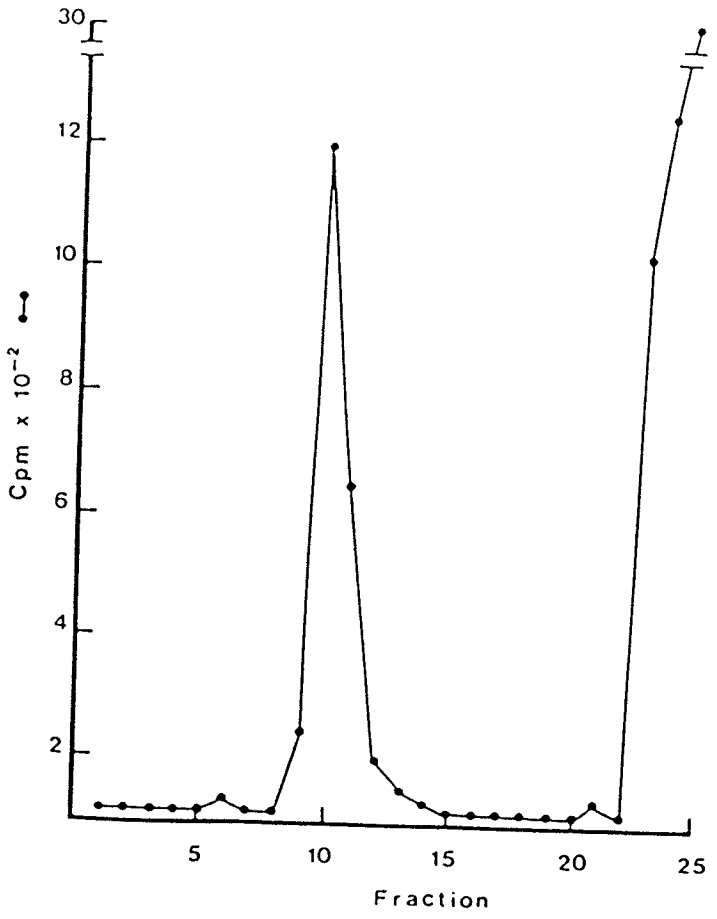
Thus, both the (A) and (B) extracellular pooled regions rebanded to their original densities in the secondary gradients. Since the TCID assay of the primary CsCl gradient had established the presence of infectious virus at 1.435 g/cm^3 (Figure 12) and because this same region yielded the cleanest peak during rebanding (Figure 14A), it was selected for nucleic acid analysis.

5.17. Enzyme Susceptibility Analysis of the Nature of Genomic Material

To determine whether the genomic material of the EPV was DNA or RNA, the nucleic acid from the twice CsCl gradient purified extracellular virion at 1.435 g/cm^3 was tested for resistance to RNase A and DNase I.

The results of the RNase A assay are presented in Table 5. The control, tracer [^{3}H] uridine RNA was almost completely digested, while the [^{32}P] nucleic acid was 59.4% removed. Thus, a core of RNase A resistant material was present at 1.435 g/cm^3.

Figure 15. Velocity sedimentation analysis of the twice CsCl gradient purified EPV from density 1.435 g/cm^3.



Approximately 10,000 cpm (Cerenkov) of EPV (density 1.435 g/cm^3) was diluted in TE buffer (pH 8.0), loaded on a preformed 15 - 30% sucrose gradient (Section 4.21) and centrifuged at 40,000 rpm for 2 hours (4°C). Four drop fractions were collected after bottom puncture and radioactivity estimated by Cerenkov counting in the tritium window of the LKB Rackbeta.

The same nucleic acid was tested for its susceptibility to DNase I, together with [^{3}H] DNA as a tracer. There was a 99.7% reduction of the TCA-precipitable counts of [^{3}H] DNA, whereas 57.5% of the [^{32}P] nucleic acid was digested (Table 6).

It was concluded that the nucleic acid extracted from the 1.435 g/cm^{3} virion peak contained both DNA and RNA.

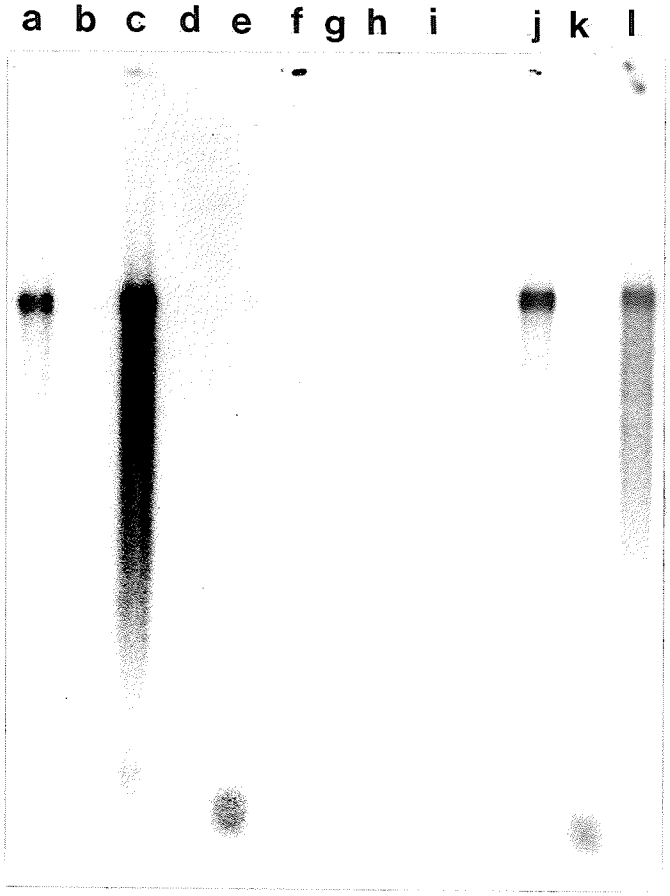
5.18. Velocity Sedimentation Analysis of the [^{32}P] Labelled Virion Peak Fraction

The initial nuclease susceptibility studies, with RNase A and DNase I, suggested that the twice CsCl gradient purified virion may not be homogeneous. Further purification by velocity sedimentation in sucrose gradients (Myers, 1980) was carried out. The radioactivity profile of the gradient is presented in Figure 15. A single sharp sedimenting peak was observed at fraction 10, which comprised about 25% of the total applied radioactivity. The remaining counts did not enter the gradient. Thus, it appeared that the labelled material at 1.435 g/cm^{3} had both particulate and soluble components.

To determine their nature, phenol extracted fractions, 10, 24, and annealed 10, were separately treated with RNase A, DNase I, and nuclease S1, as described (Section 22). The digests were analyzed by electrophoresis in a 1.4% agarose gel. The autoradiogram is shown in Figure 16.

Fraction 10 possessed what appeared (they were resolved more clearly in Figure 27) as two bands of equal intensity (Figure 16, lane j). The material from the gradient top (fraction 24) did not form discrete bands in the agarose gel, but migrated near the dye front as a smear (lane k). High salt conditions had not caused the two fraction 10 bands to hybridize (lane 1).

Figure 16. Susceptibility of velocity sedimentation resolved nucleic acids to RNase A, DNase I, and nuclease S1.



Native fractions: 10, 24, and annealed 10 (lanes j,k,l, respectively) were digested with RNase A (lanes a,b,c), DNase I (lanes d,e,f) and nuclease S1 (lanes g,h,i).

The two bands resolved from fraction 10 material were resistant to RNase A (lane a) but completely digested by DNase I (lane d). Similarly, the fraction 10 material which has been treated under annealing conditions demonstrated the same RNase A resistance (lane c) and DNase I sensitivity (lane f). However, material from fraction 24 was RNase A sensitive (lane b) and DNase I resistant (lane e). Both the nucleic acids in fractions 10 and 24 were sensitive to S1 (lanes g,h,i).

It was concluded that the material at the gradient top (fraction 24) contained RNA. By virtue of its resistance to RNase A, and sensitivity to both DNase I and S1, it was concluded that sedimenting material (fraction 10) contained single-stranded DNA which was resolved into two species by electrophoresis in a 1.4% agarose gel. Further, these two bands were either of the same polarity or unrelated molecules as annealing was not achieved. On the basis of the nuclease susceptibility results on equine viral genome, which indicate a ssDNA, it is concluded that the equine virus has a single strand DNA. Its isolation from the equine fetus liver tissue represents the first occurrence of a parvovirus in the equine species.

5.19. Quantitation of EPV Genome Susceptibility to Nuclease S1

The EPV genome was shown to be sensitive to DNase I and nuclease S1 (Figure 16). To determine if the purified genome was completely single stranded incubations were carried out (in duplicate) on both the native and heat-quenched molecule. Percent reduction in TCA precipitable counts was calculated from:

$$\frac{\text{cpm (no enzyme)} - \text{cpm (enzyme)}}{\text{cpm (no enzyme)}} \times 100\%$$

The results from three trials are presented in Table 7. In each of these, the native material was hydrolyzed to a lesser extent than the

Table 7. Measurement of [^{32}P] labelled EV genome susceptibility to neuclease S1 in native and denatured states.

Trial	Sample	Acid precipitable cmp[a]	Mean	% Reduction[b]
1	No enzyme	1595		
		1770	1682	
	Native	129		
		132	130	92.3
	Heat-quenched	93		
		61	77	95.4
2	No enzyme	682		
		696	689	-
	Native	73		
		57	65	90.6
	Heat-quenched	33		
		34	34	95.1
3	No enzyme	1482		
		1511	1496	-
	Native	112		
		113	112	92.5
	Heat-quenched	59		
		57	58	96.1

[a] Acid precipitable cpm, counts were adjusted by subtraction of 12 cpm in the [^{32}P] window, of the LKB Rackbeta.

[b] % Reduction, calculated from:

$$\frac{\text{cpm (no enzyme)} - \text{cpm (enzyme)}}{\text{cpm (no enzyme)}} \times 100\%$$

Table 8. Summation of the reduction in [^{32}P] labelled viral genome digested with nuclease S1.

Trial	Native	Heat-quenched
1	92.3	95.4
2	90.6	95.1
3	92.5	96.1
Mean	91.8 ± 1.09	95.5 ± 0.265

Table 9. Susceptibility of heat-quenched [^{3}H] thymidine DNA to nuclease S1 digestion.

Trial	Nuclease S1	Acid precipitable cmp[a]	Mean	% Reduction[b]
1	-	945		
	-	976	960	-
	+	15		
	+	7	11	98.9
2	-	911		
	-	869	890	-
	+	12		
	+	14	13	98.5

[a] Acid precipitable cpm, counts were adjusted by subtraction of 12 cpm in the [^{32}P] window, of the LKB Rackbeta.

[b] % Reduction, calculated from:

$$\frac{\text{cpm (no enzyme)} - \text{cpm (enzyme)}}{\text{cpm (no enzyme)}} \times 100\%$$

denatured molecule. The means and standard deviations are summarized in Table 8. They show that a further 3.7% of the heat-quenched form became sensitive to S1. Using the students t-test, the two values were shown to be significantly different at $p<0.05$.

When denatured tracer [^{3}H] thymidine DNA was assayed with S1, as a control, an average of 98.7% was hydrolyzed (Table 9). When the equine virus data were compared to this baseline value, it was apparent that about 6.9% of the native genome was resistant to nuclease S1, and therefore in a duplex form. The level of digestion failed to reach that of the control DNA, even after heat-quenching. This analysis strongly implies that the genome possesses duplex regions, which are capable of substantial spontaneous reannealing following disruption.

5.20. Analysis of Unlabelled Intracellular Replicative Form Extracts

Aliquots of mock and infected Hirt material were electrophoresed on either 1 or 1.4% agarose gels. Plots of marker base pair length versus migration were made for each gel from which sizes could be determined. Both preparations contained from 1 to 2 slowly migrating common bands which ran near the bacteriophage λ marker band of 23,606 bp. Although the nature of these species was not thoroughly investigated, single restriction enzyme digests resulted in multiple discrete banding as well as smearing (data not shown). It was concluded that these bands were composed of heterogeneous chromosomal material possibly fragmented during the Hirt isolation.

Unlabelled infected preparations were found to possess at least 3 other bands not shared with the mock lysates. The largest, which migrated slower than the 9,636 bp marker, was approximatley 10,000 bp in length. The predominant species ran with the 5150/5000 bp doublet of lambda phage.

On most 1.4% agarose gels, a very faint separation was discerned within this band, suggesting two molecules. To facilitate further spreading, samples were electrophoresed on 1% agarose gels, from which it was clear that there were two molecular types within this position. The size differential was apparently more than the 150 bp between the bands, since their resolution was greater.

To conclude, cold infected Hirt extracts possessed three unique molecular species: one of 10,000 bp and two near 5,000 bp in length, which suggested that possibly a monomer-dimer relationship existed.

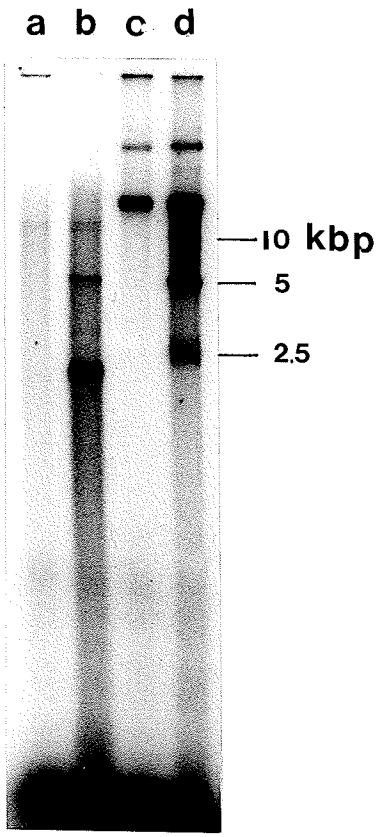
5.21. Analysis of [^{32}P] Labelled Intracellular RF Extracts

The purpose of this work was to obtain labelled viral nucleic acid intracellular replicative structures. In changing the method of detection, from UV visualization to radiography, the amount of material required for detection would lessen and other replicative molecules might possibly be identified which may have escaped ethidium bromide fluorimetry.

Mock and equine virus infected PFT cells were labelled and Hirt extracts prepared as before. Figure 17 presents the autoradiogram of a 1% agarose gel on which mock (lane c) and infected (lane d) samples were electrophoresed. As with cold preparations, common bands were present. The viral material possessed 3 molecular forms: again, a minor 10,000 bp species; a major component of near 5,000 bp size (doublet); and a previously unvisualized diffuse smear at around the 2,500 bp position. These findings confirmed the observations made with the cold material and further demonstrated the existence of a third intracellular form.

As a preliminary probe of internal features within the intracellular forms, a denaturation experiment was conducted. This was meant to test for the presence of hairpin sequences capable of holding complementary regions

Figure 17. Autoradiogram of intracellular replicative forms isolated from mock- and EPV infected cells.



Native, mock and EPV infected intracellular replicative form DNA (lanes c and d, respectively) were heat denatured at 100°C for 5 minutes (lanes a and b, respectively).

together or alternatively, to promote the spontaneous renaturation of disrupted stretches. Crude mock and infected nucleic acids were denatured by boiling at 100°C, for 5 minutes followed by quick chilling on ice. the results are presented in Figure 17. There was extensive smearing in both samples. The profile of the infected material changed as the 10,000 bp molecule disappeared completely, the 5,000 bp band's intensity diminished markedly, and the smeared material became both sharper and migrated faster (lane b). Therefore, heat-quenching had disrupted the molecular arrangements of all 3 species. However, as the extract contents were heterogeneous, no further conclusion was possible with regard to the relatedness or the observed changes, arising from the experimental conditions.

5.22. Recovery of Individual Intracellular Species

It was evident from experiments carried out with crude Hirt preparations that purified intracellular forms were necessary as substrate for the various reactions. Aliquots of total material were electrophoresed on preparative 1% agarose gels as detailed elsewhere (Section 4.25).

Recovery was optimal and reached 95% for the 5,000 bp species, was typically 80% or greater for the 2,500 bp material and lowest for the 10,000 bp molecule at about 60%. Percentages are given relative to the material in the original gel slice, quantified by Cerekov counting in the tritium window of the LKB Rackbeta.

5.23. Analysis of Recovered Replicative Forms: Heat Denaturation and Nuclease S1 Susceptibility

The 3 purified intracellular components were probed for structural features and the results are presented in Figure 18.

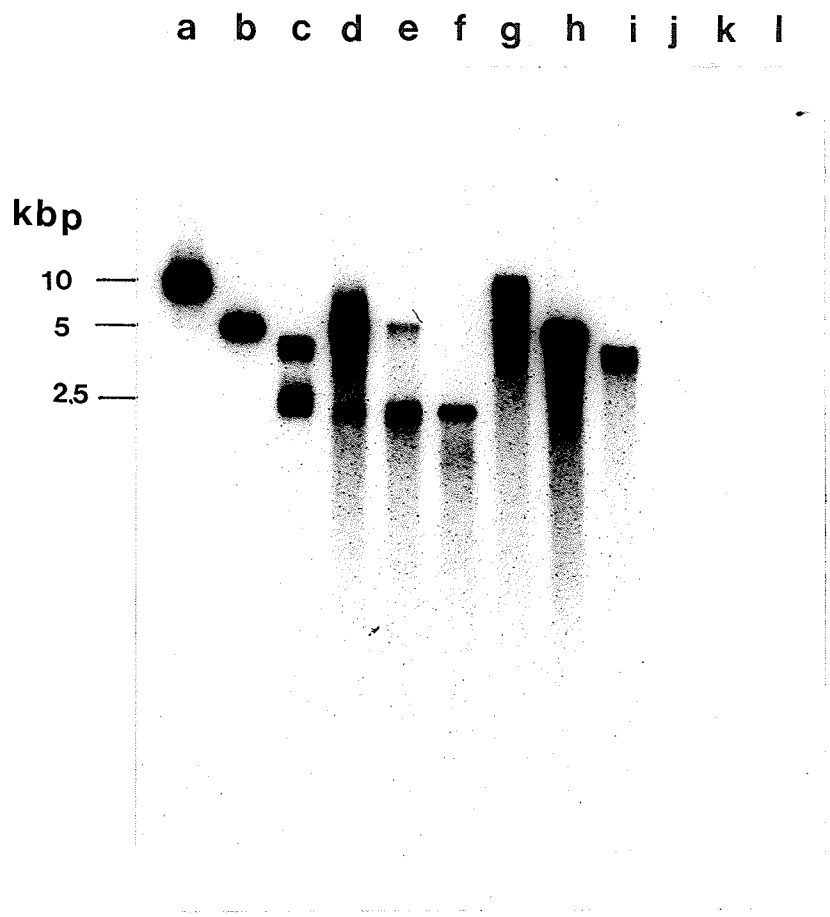
The 10 Kb molecule disappeared completely when denatured, being replaced primarily by a band which comigrated with the 5 Kb, plus 2 closely migrating species running to the 2.5 Kb position (lane d). This form might therefore be composed of molecules, nicked at or near a unique unit length site, and secondly, those having both copy and viral strands covalently linked.

When the 5 Kb form was heated, it presented the same results as the 10 Kb. Three molecular species were found: one remaining the same size as the original and two bands roughly half the size of the original (lane e). The autoradiograph indicated that the 2 latter molecules were equimolar and the predominant components. The results suggested that the 5 Kb pool was composed of 2 distinct forms. In the first of these, the viral and its complementary strands are covalently coupled. The fact that a portion of the molecules are denatured to one-half the original size and having an equimolar ratio suggests that they represent open, linear, and opposing single strands.

Melting and quick cooling of the smallest species yielded a single, distinct band with an apparent shift to smaller size (lane f). Its uniqueness contrasted with the data generated from both of its larger counterparts. There also appeared to be contamination above the 2.5 Kb form, by a slower migrating band (lane c). It was believed that this arose at the slicing step from the original preparative gel.

As an adjunct to denaturation experiments, both native and heat-quenched samples were tested for susceptibility to nuclease S1. As observed for the crude preparations, background counts increased in both native 10 Kb (lane g) and 5 Kb (lane h). Again, the 10 Kb form was found to be slightly unstable at the 50°C incubation temperature, used in the S1 reactions, evidenced by a partial breakdown to 5 Kb structures (lane g).

Figure 18. Heat denaturation and nuclease S1 susceptibility analysis of purified EPV replicative forms.



Sample order was native: 10 Kb, 5 Kb, and 2.5 Kb (lanes a, b, and c, respectively), treated by heat-quenching (lanes d, e, and f), native with nuclease S1 (lanes g, h, and i), and heat-quenched with nuclease S1 (lanes j, k, and l).

Most of the 5 Kb form remained unaltered, as expected, since the reaction conditions were not sufficient to disrupt double stranded molecules.

Evidence of single strandedness in the 2.5 Kb species was afforded when native material was completely digested (lane i). Also noted was that the contaminating higher molecular weight material remained untouched.

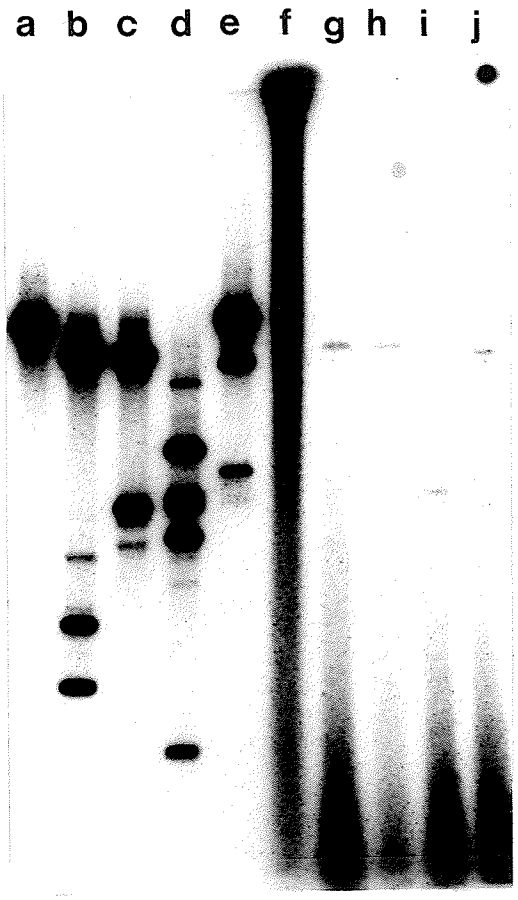
Interestingly, all 3 forms were completely digested following denaturation (lanes j,k,l). This observation may reflect the method used and will be explored in the discussion.

To summarize then, heat denaturation and S1-susceptibility trials indicate that the 3 intracellular replicative species isolated from equine virus infected cells are related. The largest form, 10 Kb, is probably a dimer of 5 Kb (diRF), being nicked at unit length sites and also containing a closed hairpin terminus. The double stranded monomeric replicative form (mRF) of 5 Kb was composed of 2 structures: open linear and snapback molecules. The smallest species was single stranded and had a heterogeneous agarose gel migration, possibly due to conformational differences, since denaturation generated a single distinct band. Since sizes were determined using double stranded (ds) fragments, and because ss molecules migrate at roughly one-half their ds counterparts, the smallest equine viral intracellular form is probably 5,000 nucleotides in length rather than 2,500 base pairs.

5.24. Orientation of Restriction Enzyme Sites Relative to the Kinased Terminus

To map the monomeric replicative form, it was necessary to establish a reference point from which restriction enzyme cleavage sites could be oriented. Denaturation studies had demonstrated that total mRF was composed of two types of molecules, fully extended and hairpin containing

Figure 19. Orientation of restriction enzyme cleavage sites relative to the kinased 5' terminus.



Uniformly labelled (lane a) and kinased mRF (lane f) were restricted with: BglII (lanes b,g); EcoRI (lanes c,h); HaeIII (lanes d,i); and HindIII (lanes e,j).

(Section 5.22, Figure 18). This experiment took advantage of the fact that the hairpin type could be selected from the mixture following heat denaturation and quick cooling. It would possess a single 5' end, suitable for labelling.

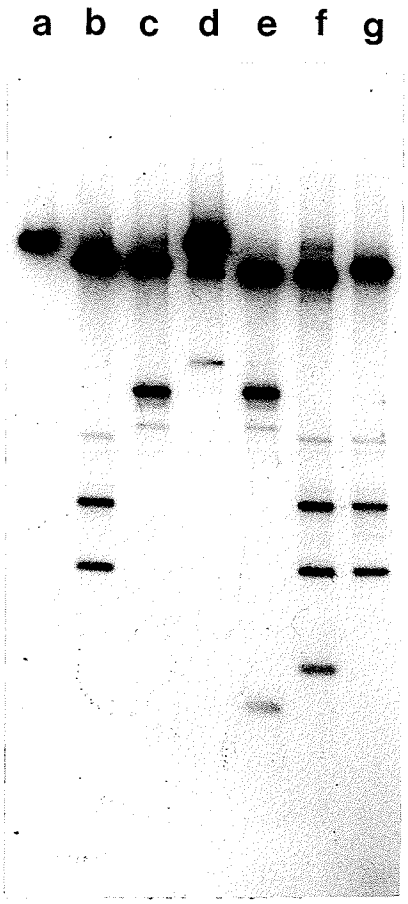
Preliminary mapping of the mRF species had established a host of enzymes which cut the molecule and these were employed in the digestion of 5' end labelled mRF. The results of uniformly and end-labelled mRF digests are presented in Figure 19.

Digests of the uniformly labelled molecule were consistent with data from several other experiments. The data indicated that the 5' labelled terminus was on the large fragment produced by cleavage with BglII (lane g), EcoRI (lane h), and HindIII (lane j). From these, it was apparent that the hairpin mRF had been the substrate for the kinase reaction, as a single fragment was observed. In the HaeIII digest of the kinased material (lane i), it was possible to detect three bands, with the 1,500 bp being the most intense. The other two were accounted for as incomplete digestion products.

Most of the untreated control kinased material failed to enter the gel (lane f). The basis for this result is unclear, perhaps aggregates or network structures were formed during storage, since the original material had migrated as mRF did (data not shown). That this material was still mRF was illustrated in the reaction lanes g-j, as nothing remained at the slot following incubation.

Thus, four restriction enzyme sites were oriented relative to the 5' terminus of the EV mRF hairpin molecule.

Figure 20. Single and double restriction enzyme digests of uniformly labelled EPV mRF.



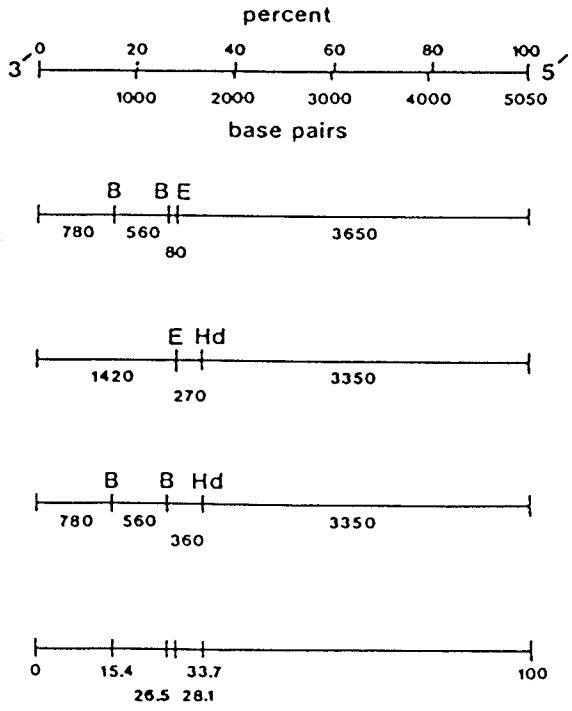
Uncleaved mRF (lane a) was digested with: BglII (lane b), EcoRI (lane c), HindIII (lane d), EcoRI/HindIII (lane e), BglII/HindIII (lane f), and BglII/EcoRI (lane g).

Table 10. Restriction enzyme fragments from single and double digests with BglII, EcoRI, and HindIII.

Restriction enzyme	BglII	EcoRI	HindIII
BglII	780, 560, 3710	-	-
EcoRI	780, 560, 80, 3650	1420(1200), 3650	-
HindIII	780, 560, 360, 3350	1420(1200), 270, 3350	1700(1500), 3350

Single and double digests were conducted on uniformly labelled mRF. Fragment sizes are given in base pairs. Ordering is relative to the mRF hairpin 5' terminus. Bracketed fragments represent 3' terminal heterogeneity.

Figure 21. Ordering of restriction sites from the BglII, EcoRI, and HindIII double digests.



Restriction fragments from Table 10 were ordered relative to the mRF hairpin's 5' terminus. Enzymes were B, BglII; E, EcoRI; and Hd, HindIII. Fragment size is given in base pairs. The bottom plot indicates the restriction sites given as percentage of genome length.

5.25. Mapping the mRF

The purpose of this experiment was to map the EPV monomeric replicative form. The mRF was screened with a compendium of restriction enzymes, including: Bam HI, BglII, ClaI, EcoRI, HaeIII, HindIII, HinfI, KpnI, MspI, PstI, PvuI, SmaI, TaqI, XbaI, and XhoI. Of these, BglII, EcoRI, HaeIII, HindIII, and MspI, were chosen for mapping as they generated four or less fragments. The enzyme HinfI was found to produce at least 12 pieces but was not used in mapping. Of the remainder, only PstI and TaqI had sites.

An autoradiogram of the BglII, EcoRI, and HindIII, single and double digests, is presented in Figure 20. The sizes of the restriction products and their ordering is displayed in Table 10 and the accompanying Figure 21. Note that in both EcoRI and HindIII single digests, a fragment is bracketed, which is 200-220 bp shorter than the next largest. In each case, when the lengths of all three bands were totalled, the sum exceeded the established mRF size (5050 bp) by the small bracketed fragment. Also, the autoradiographic intensity of the shortest piece was much less than its counterpart, with the degree too great to be accounted for by the length differential, but rather a stoichiometric amount. It was suspected that this phenomenon was also present in the BglII digest (lane b) but masked, because the 560 bp fragment was internal.

The kinase experiment had established that these three enzymes cut nearer the hairpin than the labelled 5' end. It was believed that the dual small fragments represented the extended and hairpin termini, with the latter shorter by the 200-220 bp.

In each of the double digests (lanes e-g), a unique band was produced which was not observed in the single pattern of either enzyme. For example, the individual profiles were: BglII (3710, 780, and 560 bp) and

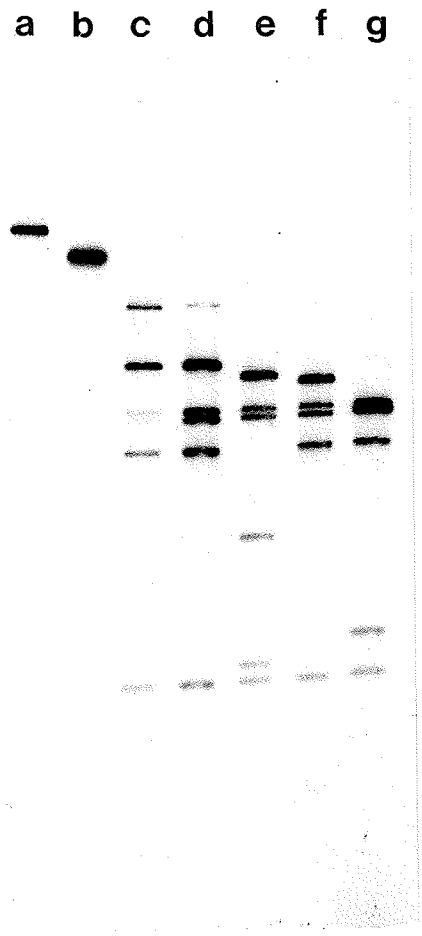
HindIII (3350 and 1700 bp). The double reaction yielded bands of: 3350, 780, and 560, all common to either BglII or HindIII alone, plus a new 360 bp fragment (lane f). It was concluded that this piece constituted the differences between the 3710 bp-BglII and 3350 bp-HindIII cuts. In a like fashion, the BglII/EcoRI reaction produced a novel fragment which migrated near the bromophenol blue dye front, with an estimated size of 80-100 bp. Due to its size, it was difficult to detect (lane g).

The sum of the unique BglII/EcoRI and EcoRI/HindIII fragments (80 and 270 bp), equalled the BglII/HindIII band of 360 bp. Thus, the fragments were ordered and a partial restriction map constructed (Figure 21).

Thus far, all four sites were clustered within a third of the mRF distal to the 5' reference which left about 70% of the molecule unmapped. The enzyme HaeIII was also identified as a suitable mapping instrument. Figure 22 is an autoradiogram of single and double digests conducted with HaeIII. Fragments are summarized in Table 11 and the order is given in Figure 23. Included on this gel was the dimer RF. The relatedness of native diRF (lane a) and mRF (lane b) was demonstrated with HaeIII on diRF (lane c) and mRF (lane d).

HaeIII cleaved mRF into 5 fragments: 1930, 1500, 1450, 1230, and 380 bp (lane d). A sixth, 2850 bp, was immediately recognized as a incomplete product, as its relative autoradiographic intensity was lower than the 1930 bp band. When the 5 band lengths were totalled, the size of 6490 bp was obviously about 1500 bp in excess of the accepted size. The 1500/1450 bp bands were equimolar and from the kinase results, the 1500 bp band was known to be on the 5' end. Once again, if the 3' extended and hairpin heterogeneity was to remain consistent with HaeIII (as it had with BglII, EcoRI, and HindIII), a pair of fragments which differed by about 200-220 bp

Figure 22. Single and double restriction enzyme digests of uniformly labelled EV mRF by HaeIII with BglII, EcoRI, and HindIII.



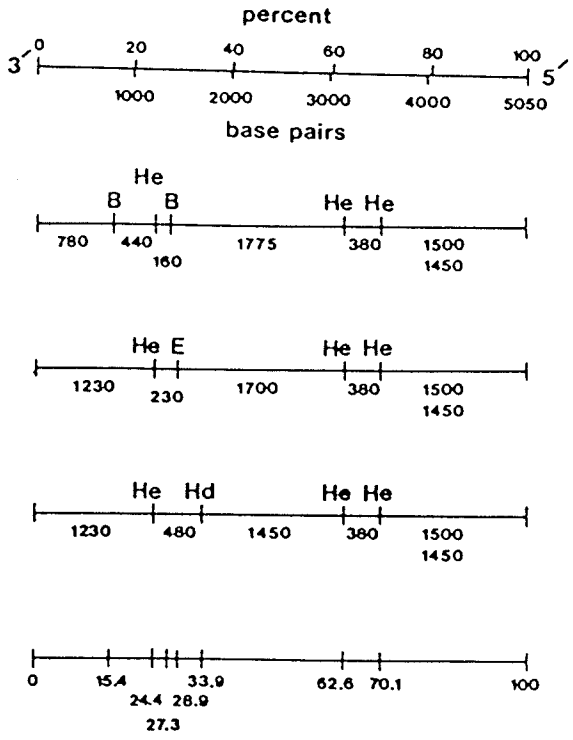
Uncleaved native diRF (lane a) and mRF (lane b) digested with HaeIII (lanes c and d, respectively). Dual reactions on mRF with HaeIII plus: BglII (lane e), EcoRI (lane f), and HindIII (lane g).

Table 11. Restriction enzyme fragments from single and double digests with HaeIII plus: BglII, EcoRI, and HindIII.

Restriction enzyme	HaeIII
HaeIII	1230, 1930, 380, 1500(1450)
BglII	780, 440, 160, 1775, 380, 1500(1450)
EcoRI	1230, 230, 1700, 380, 1500(1450)
HindIII	1230, 480, 1450, 380, 1500(1450)

Digests were conducted on uniformly labelled mRF. Fragment sizes are given in base pairs. Ordering is relative to the mRF hairpin 5' terminus. Bracketed fragments represent a 5' terminal heterogeneity.

Figure 23. Ordering of HaeIII restriction sites from double digests.



Restriction fragments from Table 11 were ordered relative to the mRF hairpin's 5' terminus labelled in the kinase reaction. Enzymes were: B, BglII; E, EcoRI; Hd, HindIII; and He, HaeIII. Fragment size is given in base pairs. The bottom plot indicates the restriction sites given as percentage of genome length.

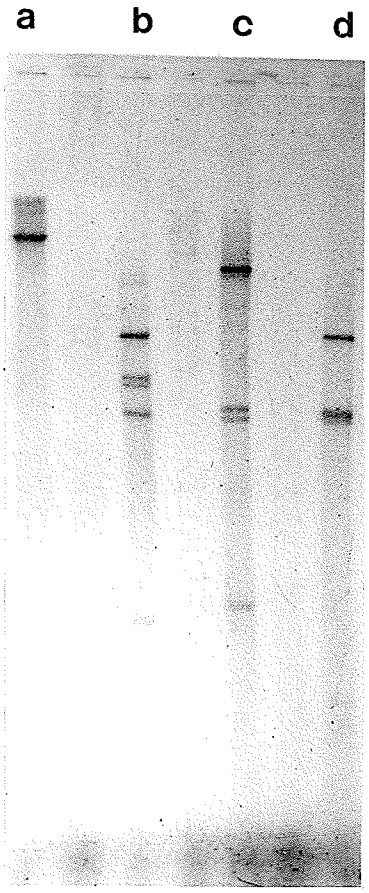
was expected. Based on this notion, a minor 1000 bp band appeared to be the counterpart of the 1230 bp (lane d).

Orientation of the HaeIII fragments was established by the double digests, and the kinase result. With BglII (lane e) both the 1930 and 230 bp were cleaved. New bands of 1775, 440, and 160 bp were produced. The origins of the largest and smallest products were assigned to 1930 bp, while the 440 bp was leftover from the 560 bp-BglII. In the parallel, EcoRI reaction (lane f) only the 1930 bp-HaeIII was cut, yielding 1700 and 230 bp. Like the previous two enzymes, HindIII cleaved within 1930 bp and generated a 480 bp piece (lane g). The second product of this reaction was located only because its comigraton with the 1450 bp-HaeIII fragment, doubled that band's intensity.

Since none of the second enzymes cleaved the HaeIII-1500, 1450, or 380 bp bands, they were placed on the 5' end (kinased) of the molecule. The 380 bp band was assigned an internal position because the 1500 bp was the kinased fragment (Figure 19, lane 1). Since the afore mentioned summation gave a length of about 1500 bp in excess of the true mRF size, it was speculated that the 50 bp differential between 1500 and 1450 bp represented a 5' terminal heterogeneity.

The previous HaeIII findings had suggested that a 1500/1450 bp heterogeneity existed on the 5' end of mRF. The enzyme MspI was also found to cut the mRF producing four fragments: 3300, 1300, 1250, and 450 bp. The same problem with the summation (total = 6300 bp), coupled with a doublet having a 50 bp differential suggested that MspI might cleave near the kinased 5' terminus as well. To confirm this, a double digest was carried out with HaeIII. The autoradiogram is presented in Figure 24. The fragment sizes and their order are summarized in Table 12 and Figure 24.

Figure 24. Single and double restriction enzyme digests of uniformly labelled EPV mRF by MspI and HaeIII.



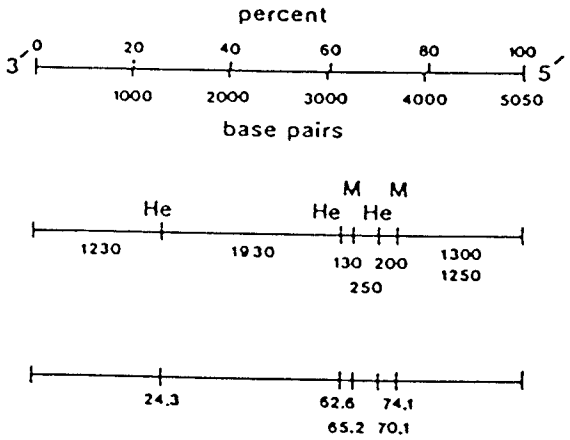
Labelled mRF (lane a) was cleaved with: HaeIII (lane b), MspI (lane c), and HaeIII/MspI (lane d).

Table 12. Restriction enzyme fragments from single and double digests with MspI and HaeIII.

Restriction enzyme	HaeIII	MspI
HaeIII	1230, 1930, 380, 1500(1450)	-
MspI	1230, 1930, 130, 250, 200, 1300(1250)	3300, 450, 1300(1250)

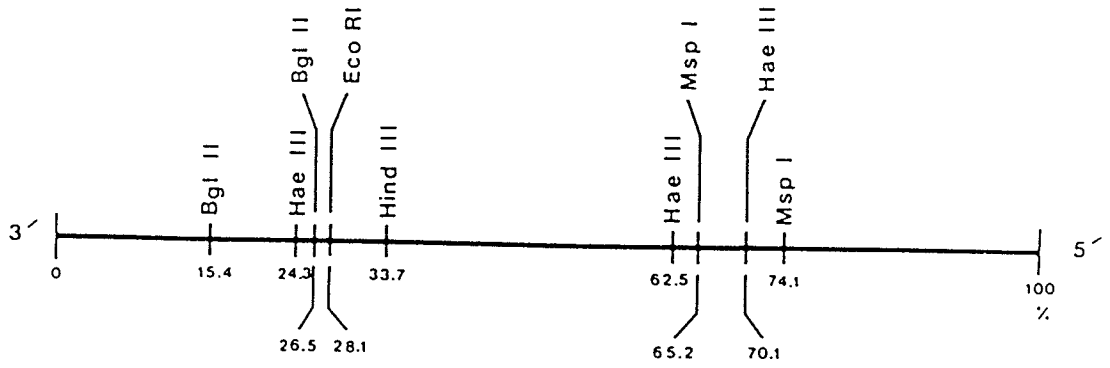
Digests were conducted on uniformly labelled mRF. Fragment sizes are given in base pairs. Ordering is relative to the mRF hairpin 5' terminus. Bracketed fragments represent a 5' terminal heterogeneity.

Figure 25. Ordering of MspI restriction sites.



Restriction fragments from Table 12 were ordered relative to the mRF hairpin's 5' terminus labelled in the kinase reaction. Enzymes were: He, HaeIII; and M, MspI. Fragment size is given in base pairs. The bottom plot indicate the restriction sites given as percentage of genome length.

Figure 26. Composite restricton enzyme cleavage map of the EPV monomeric replicative form.



Restriction enzymes are indicated above the line. Map position is given as percentage of genome length. Sites are oriented relative to the mRF hairpin 5' terminus labelled in the kinase reaction.

The HaeIII doublet was replaced by the MspI-1300/1250 bp set. In addition, three smaller fragments of approximately 130, 250, and 200 bp were generated by the proximity of the second MspI and HaeIII sites.

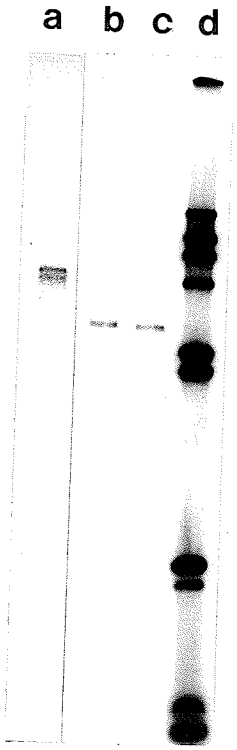
To conclude, a composite restriction enzyme cleavage map was constructed from the data of the mapping studies and is presented in Figure 26.

5.26 Changes in EPV

The high density (1.435 g/cm^3) equine parvovirus analyzed on a sucrose velocity gradient migrated as a single particle type (Figure 15) while the phenol extracted virion was shown to possess two distinct molecules. This phenomenon was further investigated using an EPV stock 16-3-84 produced, [^{32}P] labelled virus preparation (Section 4.18).

The phenol extracted genomic material was analyzed on a 1% agarose gel and compared with the material generated with EPV stock 13-11-83 in Figure 27. Both the 16-3-84, high and low density types (1.435 and 1.395 g/cm^3) possessed a single molecule (lanes b and c) while the 13-11-83 progeny had a distinct doublet (lane a). Thus it was apparent that the EPV underwent a selection during passage, in which one type of molecule was lost.

Figure 27. Changes in the viral genome character.



An examination of the nature of the EPV genome extracted from virions with density 1.435 g/cm^3 from a [^{32}P] preparation generated with, lane a, EPV stock 13-11-83 (1.4% agarose gel); lane b, EPV stock 16-3-84 (1% agarose gel); lane c, low density 1.395 g/cm^3, from EPV stock 16-3-84; and lane d, bacteriophage lambda (HindIII digest) labelled in a kinase reaction.

DISCUSSION

In spring of 1982, a high rate of abortion of nearly full term equine fetuses was reported to the Manitoba Agriculture Services, Winnipeg, by a single farm, in which more than 20% of a herd of about 70 animals, was affected. The major known pathogens for equine abortion, such as bacteria fungi, chlamydia, and herpesvirus, were not observed or isolated (Dr. F. Wong, personal communication). In the routine virology diagnostic procedure at Manitoba Agriculture Services, using a battery of cell lines the liver homogenate (but not other organs) of two of the submitted fetuses induced a mild cytopathological effect (CPE) on the porcine fallopian tube cell line (PFT). No bacterial or fungal infection was noted. Herpesvirus, which is known to be a major pathogen associated with diagnosable equine abortion was also not isoated from this series of submissions. This case history was related to us by Dr. F. Wong, Virology Section, Manitoba Agriculture Services, Winnipeg.

The culture fluids from the first passage of the liver homogenates (primary stock) of the two aborted fetuses (#21-1A and #22-1A) were submitted to this laboratory by Dr. Wong for pathogen identification and classification. Initial studies indicated that as much as 0.25 ml of the supernatant was necessary to induce a recognizable CPE in a 25 cm^2 flask culture of PFT cells. The physical-chemical stability of the infectious material of #21-1A and #22-1A was compared with the respective secondary stock. Both virus isolates were found to be resistant to Nonidet P40 and diethyl ether, suggesting a non-enveloped virus. Both isolates were resistant to inactivation at 60°C. It was tentatively decided that the two primary isolates, deriving from separate aborted equine fetuses of the same herd, on the same farm, were probably related. Subsequent electron

micrographs supported this and suggested that this was the etiological agent for the high incidence of abortions on this farm. Further epidemiological survey is a topic of study by the Manitoba Agriculture Services.

We were concerned with characterization and molecular biological studies of this equine pathogen which causes abortion of nearly full term fetuses. In view of the fact that the two isolates were so similar, only the #21-1A was subjected to detailed studies described in this thesis. The resistance to non-ionic detergent and 60°C treatment together with the morphology and particle size demonstrated in the preliminary studies allowed us to form a working hypothesis, that the equine virus was a parvovirus. If it indeed proves to be a parvovirus, these will be the first equine parvoviruses isolated.

The virus titre in the primary (1°) and secondary (2°) stocks was very low and made further study very difficult. Retrospective study indicated that the 1° stock has a titre of only 5×10^3 $TCID_{50}$/ml and 2° approximately 5×10^4 $TCID_{50}$/ml. Attempts were made to improve the virus yield. Initial studies to compare the virus yield using cultures at mid-log phase (50-70% confluency), late-log phase (80-90% confluency), and full confluency indicated that infection of cultures at mid-log phase produced substantially higher titre than those at late-log phase or at full confluency (data not shown). The dependence of this virus replication on cell growth was apparent.

If this was the case, the increased percentage of cells in the DNA synthesis period of the generation cycle may improve the infectious yield. In mammalian cells, excess thymidine results in the feedback inhibition of the pyrimidine biosynthetic pathways. When thymidine levels are elevated, TTP rises, which subsequently down regulates thymidine kinase, dCMP deaminase, and CDP reductase. The intracellular pool of dCTP diminishes,

thereby preventing DNA replication, but other cells proceed through the generation cycle until they reach the next S phase (Cleaver, 1967). Cell synchronization by double thymidine block, which arrests cells at the G1/S boundary of the cell cycle was chosen as a method to enhance virus yield.

Having obtained a culture system for the equine virus, the development of a reproducible assay for infectivity was established. The tissue culture infectious dose (TCID) assay involved the serial dilution of virus to an endpoint on a multiwell microtitre plate. Viral multiplication was detected by the cytopathological effect generated during late infection. Generally, the endpoint was both higher (about 1 $\log_{10}$) and more definite after a secondary assay, due to the amplification of virus in the primary infection. Often rows within an assay contained "skips" or wells lacking CPE. This demonstrated that one infectious unit was being pipetted during transfer. The duration of the total procedure was 5 days. The reproducibility of the method is demonstrated in the case of EPV stock 13-11-83, where the titre has been consistently $1x10^8$ $TCID_{50}$/ml (for a year).

With both cell culture and virus titration procedures operative, the physical and biological properties were defined. To examine the effect of equine virus infection on the cell, synchronized PFT cells were either mock or EPV infected and reseeded after a trypsinization step. Cell numbers were quantitated 24 hours later. It was observed that the infected cell number was less than the mock infected control and actually possessed fewer cells than had been originally seeded (Figure 2). When the cell number was adjusted based on the growth of the mock set, the inhibition was even more evident at 68%. This result is of the same order as that reported by Hampton (1970) in which mitosis was inhibited by 50-75% in H-1 infected cells. Infection in early S phase by H-1 resulted in the cessation of cell

growth due to viral disruption of nuclear chromatin (Singer and Toolan, 1975; Singer, 1976). Although no ultrastructural studies were made with EPV infected PFT cells, the reduction in cell number agrees with studies on other parvoviruses, such as H-1.

Later studies of this virus involved protein and nucleic acid labelling. It was essential therefore to have an idea of the time course of viral multiplication. The growth cycle of equine virus was examined under both asynchronized and synchronized conditions (Section 4.6). The production of infectious progeny was measured at various times post-infection (Figure 3). Virus titres were maximal at 36 hpi in asynchronous cultures but at 24 hpi in the synchronized system. The slope of titre versus hpi was steeper in synchronized cells, which may be a reflection of the cell cycle distribution. As infection proceeded, more cells became competent, ie., traversed the S/G2 phases, resulting in a gradual titre rise. That there must be a degree of synchrony achieved by the protocol (Section 4.3) is suggested by the synchronous curve. The sharp titre increase between 12-24 hpi may be an indication of the alignment at the time of infection, as near simultaneous burst occurs. The 2-4 fold increase beyond 24 hpi may therefore reflect cells elsewhere in the cycle at infection, or alternatively secondary rounds of infection in the population which escaped the initial.

Synchronization, in itself, did not prove to be of greater utility than asynchronous cultures in the production of stock virus. Infection was usually conducted at MOI 10 $TCID_{50}$/cell and the cultures were grown for 3 days to generate maximum CPE. Over such a period, there would be opportunity for a secondary round of infection in either system. Growth cycle experiments show that EPV can replicate in 24 hours which agrees with the replicative kinetics described (Section 3.10.c). However, the

influence of synchronization on the rate of virus multiplication would be of value in biological and labelling studies, especially with regard to nucleic acid and protein synthesis.

Many physical properties of the autonomous parvoviruses, as summarized by Siegl (1976), were also observed in studies with our virus; heat stability was observed after incubation at 56°C for 30 minutes. Infectivity began to decline at 70°C (by 10-100 fold) and was abolished at 80°C (Figure 4). Stocks are now routinely heated for 30 minutes at 56°C, prior to use, to inactivate other substances, such as proteolytic enzymes released as a consequence of cell disintegration.

The EPV did not possess an envelope structure necessary for infection as neither ether extraction or NP-40 treatment reduced biological activity (Tables 1,2). The virus also withstood 1% SDS during a 15 minute incubation on ice (Table 1). Incubation was conducted for a short interval to prevent the precipitation of the SDS by cooling. In later procedures, n-butanol (Section 4.12) and deoxycholate (Section 4.18) were also used for the liberation of cell debris-associated virus. High titre virus preparations were recovered (Figures 6,7).

The morphology of heavy particles (buoyant density 1.416 g/cm^3) was examined by electron microscopy. These virions excluded PTA stain, were icosahedral, and 20-23 nm in diameter. The occasional empty capsid was also detected at this density (Figure 5).

The proteins arising from EPV infection of PFT cells were studied. Their elucidation might provide an opportunity for vaccine development. Both the virion and infected cytoplasmic lysates possessed two proteins with molecular weights of 87 (VP1) and 70.5 (VP2) Kdal when analyzed by SDS-PAGE (Figures 8,9,10). The number and sizes are similar to those of MVM, H-1, and BPV (Tattersall and Ward, 1978). VP1 was always the least

abundant. Sometimes a third, minor structural protein was detected having MW 68 Kdal (VP3). It was possible to experimentally cleave VP2 to the smaller VP3 by tryptic digestion (Figure 10). However, an equimolar amount of VP2 (70.5 Kdal) remained following a 1 hour digestion. Infectivity was not affected by this treatment (Table 3). A similar modification occurs naturally during maturation in the H-1 system. Nicking of the high density, heavy full (HF) particles to the light full (LF) state, takes place during late infection. Generally, empty capsids are quite resistant to nicking, either by conditioned medium or trypsin (Kongsvik et al, 1978). It is unlikely that remaining equine virus VP2 is from empty capsids since the CsCl density was 1.416 g/cm^3, but rather one hour may not have been sufficient for complete digestion.

Both structural proteins were distributed throughout a CsCl gradient (Figure 9) and probably represented particles with varying amounts of nucleic acid, in a manner similar to LuIII (Muller et al, 1978). Velocity sedimentation analysis of [^{35}S] methionine labelled infected cell lysates on 5-20% sucrose gradients, showed that very little, if any, of the structural VP existed as soluble molecules. Rather they were present in varying degrees of aggregation (data not shown).

When EPV infected total cell lysates were prepared in SDS sample buffer, a novel protein was identified with MW 17 Kdal. Matsunaga and Matsuno (1983) have detected two NCVP of rabbit PV (MW 25 and 22 Kdal) which were predominant in the nuclear fraction. As the nuclei degenerate, the proteins gradually moved into the cytoplasm. Others have found high MW species of 92 and 84 Kdal for H-1 (Paradiso, 1984), and 60 Kdal for MVM (Astell et al, 1983a,b). The 60 Kdal protein was atached to the 5' terminus of the mRF. NCVP are believed to have enzymatic functions necessary for genome replication. Like the H-1 NCVP's, the equine viral

product appears at the same time as the structural VP (S. Luk, K. Nielsen, unpublished observations). Since, the 17 Kdal polypeptide was only found in EPV infected cells, it may be either viral encoded, or a cell derived gene product induced as a consequence of EPV infection.

Equine parvovirus biological activity was investigated with regard to virus particle types and their infectivity. Many parvoviral systems possess both HF and LF types in a precursor-product relationship which differ by 0.03-0.04 g/cm^3. The H-1 LF form is considered to be more infectious than HF (Kongsvik et al, 1978). However, Paradiso et al (1982) found that only the 1.44 g/cm^3 type of CPV was infectious. The equine virus system provided an opportunity to study this phenomenon. It was demonstrated in this report that tryptic digestion cleaved VP2 to VP3 (Figure 10), without an accompanying infectivity drop (Table 3). In addition, VP1 and VP2 were observed at all buoyant densities (Figure 9).

A number of infectivity assays were conducted on CsCl gradient purified EPV. The initial [^{3}H] thymidine labelling trial showed a peak infectivity corresponding to density 1.416 g/cm^3 (Figure 7). Virus was produced with an MOI of 0.01-0.001 $TCID_{50}$/cell, and harvested from the medium only (ie., without freeze/thaw), at 72 hpi.

A second trial was performed at MOI 10 $TCID_{50}$/cell and the total flask contents were harvested with n-butanol (48 hpi). The virus was banded in CsCl starting from a uniform density. As shown in Figure 6, a shoulder at 1.44 g/cm^3 and a predominant peak centered at 1.41 g/cm^3 were produced from the TCID assay. It was suggested that two forms were beginning to be resolved.

In an initial [^{32}P] trial, a synchronized culture was infected (MOI 10) and harvested at 48 hpi. The virus was divided into the extracellular (cell-free) and intracellular (cell debris-associated) fractions and

processed on separate gradients. The extracellular gradient gave a discernable radioactive peak at 1.43 g/cm^3 with a slight shoulder at 1.40-1.41 g/cm^3 (Figure 11). The material at 1.43 g/cm^3 was infectious. No second peak of infectivity was resolved.

A repeat [^{32}P] experiment produced the genomic material from which nuclease susceptibility measurements were made. The radioactive profile gave sound evidence for two types of particles, with densities differing by 0.04 g/cm^3. The TCID assays supported this pattern as two bands of infectivity (1.435 and 1.395 g/cm^3) were identified in each gradient (Figures 12,13). The extracellular contained more of the 1.435 g/cm^3 species (1.2 x 10^9 $TCID_{50}$/ml), than 1.395 g/cm^3 (4 x 10^8 $TCID_{50}$/ml).

The task is to rationalize these five sets of data and arrive at the EPV buoyant density. There is sound evidence that EPV has two density classes of 1.435 and 1.395 g/cm^3 from the [^{32}P] radioactivity and infectivity experiments. This is supported by the uniform density data (Figure 6), in which the 1.41 and 1.44 g/cm^3 duality was detected. These results were all arrived at using MOI's of 10 $TCID_{50}$/cell and harvesting at 48 hpi. It is probable that the method of harvest is crucial. When the virus is prepared from extracellular (cell-free) and intracellular (cell debris-associated) material, distinct peaks are clearly resolved. Perhaps this explains why the *in toto* harvest and uniform gradient (Section 4.12) failed to completely separate the two forms.

The oddity among the trials was the [^{3}H] thymidine profile. It was clear that infectivity peaked at an intermediate density of 1.416 g/cm^3. This material was only extracellular virus from clarified medium (no freeze/thaw step) and harvested at 72 hpi, rather than 48 hpi as above. What may be seen here is the actual maturation shift. The fact that the MOI was 10^4-10^5 fold lower than the other runs explains the lower output

(10^5 $TCID_{50}$/ml), and may also raise questions regarding the rate of replication.

As stated, there was no significant difference in the VP profiles from different densities at 48 hpi (Figure 9), unlike the VP2' to VP2 shift with H-1 virus (Knogsvik et al, 1978). This does not rule out the possibility of a conformational change in the absence of enzyme-directed, protein maturation.

The protocol used for labelling the EPV genome, in vivo, was developed after several trials, all of which contributed to the final successful method. An initial attempt conducted with the DNA precursor, [^{3}H] thymidine, failed to produce substantial labelled material. This was attribued to the low MOI (10^{-4}-10^{-5} $TCID_{50}$/cell) and late labelling at 24 hpi.

The [^{32}P] labelling experiments were initiated because of the higher energy level of the isotope. The first trial failed because labelling was late. Eventually, advantage was taken of the growth cycle knowledge (Figure 3). Synchronized cells were infected and after trypsinization, were suspended in the labelling medium containing isotope (Section 4.18). The [^{32}P] is incorporated into all cellular nucleic acids, in addition to the EPV, which contributed to background in the CsCl gradients. As the nature of the equine virus was not definitely known, nuclease digestion of extraparticular nucleic acids was not undertaken.

After two CsCl gradient purifications, and velocity sedimentation, the genome was phenol extracted and tested for its susceptibility to RNase A, DNase I, and nuclease S1. Both DNase I and S1 digested the genomic material while it was unaffected by RNase A (Figure 16). Quantitative measurements to determine the extent of sensitivity, fully corroborated the visual analysis (Tables 5,6,7).

For the equine virus to be classified as a parvovirus, it was expected that the genome would be single-stranded DNA (Rose, 1974). A series of incubations were performed in duplicate over a three day period. As others had detected hairpin terminal structures (Section 3.6), a set of heat quenched samples were included to check for denaturation. The means of three runs are summarized in Table 8. The data indicated that the native viral genome was digested 6.9% less than the control ssDNA (ie., 98.7 - 91.8%). Heating and quick-cooling made an additional 3.7% S1 susceptible. From the student's t-test, a significant ($p<.05$) 3.2% (ie., 98.7 - 95.5%) remained resistant. Thus, there is evidence that the EPV genome has structures which are duplex in the native state, and further, they possess an arrangement which promotes annealing, after a step which was sufficient to completely hydrolyze control ssDNA. It is concluded that the resistant sequences have an intramolecular proximity. The 6.9% value would represent approximately 350 nucleotides, based on the mRF length of 5050 bp (Sections 5.21, 5.25). Astell et al (1979, 1983a,b) have established that the MVM genome may have a total of 323 nucleotides in the duplex form. These results show that the equine viral genome has features consistent with members of the genus Parvovirus.

The EPV genomic material isolated from the twice CsCl gradient purified material (density 1.435 g/cm^3) migrated as a single peak on a velocity sedimentation gradient (Figure 15). This fraction contained two bands having equimolar intensity and of the same strand polarity, as incubation under high salt conditions failed to promote annealing (Figure 16). Bates et al (1984) showed that the DNA extracted from autonomous virus LuIII readily annealed with high salt.

Since the virus has never been plaque purified, the possibility exists that there were two strains. This presumption has been supported in a

recent EPV preparation. The first [^{32}P] experiment was conducted with EPV stock 13-11-83 which gave the doublet. A subsequent EPV stock 16-3-84, is 3 passages removed from stock 13-11-83. When the new stock was used in a [^{32}P] labelling experiment, the same density classes were detected, ie., 1.435 and 1.395 g/cm (data not shown). However, the heavy particles now contained a sole molecule (Figure 27). Thus, it appears that a selection process has taken place. The equine viral genome was single-stranded DNA which proved the agent to be a parvovirus. The virus-specific intracellular nucleic acid forms were also studied. In particular, the establishment of a genomic map of the EPV monomeric replicative form DNA was of import because it might aid in the definition of a possible route for its appearance in the horse population of Manitoba, in preliminary comparison to other published maps. In mapping studies, use was made of the heat denaturation and nuclease S1 susceptibility results presented in Figure 18. From this autoradiogram, it was apparent that the intracellular replicative species of EPV contained double-stranded, dimeric and monomeric molecules. In this preparation, a ss form was also observed (Figures 17,18).

It became apparent that heat-quenching disrupted the mRF into two closely migrating bands of half the mRF size, plus a substantial amount which retained the migration of the original (Figure 18). Figure 17 shows that native mRF was composed of a major dark band plus a faint minor component of nearly the same size. It was speculated that these two represented the open extended and hairpin forms of mRF DNA, the origin of which has been explained by the model of Astell et al (1983b) in Figure 1. Relying on this model, the conclusion was made that EPV mRF also contained these forms. It should be noted that the apparent ratio of the two denatured forms was roughly equal.

In constructing a mRF map, the first step was establishing a reference point from which restriction sites would be oriented. The employed strategy made use of the nature of the mRF pool. After heat-quenching and agarose gel electrophoresis, it was possible to isolate the hairpin-containing conformer since the extended form was denatured to faster migrating single strands (Figure 18). The 5' terminal phosphate group was removed by bacterial alkaline phosphatase (Section 4.26) and replaced with [^{32}P] by T_4 polynucleotide kinase. Restriction enzyme cleavage of this substrate in parallel with uniformly labelled mRF established that a single fragment was labelled (Figure 19).

It is very important to note that restriction enzyme site orientation was based solely on the 5' end labelling of the hairpin form. It cannot be said that this 5' marker represents the 5' end of the viral minus strand. Such supposition would rely on the model of Astell et al (1983b). As presented in Figure 1, the 3' terminus of the viral strand is transferred by nicking and displacement synthesis, coupled by nicking and ligation to the progeny viral strand (1e,f). It is believed that a nick is also introduced at the other end of dimer RF to produce an open 5' end (1f). If this fails to occur and the hairpin is not ligated, then the copy strand would possess the 5' terminus. This possibility also exists for the kinase reaction substrate, creating the chance that the orientation may be the mirror image of Figure 26.

During the restriction enzyme mapping experiments, minor bands were identified with EcoRI and Hind III which differed in length by about 200 - 220 bp from the next largest fragments (Table 10, brackets). The ratio of these bands varied between intracellular extracts. It was suggested that they were 3' terminal structures of extended and hairpin configurations,

and that the variability was attributable to the content of each mRF species within a preparation.

A second terminal heterogeneity was identified by Hae III digests (Figures 22,24) and was assigned to the 5' end of the mRF hairpin by kinase results (Figure 19). The heterogeneity was further noted by summation of the HaeIII digestion products. The total was about 1500 bp in excess of the actual mRF length. The 1500/1450 bp doublet was not cleaved in double digests, which placed them on the opposite half of the molecule. It was concluded that they represented a 5' terminal heterogeneity in a manner similar to that discussed by Astell et al (1983a,b).

Again, the purpose of this study was to describe the genome for comparison to other known parvoviral mRF maps. Burd et al (1983) compared the maps of BPV, MVM, H-1, KRV, and CPV. An EcoRI site was conserved at 16-23 map units and HindIII between 48-52 mu. All of these possessed 1-2 BglII recognition sequences. The EPV mRF also had sites for these enzymes but the map positions were somewhat different. Recently, porcine PV mRF DNA has been characterized (Molitor et al, 1984). The authors mapped the infectious (NADL-8) and less pathogenic (NADL-2') isolates. The NADL-2' genome had a 300 bp deletion near the 5' terminus (which included one of the two BglII sites). The NADL-8 and EPV maps are worth comparing. Cleavage of NADL-8 generated fragments having: BglII (3650, 850, 550), EcoRI (3600, 1400), and HindIII (3400, 1650) base pairs. These were highly similar to EPV: BglII (3710, 780, 560), EcoRI (3650, 1420), and HindIII (3350, 1700) bp. If one attaches an error of 50 bp (± 1 map unit) the difference is not great. These sites are clustered within an 18% region. Differences were detected with the restriction enzyme MspI: NADL-8 [3950, 1150 (1050)] and EPV [3300, 450, 1300 (1250)] bp. The brackets indicate the second fragment of the terminal heterogeneity, as with HaeIII.

The two physical maps are the mirror images of one another, ie., the 5' and 3' positions are reversed with respect to the BglII, EcoRI, and Hind III cluster. To arrive at their orientation, PPV genome specifically labelled at the 3' terminus was hybridized to denatured ss mRF. It was assumed that the virion material was minus sense. Thus, it is conceivable that the EPV and PPV maps may have the same orientaton. Assuming that the assignment of Molitor et al (1984) is correct, then the terminus labelled in the EPV kinase reaction (Figure 19) may be the 5' end of the copy sense.

The less pathogenic NDAL-2' strain differed from NADL-8 in that the smaller of the two bands (from EcoRI, Hind III) were the predominant (Molitor et al, 1984). It is interesting that the length differential was 300 bp, while the EPV was 200-220 bp.

When PPV strain NADL-8 is grown in fetuses (in utero) and isolated from infected tissues, the virus density is 1.39 g/cm^3 (Molitor et al, 1983). Both full and empty virions contained three structural proteins: 83, 64, and 60 Kdal. The EPV prepared in [^{32}P] labelling experiments (Section 4.18) permitted the identification of two types of infectious, full particles at 1.435 and 1.395 g/cm^3 (Figures 12,13,14). The uniform density gradient confirmed this infectivity duality (Figure 6). EPV possessed two structural polypeptides, 87 and 70.5 Kdal. A third minor protein was detected at 1.40 g/cm^3 (68 Kdal), which may have represented a degration product. The EPV capsid protein profile, prepared in PFT cells, differs from PPV grown in the animal system. The CsCl buoyant densities although similar at 1.395 and 1.39 g/cm^3, differ in that EPV has a denser form of 1.435 g/cm^3. The agreement at the lower density is not suprising since most parvovirus full particles band between 1.38-1.43 g/cm^3 (Tattersall and Ward, 1978).

The physical maps of EPV and PPV would share at least four sites clustered within a segment 18% of the genomic length, if one of the orientations is reversed. Such congruity has been described for canine PV (CPV), feline panleukopenia virus (FPLV), and mink enteritis virus (MEV) systems (Tratschin et al, 1982). The physical map of CPV DNA shared 80% of the known restriction sites of FPLV and MEV. Despite this homology, host range differences were detected as FPLV/MEV failed to replicate in two permanent canine kidney cell lines.

To summarize, restriction enzyme mapping may show that EPV is related to PPV, although differences were detected with endonuclease MspI at two sites. EPV possesses an additional full particle. The number and molecular weights of the structural proteins also vary. To date, no NCVP has been reported in the PPV system, while total cell extracts identified a unique 17 Kdal protein in EPV infected PFT cells. The 5' and 3' terminal heterogeneities may be due to differences in the hairpin and extended mRF conformers. However, as EPV has not been plaque purified (its unknown if this is possible), there is a chance that two strains of equine parvovirus were isolated.

In conclusion, the characterization of the genome and intracellular replicative forms of EPV coupled with the physical and biological properties reported in this work are sufficient to classify this virus as a member of the genus Parvovirus.

SUMMARY

This investigation has involved the description of several physical and biological properties of an unknown equine virus isolate.

1. Although possessing an equine origin, the virus grows to high titre (10^8-10^9 $TCID_{50}$/ml) in a porcine fallopian tube cell line.

2. The virus had mitolytic properties, inhibiting the growth of synchronized PFT cells by some 68% relative to an uninfected control. It appeared to favour elements of the cell cycle's S phase as infection of synchronized cultures prior to release produced higher titres in a shorter period than asynchronous cells. Cytopathological effects were observed beginning at 36 hpi. High multiplicities of 10 $TCID_{50}$/cell (or greater) caused almost complete cell disintegration by 48 hpi.

3. The full 20-23 nm particles had two major (VP1, 87,000; VP2, 70,500) and one minor (VP3, 68,000) dalton proteins. Very little of the capsid polypeptides were in a soluble state at 48 hpi. A nonstructural 17,000 dalton protein was detected in the nuclei of EPV-infected PFT cells.

4. Infectivity profiles of primary CsCl gradient purified virus indicated that there were two full classes, HF and LF with respective densities of 1.435 and 1.395 g/cm^3, both of which were infectious.

5. The viral genome was single-stranded DNA, 5050 nucleotides in length. Evidence was presented for hairpin (or snapback) terminal regions.

6. The intracellular dimeric RF molecule was composed of two mRF species, the hairpin and fully extended conformers, as evident from heat denaturation data. Most of the viral specific nucleic acid was in the mRF state, in the two forms described above. The single-stranded DNA component

migrated as a heterogeneous smear on gels, possibly due to variations in secondary structure.

7. A physical map of the mRF was oriented relative to the hairpin 5' terminus. A set of four restriction enzyme sites within an 18% region ressembled a cluster on the map of an infectious porcine parvovirus isolate.

8. Restriction fragments of EcoRI and Hind III digests of mRF showed the existence of a second minor band of 200-220 bp shorter length which varied in intensity between runs and was not in a molar relationship to its larger counterparts. This observation suggested terminal heterogeneity in mRF and the possibility of two virus strains.

9. The equine virus isolated had features entirely consistent for its classification as a member of the genus Parvovirus.

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