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EMERGENCE, NATURAL HISTORY, AND VARIATION OF CANINE, MINK, AND FELINE PARVOVIRUSES

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I. Introduction

During 1978 a new parvovirus of dogs [canine parvovirus (CPV)] was recognized simultaneously as the cause of new diseases of dogs throughout the world. Within 2 years the virus had spread into and infected virtually every population of domestic and wild dogs which has been examined. The diseases were new syndromes for dogs, and serological testing has shown that the virus was not present in dogs before the mid-1970s. The sudden appearance of CPV has raised a number of important questions about the mutability and variation of the parvoviruses, and about its relationships to the closely related viruses of cats and minks. At a fundamental level the mechanisms which define the host range of the viruses were in need of examination. This chapter examines the emergence of CPV, the evidence concerning the previous emergence of mink enteritis virus (MEV) as the cause of a new disease in minks in the 1940s, and the mechanisms which determine the host ranges and other specific properties of the viruses of cats, minks, and dogs.

The host range of animal viruses is one of their most fundamental characteristics, as it determines the range of susceptible animals. Most viruses are restricted in host range and infect only a defined spectrum of susceptible species. The emergence of new viruses and the acquisition of new natural host ranges are unusual events. However, such new viruses have the potential to cause pandemics in their new nonimmune hosts. The emergence of viruses from other animal reservoirs is clearly involved in the evolution of new pandemic strains of influenza in humans (Webster *et al.,* 1982; Palese and Young, 1982) and could be the origin of human immunodeficiency virus. It is likely that such events have occurred in the past to give rise to the present diversity of animal viruses.

Feline Panleukopenia Virus, Mink Enteritis Virus, and Canine Parvovirus

The viruses are classified as the feline parvovirus subgroup of the genus *Parvovirus,* within the family Parvoviridae (Siegl *et al.,* 1985). Feline panleukopenia virus (FPV), MEV, and CPV are classified as "host range variants." In addition to the viruses of cats, minks, and dogs, similar viruses naturally infect many species within the families Felidae, Canidae, Procyonidae, Mustelidae, and possibly the Viverridae. Here, canine parvovirus is referred to as CPV, except when it is necessary to distinguish between the two antigenic forms of the virus,

which are referred to as CPV type 2 (CPV-2) and CPV type 2a (CPV-2a) (see Section V,B).

II. Historical Background

A. *Feline Panleukopenia Virus*

A disease similar to that caused by FPV has been known in both large and small cats for many years. The agent was shown to be a filterable virus during the 1920s (Verge and Cristoforoni, 1928) and was the subject of considerable study during the 1930s and 1940s (Hindle and Findlay, 1932; Lawrence and Syverton, 1938; Lawrence *et al.*, 1940, 1943; Hammon and Enders, 1939a,b; Kikuth *et al.*, 1940; Torres, 1941; Lucas and Riser, 1945). In these studies it was determined that a number of diseases, which had been variously described as feline distemper, spontaneous agranulocytosis, feline infectious enteritis, or malignant panleukopenia, were all caused by the same agent (Hammon and Enders, 1939a). Similar diseases in common raccoons (*Procyon lotor*) and Arctic (blue) foxes (*Alopex lagopus*) were known before 1940, the viruses involved being considered the same as FPV (Waller, 1940; Phillips, 1943; Goss, 1948).

B. *Mink Enteritis Virus*

In 1947 a new disease was observed among minks in the area of Fort William, Ontario, Canada (Schofield, 1949; reviewed by Burger and Gorham, 1970). Initially named Fort William disease and later called mink viral enteritis, the agent was described as MEV. The relationships between mink viral enteritis and FPV were recognized early, due to the close similarities between the clinical signs and the characteristic pathology of the two diseases (Wills, 1952), and the antigenic similarity between the viruses was demonstrated by cross-protection studies during the early 1950s (Wills, 1952; Wills and Belcher, 1956). Early studies indicated that the experimental inoculation of minks with tissues from FPV-infected cats gave a similar disease to that caused by MEV (MacPherson, 1956), although in preliminary studies inoculations of kittens with MEV-containing tissues did not show any clinical disease. More recent studies have examined the *in vivo* host ranges and virulence of the viruses in more detail (see Section II,D).

The origin of MEV in Ontario has not been determined. Within 10–12 years the disease had been observed among farmed mink

throughout the United States, Europe, and Scandinavia (Knox, 1960; Tuomi and Kangas, 1963; Kull, 1966). Studies of MEV were hampered until the mid-1960s by the inability to grow the virus *in vitro* and by a lack of sensitive or specific serological tests. It is therefore not known whether MEV was a long-existing virus which had been present in minks many years before 1947 or whether it had only recently acquired the host range for minks at that time. FPV and MEV were both isolated in tissue culture during the 1960s (Johnson, 1964, 1965; Lust *et al.,* 1965; King and Croghan, 1965). The delay in isolating the viruses was probably due to the fact that they replicate only in dividing cells and cause a slow and nondescript cytopathic effect in infected cell cultures. However, the identification of characteristic Feulgen test-positive intranuclear inclusion bodies and fluorescent antibody staining methods for identifying infected cells allowed the viruses and antibody responses to be analyzed and titrated (Johnson, 1965, 1967c; Lust *et al.,* 1965; Gorham *et al.,* 1966; Scott *et al.,* 1970).

During the 1960s and 1970s studies of FPV and MEV revealed them to be parvoviruses and to be closely related biochemically and serologically (Johnson, 1967a; Johnson *et al.,* 1974; Flagstad, 1975, 1977). Effective vaccines were developed which controlled the diseases in cats and minks (Wills and Belcher, 1956; Pridham and Wills, 1959, 1960; Burger *et al.,* 1963; Gorham *et al.,* 1965; King and Gutekunst, 1970; Davis *et al.,* 1970).

C. *Canine Parvovirus*

During 1978 and 1979 outbreaks of previously unrecognized disease syndromes were observed in dogs in a number of countries (Eugster *et al.,* 1978; Appel *et al.,* 1978, 1979a; Burtonboy *et al.,* 1979; Gagnon and Povey, 1979; Horner *et al.,* 1979; Johnson and Spradbrow, 1979). The diseases were characterized by death due to acute or chronic nonsuppurative myocarditis in pups 3–16 weeks of age or pyrexia accompanied by vomiting and/or diarrhea in older animals.

The enteritis and pathological signs observed were similar to those of FPV in cats or MEV in minks, with necrosis of the intestinal crypt epithelium, eventual loss of the villus structure in the small intestine, and necrosis and depletion of the lymphocytes in various lymphoid tissues. Within a few months of the first observation of the diseases, a parvovirus resembling FPV was isolated or observed by electron microscopy in samples from dogs with myocardial or enteric forms of the disease. The ability of the virus from a case of myocarditis to cause enteritis was also demonstrated (Robinson *et al.,* 1979a, 1980b). The close relationship of this virus to FPV was recognized early (Appel *et*

al., 1978, 1979a; Johnson and Spradbrow, 1979), and effective vaccines were developed within a short period (Eugster, 1980; Carmichael *et al.*, 1981, 1983).

The global spread of CPV has been examined by the analysis of stored sera collected from dogs during the 1970s. The earliest CPV-positive sera reported were collected in Greece during 1974, and the reactivities of the three positive sera of 28 dogs examined from that year were confirmed by virus neutralization assays (Koptopoulos *et al.*, 1986). Positive sera were collected in Belgium and The Netherlands in late 1976 and during 1977 (Schwers *et al.*, 1979; Osterhaus *et al.*, 1980). The virus spread rapidly around the world during 1978, the first positive sera reported being collected in Australia during May 1978, in the United States during June 1978, in Denmark between January and June 1978, in New Zealand between July and October 1978, and in Japan during July 1978 (Walker *et al.*, 1980; Carmichael *et al.*, 1980; Have and Andersen, 1982; Jones *et al.*, 1982; Azetaka *et al.*, 1981; Mohri *et al.*, 1982).

Studies of sera collected from 1184 wild coyotes (*Canis latrans*) in three regions of the United States between 1972 and 1983 showed the first positive sera to be present during 1979, and by 1981 the majority of the animals in all areas were seropositive (Thomas *et al.*, 1984). The first positive sera from a population of wild wolves in Alaska were collected during 1980 (Zarnke and Ballard, 1987).

The rapid global spread of the virus was most likely a consequence of the high titers of virus shed in the feces of infected dogs and the resistance of these parvoviruses to inactivation in the environment, which would have allowed its transport, probably on inanimate objects, even into countries with strict quarantine procedures for dogs (Bouillant and Hanson, 1965; Carmichael *et al.*, 1981; Pollock, 1982; Meunier *et al.*, 1985a).

III. Diseases

The pathogenesis of diseases in animals of various ages is influenced primarily by the requirement of autonomous parvoviruses for actively dividing cells (Margolis and Kilham, 1965; Tennant *et al.*, 1969; Tattersall, 1972). It is also likely that this requirement determines many of the differences seen between infections of fetal, neonatal, or older animals. However, among the dividing cells in any animal, not all are permissive for virus replication. For example, the lymphoid and intestinal epithelial tissues, which contain rapidly dividing cell populations, are targets for virus replication by FPV, MEV,

and CPV, but appear to be resistant to infections by many parvoviruses in other species, such as the minute virus of mice (MVM) and H1 virus in hamsters, indicating that developmentally regulated properties of the different dividing cell populations can restrict parvovirus replication at the cellular level (reviewed by Cotmore and Tattersall, 1987). Although the relationships between cell replication rates and the susceptibility of tissues to parvovirus infection and replication in dogs, minks, or cats have not been examined in detail, it is clear that there are differences between the infections of animals at different ages.

A. Older Animals

In animals older than 6 weeks at the time of infection, the pathogeneses of infections of cats with FPV, minks with MEV, or dogs with CPV are similar, and the diseases in all species are therefore described together. Serological studies indicate that many infections by CPV in dogs (and probably by FPV and MEV in their respective hosts) are mild or subclinical (Smith *et al.*, 1980; Meunier *et al.*, 1980; Parrish *et al.*, 1982a). Signs seen in affected animals include pyrexia, diarrhea, and vomiting, along with leukopenia and/or a relative lymphopenia. The incidence of leukopenia or lymphopenia varies among the different viruses, panleukopenia being a striking feature of FPV infections of cats, but is uncommon in CPV infections, although a relative lymphopenia is often observed in the latter case (FPV: Hammon and Enders, 1939a,b; Lawrence *et al.*, 1940; Rohovsky and Griesemer, 1967; Larsen *et al.*, 1976; Ichijo *et al.*, 1976; Carlson *et al.*, 1977; MEV: Reynolds, 1969; CPV: Robinson *et al.*, 1980b; Carmichael *et al.*, 1981; Pollock, 1982; Macartney *et al.*, 1984a). Whether this difference reflects differences in the virus or the host is not known. Death of severely affected animals is likely to be a consequence of the extensive destruction of the gut epithelium, with dehydration and endotoxic shock contributing to the disease.

Although the prominent clinical sign observed after infection is most likely to be enteritis, the virus replicates in many organs of the infected host. The course of infection is rapid, with little virus being recovered from tissues or feces after 10–14 days postinfection (FPV: Csiza *et al.*, 1971a; CPV: Macartney *et al.*, 1984b). Natural infection is most likely via the oronasal route (FPV; Csiza *et al.*, 1971a; MEV: Myers *et al.*, 1959; Reynolds, 1970; CPV: Appel *et al.*, 1979b; Carman and Povey, 1982; Pollock, 1982; Macartney *et al.*, 1984a), although animals can be infected by most parenteral routes. After oral or oronasal inoculation initial virus replication occurs in the tonsils and

the regional and mesenteric lymph nodes, and shortly thereafter in the thymus and the spleen (FPV: Csiza *et al.*, 1971a; Carlson *et al.*, 1977, 1978; CPV: Macartney *et al.*, 1984b; Meunier *et al.*, 1985a; Carman and Povey, 1985b). Between 4 and 6 days after oral inoculation, infected cells are present in the intestine, and after day 5 postinoculation virus is excreted in large amounts in the feces; in the case of CPV between 10^7 and 10^9 infectious units may be excreted per gram of feces (Carmichael *et al.*, 1981; Meunier *et al.*, 1985a,b; Carman and Povey, 1985a).

In all species, infection of the lymphoid tissues (e.g., the thymus, lymph nodes, and spleen) results in lymphocytolysis, cellular depletion, and, subsequently, tissue regeneration in surviving animals. Virus replication and cell destruction in lymphoid tissues occur mostly in areas of dividing cells, including germinal centers of the lymph nodes and the thymus cortex (Fig. 1) (FPV: Hammon and Enders, 1939a,b; Lawrence *et al.*, 1940; Kikuth *et al.*, 1940; Carlson *et al.*, 1977, 1978; MEV: Reynolds, 1970; Krunajevic, 1970; CPV: Cooper *et al.*, 1979; Robinson *et al.*, 1980b; Macartney *et al.*, 1984a,b; Carman and Povey, 1985a,b).

The bone marrow can be affected severely, with a marked decrease in cellularity and decreases in cells of the myeloid, erythroid, and megakaryocytic series (FPV: Hammon and Enders, 1939a,b; Lawrence *et al.*, 1940; CPV: Robinson *et al.*, 1980b; Boosinger *et al.*, 1982; Macartney *et al.*, 1984a; Carman and Povey, 1985b). Although endotoxemia could play a role in the development of severe bone marrow lesions, both virus and infected cells are present in the bone marrow of CPV-infected dogs (Macartney *et al.*, 1984b; O'Sullivan *et al.*, 1984; Meunier *et al.*, 1985a).

In the gut the virus replicates in and destroys the rapidly dividing cells in the intestinal crypts. The subsequent loss of newly formed epithelium results in a flattened attenuated epithelium, with shortening of the intestinal villi (Figs. 2 and 3) (FPV: Rohovsky and Griesemer, 1967; Larsen *et al.*, 1976; Okaniwa *et al.*, 1976; MEV: Landsverk and Nordstoga, 1978; CPV: Cooper *et al.*, 1979; Yasoshima *et al.*, 1982; Macartney *et al.*, 1984c). These changes might lead to a loss of osmotic regulation, resulting in diarrhea, often with blood and mucus, as well as vomiting. Animals might become dehydrated and/or pyrexic, possibly because of endotoxin uptake from the gut.

The rate of intestinal epithelial cell turnover affects the severity of the lesions, probably by determining the number of cells able to replicate virus. This effect was demonstrated during infections of germ-free cats (Rohovsky and Griesemer, 1967; Carlson *et al.*, 1977), or after the treatment of cats with acid enemas which increased cell replication and

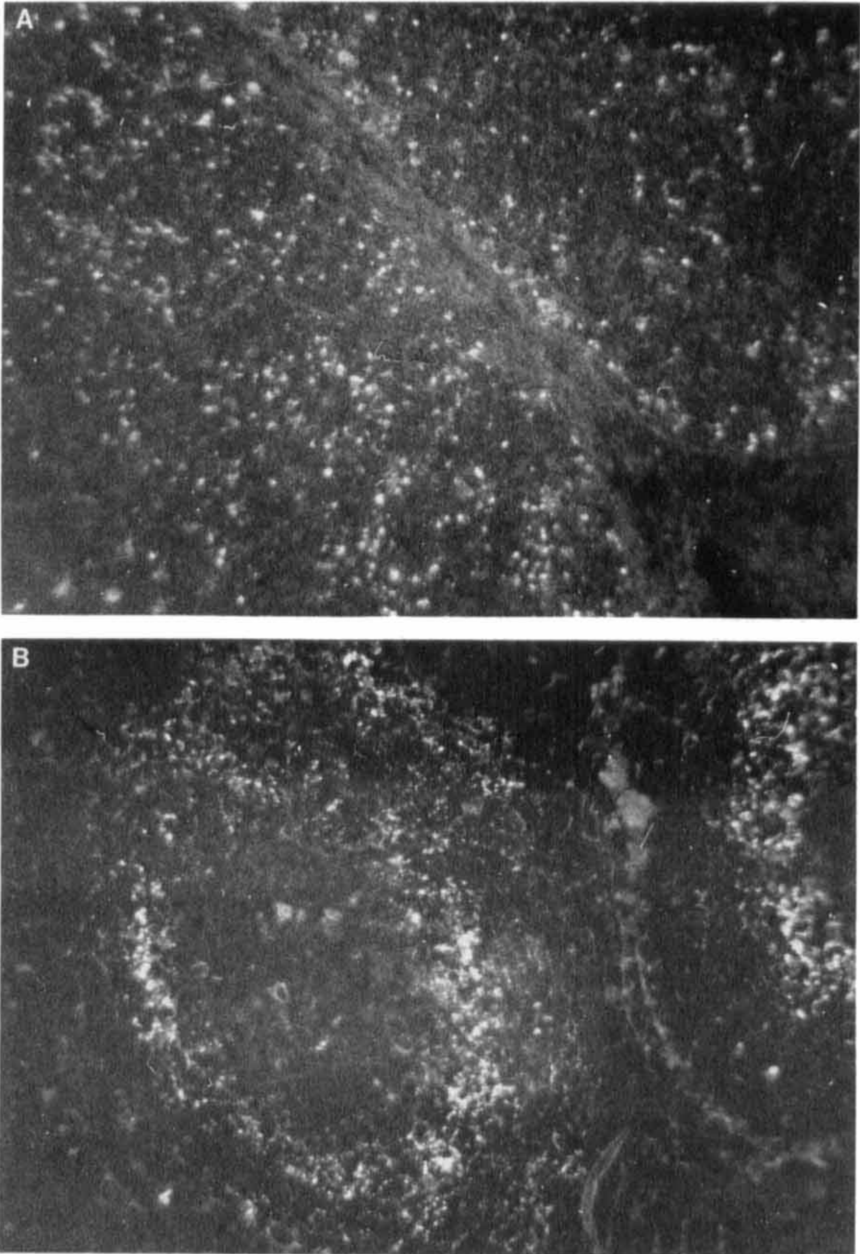
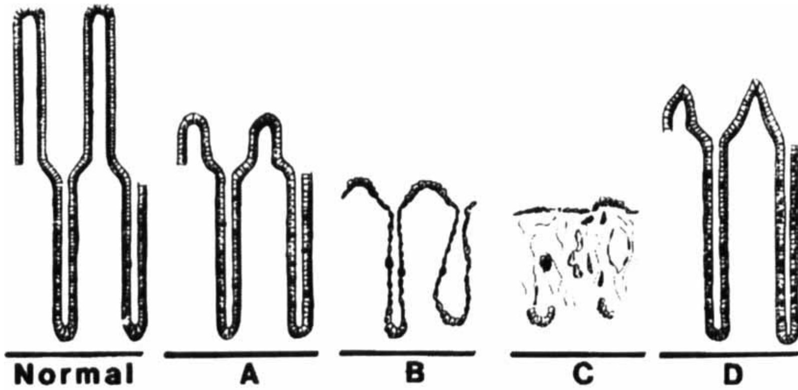


FIG. 1. Sections of (A) thymus or (B) mesenteric lymph node from dogs 5 days after parenteral inoculation with CPV and staining with a fluorescent antibody against CPV. The distribution of antigen in the thymus cortex and surrounding the lymph node germinal centers is shown. Infected cells contain intranuclear antigen, while many cells containing only cytoplasmic antigen most likely contain phagocytosed proteins.



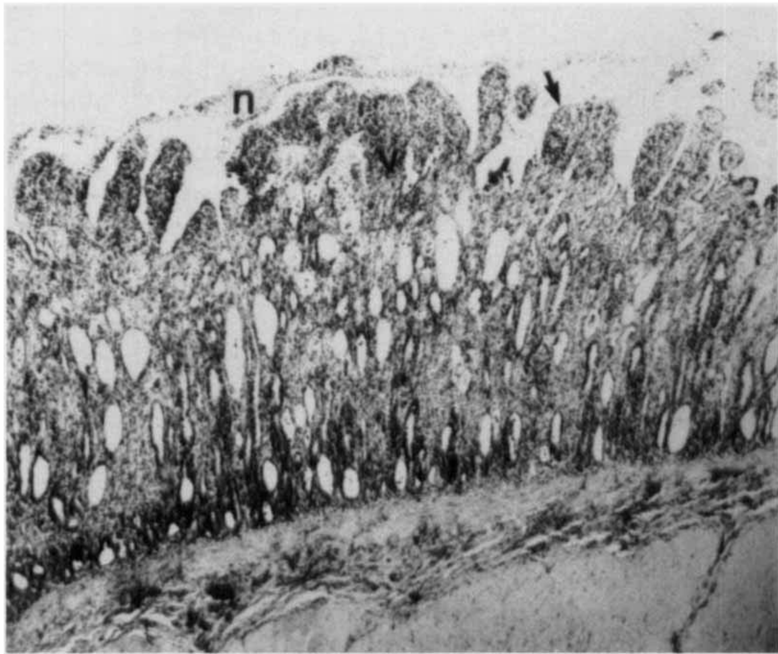


FIG. 2. Lesions in the small intestine of a dog after inoculation with CPV. (A) The observed patterns of intestinal lesions. (B) Duodenal mucosa with lesions of pattern B. Villi are stunted and occasionally fused (arrow). The crypts are dilated and lined with attenuated epithelial cells. Neutrophils (n) are present in the mucus overlying the affected mucosa. (C) Jejunal mucosa with lesions of pattern D: regenerating villi. The villi are short and covered with cuboidal epithelium, and the crypts are elongated and lined with cuboidal epithelial cells. From Macartney *et al.* (1984b) with permission.

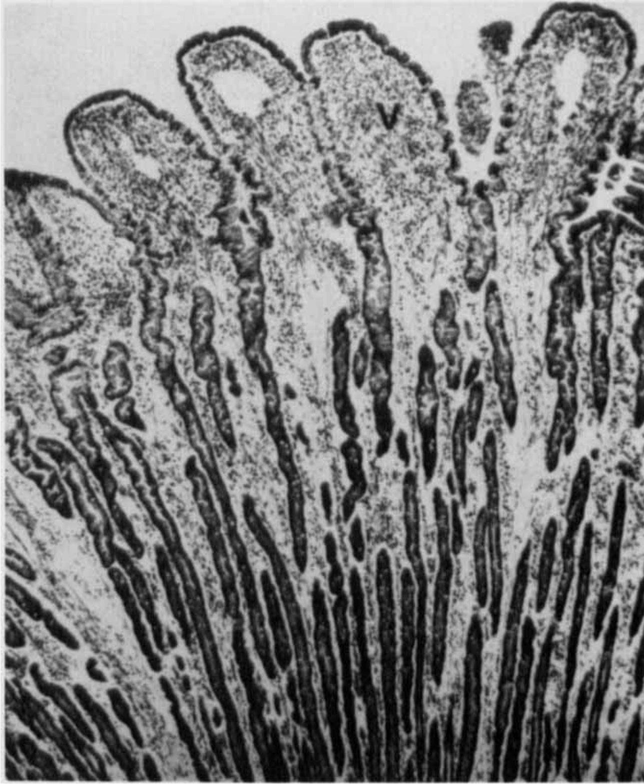


FIG. 2. *(cont.)*

subsequent FPV replication in the colon (Shindel *et al.*, 1978). A correlation was observed between the viral titers in serum and feces and the severity of the disease observed in dogs inoculated with CPV (Meunier *et al.*, 1985b). Intestinal parasites have been suggested to play a role in the natural pathogenesis of CPV infections (Chalifoux *et al.*, 1981; Pollock, 1982).

B. Fetal or Neonatal Infections

1. Feline Ataxia

In 1967 FPV was identified as the cause of an uncharacterized feline cerebellar ataxia, which had previously been thought to be a genetically inherited disease (Johnson *et al.*, 1967; Kilham *et al.*, 1967). When kittens were infected either *in utero* or shortly after birth, viral

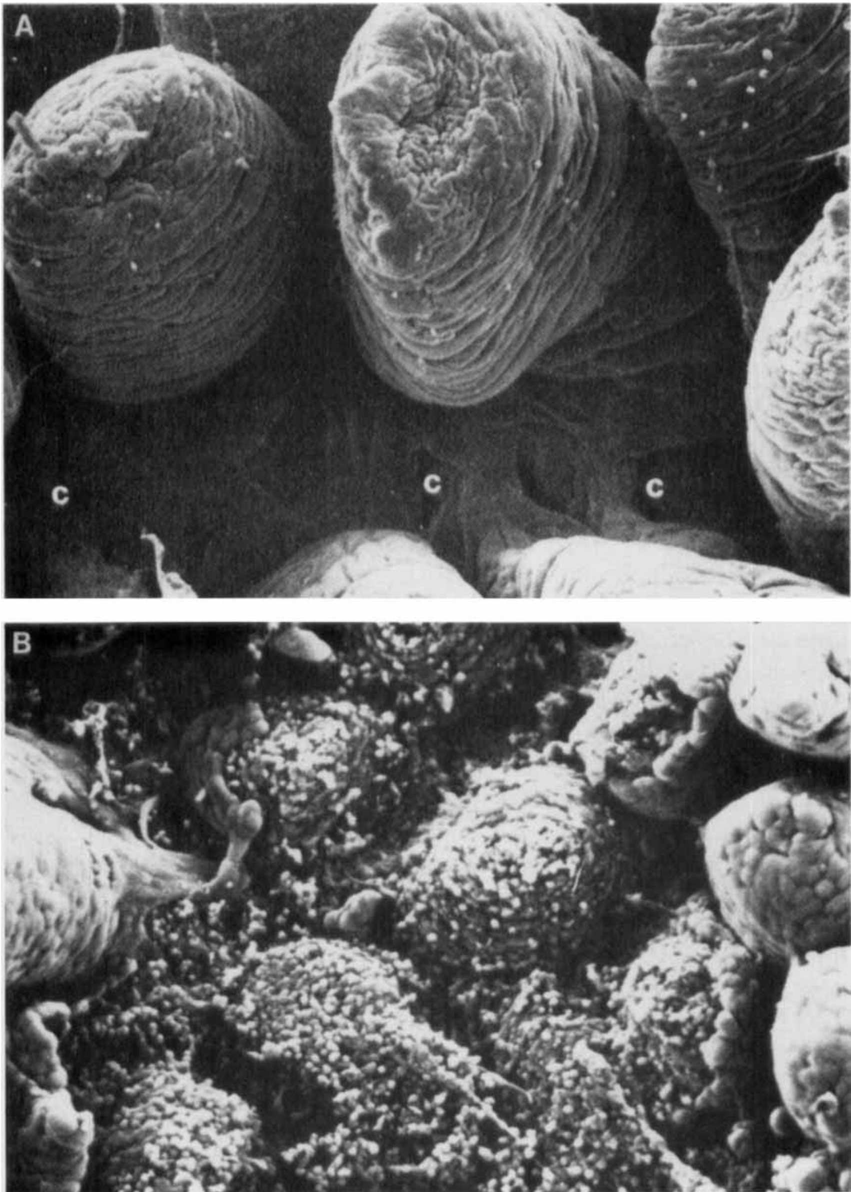


FIG. 3. Scanning electron micrographs of (A) the intestinal epithelium of the jejunum of a normal dog and (B) a dog killed 6 days after oral inoculation with CPV. In the latter case severely affected areas have completely lost the surface epithelium. Numerous crypt openings (c) are seen in the specimen from the control dog. From Macartney *et al.* (1984c) with permission.

replication in the cells of the external germinal epithelium of the developing cerebellum resulted in cerebellar hypoplasia (Csiza *et al.*, 1971a; Kilham *et al.*, 1971). While some kittens developed a widespread infection, leading to fetal death (Kilham *et al.*, 1967), most viable kittens subsequently suffered from ataxia (Kilham *et al.*, 1967; Csiza *et al.*, 1971a,b). Although ferrets are probably not a normal host for FPV (Parrish *et al.*, 1987; Veijalainen, 1986), ferrets inoculated with FPV prenatally or shortly after birth by the intracranial or other parenteral routes develop similar cerebellar lesions (Kilham *et al.*, 1967; Duenwald *et al.*, 1971).

2. Canine Myocarditis

Initial reports of CPV disease described outbreaks of acute myocarditis in puppies, mostly between 3 and 8 weeks old, although some animals were up to 16 weeks of age at the time of death (Kelly and Atwell, 1979; Huxtable *et al.*, 1979; Robinson *et al.*, 1979a,b, 1980a; Hayes *et al.*, 1979; Jesyk *et al.*, 1979; Carpenter *et al.*, 1980). Mortality in litters varied between 20% and 100%, and disease onset in apparently normal puppies was rapid, with pups dying of acute heart failure characterized by cardiac arrhythmia, dyspnea, and pulmonary edema (Robinson *et al.*, 1979b, 1980a; Hayes *et al.*, 1979; Jesyk *et al.*, 1979; Parrish *et al.*, 1982a). Electrocardiographically, affected pups showed a variety of subclinical abnormalities, and death appeared to result from progressive muscle necrosis, leading to ventricular fibrillation (Robinson *et al.*, 1979b; Carpenter *et al.*, 1980).

The primary pathological lesion observed was multifocal necrosis of the myocardium, often with a mononuclear cell infiltrate. Myocardial cells often contained intranuclear Feulgen test-positive amphophilic inclusion bodies. Lungs were diffusely edematous, with peribronchial and perivascular edema, most likely secondary to the heart failure (Robinson *et al.*, 1980a; Jesyk *et al.*, 1979; Carpenter *et al.*, 1980).

In a prospective study of CPV infections in a dog colony (Parrish *et al.*, 1982a) and in experimental infections of pups (Meunier *et al.*, 1984), it was shown that pups that developed myocarditis were infected either *in utero* or shortly after birth, often several weeks before the acute deaths due to heart failure, suggesting a chronic progression of the heart muscle damage. The age dependence of susceptibility of the myocarditis is probably related to the active cell division of the myocardial cells, which appears to occur only in pups under 15 days of age (Bishop, 1972).

It is of interest that there are no reports of cerebellar hypoplasia in dogs or myocarditis in cats infected with CPV or FPV, respectively. Although virus-infected cells have been observed within the heart

muscle cells of neonatal kittens after experimental FPV infections (Csiza *et al.*, 1971b), disease such as that in the puppies infected with CPV has not been described. It is not known whether the difference in the pathogeneses in neonatal cats and dogs is a consequence of differences in the susceptibility of the host cells and tissues or to some other difference in virus tissue tropism.

Occasional neonatal infections give rise to a generalized infection, lesions being observed in many different tissues (Lenghaus and Studdert, 1982; Johnson and Castro, 1984). *In utero* infections in cats or Arctic foxes by FPV or blue fox parvovirus (BFPV), respectively, could result in fetal death and resorption, abortion, or neonatal death (Kilham *et al.,* 1967, 1971; Veijalainen and Smeds, 1988). However, natural infections of dogs by CPV did not appear to affect reproduction (Meunier *et al.,* 1980).

C. Immunity

The functional immunity against these viruses appears to be mediated through serum antibodies. Pups or kittens which acquire maternal immunity from their dams are protected against parvovirus infection until their serum antibody titers decline to low levels [Brun *et al.,* 1979 (FPV); Parrish *et al.,* 1982a; Pollock and Carmichael, 1982; Ishibashi *et al.,* 1983]. In addition, after parenteral administration of anti-CPV antibodies to dogs, the animals were completely protected against oral challenge, and no virus replication was seen in the intestinal epithelium 4–6 days after infection (Meunier *et al.,* 1985a).

Whether secretory antibodies play any role in immunity to or recovery from disease is unclear, although it has been shown that levels of coproantibody correlated inversely with the severity of the disease in dogs naturally infected with CPV (Rice *et al.,* 1982), and the levels and classes of antibody in the jejunum collected by cannulation after either CPV infection or vaccination suggested that the antibody was being specifically secreted (Nara *et al.,* 1983).

D. Natural Host Ranges

The natural host ranges of these viruses have not been well defined. Little of the available information has been independently confirmed, and some of the results appear contradictory or unexpected. Since natural infections by contaminating viruses are an ever-present risk in experimental studies, and the viruses are not readily characterized without monoclonal antibody reagents or restriction enzyme mapping of viral DNAs, the host ranges that have been described should be

regarded as tentative in many cases until further confirmations are available.

Early studies were limited by the fact that virus could then be detected only by animal infections, so that virus or viral replication was only detected if it caused clinical disease. More recent studies have enabled the virus replication to be examined in more detail.

The natural hosts of FPV, MEV, and CPV are, by definition, the cat, mink, and dog, respectively (Siegl *et al.,* 1985). Viruses isolated from or observed in other species have not been systematically classified and are therefore named either after a virus from a related host species or after the host animal from which they were isolated; for example, raccoon parvovirus (RPV), BFPV, and raccoon dog parvoviruses (RD) have all been described. The genetic and biological relationships among the viruses from the various hosts, their natural host ranges, and pathogenic potentials are poorly understood.

All hosts are members of five families (i.e., Felidae, Canidae, Procyonidae, Mustelidae, and Viverridae) within the order Carnivora, although within the various families only certain genera or species have been reported to be susceptible. A recently described phylogeny of the Carnivora based on thermal stability of DNA/DNA hybrids is shown in Fig. 4 (Wayne *et al.,* 1989).

Virtually all large and small cats (i.e., several genera of the family Felidae) appear to be susceptible to FPV (Hindle and Findlay, 1932; Goss, 1948; Johnson, 1964). Within the family Procyonidae common raccoons (*P. lotor*) have been known to be susceptible to FPV since before 1940 (Waller, 1940; Goss, 1948; Nettles *et al.,* 1980) and are also highly susceptible to both virus replication and disease after inoculation with MEV or FPV (Barker *et al.,* 1983). Raccoons are not susceptible to CPV (Appel and Parrish, 1982). Ringtail coatis (family Procyonidae, *Nasua nasua*) are also reported to be naturally susceptible to FPV (Johnson and Halliwell, 1968). Civets (family Vivveridae) are affected by a disease similar to that caused by FPV in cats (Nain *et al.,* 1964).

Among the family Mustelidae only MEV has been described. Domesticated ferrets (*Mustela putorius*) appear to be susceptible to infection by FPV when inoculated *in utero* or by the intraperitoneal route 1–2 days after birth, and can develop cerebellar hypoplasia (Kilham *et al.,* 1967; Duenwald *et al.,* 1971). No such effect was seen when ferrets were 3 days of age or older (Duenwald *et al.,* 1971; Parrish *et al.,* 1987), and no serological evidence for natural infections by other similar parvoviruses has been reported (Veijalainen, 1986). Serological evidence for infection of wild striped skunks (*Mephitis mephitis*) has been reported, but a virus has not been isolated or characterized. CPV, FPV,

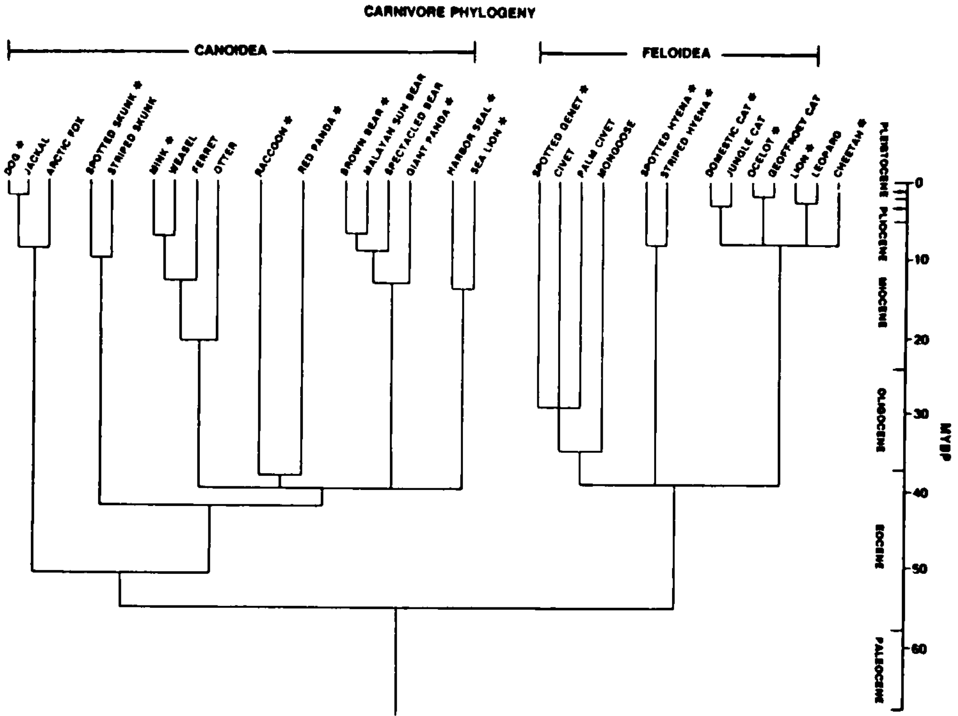


FIG. 4. Phenetic tree of members of the order Carnivora, based on the thermal stability of DNA/DNA hybrids. The time scale is based on fossil divergence times of about 40 million years before the present (MYBP) for all modern carnivore families, with the exception of the Canidae. The hosts of the FPV subgroup of parvoviruses include members of the families Canidae [represented, for example, by the dog, black-backed jackal (*Canis mesomelas*), and Arctic fox], Mustelidae (e.g., mink), Procyonidae (e.g., raccoon), Viverridae (e.g., civet), and Felidae (e.g., domestic and other cats). From Wayne *et al.* (1989) with permission.

or MEV isolates did not replicate in skunks after experimental inoculation, and the animals developed only low or undetectable antibody responses (Barker *et al.,* 1983).

Farmed American minks (*Mustela vison*) are susceptible to experimental infection by both FPV and MEV. The viruses replicate in a number of organs and are shed in the feces of many animals. Although early reports suggested that FPV isolates could cause clinical disease in mink (MacPherson, 1956), more recent studies, in which virus replication was more carefully monitored by titration, showed that only actual isolates from minks caused significant clinical disease in minks, while most infections by FPV or RPV were subclinical. In the

same studies CPV isolates replicated to only low titers in minks after experimental parenteral inoculation (Barker *et al.*, 1983; Parrish *et al.*, 1987). It therefore appears that the principal difference between FPV and MEV in minks is manifested by a difference in virulence for minks, rather than host range.

The susceptibility of the members of the family Canidae has not been clearly defined, although it appears that, besides domestic dogs, most, if not all, members of the genus *Canis* are susceptible to CPV (coyote: Evermann *et al.*, 1980; Thomas *et al.*, 1984; gray wolf: Mech *et al.*, 1986; Zarnke and Ballard, 1987). Other Canidae that are naturally susceptible to CPV include the maned wolf (*Chrysocycon brachyurus*) (Fletcher *et al.*, 1979), the bushdog (*Speothos venaticus*) (Mann *et al.*, 1980; Janssen *et al.*, 1982), the crab-eating fox (*Cerdocyon thous*) (Mann *et al.*, 1980), and the raccoon dog (*Nyctereutes procyonoides*) (Neuvonen *et al.*, 1982; Veijalainen, 1986).

Among the foxes, which are all members of the family Canidae, the red fox (*Vulpes vulpes*) and Arctic fox (*A. lagopus*) appear to be susceptible to infection by a virus distinct from CPV, which might be FPV or a virus closely related to it (Phillips, 1943; Neuvonen *et al.*, 1982; Barker *et al.*, 1983; Veijalainen, 1986; Veijalainen and Smeds, 1988). In a serological survey of wild red foxes in Ontario, Canada, 79.4% had antibodies to a virus related to CPV or FPV (Barker *et al.*, 1983). On experimental oral inoculation with CPV, MEV, or FPV isolates, red foxes did not develop clinical disease and all viruses were shed only in low titers, but the animals developed strongest serological responses against FPV (Barker *et al.*, 1983).

IV. Antigenic Relationships

A. *Polyclonal Sera*

Early studies of MEV and FPV indicated that antibodies formed to the two viruses protected animals against infection by CPV (Schofield, 1949; Wills, 1952; Wills and Belcher, 1956; Meyers *et al.*, 1959). These studies depended on the production of clinical signs by the viruses as the indication of infection.

Cross-neutralization studies of MEV and FPV using *in vitro* culture revealed that antisera against the FPV and MEV strains examined gave similar neutralization titers against the alternative virus strain (Johnson, 1967a; Flagstad, 1975, 1977).

Several studies of the antigenic relationships between FPV or MEV and CPV were performed using polyclonal sera and revealed that the

viruses all gave similar titers in neutralization or hemagglutination inhibition tests, although there was an indication of higher titers in the homologous than in the heterologous reactions (Flower *et al.*, 1980; Lenghaus and Studdert, 1980; Parrish *et al.*, 1982b; Tratschin *et al.*, 1982).

B. Monoclonal Antibodies: Antigenic Structure and Variation among Virus Types

The production of monoclonal antibodies (mAbs) against the various viruses has illuminated much of the complexity of the relationships among the various viruses.

Mouse mAbs against a purified 1978 CPV isolate revealed CPV-specific determinants, as well as determinants in common among CPV, FPV, RPV, and MEV (Parrish *et al.*, 1982b; Burtonboy *et al.*, 1982; Parrish and Carmichael, 1983; Surleraux *et al.*, 1987). Six of eight rat mAbs made against a 1973 FPV isolate reacted with CPV, RPV, FPV, and some MEV isolates, while two reacted more strongly with the FPV and MEV isolates than with the CPV isolates (Fig. 5) (Parrish and Carmichael, 1983).

When the mAbs were used to examine the antigenic structure of the viral capsid, the results revealed a variety of overlapping epitopes (Parrish and Carmichael, 1983). The CPV-specific mAbs appeared to recognize a single antigenic determinant, since all CPV isolates gave similar reactivities (Fig. 5). Antigenically variant neutralization escape mutants of CPV selected with two CPV-specific mAbs lost reactivity to all five CPV-specific mAbs, as well as to a group of five virus type-common mAbs (Fig. 6). Selection of mutant viruses with three of these type-common mAbs revealed two types of variation. Selection with mAb 6 or 12 gave variants which lost reactivity only to the three virus type-common mAbs prepared against CPV (mAbs 6, 12, and 18). In contrast, selecting with mAb B resulted in viruses which had lost reactivity to the same group of five virus type-common mAbs that were selected against by the CPV-specific mAbs, as well as the five CPV-specific mAbs (Fig. 6) (Parrish and Carmichael, 1983).

The two FPV-specific mAbs (i.e., G and H) recognized two different epitopes, as they reacted differently with various MEV or RPV strains (Fig. 5). In addition these mAbs selected mutant viruses with different patterns of reactivity, and the escape mutants selected by one mAb did not lose reactivity to the other (Fig. 6). mAb G cross-reacted with CPV, although to a much lower titer than to FPV (Parrish and Carmichael, 1983).

The remaining virus type-common mAbs in this study (Parrish and

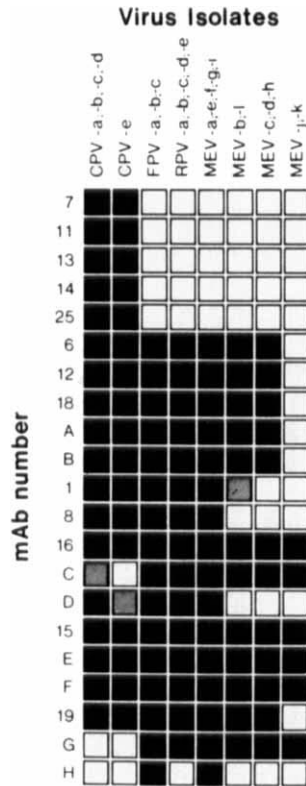


FIG. 5. Antigenic typing of FPV subgroup isolates. CPV [CPV-2 (a–d) and CPV-2a (e) strains], FPV, RPV, and MEV were titrated against mAbs, using the hemagglutination inhibition test. mAbs identified by numbers were prepared against CPV-a, and those identified by letters were prepared against FPV-b. Solid squares, at least 25% of the titer with the homologous virus; crosshatched squares, 5% to less than 25% of the titer with the homologous virus; open squares, less than 5% of the titer with the homologous virus, or no reaction. From Parrish and Carmichael (1983) with permission.

Carmichael, 1983) revealed a number of distinguishable epitopes when used to select variants. However, the patterns revealed by the analysis of naturally variant viruses (the MEV type 2 isolates, the *CPV-102/10* mutant, and the CPV-2a variant) (Parrish *et al.,* 1984, 1985; Parrish and Carmichael, 1983, 1986) suggest that these epitopes comprise only one or two distinct determinants (see Section V).

Competitive binding studies between ^{125}I-labeled and unlabeled mAbs revealed the surface of the viruses to be comprised of two non-cross-competing determinants, one recognized by mAbs 6, 12, and 18, and the other by the remaining mAbs in that panel (Fig. 7). The exten-

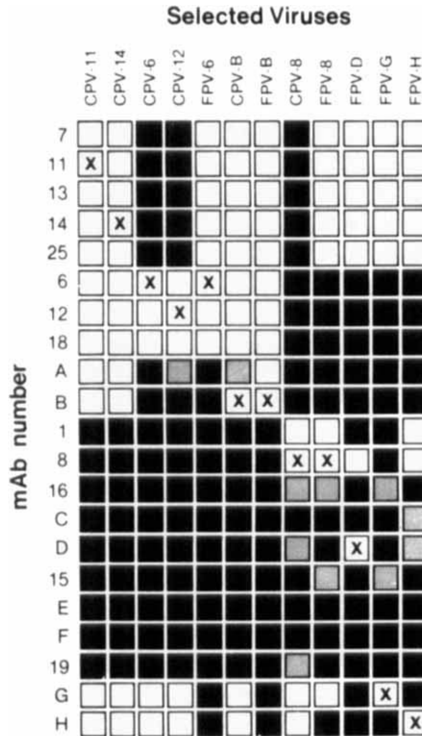


FIG. 6. Reactions of mAb-selected neutralization escape mutants of CPV-d or FPV-b in the hemagglutination inhibition assay or radioimmunoassay. X, mAb used for selection; solid squares, at least 25% of the titer with the original virus; crosshatched squares, 5% to less than 25% of the titer with the original virus; open squares, less than 5% of the titer with the original virus, or no detectable reaction. From Parrish and Carmichael (1983) with permission.

sive cross-competition between mAbs was probably a consequence of the small size of the parvovirus capsid (i.e., about 257 Å from the initial crystallographic diffraction data of CPV crystals) (Luo *et al.*, 1988)) and the number of protein subunits (most likely about 10 VP-1 and 60 VP-2) (Tattersall *et al.*, 1976; Paradiso *et al.*, 1982; Paradiso, 1983), suggesting that each epitope is repeated many times. The exposed area on each repeated subunit available for antibody binding would therefore be small compared to the size of an antibody molecule.

In the study described all mAbs both inhibited hemagglutination and neutralized the virus infectivity (Parrish *et al.*, 1982b; Parrish and Carmichael, 1983). In other studies some mAbs were nonneutralizing or were neutralizing only when an anti-rat IgG second-stage antibody was added to the neutralization reaction (Burtonboy *et al.*, 1982;

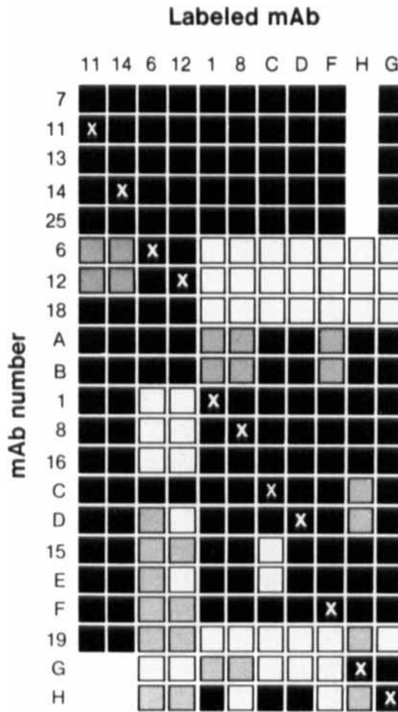


FIG. 7. Relationships between antigenic sites on CPV or FPV capsids revealed as binding of ^{125}I-labeled mAbs in the presence of dilutions of unlabeled mAbs. X, ^{125}I-labeled mAbs used in each set of tests; solid squares, efficient competition: less than 20% of the control CPM bound in the highest concentration of competing mAbs; hatched squares, partial blocking: 20–60% of the control counts per minute (CPM) bound in the highest concentration of competing mAbs; open squares, no competition: over 60% of the control CPM bound in the highest concentration of competing mAbs. From Parrish and Carmichael (1983) with permission.

Surleraux *et al.*, 1987). Thirty-nine of 47 mAbs in the latter study were either directly or indirectly neutralizing, suggesting that the determinants were mostly exposed on the surface of the intact virion. Eight of the 47 mAbs were nonneutralizing under any of the conditions tested (Surleraux *et al.*, 1987). Of the 47 mAbs 31 did not react in Western blots with proteins electrophoresed in sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose sheets, and this group included the five CPV-specific mAbs in the study.

Of the 16 mAbs which reacted in Western blots, 12 reacted with both VP-2 and VP-3 in the expected molar ratio, three reacted only with VP-1, and one reacted only with VP-2. The mAb reactive only with VP-2 did not react with the blotted proteins after the digestion of full

virions with trypsin, suggesting that it reacted with an amino-terminal region of the protein affected by the trypsin-cleavable site in the VP-2 of DNA-containing virions (Surleraux *et al.*, 1987; Clinton and Hayashi, 1976; Paradiso, 1981; Paradiso *et al.*, 1984). The mAbs with the various reactivities in Western blot reactivities included neutralizing, indirectly neutralizing, and nonneutralizing antibodies.

The antigenic relationships between a parvovirus (RD) isolated from raccoon dogs and a parvovirus isolated from an Arctic fox (BFPV) were examined by mAb analysis (Veijalainen, 1988). Of 19 mouse mAbs against the RD, two were specific in the hemagglutination inhibition test for that virus; three reacted with RD and CPV to equal titers, but to lower titers with MEV isolates; and 14 reacted to equal titers with all virus types tested. Of 20 mouse mAbs prepared against BFPV, one reacted in the hemagglutination inhibition test only with BFPV and with an MEV isolate; five reacted with BFPV, FPV, and MEV isolates, but to lower titers with a 1982 isolate of CPV; and 15 reacted to equal titers with all virus types (Veijalainen, 1988). These results suggest that RD is similar or identical to CPV, but distinct from the FPV, MEV, and BFPV isolates. Conversely, BFPV appears to be closely related antigenically to FPV and MEV but distinct from CPV.

V. Antigenic Variation and Genome Organization

A. MEV Variation

When examined with mAb panels prepared against CPV or FPV, three antigenic types of MEV were observed among the MEV isolates collected in the United States and Europe during the 1960s, 1970s, and 1980s (Parrish and Carmichael, 1983). Antigenic type 1 MEV appeared to be similar to the FPV isolates examined, while MEV types 2 and 3 differed in their reactivities to various CPV, FPV, or MEV type-common mAbs. The type 3 MEV isolates reacted with only six of 16 FPV-reactive mAbs in the panel used, while the type 2 MEVs reacted with 12 of the 16 mAbs (see Fig. 5).

The MEV types 2 and 3 isolates could be readily distinguished from the type 1 viruses by digestion of viral replicative-form DNA with various restriction endonucleases (Parrish *et al.*, 1984). The *Taq*I restriction patterns are shown in Fig. 8. The epidemiological significance of the MEV antigenic variation is not known. In cross-protection studies minks immunized with inactivated preparations of any of the three MEV types were protected against parenteral challenge by any of the alternative antigenic types (Parrish *et al.*, 1984).

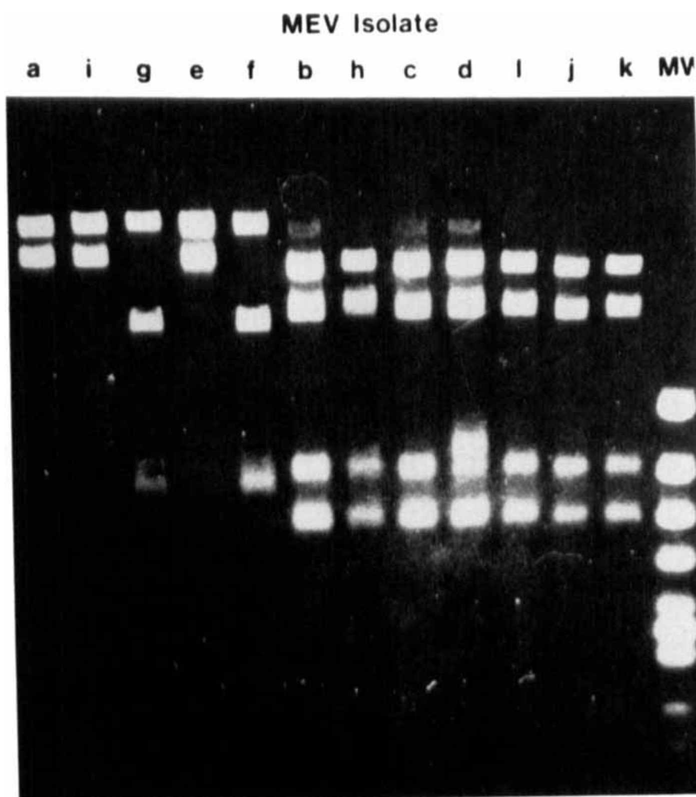


FIG. 8. Digests of replicative-form DNA from various MEV isolates with the *Taq*I restriction enzyme. The DNA was electrophoresed in a 2% agarose gel and stained with ethidium bromide. Antigenic type 1 MEV isolates (strains a, i, g, e, and f) can be distinguished from the type 2 (strains b, h, c, d, and l) and type 3 (strains j and k) isolates. From Parrish *et al.* (1984) with permission.

B. Natural Variation of CPV Strains

Analysis of CPV isolates collected after 1978 revealed that viruses collected after 1980 did not react with several mAbs which reacted with the pre-1980 virus isolates (Parrish *et al.*, 1985). Originally described as pre-1980 and post-1980, or old and new, antigenic types, these types were later designated CPV-2 and CPV-2a, respectively (Parrish *et al.*, 1988b,c). A panel of nine mouse mAbs prepared against a 1984 isolate of CPV-2a contained three mAbs which reacted with CPV-2a isolates but not with CPV-2 isolates (Fig. 9). The CPV-2a and CPV-2 isolates also differed in the patterns of their DNA after digestion with the *Hph*I restriction endonuclease (Fig. 10) (Parrish *et al.*,

mAb	1978		1979		1980		1981		1982		1983		1984		FPV-c
	b	17	d	18	21	22	43	44	25	26	14	31	15	39	
A)															
7	512	256	512	1024	512	256	512	128	128	128	128	128	128	128	<2
14	128	64	32	64	64	64	256	128	128	256	128	256	128	256	<2
25	64	64	32	64	64	64	4	4	2	4	2	2	4	<2	2
C	128	64	64	64	128	128	<2	<2	<2	<2	<2	<2	<2	<2	64
D	256	256	256	256	256	256	2	4	4	4	2	2	2	2	256
E	256	256	256	256	128	256	8	8	8	8	8	4	8	4	128
J	128	64	128	32	128	128	<2	<2	<2	<2	<2	<2	<2	<2	64
6	64	64	128	128	128	256	256	128	128	256	64	128	256	64	64
12	64	128	64	64	64	64	64	32	64	64	32	64	64	64	64
A	32	64	64	64	128	256	256	128	128	256	256	256	256	<2	256
I	32	64	32	32	32	32	64	32	64	64	64	64	64	<2	64
1	1024	512	1024	1024	512	512	512	256	256	256	512	256	256	512	256
8	128	256	256	256	256	256	256	256	128	256	256	256	256	256	256
16	128	256	256	256	256	256	128	64	64	128	64	64	64	64	128
15	64	256	128	256	256	256	256	128	128	256	32	32	128	32	64
F	2048	1024	1024	2048	1024	2048	1024	512	1024	512	512	512	512	1024	1024
B)															
1D1	<2	<2	<2	<2	<2	<2	2048	1024	1024	1024	512	512	512	512	4
7D6	4	2	2	2	<2	<2	1024	2048	1024	1024	1024	2048	2048	1024	4
7E2	<2	<2	<2	<2	<2	<2	128	128	128	64	128	128	128	64	2
2A9	64	64	32	64	64	128	64	64	64	128	32	32	32	32	2
2E2	16	32	16	16	16	16	32	32	32	32	16	16	32	32	16
2E12	128	256	64	128	128	256	64	128	256	128	64	64	64	128	256
3G6	64	64	32	64	64	64	64	64	32	32	64	64	32	128	32
4A12	128	256	128	256	128	256	128	128	128	256	64	64	128	128	256
4E9	64	128	32	128	64	128	64	128	128	128	64	32	128	256	64

FIG. 9. Reactions of CPV isolates from 1978 to 1984 with mAbs made against either a 1978 isolate of CPV (CPV-a) or FPV-b (A) or against a 1984 CPV isolate (CPV-39). Shaded are those reactions of mAbs which distinguish CPV-2 (1978–1980) from CPV-2a (1981–1984) isolates. The reactions of FPV-c are also shown. From Parrish *et al.* (1985) with permission. Copyright 1985 by the AAAS.

1985). Typing of CPV isolates from various countries with mAbs which distinguished CPV-2 and CPV-2a showed that the CPV-2a isolates among viruses collected in Japan and the United States during 1979 and 121 of 125 CPV isolates collected in Denmark during 1980 were of the CPV-2a type (Table I). By 1981 CPV-2a was the predominant virus in all countries (Parrish *et al.*, 1988b).

Testing sera collected from wild (hence, nonvaccinated) coyotes in Utah, Texas, and Idaho (Thomas *et al.*, 1984), using an Ouchterlony agar gel immunodiffusion test, showed that the same change in virus types occurred among the CPV viruses infecting these populations between 1980 and 1981 (Parrish *et al.*, 1988b). This indicates that the replacement of CPV-2 with CPV-2a between 1979 and 1981 was most

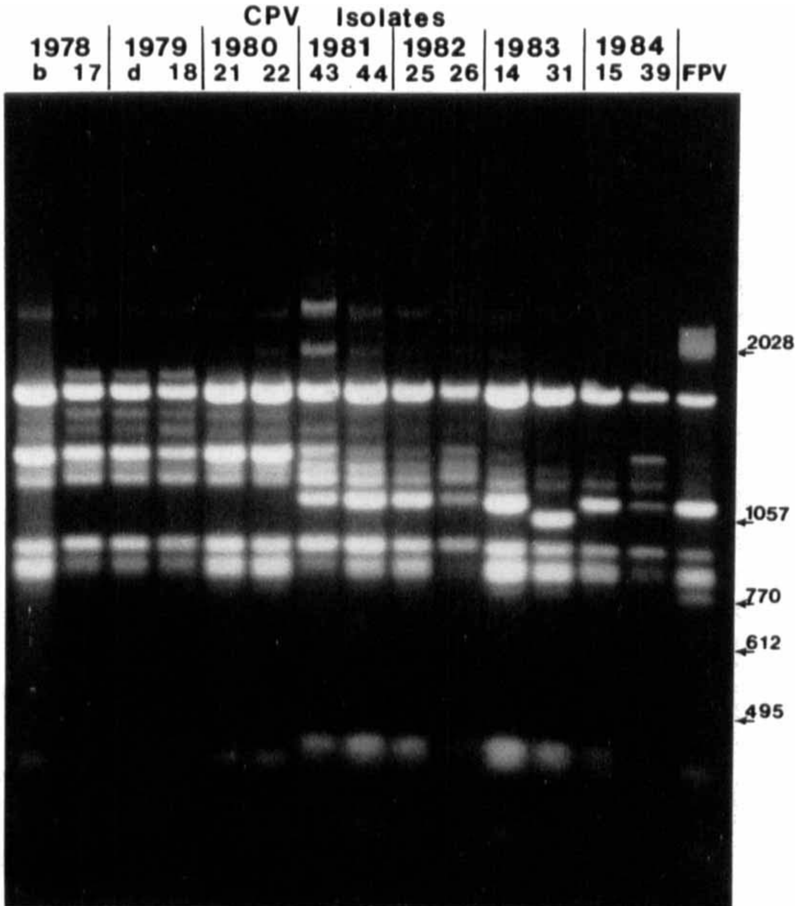


FIG. 10. Digests of replicative-form DNA from CPV isolates from 1978 to 1984 with the *HphI* restriction enzyme, electrophoresed in a 2% agarose gel. Patterns show the difference in restriction digests between CPV-2 (1978–1980) and CPV-2a (1981–1984) isolates. Size standards (MW) are $\phi \times 174$ DNA digested with *Hae*III restriction enzyme. From Parrish *et al.* (1985) with permission. Copyright 1985 by the AAAS.

likely due to some natural advantage of the latter virus, not to any human intervention, such as vaccination.

CPV isolates from Japan which were characterized by mAbs as CPV-2 or CPV-2a were examined for their abilities to hemagglutinate at 37°C or 4°C. The CPV-2 isolates were found to hemagglutinate only at 4°C, while most of the CPV-2a isolates were able to hemagglutinate at both 4°C and 37°C, although there were some virus isolates which were antigenically characterized as CPV-2a isolates, but which only

TABLE I

ANTIGENIC TYPES OF CPV ISOLATES COLLECTED IN VARIOUS COUNTRIES BETWEEN 1978 AND 1984 AND TESTED WITH MONOCLONAL ANTIBODIES[a]

Country of origin	Year of virus isolation													
	1978		1979		1980		1981		1982		1983		1984	
	2[b]	2a[b]	2	2a	2	2a	2	2a	2	2a	2	2a	2	2a
United States	4	0	5	1	5	6	2	9	1	2	0	7	0	7
Denmark					4	121	0	110	3	84	0	3	0	2
France					0	1	0	2			0	1	0	1
Belgium	1	0									0	1	0	1
Japan			3	1	5	1	2	1	1	5	0	3	0	6
Australia	2	0	2	0			0	1	0	1			0	2

[a] From Parrish *et al.* (1988b) with permission.

[b] Antigenic type of virus determined by reactivity with monoclonal antibodies specific for either CPV-2 or CPV-2a.

hemagglutinated at 4°C (Table II). The significance of these findings in relation to any selective advantage or other properties of CPV-2a has not been defined (Senda *et al.,* 1988).

C. Mutants of Viruses

Few mutants of the FPV subgroup viruses have been described or characterized (Rivera and Sundquist, 1984; Parrish and Carmichael, 1986; Parrish *et al.,* 1988a). An isolate of MEV was reported that did not hemagglutinate pig, rhesus monkey, or horse erythrocytes at pH 6.8 at 4°C (Rivera and Sundquist, 1984). As the pH used in this study was near the upper range of the conditions reported by others for the hemagglutination of FPV (Carmichael *et al.,* 1980; Moraillon and Moraillon, 1982; Veijalainan, 1988; Parrish *et al.,* 1988c), it is not clear whether that mutant was truly nonhemagglutinating.

A nonhemagglutinating strain of CPV was derived after 12–22 passages of a 1978 isolate of the virus in the NLFK feline kidney cell line. The nonhemagglutinating virus showed no antigenic differences from wild-type CPV-2 when examined with a total of 82 mAbs prepared against CPV-2, CPV-2a, or FPV, and contained only two specific nucleotide and predicted amino acid sequence differences within the *VP-1/VP-2* gene, at nucleotides 1631 and 2185 in the *VP-1* gene [around 77 and 88 map units (m.u.) (Parrish *et al.,* 1988a,c). Of these two differences the sequence at nucleotide 2185 was also present in two FPV isolates, FPV-Carl and FPV-a (Carlson *et al.,* 1985; Parrish *et*

TABLE II

RELATIONSHIPS BETWEEN TEMPERATURE DEPENDENCE OF HEMAGGLUTINATION AND THE ANTIGENIC TYPE WITH CPV-2- OR CPV-2a-SPECIFIC mAbs[a]

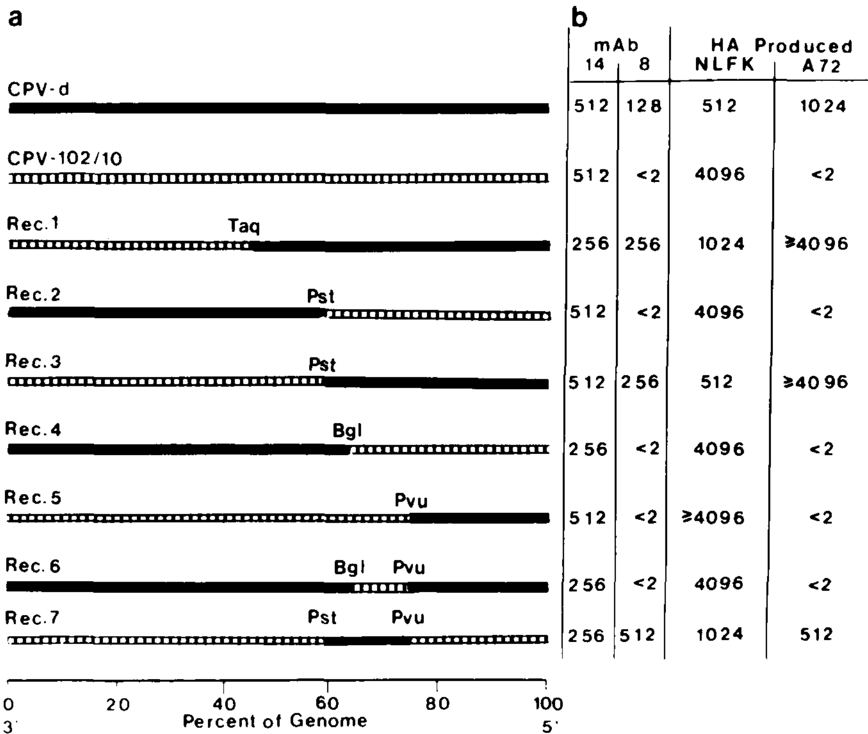
Year of isolation	Number of isolates	4°C/37°C[b]	Dependence	Antigenic CPV type[c]
1978	1	≥64	+	2
1979	3	≥64	+	2
	1	2	−	2a
1980	4	≥64	+	2
	2	8	−	2
	1	2	−	2a
1981	2	8	−	2
	1	2	−	2a
1982	1	≥64	+	2
	5	2	−	2a
1983	3	1–2	−	2a
1984	7	2–8	−	2a

[a] Adapted from Senda *et al.* (1988) with permission.
[b] The ratio between the hemagglutination titer of the virus isolate when assayed at 4°C or 37°C.
[c] Antigenic type determined by testing with CPV-2- or CPV-2a-specific monoclonal antibodies in the hemagglutination inhibition test.

al., 1988c). It is known that FPV-a hemagglutinates under standard conditions, indicating that the functional difference in the nonhemagglutinated mutant is most likely due to the sequence at nucleotide 1631, suggesting that an amino acid affects the structure of the viral ligand which binds to the erythrocyte virus-binding receptor. Whether the nonhemagglutinating virus exhibits other differences from the wild-type virus is not known.

Another mutant of CPV-2 was derived during passage of a 1978 isolate of CPV-2 102 times in primary canine kidney cells and an additional 10 times in the NLFK feline kidney-derived cell line. During the passages in NLFK cells, a mutant (*CPV-102/10*) was selected which no longer replicated in dogs or in dog cells and which was variant antigenically when tested in the hemagglutination inhibition test with mAbs prepared against CPV-2 or FPV (Parrish and Carmichael, 1986). The mutation in *CPV-102/10* was identified by recombination mapping, in which restriction fragments prepared from replicative-form DNA of each virus were religated to form the entire genome and then transfected into NLFK cells. By examination of seven recombi-

nant viruses prepared between *CPV-102/10* and a wild-type CPV strain, the antigenic and host range differences of *CPV-102/10* were mapped to a 438-base pair 64–73 m.u. *Bgl*II–*Pvu*II fragment of the viral genome (Fig. 11) (Parrish and Carmichael, 1986). Within this region there were two *CPV-102/10*-specific nucleotide and amino acid sequence differences at adjacent codons (i.e., nucleotides 1400 and 1403 in the *VP-1/VP-2* gene sequence) (Parrish *et al.*, 1988c).



	mAb 14	mAb 8	HA Produced NLFK	HA Produced A72
CPV-d	512	128	512	1024
CPV-102/10	512	<2	4096	<2
Rec.1	256	256	1024	≥4096
Rec.2	512	<2	4096	<2
Rec.3	512	256	512	≥4096
Rec.4	256	<2	4096	<2
Rec.5	512	<2	≥4096	<2
Rec.6	256	<2	4096	<2
Rec.7	256	512	1024	512

FIG. 11. (a) Analysis of recombinants (Rec.) between a wild-type CPV-2 isolate (CPV-d) and the antigenic and host range mutant *CPV-102/10*. Recombinants were formed by ligating genome fragments from these two viruses at various restriction sites (i.e., *Taq*I, *Pst*I, *Bgl*II, and *Pvu*II). Viruses produced after transfection of cell cultures were examined for recombinant genome type. (b) Antigenic and host range types of CPV-d and *CPV-102/10* and the recombinants between them. Antigenic types are represented by reactions with mAb 14 (control) or mAb 8 (which reacts with CPV-d, but not with *CPV-102/10*) in the hemagglutination inhibition assay, while host ranges are represented by NLFK (feline) or A72 (canine) cell culture supernatant hemagglutination titers 4 days after inoculation with 0.02 pfu of virus per cell. The antigenic type and host range of the mutant virus map between the *Bgl*II and *Pvu*II sites (64–73 m.u.). From Parrish and Carmichael (1986) with permission.

D. Genome Organization

The genome organization appears to be similar to that described for several other related autonomous parvoviruses, including H1, MVM, and bovine parvovirus (Rhode and Paradiso, 1983; Astell *et al.*, 1983; Chen *et al.*, 1986). The proposed genome organizations of MVM and CPV are shown in Fig. 12. The sequence contains an open reading frame DNA from a promoter at 4 m.u. (i.e., p4), which encodes the NS-1 protein which has been extensively characterized for H1 and MVM (Cotmore *et al.*, 1983; Paradiso, 1984; Cotmore and Tattersall, 1986, 1988).

An 83-kDa protein was precipitated from *in vitro* translated CPV mRNA, using rabbit antisera prepared against bacterially expressed fusion proteins from the middle portion of the *NS-1* gene of MVM. However, no translation product was precipitated by antiserum against a sequence from the amino terminus of the MVM NS-1 protein, which is contained in both the 83-kDa NS-1 and 25-kDa NS-2 proteins of MVM (Cotmore and Tattersall, 1986). The carboxy-terminal two-thirds of the FPV *NS-1* gene sequence was expressed in *Escherichia coli* as a fusion with the tryptophan *LE* gene product and the proteins used to produce antibodies in rabbits. These antibodies recognized proteins of 84, 60, 53, and 49 kDa, the most abundant product being the 60-kDa form (Carlson *et al.*, 1987). The relationships of the various proteins to each other and to the 83-kDa protein precipitated from the *in vitro* translated CPV mRNA have not been determined, but the smaller proteins most likely represent degraded forms of the 83-kDa NS-1 protein.

It is not clear whether CPV encodes a protein equivalent to the 25-kDa NS-2 protein of MVM and H1, which is translated from the MVM R2 transcript. In MVM this transcript contains 84 amino acids from the amino terminus of NS-1 in the third open reading frame, with a carboxy-terminal portion derived from 40–46 m.u. of the MVM sequence after mRNA splicing. This portion is encoded from open reading frame 2 and is followed by a second minor splice (i.e., 46–48 m.u.) to give a further short sequence encoding carboxy-terminal domains in open reading frame 2 (Morgan and Ward, 1986; Cotmore and Tattersall, 1986, 1987). An equivalent transcript or product in CPV or FPV has not been identified. The presence of a stop codon upstream from the second (minor) splice and the low homology between the translated open reading frames in this region between the sequences of MVM and CPV or FPV suggests that the latter viruses have different patterns of transcription and translation of the NS proteins from those seen for MVM and H1 (Cotmore and Tattersall, 1986).

The genomic 5′ 60% of the parvovirus genome encodes the VP-1 and

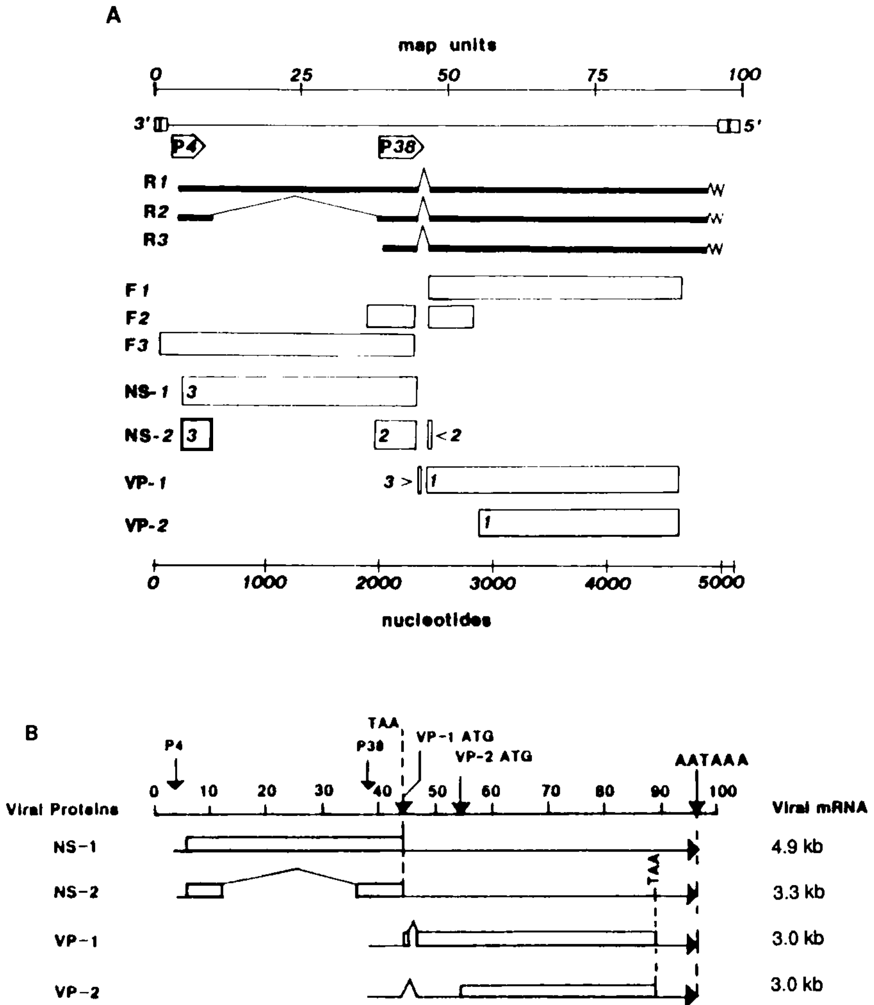


FIG. 12. Coding strategy of the minute virus of mice (MVM) genome (A) and that proposed for the the CPV genome (B). The cytoplasmic transcripts are aligned with the viral DNA strand (orientation 3′ to 5′), showing the promoters at 4 and 38–40 m.u. Major blocks of open reading frame are shown for MVM in each of the three reading frames (F1, F2, and F3) and the sequences which encode NS-1, NS-2 (which has not been identified in CPV), and the capsid proteins VP-1 and VP-2. (A) The open reading frames used to encode the various parts of each virus are shown as numbers in the transcript map. From Cotmore and Tattersall (1987) and Reed *et al.* (1988) with permission.

VP-2 from mRNAs transcribed from a promoter around 40 m.u. (Fig. 12) (Carlson *et al.,* 1985). The two proteins are most likely translated from differently spliced forms of the R3 transcript, as described in detail for MVM (Morgan and Ward, 1986), although it is possible that R1 and R2 transcripts can also be translated to form VP-1 or VP-2 (Cotmore and Tattersall, 1987). Messages from the CPV p40 promoter are able to direct the translation of both VP-1 and VP-2 when cloned in a bovine papillomavirus expression vector (Mazzara *et al.,* 1987).

Alternative splices predicted to generate VP-1 and VP-2 messages are most likely derived from two different splice donors. One, prior to the methionine codon thought to be the initiation codon of VP-1, would give rise to an mRNA in which the first AUG used would be that initiating VP-2 (Rhode, 1985, Carlson *et al.,* 1985; Morgan and Ward, 1986). The alternatively spliced mRNA would most likely use a predicted splice donor after the predicted ATG initiating VP-1, giving 10 codons prior to the splice, then the mRNA could be spliced to the same acceptor as for the VP-2 message. The outcome of the alternative splices would encode an additional 153 amino acids in VP-1, to give VP-1 and VP-2 of predicted sizes of 79,845 and 64,661 Da, respectively, for FPV (Carlson *et al.,* 1985) and similarly sized proteins for CPV (Rhode, 1985; Reed *et al.,* 1988). Whether these mRNA splicing reactions actually occur in CPV and FPV has not yet been determined.

The genomes of CPV, FPV, MEV, and RPV isolates contain various directly repeated units of unknown significance in the genomic 5′ (largely noncoding) region. Some of the rearrangements could have arisen during passage of the viruses in tissue culture, although some of the variable sequences are present in wild-type low-passage isolates of CPV (Fig. 13) (Rhode, 1985; Carlson *et al.,* 1985; Parrish *et al.,* 1988c). No correlation between the form of the repeat units and any biological function has been described, but all viruses examined in one study had at least one copy of each repeat, and the DNA sequences were conserved, suggesting that the sequences play some role in the virus life cycle (Cotmore and Tattersall, 1987; Parrish *et al.,* 1988c).

VI. Other Properties of FPV, MEV, and CPV

A. *Hemagglutination Dependence on pH*

All virus types hemagglutinate erythrocytes from a variety of species at 4°C in buffers with pH values below 7.0 (Johnson, 1971; Goto *et al.,* 1974; Goto, 1975; Mochizuki *et al.,* 1978; Senda *et al.,* 1988). FPV and MEV isolates hemagglutinated pig or rhesus monkey erythrocytes

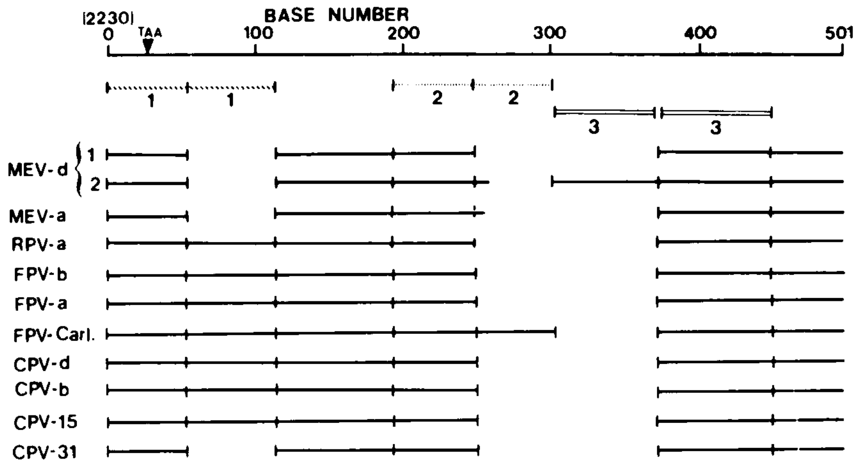


FIG. 13. The sequences between about 88 and 95 m.u. in the genomes of MEV, RPV, FPV, and CPV, isolates, showing the structure of the three repeated units of 50–60 base pairs, and the form of this region in each of the virus isolates examined. The MEV-d stock examined contained two genome forms, shown as 1 and 2. CPV-d and CPV-b are antigenic type 2 isolates, and CPV-15 and CPV-31 are type 2a isolates. From Parrish *et al.* (1988c) with permission.

only at pH values below 7.0, and the pH optimum for most strains is below pH 6.6 (Carmichael *et al.*, 1980; Moraillon and Moraillon, 1982). In contrast, CPV isolates hemagglutinated with approximately equal titers in buffers with pH values up to 8.0 (Carmichael *et al.*, 1980; Parrish *et al.*, 1988c). RD showed a hemagglutination dependence on pH similar to that of CPV isolates, while the BFPV was similar to FPV or MEV isolates, supporting the mAb antigenic typing results of these viruses (Veijalainen, 1988).

B. Experimental Host Range Properties

Isolates of CPV, FPV, and MEV all replicate in feline kidney cells and many other feline cell lines in culture (Johnson, 1965; Appel *et al.*, 1979b; Tratschin *et al.*, 1982; Parrish *et al.*, 1988c). CPV isolates replicate in canine cells in culture, but FPV and MEV isolates do not (Tratschin *et al.*, 1982; Parrish *et al.*, 1988c). Studies with RD and BFPV in tissue culture show these viruses to differ and to have host ranges similar to those for CPV and FPV, respectively (Veijalainen, 1988).

The experimental host ranges of the FPV subgroup viruses have not been well defined, and the many viruses and hosts make definitive

analysis of the various host ranges difficult. In early studies feline tissues containing FPV were inoculated into dogs, ferrets, ground squirrels (*Citellus richardsonii*), mongooses, rhesus monkeys, hamsters, chick embryos, rabbits, guinea pigs, mice, and rats without producing any signs of disease (Hindle and Findlay, 1932; Hammon and Enders, 1939a; Lawrence *et al.*, 1940; Kikuth *et al.*, 1940). FPV isolates inoculated into minks in a number of studies caused little clinical disease (Myers *et al.*, 1959; Barker *et al.*, 1983; Parrish *et al.*, 1987). An FPV or RPV isolate was passaged repeatedly through minks in one study, but only one animal in each series of RPV- or FPV-inoculated minks showed any signs of clinical disease, in contrast to the MEV isolates which caused clinical disease in many of the inoculated minks (Parrish *et al.*, 1987). Inoculation of an MEV isolate into a single cat resulted in clinical disease (Higashihara *et al.*, 1981).

Inoculation of FPV isolates into dogs gave a limited replication in a number of lymphoid tissues, but the viruses could not be propagated repeatedly in this host (Parrish *et al.*, 1988c). CPV isolates inoculated into cats showed little or no replication (Goto *et al.*, 1984b; C. R. Parrish, unpublished observations).

VII. Genetic Analysis and Variation among the Viruses

A. *Restriction Mapping*

Comparisons of the restriction enzyme cleavage maps of CPV, FPV, and MEV replicative-form DNAs revealed a number of differences in restriction sites (McMaster *et al.*, 1981; Tratschin *et al.*, 1982). Most of the variable sites mapped to the right-hand side of the genome, within the *VP-1*/*VP-2* gene(s) (Fig. 14). The restriction maps of various wild-type and vaccine strains of FPV, MEV, and CPV were compared, using seven restriction enzymes (Tratschin *et al.*, 1982). A Swiss isolate of CPV (*Ka/BE*, discovered in 1979) differed from the French V1 strain of MEV in 11 of 79 restriction enzyme sites examined, allowing unambiguous identification of the CPV isolates. Low-passage MEV and FPV isolates appeared to be similar in this analysis, differing in two or fewer restriction sites (Fig. 14) (Tratschin *et al.*, 1982).

Two or three *Hin*fI sites in two of the vaccine strains of FPV were similar to CPV-specific sites in the CPV isolates (Fig. 14) (Tratschin *et al.*, 1982). Although this finding suggested a possible origin for CPV in the FPV vaccine viruses (Siegl, 1984), other studies comparing *VP-1*/*VP-2* gene sequences of various CPV isolates with the two FPV vaccine strains indicated that these FPV vaccine strains were not progenitors of CPV (Parrish *et al.*, 1988c; see Section IX).

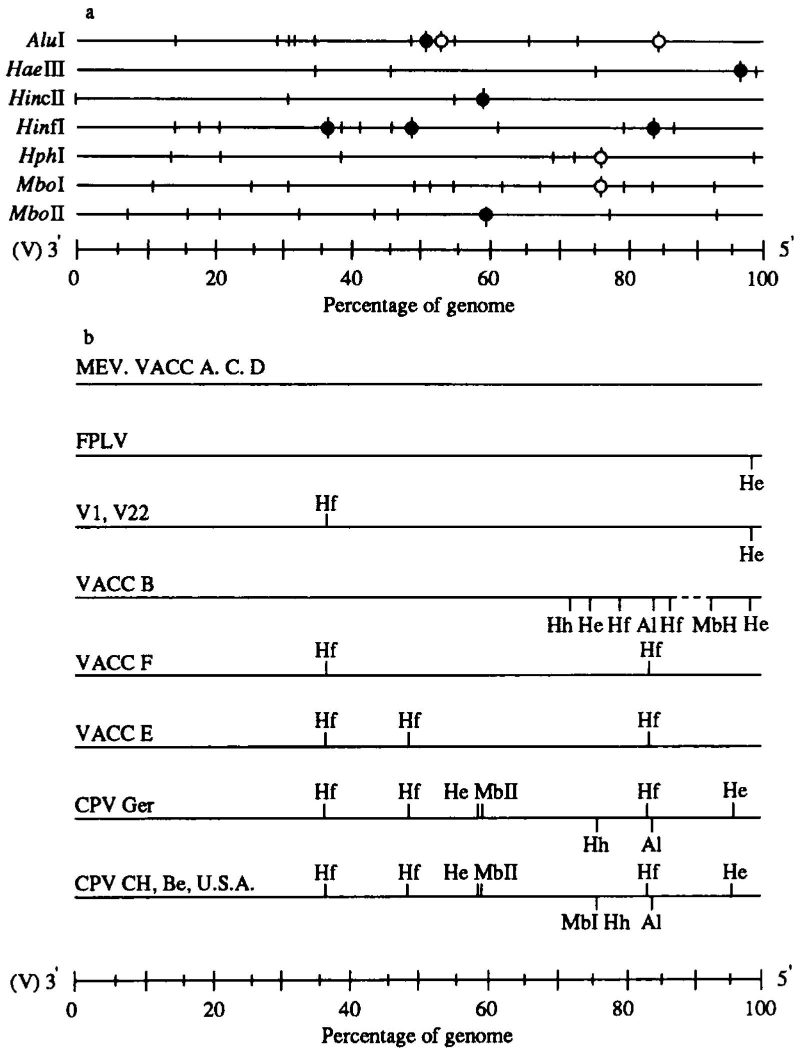


FIG. 14. (a) Restriction enzyme maps of MEV and CPV virus replicative-form (RF) DNAs, aligned with the viral genomic (−) DNA shown as the 3′–5′ orientation. Vertical lines indicate sites present in both CPV and MEV, open circles show sites present only in MEV, and closed circles indicate sites present only in CPV (Swiss isolate). (b) Comparative restriction enzyme digestion maps of the RF DNAs of various CPV, FPV, and MEV isolates, along with those of six FPV vaccine strains. The map of the MEV isolate is used for comparison. Additional restriction sites are shown above the line, while sites absent from a virus strain are shown below it. Sites common to all viruses are not shown. Live attenuated FPV vaccine strains examined were from the following manufacturers' vaccines—strain A, Iffa-Merieux; strain B, Norden; strain C, Frieosythe/Wellcome; strain E, Phillips Roxanne; and strain F, Dellen—as well as one strain (strain D) derived from MEV from Connaught. Hf, *Hin*fI; He, *Hae*III; Hh, *Hph*I; Al, *Alu*I; MbII, *Mbo*II; Hc, *Hinc*II; MbI, *Mbo*I. Comparisons of the DNA sequences of strains E and F are also shown in Fig. 17. From Tratschin *et al.* (1982) with permission.

B. DNA Sequence Analysis

An almost complete DNA sequence of one tissue culture-passaged vaccine strain of CPV isolated in 1978 has been reported (Reed *et al.*, 1988). Partial nucleotide sequences of a tissue culture-passaged vaccine strain of FPV (about 22–97 m.u.) (Carlson *et al.*, 1985) and a wild-type 1978 isolate of CPV (about 33–95 m.u.) (Rhode, 1985), as well as the *VP-1* and *VP-2* gene sequences of eight further isolates of CPV-2, CPV-2a, FPV, RPV, or MEV have also been reported (Parrish *et al.*, 1988c).

VIII. Genetic Analysis of Antigenic, Host Range, and Other Functions within the *VP-1/VP-2* Genes

Mapping CPV-Specific Properties

Recombinants between a CPV-2 isolate and an FPV isolate were prepared from purified viral replicative-form DNA, using methods described above for analysis of the *CPV-102/10* mutant (Parrish and Carmichael, 1986; Parrish *et al.*, 1988c). Recombinant genomes prepared at the *Pst*I, *Bgl*II, and *Pvu*II sites (at 59, 64, and 73 m.u. in the viral genome, respectively) were transfected into cell cultures, and recombinant viruses recovered were examined for the CPV-specific antigenic epitope, the pH dependence of hemagglutination, and the canine host range (Fig. 15 and Table III). These analyses showed that the CPV-specific pH dependence of hemagglutination and a sequence difference determining the CPV-specific antigenic epitope both mapped within the 300-base pair 59–64 m.u. region. Within that region there were only two CPV- or FPV-specific sequence differences—an asparagine–lysine change coded for by nucleotide 780 and an alanine–valine difference coded for by nucleotide 809—and therefore one or both of those differences gave rise to the differences in antigenic type and in the pH dependence of hemagglutination (Parrish *et al.*, 1988c).

The host range differences between CPV and FPV proved genetically more complex, and only those properties that allow CPV to replicate in dogs have been defined. When the 59–73 m.u. region of FPV was replaced by the same region of CPV, the resulting virus replicated in dogs to a titer as high as that for the wild-type virus, identifying this region as being responsible for the canine host range of CPV (Fig. 15 and Table III). This region contained the two sequence differences associated with the CPV-specific epitope and pH dependence of hemagglutination in the 59–64 m.u. region, as well as one further CPV-specific coding change at 71 m.u. (asparagine–aspartic acid at nu-

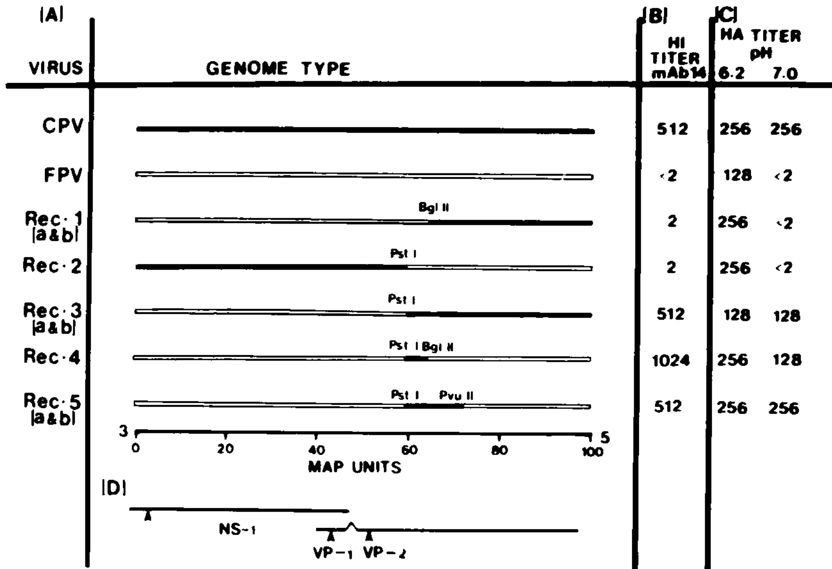


FIG. 15. Analysis of recombinants between CPV-d and FPV-b prepared from replicative-form viral DNA and transfected into NLFK cells. Viruses recovered were confirmed by restriction enzyme analysis and DNA sequencing. (A) CPV, FPV, and five types of recombinants (Rec.) between these viruses prepared using the *Pst*I, *Bgl*II, and *Pvu*II sites at 59, 64, and 73 m.u., respectively, in the virus genome. (B) Hemagglutination inhibition titers of recombinant viruses with CPV-specific mAb 14. (C) The pH dependence of hemagglutination of the parental viruses and of the recombinants. Hemagglutination assays were performed in buffers with pH values of 6.2 and 7.0. From Parrish *et al.* (1988c) with permission.

cleotide 1468 in the *VP-1/VP-2* gene) and a virus-specific noncoding difference at nucleotide 1200 (Fig. 16).

The requirements for and the relative importance of the various sequence differences within that region are not known. However, the region between 64 and 73 m.u. does not itself contain the host range function since Rec 1, which contained the 0–64 m.u. region from FPV and the 64–100 m.u. region from CPV, was not able to replicate in dogs (Fig. 15 and Table III). It appears that differences in both the 59–64 and 64–73 m.u. regions are required to endow the canine host range (Parrish *et al.*, 1988c).

A similar region within the capsid proteins genes is also important for determining the host range differences between two strains of MVM which differ in their host ranges for various differentiated cells: MVM(p), replicating in fibroblast-derived cells, and MVM(i), replicating in lymphocyte-derived cells (Engers *et al.*, 1981; Spalholz and Tattersall, 1983; Tattersall and Bratton, 1983; Kimsey *et al.*, 1986). This

TABLE III

REPLICATION OF CPV-d, FPV-b, AND RECOMBINANTS BETWEEN THEM IN SPECIFIC PATHOGEN-FREE DOGS

	Virus titer per 0.03 g of tissue		
Virus	Thymus	Mesenteric lymph node	Ileum
CPV-d	1.2×10^5	1.6×10^3	7.0×10^4
	3.0×10^4	1.7×10^3	2.2×10^4
FPV-b	1.4×10^3	<10	<10
	8.0×10	<10	<10
Rec 1a	10	1.0×10^2	3.0×10
	10	<10	<10
Rec 2	<10	<10	<10
	<10	<10	<10
Rec 3a	2.2×10^5	1.9×10^4	1.2×10^3
	2.8×10^4	2.4×10^4	4.0×10^2
Rec 3b	1.7×10^4	2.2×10^4	1.1×10^3
Rec 4			
Experiment 1	9.1×10^4	<10	<10
	<10	5.0×10	<10
Experiment 2	2.7×10^6	<10	10
	<10	<10	<10
Experiment 3	<10	<10	<10
	<10	<10	<10
Rec 5a	1.3×10^4	2.8×10^6	3.1×10^3
	2.7×10^6	2.0×10^3	4.1×10^2
	8.0×10^6	$>4.0 \times 10^7$	4.0×10^2
	2.2×10^6	<10	<10
Rec 5b	1.4×10^6	2.2×10^6	2.6×10^5
	6.0×10^4	4.0×10^3	1.4×10^5

[a] Virus titers in various tissues of dogs 4 days after intramuscular inoculation with FPV, CPV, or recombinants (Rec). The dogs were inoculated with preparations of Rec 4 from tissue culture (experiments 1 and 3), while the dogs in experiment 2 were inoculated with virus recovered from the thymus of a dog inoculated with Rec 4 in experiment 1. From Parrish *et al.* (1988c) with permission.

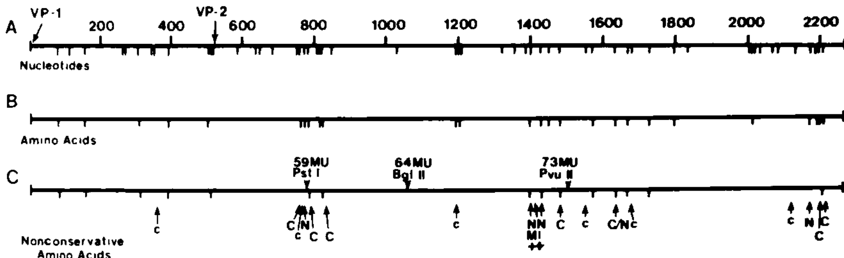


FIG. 16. Sequence variation within the *VP-1/VP-2* gene sequences, compared to a wild-type CPV type 2 sequence (CPV-d). (A) Positions of variable nucleotides. (B) Predicted amino acid sequence differences. (C) Amino acid differences which gave changes in charge or polarity. C, c, amino acid, or "silent," sequence differences which distinguished CPV from the other viruses; N, sequence differences specific for CPV-2a isolates; M, amino acid sequence difference between MEV-a and MEV-b (antigenic types 1 and 2 MEV isolates); +, mutations in *CPV-102/10* associated with host range and antigenic differences. Adapted from Parrish *et al.* (1988c) with permission.

difference in the host range mapped between 68 and 73 m.u. in these viruses and involved residues similar to those affecting the host range defined in this region for CPV or FPV (Figs. 11 and 15) (Antionetti *et al.*, 1988; Gardiner and Tattersall, 1988a,b).

Nothing is known about the specific function by which the CPV-specific differences in the *VP-1/VP-2* gene determine the canine host range. However, between MVM(i) and MVM(p) the functional difference within the *VP-1/VP-2* gene does not affect virus binding to cell receptors, but influences early events in the replication of the virus, in either mRNA transcription or DNA replication (Spalholz and Tattersall, 1983; Antionetti *et al.*, 1988; Gardiner and Tattersall, 1988a).

IX. Virus Sequence Variation and Phylogenetic Relationships

The *VP-1/VP-2* gene sequences of a total of 10 isolates have been compared: two CPV-2, two CPV-2a, two MEV (types 1 and 2), one RPV, and three FPV isolates. An FPV and a CPV sequence were obtained from published sequences (Carlson *et al.*, 1985; Rhode, 1985). Others were obtained from M13 phage clones of viral replicative-form DNA, which were sequenced using a series of primers complementary to various positions within the *VP-1/VP-2* gene (Parrish *et al.*, 1988c). The distribution of the variant positions within the *VP-1/VP-2* gene is shown in Fig. 16. Although the differences in nucleotide sequences were evenly distributed through the genes, the coding changes were less evenly distributed. Changes that were FPV or CPV specific or that were specific for CPV-2a viruses tended to cluster into three regions

within the gene: around 60 m.u., between 70 and 77 m.u., and around 85 m.u, near the carboxy terminus (Fig. 16) (Parrish *et al.*, 1988c).

The differences in nucleotide sequences within the *VP-1/VP-2* gene were used to prepare a network of the phylogenetic relationships between the viruses, assuming maximum parsimony (Fig. 17) (Fitch, 1977). Among the FPV, MEV, and RPV viruses there was a certain amount of apparently random sequence variation, but the viruses isolated from the different hosts could not be readily distinguished. However, the CPV isolates formed a separate cluster, which subdivided into the CPV-2 and the CPV-2a types that had previously been distinguished antigenically and by restriction enzyme analysis (Parrish *et al.*, 1985). It is apparent from this analysis that CPV-2 and CPV-2a most likely derived from some common ancestor virus prior to 1978 and that CPV-2a was not simply a linear descendent of CPV-2 (Parrish *et al.*, 1988c).

X. Conclusions

The emergence of MEV in minks during the 1940s and the recent emergence of CPV in dogs during the 1970s are unusual events, which raise a number of questions about the evolution of viruses and the ways in which host ranges of the viruses are determined and restricted. By examining these phenomena, we hope to derive lessons about the natural history of viruses and the interactions of these viruses with their hosts.

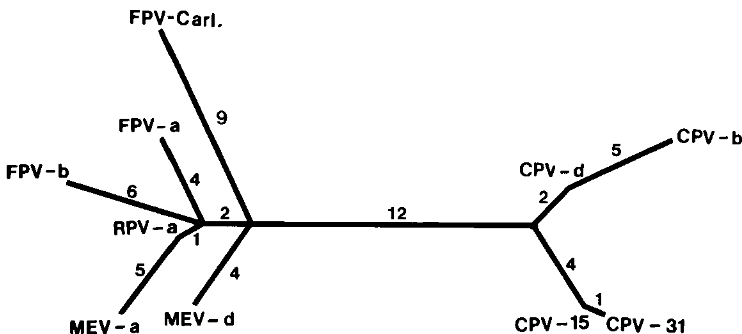


FIG. 17. An unrooted network showing the phylogenetic relationships between virus isolates based on the nucleotide sequences of the *VP-1/VP-2* gene between nucleotide 203 and the termination codon at nucleotide 2254. Isolates are feline panleukopenia virus (FPV-a, -b, and -Carl), mink enteritis virus (MEV-a and -d), raccoon parvovirus (RPV-a), canine parvovirus type 2 (CPV-d and -b), and CPV type 2a (CPV-15 and -31). FPV-Carl and FPV-a represent the Dellen Laboratories and Phillips Roxanne vaccine strains of FPV, respectively. From Parrish *et al.* (1988c) with permission.

The emergence of MEV appears to be an example not of altered host range, but was most likely due to the recognition of a long-existing virus which might have altered in virulence for minks. It is also possible that the disease was recognized at that time due to some change in the farming practices or possibly by a change in minks due to genetic selection. The differences in virulence for minks observed after inoculation of MEV or FPV suggests that there are subtle differences between FPV and MEV which have yet to be defined.

The emergence of CPV probably represents a different type of event. It is clear from the retrospective serological studies that CPV is a new virus of dogs and that it was first present in domestic dogs in Europe only during the mid-1970s. The widespread outbreaks and global spread of the virus did not occur until 1978. It is possible that this was due to some change in the virus at that time which gave it an epidemiological advantage. Genetic mapping studies indicate that only three or four sequence differences between the FPV and CPV-2 isolates within the *VP-1/VP-2* gene determine all of the specific properties of CPV that have been defined: the pH dependence of hemagglutination, the CPV-specific epitope, and the host range for canine cells and dogs. Whether these changes are all that would be required to convert FPV into a successful canine pathogen is not known, but it is clear that CPV could have been derived from FPV or some closely related virus by only a few specific changes. How this occurred and which virus was the ancestor are unknown and are subjects for future studies.

These parvoviruses, therefore, present a unique opportunity for understanding the natural evolution and variation of viruses and for determining the ways in which viruses can gain new host range and other functions. In this case the viruses apparently emerged by two different mechanisms as pathogens of the members of two families (i.e., Mustelidae and Canidae) among the Carnivora which had previously been resistant to infection or disease. Further examination of the viruses and further definition of the specific genetic properties involved in the changes will allow the details of these unusual events and their implications for virus evolution and host range stability to be understood.

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