Basic studies for canine contagious viruses

（イヌの病原性ウイルスに関する基礎的研究）

——————— *Basic studies for persistent infection and for development of novel recombinant vaccines using a CDV reverse genetics system*

（CDV リバースジェネティクス法を用いた持続感染機構の解析と新型ワクチン開発の基礎的研究）

土器　美穂

CONTENTS

GENERAL INTRODUCTION

Canine contagious virus diseases, in particular, canine distemper, parvovirosis, infectious canine hepatitis and rabies are serious health threats to dogs. With forcusing on the causative agents, canine distemper virus (CDV), canine parvovirus (CPV), and rabies virus (RaV), in chapter 1, the author investigated the mechanisms of CDV persistence in lymphocytes. In chapter 2-1, the author performed phylogenetic analysis of VP2 gene of CPV isolated from domestic dogs in Japan in 1999-2000. In chapter 2-2, the author tried to generate recombinant CDV harboring VP2 gene derived from CPV. In chapter 2-3, the author attempted to generate recombinant CDV expressing G protein of RaV.

Canine Distemper Virus

Canine distemper is a fatal disease, which is caused by the CDV infection, in dogs and many other carnivores including raccoons, ferrets, and foxes. CDV induces fever, gastroenteritis, pneumonia, conjunctivitis and encephalomyelitis with high mortality in puppies. Transmission generally occurs via an aerosol-droplet route, direct contact, or possibly by contact with contaminated objects. The virus is shed in the feces and urine of infected individuals and some evidence exists for transplacental transmission [Krakowka et al., 1977]. The

usual route of infection is through the upper respiratory tract, following inhalation of the virus. Infected dogs may continue to shed the virus for several weeks after symptoms disappear, but they no longer shed the virus after once they are fully recovered. Macrophages carry the inhaled virus to nearby lymph nodes where it begins replicating. It spreads rapidly to all the lymphoid organs within 2 to 5 days. By day six to nine, the virus spreads through blood. It then spreads to the surface epithelium of the respiratory, gastrointestinal, urogenital, and central nervous systems, where virus begins damaging and causes the symptoms.

Live attenuated vaccines have been used successfully for many years to control canine distemper (CD) in the world. However, there were CD-outbreaks in several parts of the world [Blixenkrone-Møller et al., 1993; Bolt et al., 1997; Haas et al., 1997; Ek-Kommone et al., 1997; Jozwik and Frymus, 2002]. Similarly, incidence of CD both in unvaccinated and vaccinated dogs had rapidly increased in 1989 in Tokyo area [Gemma et al., 1996b]. Clinically, the affected dogs showed neurological, respiratory and gastrointestinal signs of varying degrees. Some dogs showed severe central nervous system signs and mild respiratory signs but no gastrointestinal signs. Comparison of the antigenicities between the Onderstepoort vaccine strain and Japanese field isolates of CDV suggested that the generation of different types of CDV population have been associated with the current outbreaks of CD in Tokyo. Okita et al. (1997) also suggested that there were two distinct types of CD, with or without

cytopahogenic effects in the gastrointestinal tissues, in Japan. Following these clinical and serological studies, molecular approaches identified the differences of vaccine strains and Japanese recent field isolates of CDV [Gemma et al., 1996a, b; Iwatsuki et al., 1997, 2000]. A numbers of CDV strains were successfully isolated from affected dogs in recent years. On their genetic and antigenic characteristics, recent field isolates were clearly distinct from vaccine strains of CDV, and Japanese isolates formed one group which is distinct from European or American groups [Iwatsuki et al., 1997, 1998, 2000; Yoshida et al., 1998].

Canine distemper virus (CDV) is a non segmented single stranded RNA virus, which belongs to the genus morbillivirus with the family of *Paramyxoviridae*. Paramyxoviruses contain non-segmented single stranded RNA genomes of negative polarity, and they replicate entirely in the cytoplasm. The genomes of morbilliviruses are approximately 16 kbp in length, and the genomes contain six tandemly linked genes. A lipid envelope containing two surface glycoproteins, fusion (F) protein and hemagglutinin (H) protein, surrounds the virions. F protien is responsible for virus-cell fusion and cell-cell fusion, which facilitates virus penetration into cells and viral spread between cells. H protein interacts with cellular receptors and plays a role of virus attachment to the cells. These two envelope proteins are of primary importance

in inducing neutralizing antibodies and immunity against reinfection. The viral matrix (M) protein localizes at the inner layer of plasma membrane of the infected cells and of envelope of the virus particles, and is involved in virus budding from the infected cells. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleocapsid- (N), phospho- (P) and large (L) proteins, which initiate intracellular virus replication. The RNA genome are tightly associated with N protein to form ribonucleocapsid (RNP) complex with P and L proteins, and the RNP but not naked RNA can be the template for transcription and replication. The viral P and L proteins form RNA-dependent RNA polymerase complex, and are responsible for viral transcription and replication. The RNP is packaged in viral particle, and then released from the cells. M protein is thought to bridge the RNP and glycoproteins on cell surface, where the budding of virus occurs.

It had been impossible to recover genetically engineered recombinant viruses for Mononegaviruses, because unlike positive sense RNA viruses, the naked RNA of Mononegaviruses alone can not be the template for viral transcription and replication. In 1994, Conzelmann and colleagues [Schnell et al., 1994] generated recombinant rabies virus (RaV), demonstrating the feasibility of producing a negative-sense RNA virus entirely from cloned cDNA. Cells were cotransfected with protein expression constructs for the L, P and N proteins and with a cDNA construct encoding the full-length RaV antigenome, all under control

of the T7 RNA polymerase promoter. Infection with recombinant vaccinia virus (VV), which provided T7 RNA polymerase, was the final step needed to produce infectious RaV. The key element to this success was the synthesis of a positive-sense antigenomic RNA from cloned DNA. Positive-sense antigenomic RNA, in contrast to negative-sense genomic RNA, cannot hybridize to positive-sense mRNAs encoding the L, P and nucleoproteins and thus does not interfere with virus generation. Moreover, the genomic RNAs of some negative-sense RNA viruses contain stretches of uridine residues followed by hairpin structures that resemble T7 RNA terminator elements, which may cause premature abortion of T7 RNA polymerase transcription [Whelan et al., 1995]. Since the initial report by Schnell et al. (1994), the generation of an ever-growing number of rhabdo- and paramyxoviruses by reverse genetics was carried out [MV: Radecke et al., 1995; Schneider et al., 1997; Takeda et al., 2000; RPV: Baron and Barret, 1997; CDV: Gassen et al., 2000; Fujita, 2002; Parks et al., 2002; Plattet et al., 2004; SeV: Garcin et al., 1995; Kato et al., 1996; hPIV3: Durbin et al., 1997; Hoffman and Banerjee, 1997; bPIV3: Haller et al., 2000; SV5: He et al., 1997; Mumps V.: Clarke et al., 2000; hPIV2: Kawano et al., 2001; NDV: Peeters et al., 1999; Romer-Oberdorfer et al., 1999; Krishnamurthy et al., 2000; hRSV: Collins et al., 1995; bRSV: Buchholz et al., 1999]. Refinements of the original rescue procedure included the expression of T7 RNA polymerase from stably transfected cell lines [Radecke et al., 1995], protein expression

plasmids [Lawson et al., 1995] or heat shock procedures to increase rescue efficiencies [Parks et al., 1999] were performed. Recently, Ebola virus, a member of the family *Filoviridae*, was also generated from cDNA [Volchkov et al., 2001; Neumann et al., 2002]. Recently, we succeeded the establishment of reverse genetics of CDV Yanaka strain [Fujita, 2002], which was isolated in Japan from a dog clinically diagnosed with distemper, and produced the recombinant CDVs which express enhanced green fluorescence protein (EGFP) and firefly luciferase [Fujita, 2002].

Persistent infection of morbilliviruses

Replication of morbillivirus including MV and CDV usually causes cell death in culture. However, MV is well known to cause persistent infection as observed in subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis. The mechanism of persistent noncytolytic infection is not clarified.

Methods used to establish persistently infected cell line were a number of passage of virus at high multiplicity with generation of defective interfering particles, passage of infected cells in the presence of antibodies, cultivation of cells surviving lytic infection and co-cultivation of cells with virus infected brain

cells from patients with SSPE or from persistently infected animals. Persistent infection is most easily established in neuronal cells, but it has also been established in lymphoid, epithelial and glial cells. The appearance of mutants in persistently infected cultures was demonstrated by changes in patterns of antigenicity of the H protein [Robbins et al., 1991], N protein [Boriskin et al., 1986], the P or V protein [Bellini et al., 1986] after cultured with human convalescent and SSPE sera. Reduced amounts of M protein and altered processing of the H protein in persistently infected HeLa cell cultures were shown by pulse-chase experiments [Young et al., 1985]. Lack of the M protein in particular restricts viral budding and could be a mechanism of viral persistence. However, in a persistent infection of Vero cells with the hamster neurotropic (HNT) strain of MV, restriction in the expression of the fusion protein was suggested as a mechanism for the maintenance of persistence [Hummel et al., 1994]. The lack of F protein expression in these cells was attributed to the overabundance of M/F read-through transcripts (5:1 over F transcripts). These reports indicate that diverse mechanisms mediate achievement of persistence.

In the chapter 1, the author established a persistent CDV based on CDV-EGFP and analyzed a mechanism of persistence of CDV in lymphocyte.

Canine Parvoviruses

Canine parvovirus (CPV) was first observed in the late 1970s when a new disease of dogs were observed: myocarditis leading to sudden death in neonatal pups, and enteritis accompanied by diarrhea in dogs older than 2 months. Rapidly the virus infection virtually spread all of the wild and domestic dogs in every region of the globe [Parrish et al., 1990]. After the emergence, it was apparent that the CPV isolated was antigenically closely related to feline parvovirus (FPV), and subsequent sequence analysis revealed that the viruses were more than 99% identical [Horiuchi et al., 1994, Parrish, 1991, Shackelton et al., 2005]. FPV has been known since the 1920s as the cause of diseases of cats [Hindle and Findlay, 1932, Verge and Cristoforni, 1928], and since the 1940s has been recognized as a natural pathogen of minks, foxes, raccoons, and some other carnivores, giving the virus various names including mink enteritis virus, blue (Arctic) fox parvovirus, and raccoon parvovirus [Parrish et al., 1990]. CPV has remained endemic in dogs throughout the world and is still a frequent cause of disease in puppies. Although antigenic variation has been detected in the viruses isolated from dogs, the immunity generated lasts for many years and reinfection of recovered dogs is not reported. Although vaccination can frequently protect puppies, it has not controled the wild-type viruses.

CPV belongs to the family *Parvoviridae*, Subfamily *Parvovirinae*, genus Parvovirus. The first CPV spread worldwide was termed CPV type-2 to distinguish it from the distantly related parvovirus, minute virus of canis. In 1979 a variant strain of CPV emerged that contained several sequences different from CPV-2, and the new virus was antigenically distinct and was therefore termed CPV-2a. Within one year CPV-2 had been replaced by CPV-2a throughout the world [Parrish, 1991; Parrish et al., 1988a]. CPV-2a strains also differed from CPV-2 in host range, as they had gained the ability to infect cats, generally causing a mild disease [Mochizuki et al., 1996, Nakamura et al., 2001, Truyen et al., 1996]. In subsequent years further variants emerged from the CPV-2a viruses that distributed globally, including additional antigenic variants with single-sequence substitutions in the capsid protein gene recognized in 1984, the late 1990s, and the early 2000s [Battilani et al., 2002, Ikeda et al., 2000, Martella et al., 2004, Mochizuki et al., 1993, Shackelton et al., 2005]. New genetic and antigenic variants, type 2a (CPV-2a), type 2b (CPV-2b) and type 2c (CPV-2c) [Parrish, 1991; Parrish et al., 1991; Mochizuki et al., 1996; Ikeda et al., 2000] emerged. The CPV-2a and 2b have gained the infectivity in cats, although CPV-2 did not have infectivity to cats. In the chapter 2-1, the author performed phylogenetic analysis of VP2 gene of CPV isolated from domestic dogs in Japan in 1999-2000.

CPV has non-enveloped and isometric particles with a capsid formed by three structural proteins VP1, VP2 and VP3, and the genome is a linear molecule of single-stranded DNA (ssDNA), 4-6 kb in size. CPV replicates in the nucleus. VP1 and VP2 are different splicing products from the same gene [Jongeneel et al., 1986], and VP3 results from proteolytic removal of the amino terminus from VP2 [Cotmore and Tattersall, 1987]. VP2 protein is the major protein of virus surface and the target of the host immunity. VP2 gene encoded 585 amino acids and the first half is sufficient for protective immunity because the 2L21 peptide [Langeveld et al., 1994] or the 3L17 peptide epitope [Casal et al., 1995] generated high titers of VP2 peptide-specific neutralizing antibody in vaccinated dogs and were completely protected dogs from clinical disease.

Rabies virus

Rabies virus (Rav) belongs to the order Mononegavirales, viruses with a nonsegmented, negative-stranded RNA genomes as well as CDV. Within this group, viruses with a distinct "bullet" shape are classified in the *Rhabdoviridae* family, which includes at least three genera of animal viruses, Lyssavirus, Ephemerovirus, and Vesiculovirus. The genus Lyssavirus includes rabies virus, Lagos bat, Mokola virus, Duvenhage virus, European bat virus 1 and 2 and

Australian bat virus. Rabies is a preventable viral disease of mammals most often transmitted through the bite of a rabid animal. Rabies virus infects the central nervous system, causing encephalopathy and ultimately death. Rabies has been the object of human fascination, torment, and fear since the disease was recognized in antiquity [Fishbein and Robinson, 1993]. The World Health Organization estimates that 40,000 - 100,000 human deaths are caused by rabies each year [Meslin et al., 1994], and that 10–12 million people receive one or more doses of rabies vaccine annually after exposure to the virus [Dressen, 1997]. The dog has long been known to be a principal transmitter of rabies. The first rabies vaccine was developed by Pasteur in the early 1880s when he adapted "street" virus to rabbits by serial intracerebral passage [Pasteur, 1884]. The Pasteur vaccine has predominantly used for human vaccination. Chloroform or either inactivated virus vaccines for dogs prepared from infected brain suspensions became available in the 1920s. The development of live attenuated rabies virus, vaccines in low egg passage (LEP) and high egg passage (HEP) [Koprowski, 1954] led to effective vaccination of dogs [Kaplan et al., 1954; Sikes et al., 1971]. However, on rare occasions the live attenuated vaccines caused rabies-like disease in the dogs. Currently, improved inactivated virus vaccines prepared from rabies virus and controlled onset of rabies in domestic dogs. However, it is impossible to immunize inactivated vaccines to wild animals. To resolve this problem, the recombinant vaccine based on vaccinia virus

expressing rabies virus transmembrane G protein was developed in 1984 [Wiktor et al., 1984]. In Europe, this vaccine is used for wild animals in practice, using the bait containing this recombinant vaccinia virus, and obtained certain results [Brochier et al., 1991].

Vaccines

During the past 50 years many vaccines have been developed to prevent a variety of infectious diseases of dogs. Vaccines consist of either attenuated or inactivated microorganisms. Despite avirulent strains used, attenuated vaccines may cause severe diseases in immunocompromised individuals, and inactivated vaccines sometimes cause side effects by adjuvant and are unable to induce long term immunity. To resolve these problems, new generation vaccines are researched, such as subunit vaccines and recombinant vaccines. Subunit vaccine has an advantage for safety. However, the production of subunit vaccines cost much and needs stringent purification processes. Whereas recombinant vaccines have an advantage to induce stronger and more prolonged immune responses and be effective for eliciting protective cell-mediated immunity. In addition, the appearance of new viruses with pathogenic potential is recently a constant threat to humans and animals.

Recombinant vaccines will be available for prevention of such emerging and re-emerging infectious diseases for which attenuated or inactivated vaccines are unable to be rapidly prepared.

The power to manipulate the genome of negative-strand RNA viruses, including the insertion of additional viral or non-viral genes, has led us to the develop a new type of viral vectors for gene transfer approaches. RaV, vesicular stomatitis virus, Sendai virus, human parainfluenza virus type 3, MV, rinderpest virus, siminan virus 5, Newcastle disease virus, mumps virus, respiratory syncytial virus or Ebola virus has been known as a potential virus [Schnell et al., 1994, Lawson et al., 1995, Kato et al., 1996, Durbin et al., 1997, Radecke et al., 1995, Baron and Barrett 1997, He et al., 1997, Peeters et al., 1999, Clarke et al., 2000, Collins et al., 1995, Volchkov et al., 2001]. Since reverse genetics of CDV [Kooriyama, 2004, Plattet et al., 2004, Parks et al., 2002, Gassen et al., 2000, Jung et al., 2005] were established, CDV started to be used for the development of a new type of vaccine vectors, transfer vectors and oncolytic vectors [Suter et al., 2005]. Since the negative-strand RNA viruses have the features such as efficient infection in the respiratory tract, an exclusively cytoplasmic replication cycle without any risk of chromosomal integration, and induction of strong humoral and cellular immune responses, they should be new and excellent candidates for vaccine vectors.

In the chapter 2-2 and 2-3, the author performed fundamental studies to develop a polyvalent vaccine based on CDV.

CHAPTER 1

Generation and characterization of a persist mutant of recombinant canine distemper virus expressing enhanced green fluorescence protein

SUMMARY

We previously showed that CDV has a potential to cause persistent infection in lymphocytes in vitro. However the mechanism of persistent infection of CDV is poorly understood. In this chapter, by using CDV-EGFP the author has newly established a noncytopathic CDV strain (CDV-EGFP-BP2) that caused persistent infection in B95a cells and investigated the mechanism of persistence in lymphocyte. CDV-EGFP-BP2 infected cells survived and the virus was continuously produced and extracellularly released. There was no significant difference of H and F expression compared to parental CDV, but analysis using stably SLAM-expressing cells showed that CDV-EGFP-BP2 reduced the fusogenicity compared with CDV-EGFP. Amino acid changes of CDV-EGFP-BP2 were only found in M and H in comparison with CDV-EGFP. Since cell fusion requires the interaction of H and F, these results indicate that the substitution of H protein may be responsible for the interaction with F protein.

INTRODUCTION

Canine distemper virus (CDV) is classified in the genus *Morbillivirus* of the family *Paramyxoviridae* containing a nonsegmented, negative-strand RNA as the genome. Clinically, CDV causes an acute to subacute contagious systemic disease with a high mortality rate in dogs [Appel, 1987]. After the live attenuated vaccines were developed and have been introduced since the 1960s, the incidence of CD in dogs has been reduced. Although it was unclear how the predictive immunity can be sustained, dogs that recover from virulent CDV infection are known to resist challenge by virulent virus for up to seven years after the first infection [Appel, 1987]. Additionally, while measurable cell-mediated immune responses are of short duration, the humoral immune response persists in recovered dogs for at least several years [Appel et al., 1982]. These results indicated lifelong immunity against CDV infection, similar to that of humans who have recovered from MV infection.

CDV causes lytic infection in vivo and in vitro. CDV has also a potential to induce persistent infection in neural cells [Zurbriggen et al., 1995] and lymphoid cells [Friedlander et al., 1985], but the mechanism of persistent infection of CDV is poorly understood. CDV persistence in the central nervous system (CNS) appears to operate the chronic progression of the disease. Demyelination in dogs induced by CDV is considered to be an animal model for

multiple sclerosis in human.

Since it has been reported that B95a cells, a marmoset B lymphoid cell line, are highly susceptible to wild-type MV and that the isolated viruses using the cells retain their pathogenicity [Kobune et al., 1990], we used B95a cells for the isolation of CDV and found that they are also more susceptible than Vero cells to CDV infection [Kai et al., 1993]. Thus, using B95a cells, the Yanaka strain of CDV was isolated from dogs clinically diagnosed with canine distemper in Japan [Gemma et al., 1996a]. This Yanaka strain produces extensive syncytia followed by cell death in B95a cells. By numerous blind passages of the Yanaka strain and following observation of no detectable syncytia in B95a cells, a persistent virus mutant, the Yanaka-BP strain, was established [Iwatsuki et al., 1999]. The virus was expected to be helpful to elucidate the mechanism for CDV persistence in lymphocytes. The virus infected cells were, however, indistinguishable from uninfected cells unless immunostaining was performed.

We established reverse genetics of CDV Yanaka strain and produced the recombinant CDV that expresses enhanced green fluorescence protein (CDV-EGFP) [Fujita, 2002]. By using CDV-EGFP, infected cells can be observed without immunostaining under the confocal microscopy. In this chapter, the author newly established a noncytopathic CDV strain that caused persistent infection in B95a cells by use of the CDV-EGFP and investigated the mechanism of persistence in lymphocyte.

MATERIALS AND METHODS

Cells and viruses

B95a cells, an Epstein-Barr virus transformed marmoset lymphoblastoid cell line [Kobune et al., 1990], were maintained in RPMI1640 (Sigma) containing 5% fetal calf serum (FCS). 293 cells, a human embryonal kidney cell line, and CHO cells, chinese hamster ovarian cell line, were maintained in Dullbecco's modified Eagle's medium (DMEM) (Sigma) containing 5% FCS. SLAM, derived from B95a cells, stably expressing 293 and CHO cells (293-SLAM and CHO-SLAM cells) maintained as well as these parental cells. Cell viability was determined by trypan blue exclusion assay. CDV-EGFP, established from the CDV-Yanaka strain (Fig. 1.1) [Fujita, 2002] was propagated in B95a cells.

Virus characterization

Multistep growth of viruses were analyzed by infecting B95a cells at an moi (multiplicity of infection) of 0.01 in 12-well plates. After virus was adsorbed to the cells for 30 min, inoculum was removed, and the 1 ml of medium was supplied into each well. The cells were incubated and collected for various times. Virus induced cytophasic effect (CPE) or EGFP expression was visualized by infecting B95a cells at 48hrs post infection. At 6 hr, 12 hr, and

every 24 hr from 1 day to 7 days post infection, the cells and medium were separately harvested and frozen at -80°C. The cells were subjected to three cycles of freezing and thawing. Virus titers of the cell-associated viruses and released viruses in media were determined by endpoint dilution assays to calculate 50% tissue culture infectious dose ($TCID_{50}$) values using a standard method.

Northern blot analysis

Total cellular RNA of infected or mock-infected B95a cells was prepared by guanidine thiocyanate method using Isogen (Nippongene) as an extraction reagent. Thirty μg of total RNA was separated in 1% agarose/formaldehyde gel, and subsequently transferred to Hybond-N+ (Amersham bioscience). The membrane was UV-crosslinked for 3 min and baked at 80 °C for 2 hrs, and hybridized with specific probes for CDV H or F gene. The probes were prepared by PCR amplification with specific primers of ORF region and then purification by Wizard SV gel and PCR clean-up system (Promega). The probes were labeled with ^{32}P-dCTP using Alk Phos labeling kit (Rediprime; Amasham Bioscience). The hybridization was carried out according to the manufacturer's instruction. The membranes were exposed to imaging plates and the detection was performed using FLA5000 (Fuji film).

Indirect immunofluorescence staining

B95a cells were grown in 6-well plates and infected with recombinant CDVs or the parental Yanaka strain. After 3 days, the cells were washed once with PBS and fixed by 4% paraformealdehyde for 30 min. The cells were consecutively incubated for 120 min with monoclonal antibody (mAb), d-7 for the H protein or a-8 for the F protein, which was raised against the CDV vaccine FXNO strain [Hirayama et al, 1991]. The cells were washed with PBS three times and then incubated with goat anti-mouse antibody-sulforhodamine (diluted at 1:1000 in PBS) (Alexa Flour 548; Molecular probes, Cochranville, PA) for 60 min. After washing 3 times with PBS, imaging analysis was performed with confocal microscopy, Fluoview FV500 system (Olympus Optical Co. Ltd, Tokyo).

Radioimmunoprecipitation assay

Virus-infected or mock-infected B95a cells were radiolabelled with 0.2 μCi [^{35}S] methionine (DuPont NEN research products) for 4 hrs. The radiolabelled cells were lysed by RIPA buffer (1% Triton X-100, 1 mM iodoacetamide, 0.2 U/ml of the aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1% sodium deoxycholate, 0.14 M NaCl, and 10 mM Tris-HCl at pH 8.0). The lysate was reacted with mixture of the mAbs (diluted at 1:1000 in PBS), d-7 against CDV H protein and a-8 against CDV F protein for overnight at 4°C,

followed by incubation with protein A beads (Amersham biosciences). The beads were washed in PBS three times and loading dye was then directly added. The immunoprecipitates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography.

Cell surface protein biotinylation and western blot analysis

To perform biotin labeling of cell surface proteins, Cellular labeling and immunoprecipitation kit (Roche) were used. B95a cells were infected with recombinant CDVs in the 6-well culture dish. Biotinylation was carried out according to the manufacturer's instruction. Briefly, the cell monolayer was washed with PBS and incubated with biotin-7-NHS (D-biotinoyl-e-aminocaproic acid-N-hydroxysuccini-mide ester) solution. After incubation, the cells were washed by PBS and lysed. The suspension was sonicated and centrifuged. The supernatant was divided into two equally aliquots and adjusted the volume of each aliquot to 1.0 ml with dilution buffer. After the supernatants were pre-incubated with 50 μl of protein A-agarose suspension, the samples were incubated with 150 μl of the MAbs against CDV H or F protein described above (diluted at 1:1000 in PBS) for overnight at 4°C with gently rocking. After washed, the samples were separated by 10% SDS-PAGE and transferred onto Immobilon-P nylon membranes (Millipore). After blocked by PBS containing 4%

block ace (Dainippon pharmachemical), the membranes were incubated with peroxidase-conjugated streptavidin (Pierce) for 1hr at room temperature and visualized by DAB solution.

Sequence analysis

The DNA sequences were determined using the Dye terminator cycle sequencing method and analyzed with an autosequencing system (Applied Biosystem, Foster City, CA). For the amino acid translation and comparison, the GENETYX-MAC software (ver. 11.0) was used.

RESULTS

Establishment of a persistent mutant of CDV-EGFP

Previously, Iwatsuki et al succeeded in establishment of a persistent mutant virus, designated Yanaka-BP, derived from CDV-Yanaka strain by a number of blind passages in B95a cells [Iwatsuki et al., 1999]. Since the persistent mutant infected B95a cells showed no detectable syncytia and can proliferate as well as uninfected cells, the infected cells were undistinguishable without immunostaining using antibodies against virus antigens. Fujita et al produced EGFP expressing recombinant CDV (CDV-EGFP) by reverse genetics [Fujita, 2002]. The virus produced extensive CPE in B95a cells and the infected cells in culture can be found by EGFP fluorescence under confocal microscopy (Fig. 1.2). Therefore, using the CDV-EGFP, the author attempted to produce a new persistent mutant by passages as an indicator of the EGFP fluorescence.

After B95a cells were inoculated with CDV-EGFP, cell fusion and syncytia formation were induced in a couple of days and most of dead cells were floating. However, a few EGFP-positive cells survived and started to propagate. When the cells became nearly confluent, they were passaged ten times at an interval of 4-5 days until detectable syncytia disappeared. From the EGFP-positive cells, cell cloning was performed by limiting dilution, and finally

we obtained several EGFP-positive cell clones. Extract was obtained from the confluent cells by three cycles of freezing and thawing followed by sonication. When B95a cells were inoculated with the extract, green fluorescence without syncytium formation was observed in a number of cells (Fig. 1.2). Thus, a persistent CDV derived from CDV-EGFP is considered to be established, which was designated as the CDV-EGFP-BP2.

Viabilities of CDV-EGFP-BP2 infected B95a cells

The CDV-EGFP and the parental strain of CDV-Yanaka infected B95a cells induced syncytium formation and killed most of cells rapidly. Viability of B95a cells infected with CDV-EGFP-BP2 was analyzed by trypan blue exclusion assay (Fig. 1.3 (A)). Confluent B95a cells were infected with each virus at an moi of 0.01 and were serially monitored without medium change during 4 days. At 4 day after infection, 80% of both mock and CDV-EGFP-BP2 infected cells survived similarly, while viable cells in both CDV-EGFP and CDV-Yanaka infected cells were markedly decreased to approximately 60%. Most of syncytia were almost dead. In time-lapse imaging analysis under the confocal microscopy for 18 hrs, cell division normally occurred in CDV-EGFP-BP2 infected cells, which were visualized by fluorescence (data not shown).

Growth kinetics of CDV-EGFP-BP2

Growth kinetics of the CDV-BP2-EGFP was compared with CDV-EGFP and CDV-Yanaka using B95a cells. B95a cells were infected with each virus at an moi of 0.01 to allow multiple cycles of replication, and media and cells were kinetically harvested. Released virus titers were shown as viruses in the media (Fig. 1.3 (B)) and cell associated virus titers as viruses in the extracts (Fig. 1.3 (C)). Virus titers of CDV-EGFP and CDV-EGFP-BP2 were determined by monitoring EGFP expression. Until 3 day post infection (d.p.i.), three kind of viruses exhibited similar titers probably due to the lack of cell death before syncytium formation. Therefore, both released virus and cell associated virus of CDV-EGFP-BP2 kept higher titers than other viruses, indicating that CDV-EGFP-BP2 infected cells survived and the virus was continuously produced and extracellularly released.

Expression of CDV H and F envelope glycoproteins in B95a cells

In morbilliviruses, H glycoprotein is involved in cell attachment and F glycoprotein in fusion process of the viral envelope with the plasma membrane of the host cells. It is generally thought that cell-to-cell fusion in which virus infected cells are attached and fused to neighbor uninfected cells, also occurred by the same mechanism as virus infection. Since the persistent CDV did not induce syncytium formation in B95a cells, the author examined expression of H

and F glycoproteins of CDV-EGFP-BP2, in comparison with CDV-Yanaka and CDV-EGFP. Northern blot analysis showed that CDV-EGFP-BP2 infected cells sufficiently produced both H and F mRNAs (Fig. 1.4). Both H and F glycoproteins of CDV-EGFP-BP2 infected cells were synthesized as well as those of CDV-EGFP (Fig. 1.5 (A)) and these proteins were transported to the cell surface (Fig. 1.5 (B)). In immunofluorecence staining, H and F were located at the cell surface (Fig. 1.6). These results showed no significant difference of H and F expression between these viruses.

Analysis of SLAM-dependent infection with CDV-EGFP-BP2

SLAM (also known as CDw150) located at the plasma membrane of lymphocytes and dendritic cells plays a role of entry receptor for H glycoprotein mediating attachment of morbilliviruses [Tatsuo et al., 2001]. CDV-Yanaka also uses this molecule as a receptor on B95a cells like other CDV, MV and RPV [Fujita, 2002]. Although both 293 and CHO cells were hardly infected with morbilliviruses including CDV, the cells, in which marmoset SLAM cDNA derived from B95a cells was transiently expressed, acquired the susceptibility to these viruses (data not shown). Since virus infection and fusion formation host cells are closely related, no syncytium formation of CDV-EGFP-BP2 raised the possibility of the change of reactivity to SLAM. Therefore, the author established cell lines stably expressing SLAM and investigated the infectivity of

CDV-EGFP-BP2 to these cells.

CHO and 293 cells were transfected with the marmoset SLAM cDNA inserted into pCAGGS plasmid. The stable cell clones, 293-SLAM and CHO-SLAM cells were selected by G418 (Gibco-BRL) and confirmed to be highly susceptive to CDV-Yanaka (Fig. 1.7). Infections with CDV-EGFP and CDV-EGFP-BP2 were barely detectable in original 293 and CHO cells and the EGFP-positive cells formed no syncytium. In contrast, 293-SLAM and CHO-SLAM cells infected with these viruses obviously induced formation of syncytia. Especially, more invasive cell fusion and a larger size of syncytia emerged in 293-SLAM cells infected with CDV-EGFP and CDV-Yanaka, as compared with CDV-EGFP-BP2 (Fig. 1.7). These results imply that the alternation between CDV-EGFP and CDV-EGFP-BP2 may cause the reduced fusogenicity.

Sequence analysis of CDV-BP2-EGFP

CDV genome consists of six viral genes, N, P/V/C, M, F, H and L. The author determined the nucleotide sequences of open reading frame of the M, F, H genes in CDV-EGFP-BP2 and compared the deduced amino acid sequences with CDV-EGFP. Reverse transcription PCR products from a preparation of fresh total RNA from the CDV-BP2-EGFP-infected B95a cells were directly sequenced to exclude the possibility of nucleotide misincorporation by

polymerase error. In F gene, there was no change of nucleotide sequence. On the other hand, amino acid changes were found in M (13th D to N) (Fig. 1.8 (A)) and H (215th E to D) (Fig. 1.8 (B)). Compared with the persistent CDV, Yanaka-BP, which was previously established [Iwatsuki et al., 1999], no common mutation was found.

DISCUSSION

In this chapter, the author newly established a persistent mutant virus (CDV-EGFP-BP2) derived from EGFP expressing recombinant CDV (CDV-EGFP) [Fujita, 2002]. The persistent mutant infected B95a cells showed no detectable syncytia and can proliferate as well as uninfected cells. Since living cells infected with CDV-EGFP-BP2 were visualized under the confocal microscopy, it is more useful to analyze the mechanism of persistent infection in detail. Only two mutations were found in the deduced amino acid sequence of the viral proteins of CDV-EGFP-BP2 compared with CDV-EGFP, while there were totally 11 amino acids mutations in comparison between Yanaka and Yanaka-BP probably due to a large number of blind passages [Iwatuski et al., 1999; Nishi, 2002; Wakasa, 2000; Funabashi, 2003]. Since we could obtain the persistent mutant by only ten times of passages, it may be the minimum number of mutations in the genome for generation of a persistence mutant.

Numerous viruses are able to induce apoptotic cell death in target cells. On the contraly many viruses potential to persist in infected cells with restricting apoptosis formation. It has been suggested that inhibition of apoptosis may favor the development of persistent rather than lytic infection [Hummel et al., 1992]. The CDV-EGFP-BP2 infected B95a cells grew as well as uninfected cells.

Cell division normally occurred in the infected cells. Nishi et al showed that CDV Yanaka infection caused extensive syncytia formation and apoptosis significantly and that the Yanaka-BP strain only induced an early sign of apoptosis in a small population of infected cells but did not induce a typical apoptosis [Nishi et al., 2004]. These results suggest that cell fusion is one of main factors for induction of apoptosis by CDV infection.

As compared the amino acids of the Yanaka-BP with the parental virus, the Yanaka strain, there were one amino acid difference in M protein, 8 amino acids in F protein, and 4 amino acids in H protein [Nishi, 2002]. Surprisingly, only 2 amino acid changes were found within the viral surface protein genes of CDV-EGFP-BP2; one in the H protein and the other in M protein. These changes were not identical to those of Yanaka-BP. These results show that different mutations can lead to similar persistence. A75/15 CDV, a strain which induce a persistent infection in CNS [Imagawa et al., 1980; Bollo et al., 1986; Higgins et al., 1989] and its Vero-adapted CDV strain (A75/15 CDV-V) [Hamburger et al., 1991] were reported. A75/15 CDV-V induced persistent infection and spreads in a cell-to-cell manner without obvious syncytium formation [Hamburger et al., 1991; Meertens et al., 2003; Plattet et al., 2004]. A75/15 CDV-V made smaller syncytia in Vero-dogSLAMtag cells than CDV Ondersteproot laboratory strain (OP-CDV) [Plattet et al., 2005], similar to

CDV-EGFP-BP infected 293-SLAM cells in this study. However, in analysis of H and F chimeric CDVs between A75/15 CDV-V and OP-CDV, F protein derived from OP-CDV was necessary to induce large syncytium formation and the authors described that the mechanism is yet known. This strain was established using dog brain cells and Vero cells that are devoid of SLAM expression. This inconsistence between CDV-EGFP-BP2 and A75/15 CDV-V implies that the different mechanism may exist for persistence of CDV, dependent on host cells.

The structures of H proteins in Paramyoviruses are a superbarrel comprising six similarly folded antiparallel beta-sheets of four strands each [Crennell et al., 2000]. In the superbarrel, the six sheets are arranged cyclically around an axis through the center of the molecule like blades of a propeller. Recently, the model of three dimensional structure of MV-H were proposed and residues which are relative to SLAM-binding were reported [Vongpunsawad et al., 2004; Masse et al., 2004]. Vougpunsawad et al. identified seven SLAM-relevant residues in the propeller beta sheet 5: Y529, D530, T531, R533, F552, Y553 and P554 and Masse et al showed residues affecting SLAM-down regulation and SLAM-dependent fusion: D505, D507 and R533. The amino acid substitution of H protein of CDV-EGFP-BP2 (E205D) resided in beta sheet 2 and seemed not to be related to SLAM-binding. Cross-linking analysis showed that MV-F and MV-H are closely associated in the plasma membrane [Malvoisin

and Wild, 1993; Plemper et al., 2002] and a specific interaction between the two glycoproteins is apparently required in order for fusion to occur. Furthermore, only CDV H and F expression are sufficient for formation of syncytium, since B95a and 293-SLAM cells transiently transfected with both H and F cDNA, exhibited typical syncytia (data not shown). When either the H or F were exchanged to MV-H or F which has an ability of syncytium formation in B95a cells, no syncytium were observed, demonstrating that cell fusion are necessary for acceptable interaction between H and F. Taken together, these results suggest that the substitution of H protein may be responsible for the interaction with F protein.

Released particles of CDV-EGFP-BP2 was infected and replicated in B95a cells. H and F were sufficiently expressed and located at the surface of plasma membrane of infected B95a cells, but cell fusion was not observed. Although it is unclear why the virus entry to cell was succeeded but cell fusion did not occur, it may depend on the affinity of each responsible proteins or on the numbers of expressed proteins on the cell surface. Cell to cell fusion supposed to mediate a larger number of molecules as compared to virus entry to cell. On the other hand, in analysis using 293-SLAM cells, both CDV-EGFP and CDV-EGFP-BP2 made typical syncytia, although the size was different. Thus, the affinity between viral proteins or between viral proteins and cell

receptors may be important for fusogenicity.

Aberrant M protein was believed to be the hallmark of persistent MV infections in SSPE patients [Giraudon and Wild, 1985]. Mutaiton or deletion of M increases cell-to-cell fusion and decreases release of virus particles due to the failure of virus budding [Mottet et al., 1999; Naim et al., 2000; Cathomen et al., 1998; Inoue et al., 2003]. Since CDV-EGFP-BP2 was normally released from the infected cells, the persistent mechanism is different from SSPE viruses.

The long-term maintenance of protective antibodies which immediately neutralize viruses has been proposed to depend on antigen stimulation provided by repeated subclinical infections, viral persistence, cross-reactive antigens or antigen-antibody complexes [Zinkernagel et al., 1996]. Some of the antigen-antibody complexes are probably stably bound to follicular dendritic cells for weeks or months, as observed in vesicular stomatitis virus infection, and act to maintain memory T and B cells [Zinkernagel, 1997]. Cytopathic infectious agents are in general rapidly eliminated completely, but reexposure or the persistence of incomplete viral information may boost and maintain the response. Thus, the potential of CDV to induce persistent infection in lymphocytes observed in this study may be implicated in long term immunity,

and the established virus in the present study could be a valuable tool to investigate the mechanisms of persistence. In addition, such uncytopathic viruses would be available for development of a new type of vaccines and virus vectors.

FIGURE LEGENDS

Figure 1.1 Establishment of CDV-EGFP-BP2

The original infectious cDNA clone was based on the Yanaka strain of CDV. The EGFP cDNA attached downstream of a transcriptional unit of CDV was inserted between N and P genes of the infectious clone [Fujita, 2002]. The rescued virus was designated as CDV-EGFP and the fluorescence was observed in the infected B95a cells. CDV-EGFP infected B95a cells were repeatedly passaged until CPE disappeared. Then the infected cells were harvested and the virus was collected. After the cloning, the virus was designated as CDV-EGFP-BP2.

Figure 1.2 Comparison of viral infected B95a cells

B95a cells were infected with either CDV-EGFP or CDV-EGFP-BP2 and incubated for 3days. Syncytium formation and EGFP expression in the infected cells were analyzed by light microscope (phase contrast) and confocal fluorescence microscopy (EGFP) respectively. Whereas typical syncytia were formed in CDV-EGFP infected cells, no observable syncytium was detected in CDV-EGFP-BP2 infected cells.

Figure 1.3 Cell viability of viral infected cells and viral growth kinetics

(A) B95a cells were infected with CDV-Yanaka (▲, green), CDV-EGFP (●, blue), CDV-EGFP-BP2 (■, pink) or mock (◆, light blue), and the viable cell number was determined using trypan blue dye exclusive assay in comparison with mock infected cells. N=5. (B), (C) Viral growth kinetics of CDV-EGFP-BP2 was compared with CDV-Yanaka and CDV-EGFP. These viruses were inoculated with B95a cells at an moi of 0.01.

Figure 1.4 Comparison of expression of H and F mRNAs in virus infected B95a cells

Northern blot analysis was performed using CDV H and F cDNA. B95a cells infected with each virus were harvested sequentially and the total RNA was collected. These RNAs were separated by 1% agarose/formaldehyde gel electrophoresis and transferred to nylon membrane. Each cDNA of CDV-H or F cDNA was labeled by ^{32}P-dCTP and used as probes. Expression of both CDV H and F mRNA were closely similar to each other. Mock: no virus infected B95a cells.

Figure 1.5 Comparison of synthesis (A) and cell surface expression (B) of CDV- H and F proteins in virus infected B95a cells

B95a cells were infected with CDV-EGFP or CDV-EGFP-BP2 at an moi of 0.01. (A) The syntheses of CDV-H and F proteins at various times were

analyzed by radio-immunoprecipitation. The cells were radiolabeled for 4 h and then immunoprecipitated using MAb d-7, against CDV-H protein, or MAb a-8 against CDV-F protein. Both CDV-EGFP and CDV-EGFP-BP2 infected cells were almost similar in the manner and the quantity. (B) Cell surface expression of CDV-H and -F proteins were analyzed by surface protein biotinylation and immunoprecipitation. The accumulation of CDV H or F proteins was observed by western blot analysis. Immunoprecipitated samples were separated by SDS-PAGE and transferred onto nylon membranes. The membrane was reacted with peroxidase-coupled streptavidin and detected by DAB.

Figure 1.6 Immunofluorescence staining of CDV viral proteins in the infected cells

CDV-EGFP or CDV-EGFP-BP2 infected B95a cells were fixed and reacted with MAbs against CDV-H (A) or -F (B). Then the cells were reacted with sulforhodamine-conjugated secondary antibody and observed under confocal microscopy. Both H and F proteins were also localized on the surface of CDV-EGFP-BP2 infected cells.

Figure 1.7 Infectivity of SLAM stably expressing cells.

293, 293-SLAM, CHO and CHO-SLAM cells were infected with CDV-EGFP and CDV-EGFP-BP2 at an moi of 0.01. After the 2 days incubation,

the living cells were observed under the confocal microscopy. The both cells infected with CDV-EGFP were more invasive than with CDV-EGFP-BP2. The microscopy with magnifications of x30 (293, CHO and CHO-SLAM) and x10 (293-SLAM) were used.

Figure 1.8 Comparison of deduced amino acid sequences

The deduced amino acid sequences of M (A) and H (B) were compared among CDV-EGFP, CDV-EGFP-BP2 and CDV-BP. The sequence of CDV-EGFP-BP (top line) were compared with that of the Yanaka-BP (middle line) or Yanaka strain (bottom line). Asterisks (*) below the alignment represent identical amino acids.

Fig. 1.1

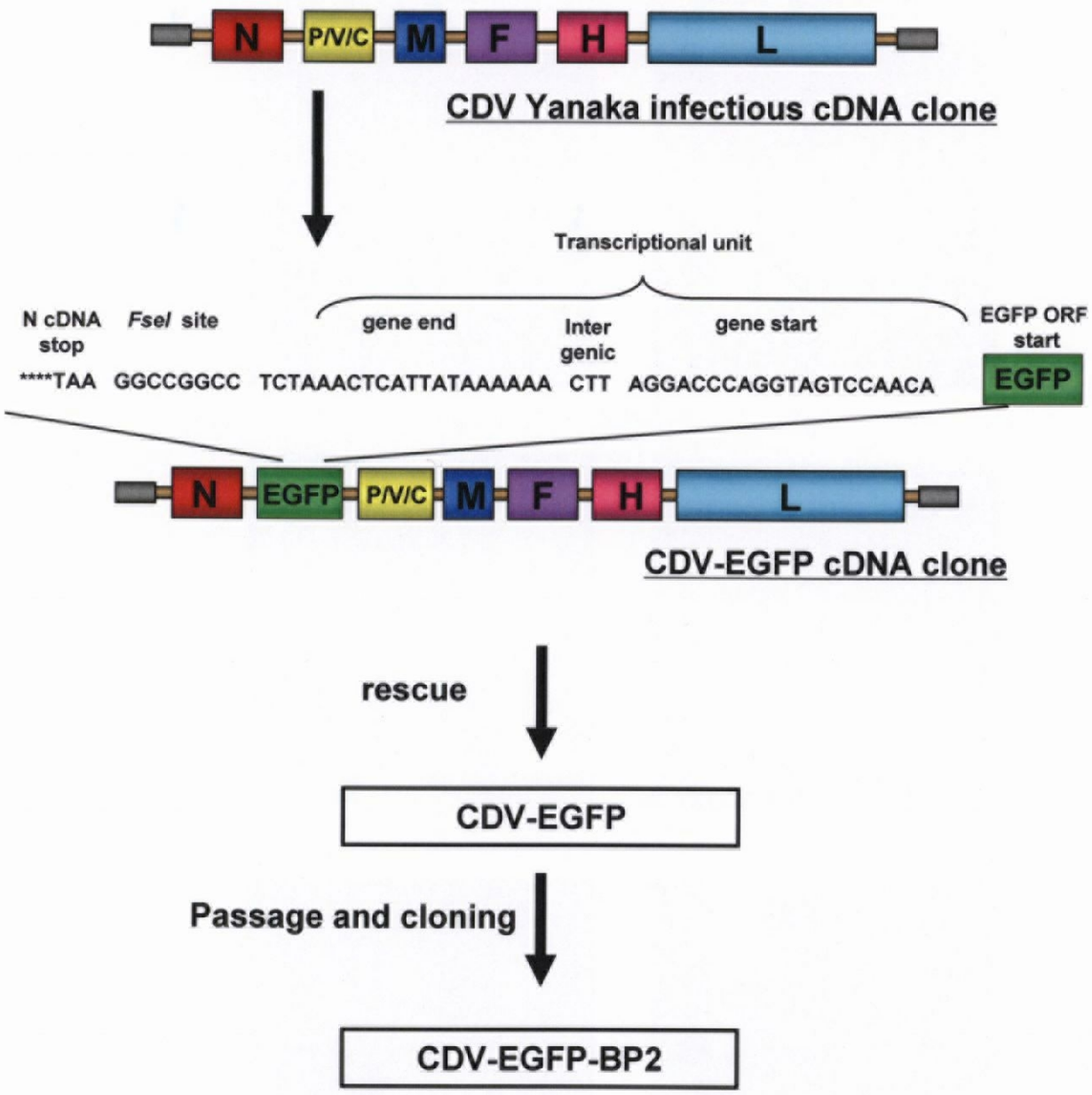

N
P/V/C
M
F
H
L
CDV Yanaka infectious cDNA clone
Transcriptional unit
N cDNA stop
FseI site
gene end
Inter genic
gene start
EGFP ORF start
****TAA GGCCGGCC TCTAAACTCATTATAAAAAA CTT AGGACCCAGGTAGTCCAACA
EGFP
N
EGFP
P/V/C
M
F
H
L
CDV-EGFP cDNA clone
rescue
CDV-EGFP
Passage and cloning
CDV-EGFP-BP2

Fig. 1.2

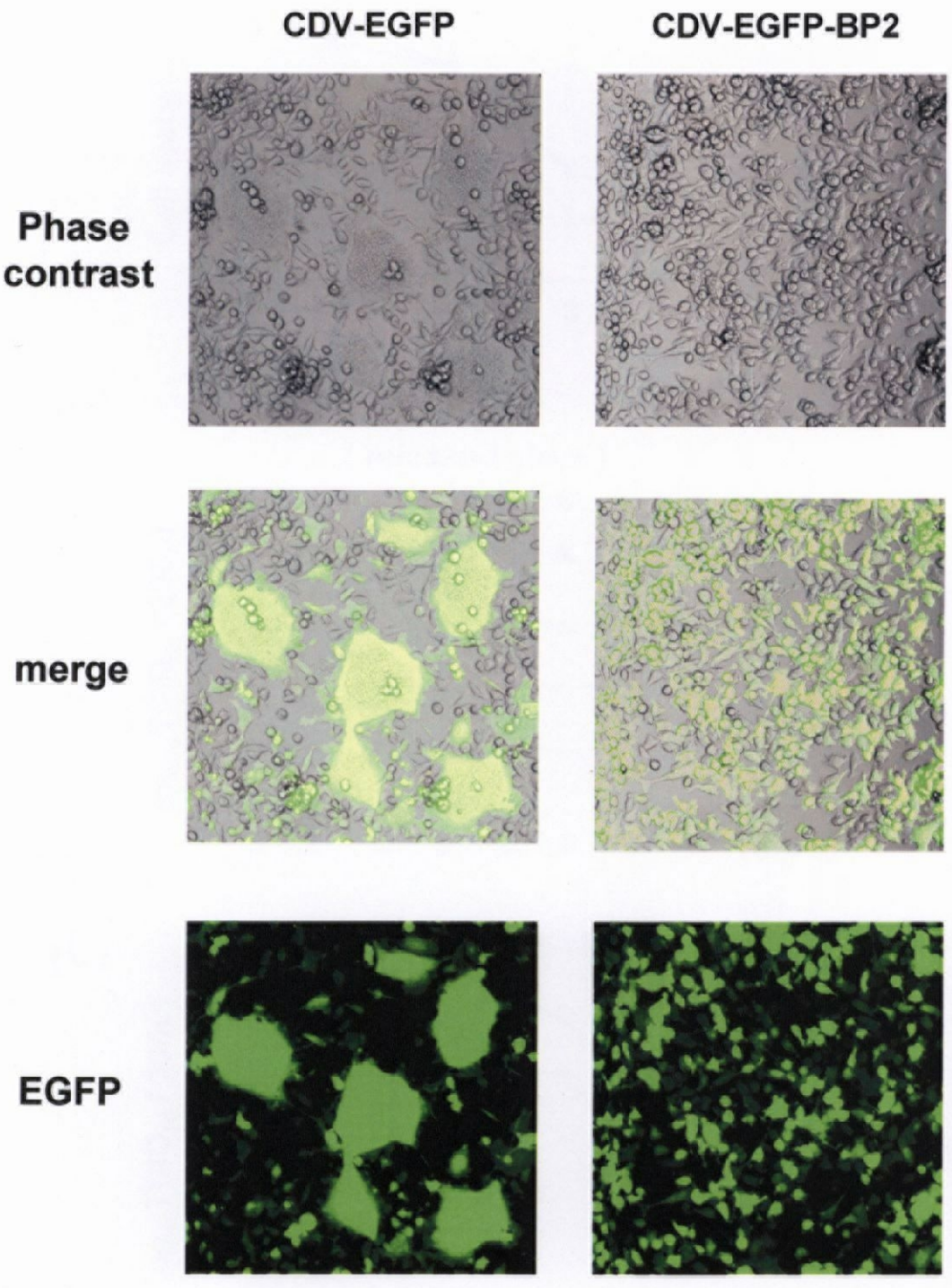


Fig. 1.3

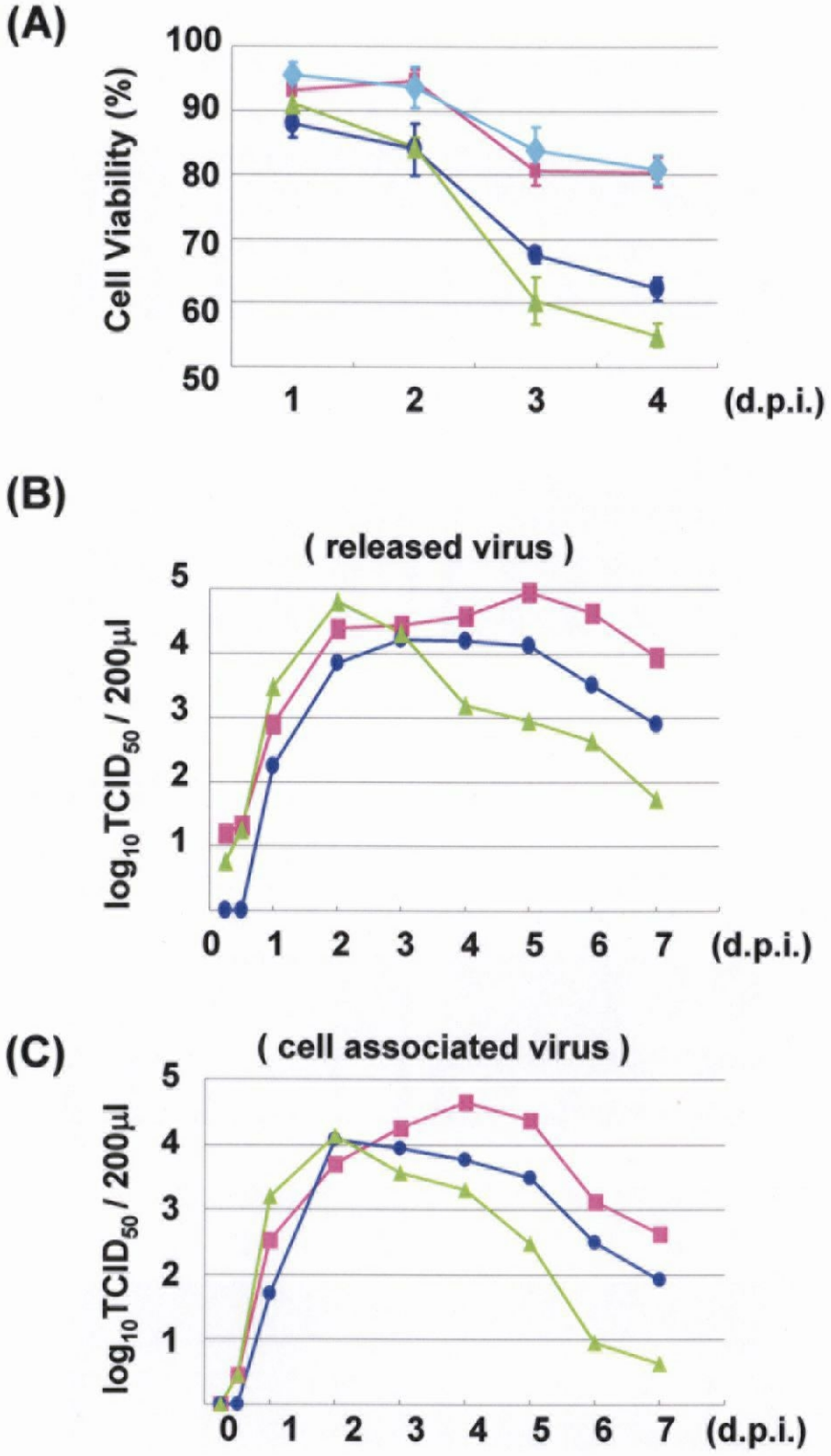
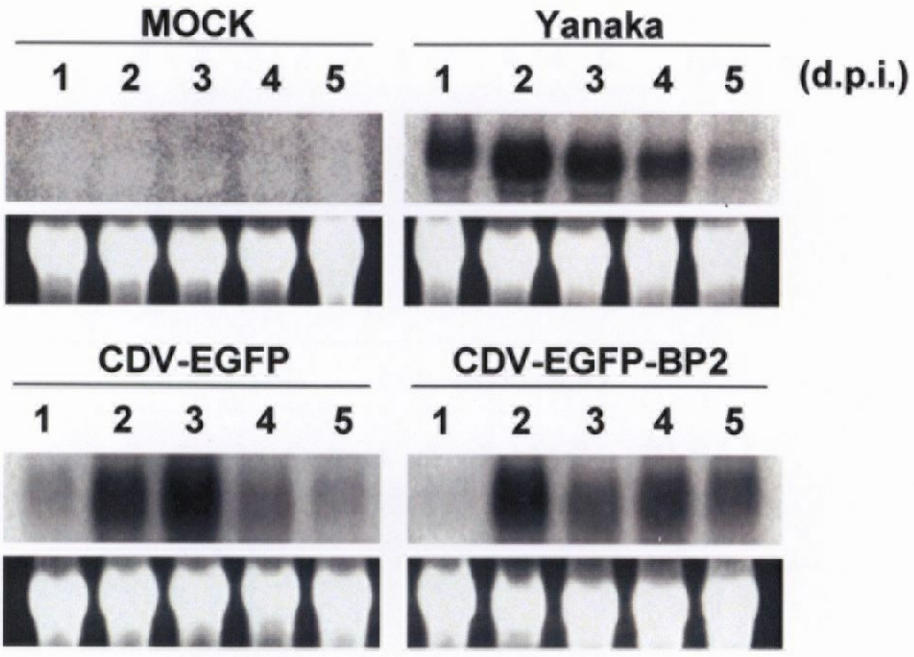
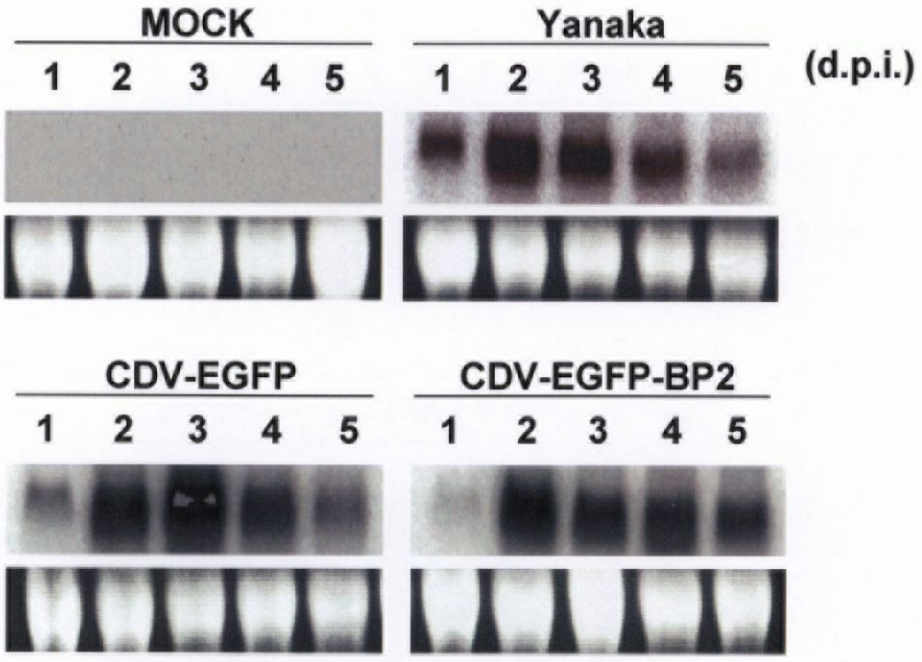

(A)
Cell Viability (%)
100
90
80
70
60
50
1
2
3
4
(d.p.i.)
(B)
(released virus)
log10TCID50 / 200µl
5
4
3
2
1
0
1
2
3
4
5
6
7
(d.p.i.)
(C)
(cell associated virus)
log10TCID50 / 200µl
5
4
3
2
1
0
1
2
3
4
5
6
7
(d.p.i.)

Fig. 1.4

(A) CDV H



(B) CDV F



(A) whole lysate

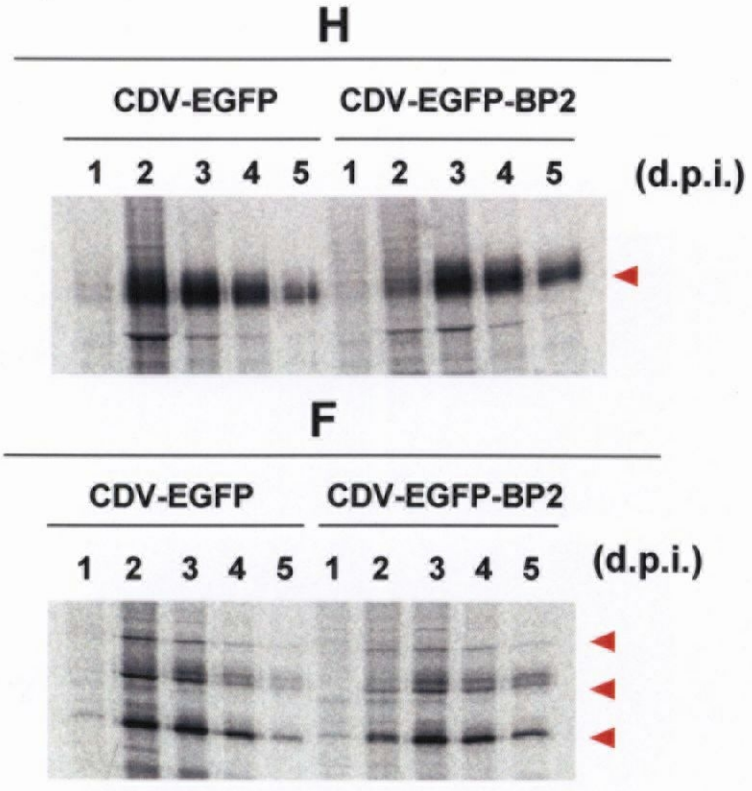


Fig. 1.5

(B) cell surface

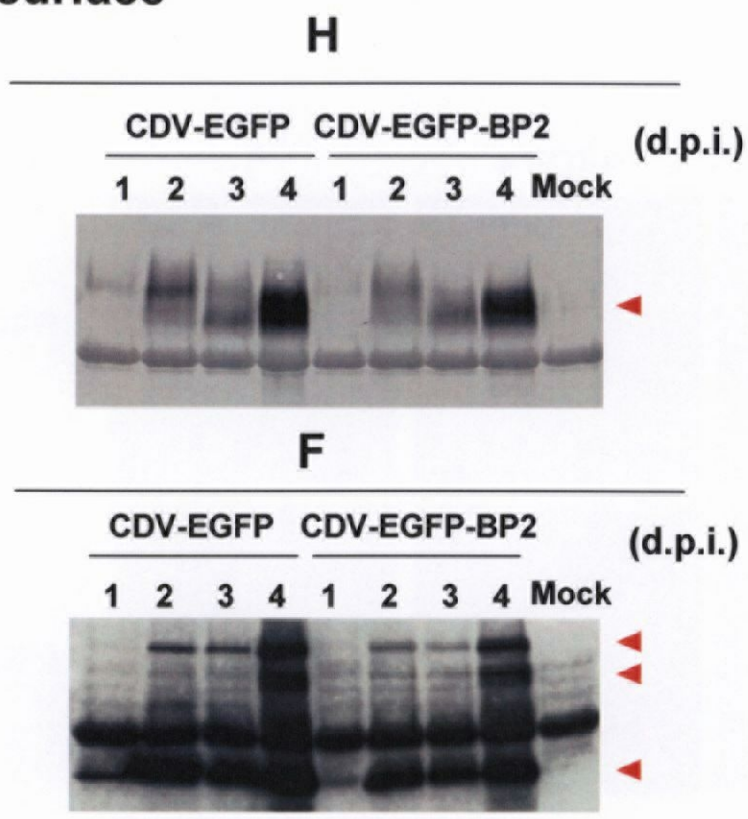


Fig. 1.6

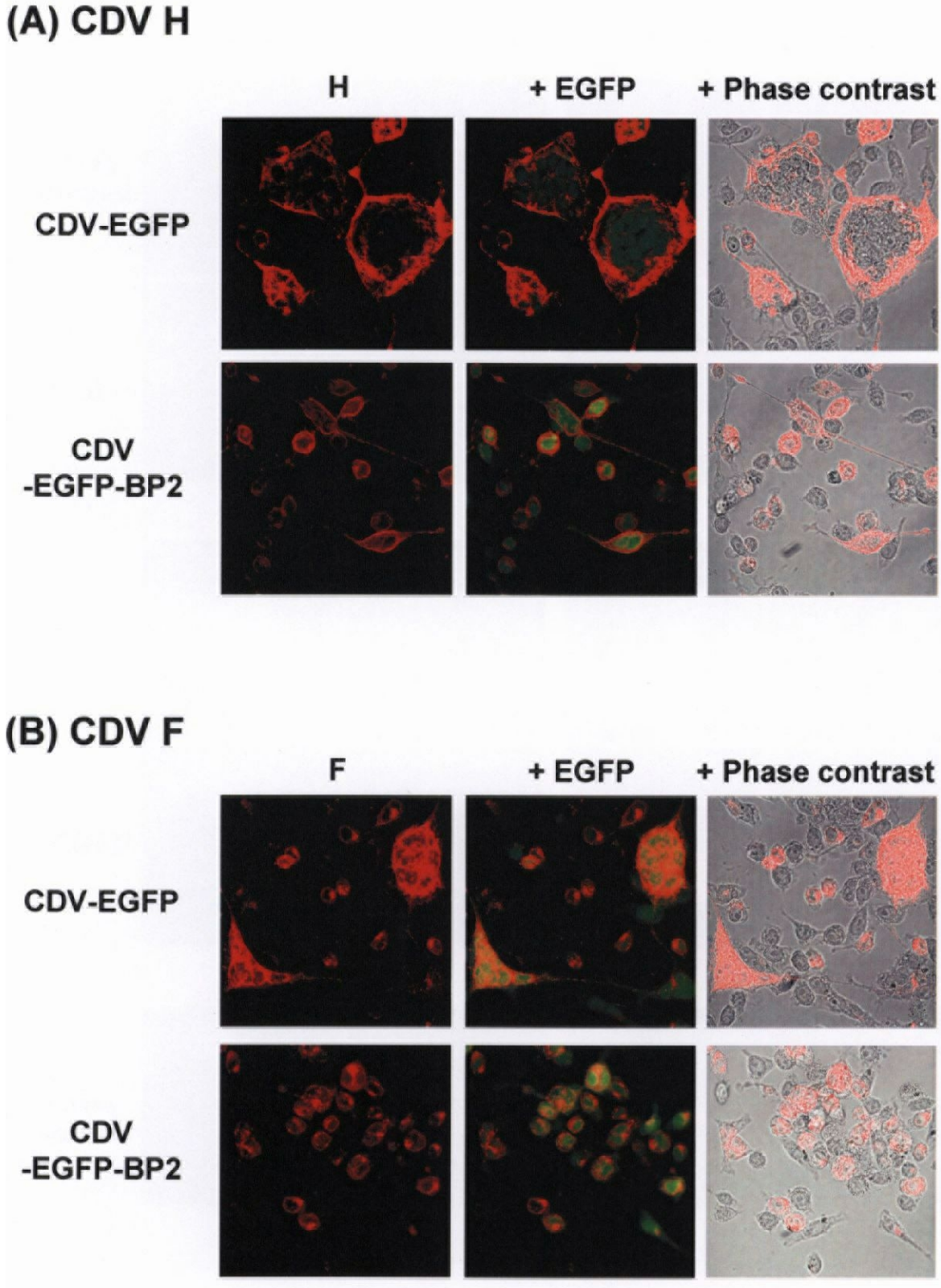

(A) CDV H
H
+ EGFP
+ Phase contrast
CDV-EGFP
CDV
-EGFP-BP2
(B) CDV F
F
+ EGFP
+ Phase contrast
CDV-EGFP
CDV
-EGFP-BP2

Fig. 1.7

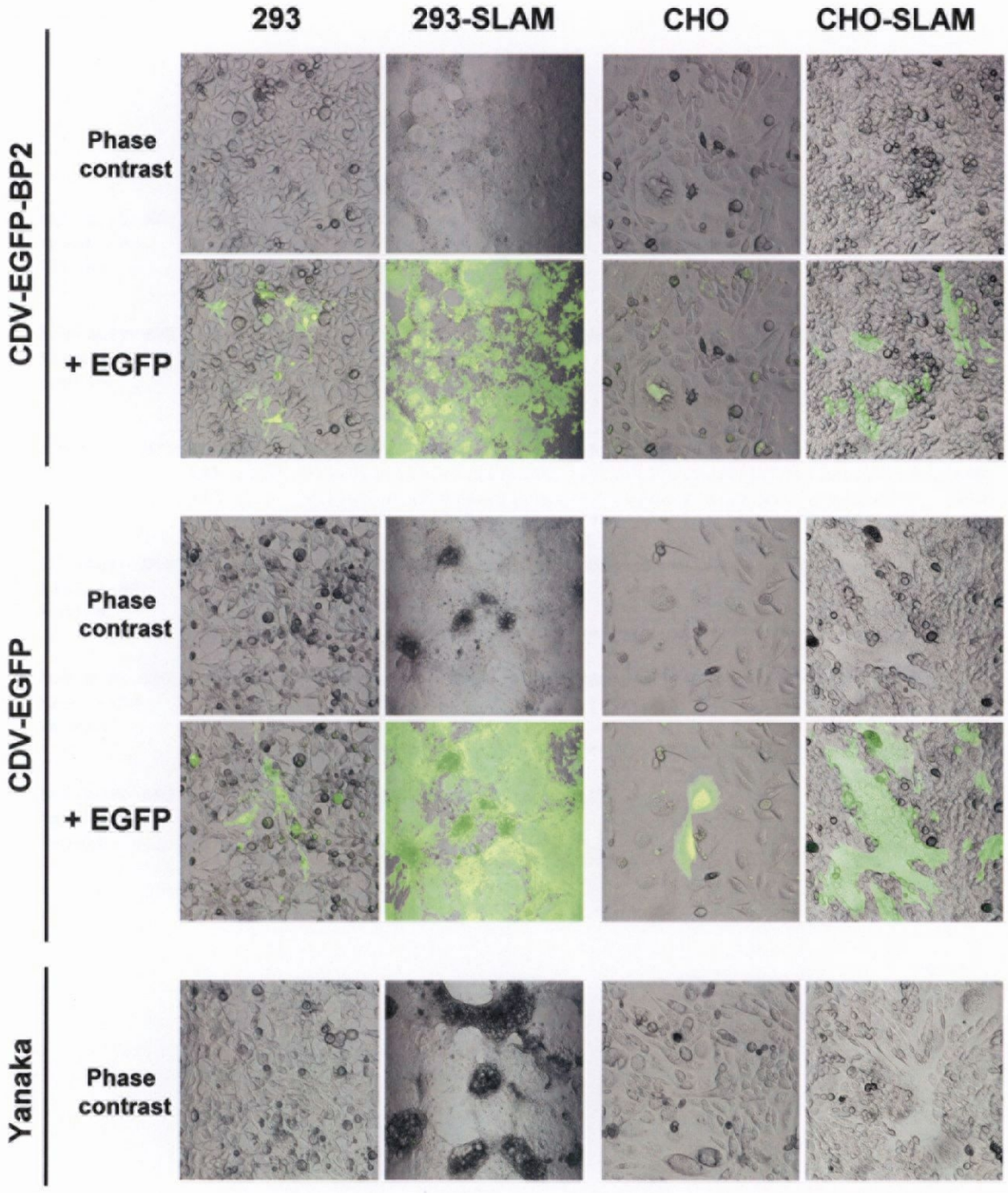

293
293-SLAM
CHO
CHO-SLAM
CDV-EGFP-BP2
Phase contrast
+ EGFP
CDV-EGFP
Phase contrast
+ EGFP
Yanaka
Phase contrast

Fig. 1.8 (A)


CDV-EGFP-BP2 1 MTEVYDFDQSSWNTKGSLAPILPTTYPDGRLVPQVRVIDPGLGDRKDECFMYIFLLGIIE 60
Yanaka-BP 1 .....D..... 60
Yanaka 1 .....D..... 60
CDV-EGFP-BP2 61 DNDGLGPPIGRTFGSLPLGVGRTTARPEELLKEATLLDIVVRRTAGVKEQLVFYNNTPLH 120
Yanaka-BP 61 .....I..... 120
Yanaka 61 .....T..... 120
CDV-EGFP-BP2 121 ILTPWKKVLTSGSVFSANQVCNAVNLIPLDIAQRFRVVYMSITRLSDDGSYRIPRGMFEF 180
Yanaka-BP 121 180
Yanaka 121 180
CDV-EGFP-BP2 181 RSRNALAFNILVTIQVEGDVCSSRGNLSMFKDHQVTFMVHIGNFSRKKNQAYSADYCKLK 240
Yanaka-BP 181 240
Yanaka 181 240
CDV-EGFP-BP2 241 IEKMGLVFALGGIGGTSLHIRCTGKMSKALNAQLGFKKILCYPLMEINEDLNRFLWRLEC 300
Yanaka-BP 241 300
Yanaka 241 300
CDV-EGFP-BP2 301 KIVRIQAVLQPSVPQDFRIYNDVIISDDQGLFKIL* 336
Yanaka-BP 301 .....* 336
Yanaka 301 .....* 336

Fig. 1.8 (B)

```
CDV-EGFP-BP2    1 MLSYQDKVGAFYKDNARANSSKLSLVTEEQGGRRPPYLLFVLLILLIGILALLAITGVRF   60
Yanaka-BP       1 ........................P.............L............S.......   60
Yanaka          1 ........................L.............F............L.......   60
                  ************************ ************ ************ *******

CDV-EGFP-BP2   61 HQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIGDEIGLRLPQKLNEIKQFILQ   120
Yanaka-BP      61 ............................................................   120
Yanaka         61 ............................................................   120
                  ************************************************************

CDV-EGFP-BP2  121 KTNFFNPNREFDFRDLHWCINPPSKIKVNFTNYCDTVGVKKSIASAANPIILSALSGARG   180
Yanaka-BP     121 ............................................................   180
Yanaka        121 ............................................................   180
                  ************************************************************

CDV-EGFP-BP2  181 DIFPPYRCSGATTSVGRVFPLSVSLSMSLISRTSDIINMLTAISDGVYGKTYLLVPDYIE   240
Yanaka-BP     181 ..................................E.........................   240
Yanaka        181 ..................................E.........................   240
                  ********************************* ************************

CDV-EGFP-BP2  241 GEFDSQEIRVFEIGFIKRWLNDMPLLQTTNYMVLPETSKAKVCTIAVGELTLASLCVDES   300
Yanaka-BP     241 ............................................................   300
Yanaka        241 ............................................................   300
                  ************************************************************

CDV-EGFP-BP2  301 TVLLYHDSNGSQNGILVVTLGIFGATPMDQVEEVIPVAHPSVERIHITNHRGFIKDSIVT   360
Yanaka-BP     301 ............................................................   360
Yanaka        301 ............................................................   360
                  ************************************************************

CDV-EGFP-BP2  361 WMVPALVSEKQEEQKNCLESACQRKSYPMCNQTSWEPFGGGQLPSYGRLTLLLDPSIDLQ   420
Yanaka-BP     361 ............................................................   420
Yanaka        361 ............................................................   420
                  ************************************************************

CDV-EGFP-BP2  421 LNISFTYGPVILNGDGMDYYESPLLDSGWLTVPPKNGTVLGLINKASRGDQFTVTPHVLT   480
Yanaka-BP     421 ............................................................   480
Yanaka        421 ............................................................   480
                  ************************************************************

CDV-DGFP-BP2  481 FAPRESSGNCYLPIQTSQIMDKDVLTESNLVVLPTQNFRYVIATYDISRGDHAIVYYVYG   540
Yanaka-BP     481 ............................................................   540
Yanaka        481 ............................................................   540
                  ************************************************************

CDV-EGFP-BP2  541 PIRTISYMYPFRLTTKGRPDFLRIECFVWDDDLWCHQFYRFEANITNSTTSVENLVRIRF   600
Yanaka-BP     541 .......T....................................................   600
Yanaka        541 .......M....................................................   600
                  ******* ********************************************************

CDV-EGFP-BP2  601 SCNRSKP*                                                      608
Yanaka-BP     601 .......*                                                     608
Yanaka        601 .......*                                                     608
                  *******
```

CHAPTER 2-1

Sequence Analysis of VP2 Gene of Canine Parvovirus isolated from Domestic Dogs in Japan in 1999 and 2000

SUMMARY

The VP2 genes of canine parvovirus (CPV) isolated in 1999 and 2000 from 7 domestic dogs in Japan were genetically analyzed. The predicted amino acid sequences of three virus isolates were corresponding to those of CPV type 2a and the others to CPV type 2b. The phylogenetic tree constructed from the VP2 genes showed that the isolated strains were classified into the cluster of the previous Japanese and Taiwanese field isolates, which were different from Vietnamese isolates and CPV type 2.

INTRODUCTION

Canine parvovirus (CPV) is a member of the feline parvovirus (FPV) subgroup and is classified into autonomous parvoviruses of the family *Parvoviridae* (genus *parvovirus*, subfamily *parvovirinae*). CPV is an important pathogen in domestic dogs and some wild carnivore animals. It causes an acute hemorrhagic gastroenteritis, leukopenia, nausea and occasionally fatal myocarditis in young puppies. It was first identified in 1978 in the USA [Appel et al., 1979] and was named CPV type 2 (CPV-2) to distinguish it from the previously recognized parvovirus known as canine minute virus (CMV or CPV-1), which is antigenically different from CPV-2 [Carmichael and Binn, 1981; Carmichael et al., 1994].

CPV appeared to be a host-range variant of feline panleukopenia virus (FPV) or a closely related virus of another carnivore [Parrish, 1990; Truyen et al., 1995]. After the initial appearance, antigenic drift was continuously observed. CPV-2 spread worldwide within a few years after that (during 1978 to 1981) and then the original type 2 was replaced by new genetic and antigenic variants, type 2a (CPV-2a), type 2b (CPV-2b) and type 2c (CPV-2c) [Parrish, 1991; Parrish et al., 1991; Mochizuki et al., 1996; Ikeda et al., 2000]. The variants CPV-2a and 2b have gained the infectivity in cats, although CPV-2 did not have a feline host range.

The new types of CPV have some nucleotide changes in the gene encoding the VP2 coat protein compared to the nucleotide sequence of the original CPV-2 [Parrish, 1991; Truyen et al., 1995]. The icosahedral non-enveloped capsid of CPV is formed of multiple copies of VP1, VP2 and VP3 [Mengeling et al., 1988]. VP2 protein is the major capsid protein and induces neutralizing antibody. Since only a few amino acids substitutions of VP2 are responsible for the antigenic properties [Truyen and Parrish, 1992; Strassheim et al., 1994; Yuan and Parrish, 2000], the amino acid sequence of VP2 could be a good candidate as a basis for the classification of CPV.

In this chapter, the author conducted genetic analysis of CPV VP2 gene of 7 recent CPV strains isolated from domestic dogs in Japan between 1999 and 2000 comparing to various strains of CPV in worldwide for better understanding of the recent epidemic of canine viral infectious disease.

MATERIALS AND METHODS

Virus isolation

CPV was isolated from 7 puppies aged 60-90 days old in various areas of Japan (Table 2-1.1). The dogs were clinically diagnosed with canine parvovirus infection with symptoms of nausea and hemorrhagic diarrhea and two of them died. All of the puppies were vaccinated with live attenuated vaccine based on CPV-2 3 to 27 days before showing these symptoms. For the virus isolation, the supernatant of feces was used to inoculate onto Crandell feline kidney cell line (CRFK, derived from feline kidney cortex) [Crandell et al., 1973], and within 5 days, cytopathic effect (CPE) was observed in all of the cells. The isolates were propagated in CRFK cells and the viral titers were measured by hemagglutination test.

VP2 gene amplification and sequencing

The VP2 gene was amplified by PCR using a set of primers, F (5'-^{2786} AAT GAG TGA TGG AGC AGT TC -3') and R (5'-^{4590} TTC TAG GTG CTA GTT GAT ATG -3'), whose sequences correspond to the 5' and 3' regions of the VP2 genes in the CPV-b genome, respectively [GenBank accession no. M38245] [Parrish, 1991]. The PCR products were separated by 1 % agarose gel electrophoresis and the desired DNA fragment (1744 bp) was extracted and then

directly sequenced using a Big Dye Terminator cycle sequence kit (Applied Biosystems Inc., CA, USA) and using internal VP2 gene sequence primers: Fc1 (5'-^{3115} CAT TGG TTG ATG CAA ATG CT -3'), Fc2 (5'-^{3449} TCA TAC TGG AAC TAG TGG CA -3'), Rc (5'-^{4179} TCC CAA ATT TGA CCA TTT GG -3'), VP2 start (5'-^{3096} GTG CAT GGG TAT CAT CTA AAG C -3') and VP2 stop (5'-^{4224} CCA AGA CTT CAT GTA AAT GCA CC -3') .

Construction of the Phylogenetic trees based on the VP2 gene

Phylogenetic trees were constructed from the VP2 gene nucleotide sequences of the CPV strains isolated in this study and other sequences obtained from the GenBank database with PHYLIP program (Fig. 2-1.1) [Felsenstein, 1995] (Accession number of each strains; M19296 (CPV-N), M38245 (CPV-b), D26079 (CPV-Y1), M24000 (CPV-31), M24003 (CPV-15), M74852 (CPV-133), M74849 (CPV-39), AB054214 (LCPV T1), AB054220 (V217), AB054219 (V209), AB054224 (LCPV V203), AB054221 (LCPV V204), AB054218 (V123), AB054223 (LCPV V140), AB054215 (V120), AB054217 (V154), D78585 (FPV-314), AB054213 (Taiwan 9), AY742933 (CPV-339), AY742934 (CPV-447), AY742936 (CPV-395), AY742951 (CPV-431), AY742953 (CPV-435), AY742935 (CPV-U6), AB120721 (HCM-8), AB120722 (HCM-18), AB120723 (HCM-23), AB120727 (HNI-4-1), AB128923 (Sho-nan), AY869724 (Taichung) and DQ177497 (HN-3)) [Reed et al., 1988; Parrish et al., 1988b;

Parrish, 1991; Horiuchi et al., 1994; Ikeda et al., 2000; Nakamura et al., 2004; Hirayama et al., 2005; Shackelton et al., 2005; Wang et al., 2005; Yeung et al., 2000].

RESULTS

The nucleotide sequences of the PCR products of 7 isolates were not identical to that of the CPV-2 VP2 gene. Therefore, the possibility of the appearance of a revertant virulent CPV from the vaccination strain was excluded. The disease might instead have been caused by infection with field strains of CPV before proper immunity had been developed in the puppies, and the vaccine failure might have been due to the presence of maternal antibodies. Nucleotide changes of the 7 isolates are shown in Table 2-1.2. Not a few mutations were observed among the new isolates.

Common substitutions among the 7 isolates compared with a type2 strain (CPV-b) were observed at nt 3089 and nt 3675. Sequence alignment analyses showed that there were different silent mutations and few coding changes in the VP2 gene. Comparison of our Japanese isolates with other CPV strains isolated (sequences obtained from the GenBank database accession numbers; M19296, M38245, D26079, M24000, M24003, M74852, M74849, AB054214, AB054220, AB054219, AB054224, AB054221, AB054218, AB054223, AB054215, AB054217, D78585, AB054213, AY742933, AY742934, AY742936, AY742951, AY742953, AY742935, AB120721, AB120722, AB120723, AB120727, AB128923, AY869724 and DQ177497) revealed nucleotide changes at nt 3675 and 4062 resulting in amino acid substitutions at residues 297 (from a

serine to an alanine) and 426 (from an asparagine to an aspartic acid) of VP2 protein. In particular, a coding mutation at nt 3675 was detected in almost all new Asian strains. The nucleotide mutation at nt 4062 was observed in all CPV type 2b isolates, and residue 426 could distinguish between type 2a and 2b. Synonymous mutations were commonly found at nt 2822, 2933, 3089, 3467, and 4076 in Asian strains. Especially, mutations at nt 2933, 3089 and 3467 found in the Japanese new isolates were identical to mutations found in Taiwanese strains. There were characteristic mutations at nt 3191, 3425, 3485, 3657, 4077, 4295 and 4481 in our isolates. Comparison of the sequences of our isolates with previously reported sequences indicated that 3 of our isolates (IJ-C-11, IJ-C-12, IJ-C-13) were classified into CPV type 2a strains, while the other 4 strains (IJ-C-14, IJ-C-29, IJ-C-30, IJ-C-31) were type 2b.

Comparison of the VP2 gene sequences showed 99.8 to 99.4% nucleotide identity between our isolates and each of the typical strains of CPV: CPV-b (type 2), CPV-15 (type 2a) and CPV-39 (type 2b). The predicted amino acid sequences showed 99.8-99.0% identity. Phylogenetic trees were constructed from the VP2 gene nucleotide sequences of the CPV strains isolated here and other sequences obtained from the GenBank database (Fig. 2-1.1) [Felsenstein, 1995].

DISCUSSION

Currently, CPV-2a and CPV-2b are prevalent in different countries. CPV-2a is major in Germany and Italy, while CPV-2b is commonly in United States, Taiwan and Japan [Truyen et al., 1996; Martella et al., 2004; Decaro et al., 2005; Desario et al., 2005; Hirayama et al., 2005]. Additionally, since there are not a few mutations observed among the new isolates and new antigenic type CPV-2c remarkably increasing according to the recent studies in Italy [Martella et al., 2004; Decaro et al., 2005; Desario et al., 2005]. In this phylogenetic tree constructed from the VP2 genes, there are three distinctive groups based on period and area of isolation. The isolates were classified into a cluster consisting of the Japanese and Taiwanese field isolates, which were different from Vietnamese isolates and American strains. The isolates were also classified into a cluster consisting of the recent Asian wild isolates, which were different from old American CPV strains. The mutation has been found locally, that 297th animo acid S (United States) changes to A (Germany, Japan, Taiwan) [Truyen 1999]. These results indicate that the cases of CPV infection analyzed in this study were caused by field CPV strains of East Asia.

FIGURE LEGEND

Figure 2-1.1 Phylogenetic tree constructed from the nucleotide sequences of the VP2 gene of CPV strains.

CPV strains generated in this study are underlined and other sequences were obtained from the GenBank database. JPN, Japan; TWN, Taiwan; US, United States; VTN ,Vietnam; GE, Germany; NZ, New Zealand.

Table 2-1.1 CPV isolates in Japan

Virus	Year	Region	Dog strain	Age (days)	Clinical presentation	Vaccination before crisis (days)
IJ-C-11	1999	Tokyo	Shiba	60	Nausea, hematochezia*	6
IJ-C-12	1999	Kyoto	Miniature Dachshund	60	Nausea, diarrhea	14
IJ-C-13	1999	Osaka	Mongrel	90	Nausea, diarrhea	14
IJ-C-14	1999	Shiga	Mongrel	60	Nausea, diarrhea	3
IJ-C-29	2000	Niigata	Shih Tzu	68	Fever, hematochezia*	12
IJ-C-30	2000	Aichi	Yorkshire Terrier	70	Nausea, hematochezia	27
IJ-C-31	2000	Niigata	Shiba	90	Bellyache, mucous feces	18

*The dogs were died.

Table 2-1.2 Variable nucleotides in the sequence of the VP2 genes.

		Nucleotide position														
	Complete genome	2822	2933	3065	3089	3191	3425	3467	3485	3657	3675	4062	4076	4077	4295	4481
	CPV strain VP2 gene	36	147	279	303	405	639	681	699	871	889	1276	1290	1291	1509	1695
Type 2	CPV-b(US,1978)	A	G	C	T	G	A	C	C	C	T	A	G	C	G	T
New isolates	IJ-C-11 (JPN,1999)	G	-	-	C	-	-	-	-	-	G	-	-	-	-	-
	IJ-C-12 (JPN,1999)	G	-	-	C	-	-	-	-	T	G	-	-	-	-	-
	IJ-C-13 (JPN,1999)	-	A	-	C	-	G	G	-	-	G	-	-	-	-	-
	IJ-C-14 (JPN,1999)	G	-	-	C	-	-	-	-	-	G	G	A	T	A	C
	IJ-C-29 (JPN,2000)	G	-	-	C	A	-	-	T	-	G	G	-	-	A	-
	IJ-C-30 (JPN,2000)	-	-	-	C	-	-	G	T	-	G	G	-	-	-	-
	IJ-C-31 (JPN,2000)	-	-	T	C	-	-	G	-	-	G	G	-	-	-	-
Type 2a	CPV-31 (US,1983)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CPV-Y1 (JPN,1982)	-	-	-	A	-	-	-	-	-	-	-	A	-	-	-
	FPV-314 (JPN,1993)	G	-	-	C	-	-	-	-	-	G	-	-	-	-	-
	LCPV V140 (VTN,1997)	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-
	Taiwan 9 (TWN,1998)	-	A	-	C	-	-	G	-	-	G	-	-	-	-	-
	CPV-435 (US,2003)	-	-	-	-	-	-	G	-	-	G	-	-	-	-	-
	CPV-339 (NZ,1994)	G	-	-	-	-	-	G	-	-	G	-	-	-	-	-
Type 2b	CPV-39 (US,1984)	-	-	T	-	-	-	-	-	-	-	G	-	-	-	-
	CPV-431 (US,2003)	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-
	V123 (VTN,1997)	-	A	-	-	-	-	-	-	-	G	G	-	-	-	-
	Sho-nan (JPN,2003)	G	-	-	C	-	-	-	-	-	G	G	-	T	A	C
	Taichng (TWN,2004)	G	-	-	C	-	-	-	-	-	G	G	-	-	A	-
											aa297 S-A	aa426 N-D				

*The nucleotides identical to those in CPV-b are indicated by dashes, while the nucleotides that differ from those in CPV-b are indicated by letters. Nucleotide changes that result in amino-acid substitutions are indicated by the residue position of VP2 protein and the amino the acid changes.

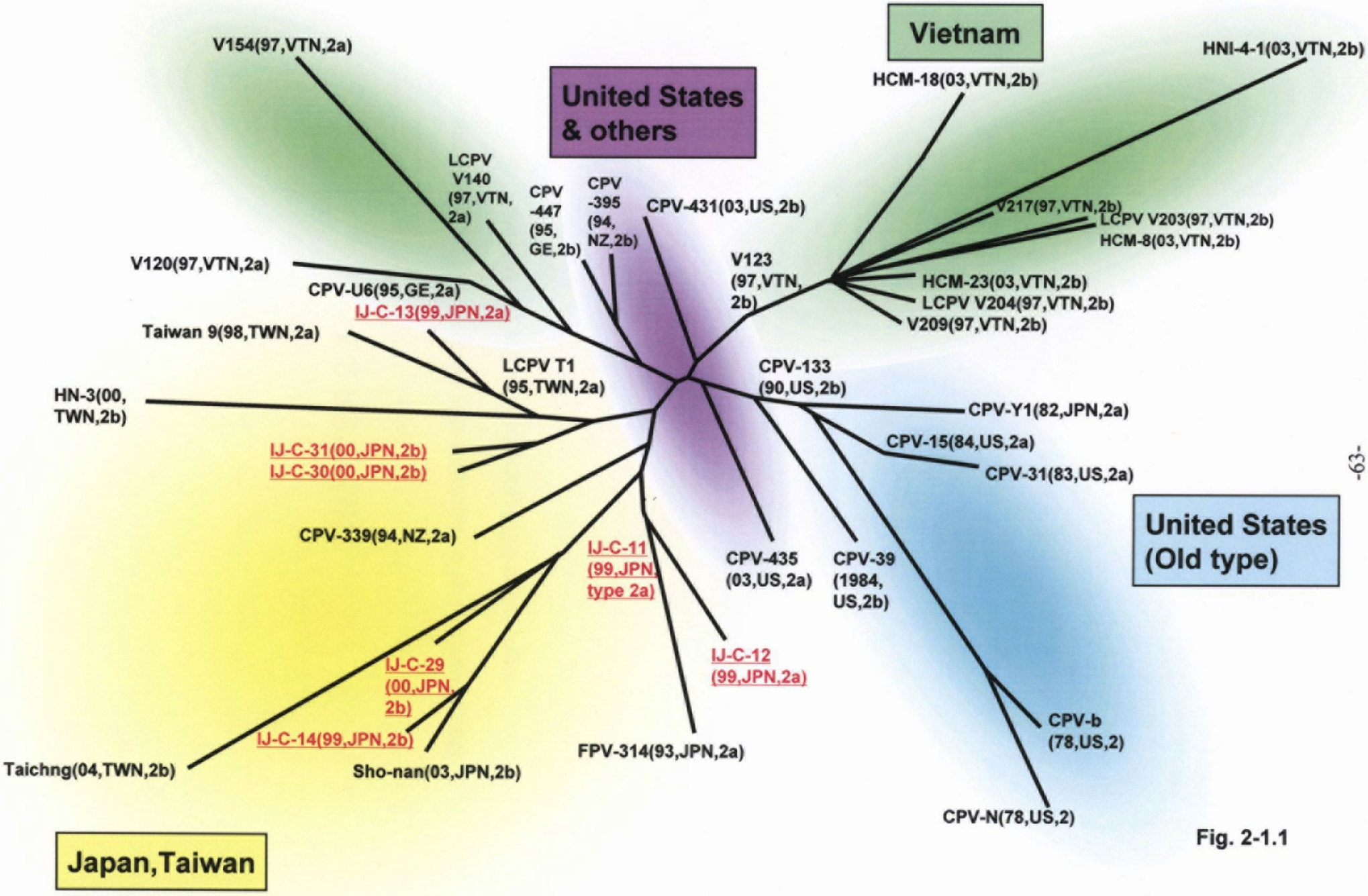

Vietnam
United States & others
United States (Old type)
Japan,Taiwan
V154(97,VTN,2a)
HCM-18(03,VTN,2b)
HNI-4-1(03,VTN,2b)
LCPV V140 (97,VTN, 2a)
CPV -447 (95, GE,2b)
CPV -395 (94, NZ,2b)
CPV-431(03,US,2b)
V217(97,VTN,2b)
LCPV V203(97,VTN,2b)
HCM-8(03,VTN,2b)
V120(97,VTN,2a)
V123 (97,VTN, 2b)
HCM-23(03,VTN,2b)
LCPV V204(97,VTN,2b)
V209(97,VTN,2b)
CPV-U6(95,GE,2a)
IJ-C-13(99,JPN,2a)
Taiwan 9(98,TWN,2a)
LCPV T1 (95,TWN,2a)
CPV-133 (90,US,2b)
HN-3(00, TWN,2b)
CPV-Y1(82,JPN,2a)
CPV-15(84,US,2a)
IJ-C-31(00,JPN,2b)
IJ-C-30(00,JPN,2b)
CPV-31(83,US,2a)
CPV-339(94,NZ,2a)
IJ-C-11 (99,JPN, type 2a)
CPV-435 (03,US,2a)
CPV-39 (1984, US,2b)
IJ-C-12 (99,JPN,2a)
IJ-C-29 (00,JPN, 2b)
CPV-b (78,US,2)
IJ-C-14(99,JPN,2b)
FPV-314(93,JPN,2a)
Taichng(04,TWN,2b)
Sho-nan(03,JPN,2b)
CPV-N(78,US,2)

Fig. 2-1.1