CHAPTER 2-2

Development of a recombinant canine distemper virus harboring VP2 gene of Canine Parvovirus

SUMMARY

In this chapter 2-2, the author generated recombinant CDVs harboring the gene of the CPV-2 VP2 protein and investigated the characteristic of the recombinant virus designated CDV-CPV-VP2. The size of CPE and growth kinetics caused by CDV-CPV-VP2 was as similar as the parental strain CDV-Yanaka. Northern blot analysis showed that VP2 mRNA was detected as almost bicistronic N-VP2 mRNA and little monocistronic VP2 mRNA expressed. By immunoprecipitation assay and immunofluorescence staining, CDV-CPV-VP2 produced no detectable VP2 probably due to inadequate bicistronic mRNA caused by read-through. Therefore, additional infectious cDNA clones that possessed DNA fragment of EGFP-VP2, VP2-transmembran domain (tm) or EGFP-VP2-tm were constructed. However, these clones had not produced recombinant CDV, suggesting that VP2 may prevent virus propagation and host cell survival. These results can contribute to the development of polyvalent CDV vaccines and a new type of viral vectors for gene transfer approaches.

INTRODUCTION

Traditional vaccines consist of either attenuated or inactivated microorganisms delivered by injection. Despite avirulent strains used, attenuated vaccines have still a potential to cause severe diseases in immunodeficient individuals, and inactivated vaccines sometimes cause side effects by adjuvant and are unable to induce long term immunity. To improve vaccines, new generation vaccine is contenuously researched, such as subunit vaccines and recombinant vaccines. Subunit vaccine has an advantage for convenient production and for safety but still has a disadvantage for induction of long term immunity. Recombinant vaccines have an advantage to induce stronger and more prolonged immune responses and be effective at eliciting protective cell-mediated immunity because of self-replicating. Additionally, recombinant vaccines will be available for prevention of emerging and re-emerging infectious diseases whereas attenuated or inactivated vaccines are unable to be rapidly prepared for them.

There are various virus vectors, such as vaccina virus, adenovirus, adeno-associated virus and retroviruses. In particular the power to manipulate the genome of negative-strand RNA viruses, including the insertion of additional viral or non-viral genes, has led to the development of a new type of viral vectors for gene transfer approaches. MV, murine parainfluenza virus type 1,

Sendai virus, rabies virus, vesicular stomatitis virus, human parainfluenza virus type 3, 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]. The negative-strand RNA viruses replicate in cytoplasma without any risk of chromosomal integration, are enable to express foreign genes well.

Canine distemper virus (CDV) belongs to the genus Morbilliviruses of the family Paramyxoviridae, and is a causative agent of canine distemper (CD). Affected dogs by CDV usually showed respiratory, gastrointestinal and neurological signs. We determined the complete sequence of the Yanaka strain, one of recent Japanese isolates [Gemma et al., 1996a] and established its reverse genetics [Fujita, 2002]. The new reverse genetics of CDV enabled us to develop a new vaccine or gene tranfer vector with CDV. [Kooriyama, 2004; Plattet et al., 2004; Parks et al., 2002; Gassen et al., 2000; Jung et al., 2005].

Canine parvovirus (CPV) was first observed in the late 1970s when a new disease of dogs was observed: myocarditis leading to sudden death in neonatal pups, and enteritis accompanied by diarrhea in dogs older than 2 months. The virus infection rapidly spread in wild and domestic dogs in every region of the globe [Parrish, 1990]. After the emergence, it was clarified that

CPVs isolated were antigenically closely related to feline parvovirus (FPV), and subsequently the viruses were more than 99% identical in DNA sequence [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 it has been also 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 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.

CPV is non-enveloped, isometric particles with a capsid formed by several proteins: VP1, VP2 and VP3, and their genome is a linear molecule of single-stranded DNA (ssDNA), and 4-6 kb in size. CPV replicates in the nucleus. The viruses possess three structural proteins VP1, VP2 and VP3. 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 for host immunity. VP2 gene encoded the full length of 1755 bp and 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 titres of VP2 peptide-specific neutralizing antibody in vaccinated dogs and were completely protected from clinical disease. In chapter 2-2, author attempted to generate a recombinant CDV expressing the half of VP2 (1-334 aa).

MATERIALS AND METHODS

Cells, viruses and genes

293 cells (a human embryonal kidney cell line) and CRFK cells (a feline kidney cell line) were maintained in Dullbecco's modified Eagle's medium (DMEM) (Sigma) containing 5% fetal calf serum (FCS). B95a cells (an Epstein-Barr virus transformed marmoset lymphoblastoid cell line) [Kobune et al., 1990] were maintained in RPMI1640 (Sigma) containing 5% FCS. The Yanaka strain of CDV [Gemma et al., 1996a] and rescued virus was grown in B95a cells. The host range mutant of recombinant vaccinia virus Ankara (MVA-T7), which expresses T7 RNA polymerase, was a kind gift from Dr G. Shutter. Canine parvovirus IJ-C-12 strain was one of Japanese field isolates described in the chapter 2-1.

Plasmid constructions and virus rescue

The author planed to generate recombinant CDV expressing a part of VP2 (1-334 aa) in canine parvovirus (Fig.2-2.1). Infectious plasmids of CDV and the procedure for virus rescue were described in chapter 1. Briefly, DNA fragments of VP2 were obtained by PCR using specific primers as follows;

VP2-F-in;

5'- TAAAAAACTTAGGACCCAGGTAGTCCAACAATGAGTGATGGAGCA -3'

and VP2-R; 5'- GGCCGGCCTTAAGCTGGTCTCATAATA -3' (*FseI* site underlined). As PCR templates, DNA extracted from CPV infected cells were used. These fragments were subcloned and these sequences were confirmed. The DNA fragments were attached with *FseI* recognition sequence and CDV transcription unit at 5' ends of the above primers (rCDV-F-out; 5'-GGCCGGCCTCTAAACTCATTATAAAAAACTTAGGACCCAGGTAG -3', *FseI* site underlined). Amplified cDNA fragment was subcloned into TA cloning vector pCR2.1^{TM} (Invitrogen). The plasmid was digested by *FseI* and then the cDNA fragment was purified by Wizard SV gel and PCR clean-up system (Promega). It was inserted into the *FseI* site located between N and P genes of a cDNA clone, pCDV coding the genome of the Yanaka strain [Fujita, 2002]. The obtained cDNA clones were applied for the virus rescue. The DNA sequences were confirmed by Big dye terminator and ABI377 or ABI310 model sequencers (Applied Biosystem). For virus rescue, 293 cells were prepared at 70% confluency in 6-well plates with 5% FCS added DMEM. The cells were infected with MVA-T7 at the MOI of 1 for 30 min. Following MVA-T7 infection, each plasmid was transfected into 293 cells with supporting plasmids (pRPV-N, pRPV-P and pRPV-L) as mixtures using Fugene 6 (Roche). After 3 days incubation at 37°C, the medium containing 2% FCS was removed and B95a cells ($1x10^6$ per well) were spread on 293 cells with RPMI1640 medium. After the appearance of cytopathic effect (CPE) under microscope, cells and

supernatants were harvested and store at -80°C. For confirmation of rescued CDV, RT-PCR was performed using primers amplified between N and P sequence and the obtained PCR fragments were determined.

Preparation of recombinant VP2 antigens and their polyclonal antibodies

VP2 cDNA (1-1055 nt) was ligated to *E.coli* protein expression vector pGEX-4T-2 (Novagen), by which recombinant protein was expressed as a fusion protein with glutathione S transferase (GST). The plasmid was transformed into BL21 competent cells for high-level protein expression and one-litter culture was induced to express the protein at mid-log phase of growth (0.2 OD_{600}) by addition of 1mM IPTG. After 3 hr incubation, *E. coli* was pelleted and washed with PBS. The pellet was lysed by lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA and 1 mM DTT) and centrifuged at 15,000 x*g* for 30 min to remove insoluble materials. Recombinant VP2 was mainly in insoluble fraction. The VP2 protein containing the C-terminal GDT in the supernatant was affinity purified with glutathione sepharose column 4B (Amersham bioscience). VP2-GST protein (2mg) were mixed with RIBI adjuvant (Corixa) and immunized to rabbits twice with two weeks interval. Sera were collected at 16 days after first immunization. Non-specific antibody in the sera were removed with liver powder for 3 hr at 4°C followed by inactivation of complements at 55°C for 30min. Reactivity of antibodies was confirmed by

ELISA and western blot analysis.

Indirect immunofluorescence staining

CDV infected B95a cells, CPV infected CRFK cells and VP2 expressing plasmids tranfected 293 cells were prepared. The cells were fixed by 4% formaldehyde for 30 min at room temperature and then washed three times in PBS. The cells were consecutively processed for 120 min with the first antibody. The polyclonal anti-VP2 rabbit sera (diluted at 1:200 in PBS) and mAb anti-CDV-H (d-7: diluted at 1:1000) were used as first antibody. After washed by PBS as well, the cells were reacted with the secondary antibody for 60 min. The goat anti-rabbit antibody-FITC (Alexa Flour 488, Molecular probes, Cochranville, PA) (diluted at 1:1000), goat anti-mouse antibody-sulforhodamine (Alexa Flour 568) (diluted at 1:1000) and goat anti-rabbit antibody-sulforhodamine (Alexa Flour 568) were used as secondary antibody. After washing in PBS 3 times, imaging analysis was performed with confocal microscopy, Fluoview FV500 system (Olympus Optical Co. Ltd, Tokyo).

Radioimmunoprecipitation assay

Virus-infected cells or mock-infected cells were radiolabelled with 0.2 μCi [^{35}S] methionine (DuPont NEN research products, Boston, MA) for 4 hrs. The radiolabelled cells were lysed by RIPA buffer (1% Triton X-100, 1 mM

iodoacetamide, 0.2 U/ml of the trypsin inhibitor aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1% sodium deoxycholate, 0.14 M NaCl, and 10 mM Tris-HCl at pH 8.0). The lysate was incubated with mixture of rabbit serum against VP2 protein (diluted at 1:500) and protein A beads (Amersham biosciences) for overnight at 4 °C with gently rocking. The beads were washed with 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.

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. Twenty 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 CPV-VP2 or CDV-N 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 manufacturer's

instruction. The membranes were exposed to imaging plate and the detection was performed using FLA5000 (Fuji film).

Virus titration

Multistep growth kinetics was analyzed by infecting B95a cells with viruses at an moi of 0.01 in 12-well plates. Virus was adsorbed to the cells for 30 min, and the inoculum removed. Then, 1ml of medium was supplied into each well. Virus induced CPE was visualized by infecting B95a cells at 48hrs post infection. At every 24 hrs from 0 hr to 144 hrs, the cells with medium were harvested and frozen at -80°C. The suspension was frozen and thawed three times and the released virus was determined by endpoint dilution assays to calculate 50% tissue culture infectious dose ($TCID_{50}$) values as standard method [Iwatsuki et al., 1995].

Construction of plasmids expressing VP2 fusion protein

DNA fragment encoding antigenic region VP2 (1-334 aa) was inserted into pEGFP-C1 or pDisplay for EGFP fusion or expression on the plasma membrane respectively. Additionally, DNA fragment encoding EGFP and VP2 was also inserted into pcDNA or pDisplay (Invitrogen). *E.coli* transformed with the plasmids grew and the plasmids were collected by standard method. The plasmids transfected cells by Fugene 6 were visualized by confocal microscopy,

Fluoview FV500 system.

RESULTS

Rescue of recombinant CDVs expressing VP2 antigen derived from CPV

The author attempted to generate recombinant virus expressing a viral antigen, derived from CPV-2a using CDV as a viral vector (Fig.2-2.1). The CDV vector was derived from a field isolate in Japan and its infected dogs had no clinical sighs [Kooriyama and Takenaka in preparation]. As an antigen, capsid protein VP2 of CPV IJ-C-12 strain (See chapter 2-1) was selected. CPV VP2 gene (1755 bp) encoded a protein of 585 amino acids and a part of VP2 encoding 1 to 334 amino acids were used because important domain such as neutralization subsites and nuclear localization signal are located in the N terminus of VP2 [Casal et al., 1995; Vihinen-Ranta et al., 1997]. At first, the author constructed *E.coli* expression system of CPV-VP2 (1-334 aa) and generated specific polyclonal antibody against VP2 by immunization of rabbit (Fig. 2-2.2). The cDNA was attached with a transcription unit of CDV and inserted into a cloning site between CDV N and P genes into infectious cDNA clone [Fujita, 2002]. The plasmid was applied for our standard CDV reverse genetics system using 293 cells and MVA-T7, a recombinant vaccinia virus expressing T7 RNA polymerase [Fujita, 2002]. At a week after overlay of B95a cells on 293 cells, the typical cytopathic effect (CPE) was observed in B95a cells transfected with the infectious clone (Fig. 2-2.3(A)). The virus was

successfully rescued, designated CDV-CPV-VP2. The size of syncytia caused by CDV-CPV-VP2 was as similar as the parental strain CDV-Yanaka.

Characteristics of the recombinant CDV

Foreign gene insertion in the recombinant virus was confirmed by RT-PCR using set of specific primers, which encoded the downstream of N gene and upstream of P gene. The amplified DNA fragment was shown in expected sizes and consistent with the sequence (Fig. 2-2.3 (B)). The CPE formation and virus growth kinetics by the CDV-CPV-VP2 was inoculated B95a cells were almost similar to the parental Yakana strain (Fig. 2-2.3 (A), (C)).

Expression of foreign antigen in the recombinant CDV infected cells

To confirm the expression of virus antigen by CPV-VP2, the infected B95a cells were analyzed by immunofluorescence staining and immunoprecipitation. However, the VP2 expression was not found in CDV-CPV-VP2 (Fig. 2-2.4 (A), (B)). Northern blot analysis showed that VP2 mRNA was detected as a large amount of bicistronic N-VP2 mRNA and little monocistronic VP2 mRNA (Fig. 2-2.5). The bicistronic mRNA is composed of N gene and the foreign gene and such skipping over transcriptional end is known to be a "read-through". The monocistronic mRNA can translate the foreign antigen protein, whereas the bicistronic mRNA can produce only N protein

without the antigen protein synthesis. Thus these results indicate that the read-through produced a large number of inadequate mRNA for VP2 expression.

Analysis of fusion proteins with CPV-VP2

As described above, CDV-CPV-VP2 produce no detectable VP2 due to the inadequate mRNA caused by read-through. One possible explanation for the outcome is that only viruses which do not express VP2 was selectively rescued because the antigen expression may hampered CDV replication or host cell survival. Another possibility is that nucleotide sequence of VP2 contained a specific sequence that induced a large number of read-through. Thus, the author constructed plasmids expressing VP2-fusion protein (Fig. 2-2.6(A)) and performed virus rescue. CDV-EGFP was also found to express both monocistronic and bicistronic mRNA in the infected cells but EGFP can be detected in infected cells [Fujita, 2002]. Thus the author constructed expression vectors which would produce C-terminally EGFP-fused VP2 (EGFP-VP2). Additionally expression plasmid which VP2 and EGFP-VP2 were attached with an additional transmembrane signal (VP2-tm, EGFP-VP2-tm) were constructed. As entire VP2 was reported to locate in nucleus [Vihinen-Ranta et al., 2002; Suikkanen et al., 2003; Gilbert et al., 2005], the VP2 and EGFP-VP2 was also strongly detected in nucleus (Fig. 2-2.6 (B)). In case of an additional

transmembrane signal attachment, VP2-tm and EGFP-VP2-tm were mainly located at cell surface of transfected cells (Fig. 2-2.6 (B)). The cells transfected with EGFP-VP2, however, tended to die and float in culture medium. After confirmation of expression of the fusion proteins, the corresponding DNA fragments were inserted into the infectious cDNA clone and virus rescue was performed by the reverse genetics methods described above. However, no recombinant CDV was obtained from these clones after multiple trials. The failure of the rescue suggests that VP2 expression may prevent virus replication and host cell survival.

DISCUSSION

In this study, CDV-CPV-VP2 was successfully rescued. However, the virus could not produce VP2 protein in the infected cells. The "read-through" recombinant CDV transcribed a large number of bicistronic mRNA coding N and VP2 genes, which have no ability of VP2 translation. Additionally, EGFP-VP2, VP2-tm or EGFP-VP2-tm inserted clones were also unable to generate the virus. It was known that some infectious clones had been unable to produce viruses. When the infectious cDNA clones containing Leishmania antigen A2 gene was constructed in our group, the clones did not produce recombinant CDV, probably due to the presence of unique repeated sequence existed in the gene [Charest and Matlashewski, 1994]. For another example, canine IFN-gamma cDNA inserted in the infectious SeV cDNA clone was also unable, because a sequence similar to SeV gene end in the canine IFN-gamma stopped the transcription [Fujita, 2002]. However, the sequence which had been reported to disturb the virus rescue reported previously has not been found in the VP2 gene. Thus, VP2 may prevent virus propagation and host cell survival by different mechanisms.

Northern blot analysis revealed that relatively large amounts of the bicitronic N-VP2 RNA were transcribed in CDV-CPV-VP2 infected cells. The

read-through product is often observed in mononegavirus infected cells. The regulation of VP2 transcription was mainly determined by a transcriptional unit that encoded transcriptional end signal, intergenic trinucleotides and transcriptional start signal attached at the upstream of VP2 gene. We previously established recombinant CDV expressing EGFP or luciferase, which has the same transcriptional unit at the upstream of each foreign gene. CDV-EGFP infected B95a cells interestingly produced as large amounts of bicistronic RNA as CDV-CPV-VP2 infected cells, whereas little bicistronic RNA was expressed in CDV-luciferase infected cells [Fujita, 2002]. These results indicate that the bicitronic mRNA production is strongly dependent on inserted foreign gene. Further study will be required for efficient expression of foreign genes using CDV as a vector.

FIGURE LEGENDS

Figure 2-2.1 Establishment of infectious cDNA clone for CDV-CPV-VP2

The original infectious cDNA clone was based on the Yanaka strain of CDV described previously [Fujita, 2002]. The foreign antigen CPV-VP2 cDNA was attached at the downstream of a transcriptional unit of CDV and inserted at *FseI* site located between N and P genes of the infectious clone. The cDNA clone was supplied to the reverse genetics system [Fujita, 2002]. The rescued virus was designated as CDV-CPV-VP2.

Figure 2-2.2 Production of rabbit polyclonal antibody against CPV VP2

(A) Recombinant proteins VP2, fused with tag protein (GST) were expressed in E.coli and analyzed by coomassie brilliant blue staining and western blot. The cultures were separated to LB medium fraction, periplasm, soluble fraction and insoluble fraction. After SDS-PAGE, one gel was visualized by coomassie staining (left panel). Lane 1: BL21 (non-transformed E.coli), 2: pGEX-VP2 in BL21; non-induction by IPTG, 3: pGEX-VP2 in BL21; induction by IPTG. *Arrows* indicate the recombinant VP2. For western blot analysis, the proteins in another gel were transferred to Hybond-C and reacted with anti-GST monoclonal antibody and then HRP-conjugated anti-mouse IgG as second antibody. The membrane was visualized by DAB. Since the tag protein is

estimated at 27kDa of molecular weight, GST-VP2 were shown as fusion protein of 65kDa (right panel). (B) Immunofluorescence staining of CPV infected CRFK cells. The polyclonal anti-VP2 rabbit sera as the first antibody, and the goat anti-rabbit antibody-FITC as the secondly antibody were used.

Figure 2-2.3 Characteristics of the recombinant CDV

(A) B95a cells were infected with CDV-CPV-VP2 and incubated for 2-3 days. Syncytium formation was observed by light microscope. The size of syncytia caused by CDV-CPV-VP2 was as similar as the parental strain CDV-Yanaka. (B) To confirm the recombinant CDV, RT-PCR was performed by a pair of specific PCR primers coding downstream of N and upstream of P sequence. The amplified DNA was detected in expected size. (C) B95a cells were infected with CDV-CPV-VP2 or CDV-Yanaka at an moi of 0.01 and serially collected. The released virus titers were determined. The titer of CDV-CPV-VP2 was closely similar to that of CDV-Yanaka.

Figure 2-2.4 Immunofluorescence staining and immunoprecipitation of VP2 in the recombinant CDV infected cells

(A) The virus infected B95a cells were stained by immunofluorescence. The polyclonal anti-VP2 rabbit sera and mAb anti-CDV-H as the first antibody mixture, and the goat anti-rabbit antibody-FITC and goat anti-mouse

antibody-sulforhodamine as the secondly antibody mixture were used. (B) The infected cells were radiolabeled for 4 hrs and then lysed by RIPA buffer. The cell extracts reacted with anti-VP2 rabbit sera (See Fig. 2-2.2) and were separated by SDS-PAGE. VP2 protein expression was undetectable.

Figure 2-2.5 Northern blot analysis of VP2 mRNA in the recombinant CDV infected cells

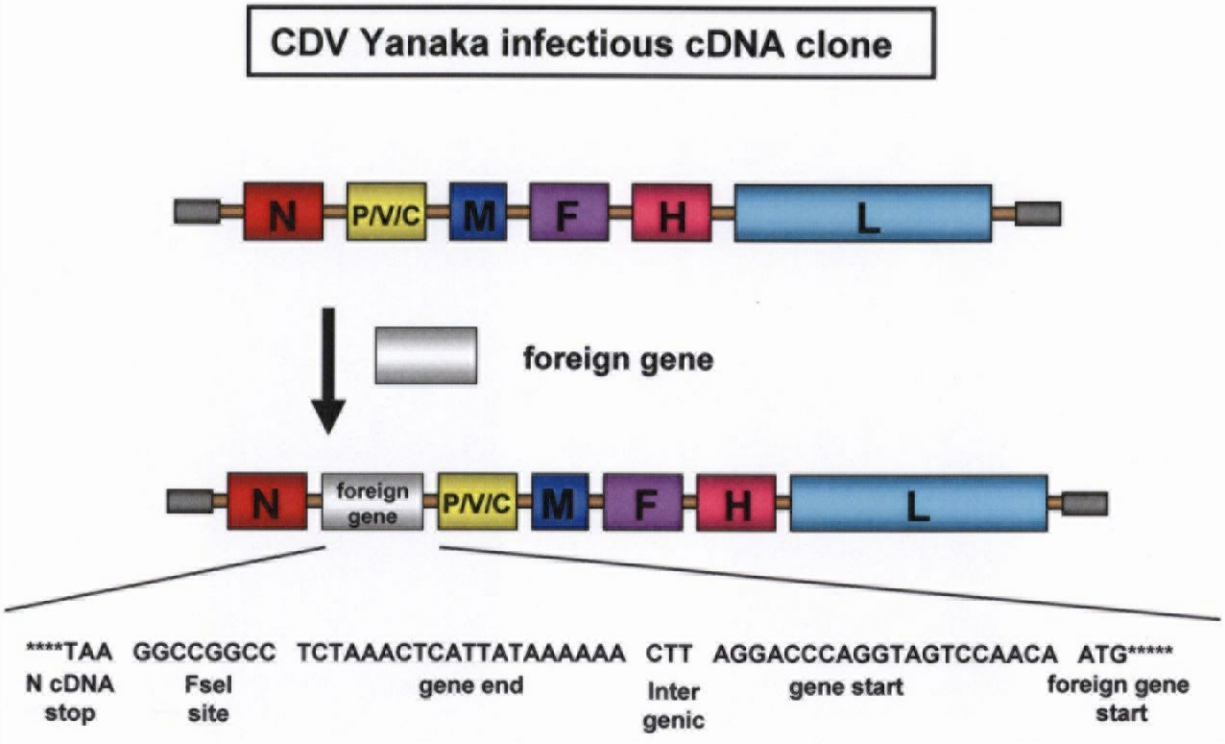
Total RNA from CDV-CPV-VP2 or Yanaka infected or mock-infected B95a cells were analyzed by northern blotting with probes encoding VP2 gene. VP2 mRNA was detected as both monocistronic VP2 mRNA (*arrows* at lower) and bicistronic N-VP2 mRNA (*arrows* at upper).

Figure 2-2.6 Expression of VP2 fusion protein in the transfected cells

(A) The expression plasmids were constructed. Original VP2 cDNA (1-334aa) was cloned into pCDNA3.1 (a). EGFP in pEGFP-N1 (b) was fused with VP2 in pCDNA3.1 (c). For additional transmembrane signal, VP2 or EGFP-VP2 was into pDisplay (d) or (e). (B) The expression plasmids were transfected into 293 cells. VP2 expression was determimed by immunofluorescence staining using the polyclonal anti-VP2 rabbit sera as the first antibody, and the goat anti-rabbit antibody- sulforhodamine as the secondly antibody. EGFP expression was observed under the confocal microscopy. The

VP2 protein and EGFP-VP2 fusion protein were strongly detectable in nuclear. VP2-tm and EGFP-VP2-tm fusion protein were mainly located at cell surface of transfected cells.

Fig. 2-2.1


CDV Yanaka infectious cDNA clone
N
P/V/C
M
F
H
L
foreign gene
N
foreign gene
P/V/C
M
F
H
L
****TAA
N cDNA stop
GGCCGGCC
FseI site
TCTAAACTCATTATAAAAAA
gene end
CTT
Inter genic
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gene start
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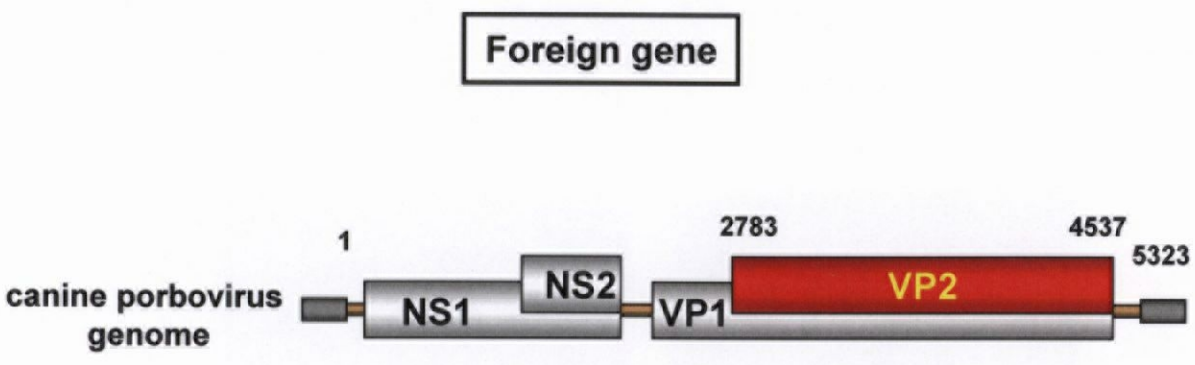
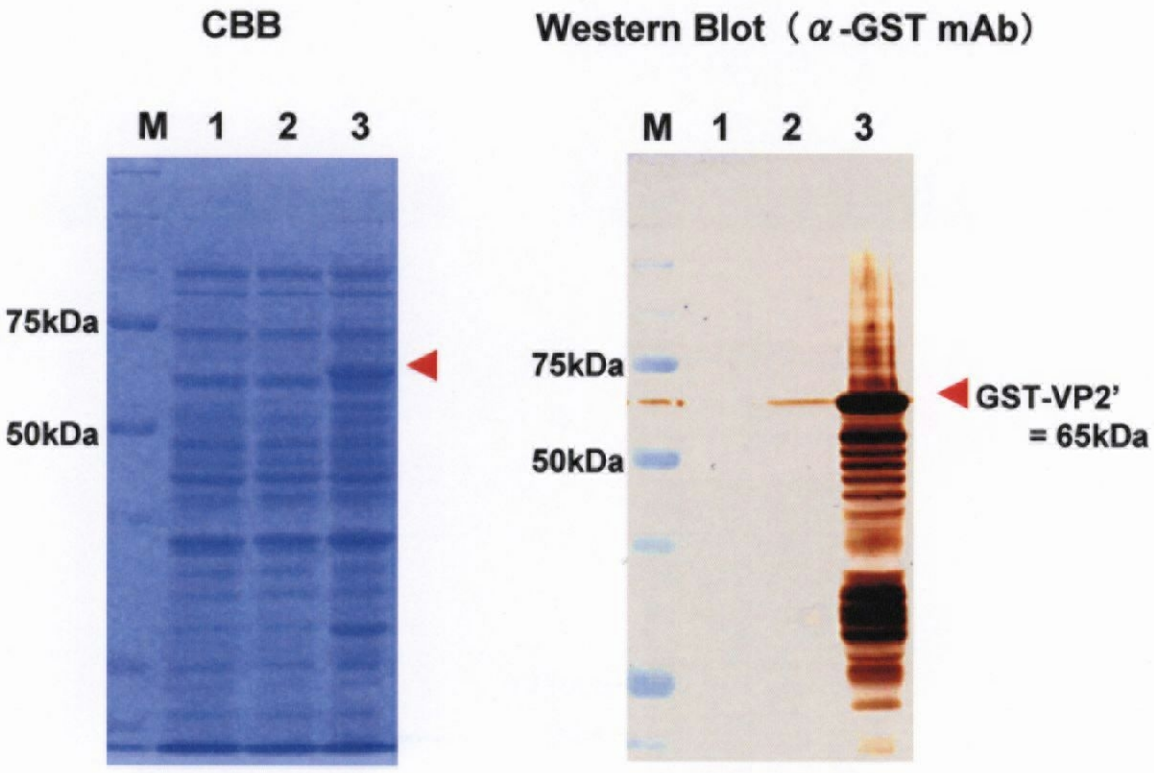

Foreign gene
canine porbovirus genome
1
2783
4537
5323
NS1
NS2
VP1
VP2

Fig. 2-2.2

(A)


CBB
Western Blot (α-GST mAb)
M 1 2 3
M 1 2 3
75kDa
50kDa
75kDa
50kDa
GST-VP2'
= 65kDa

(B)

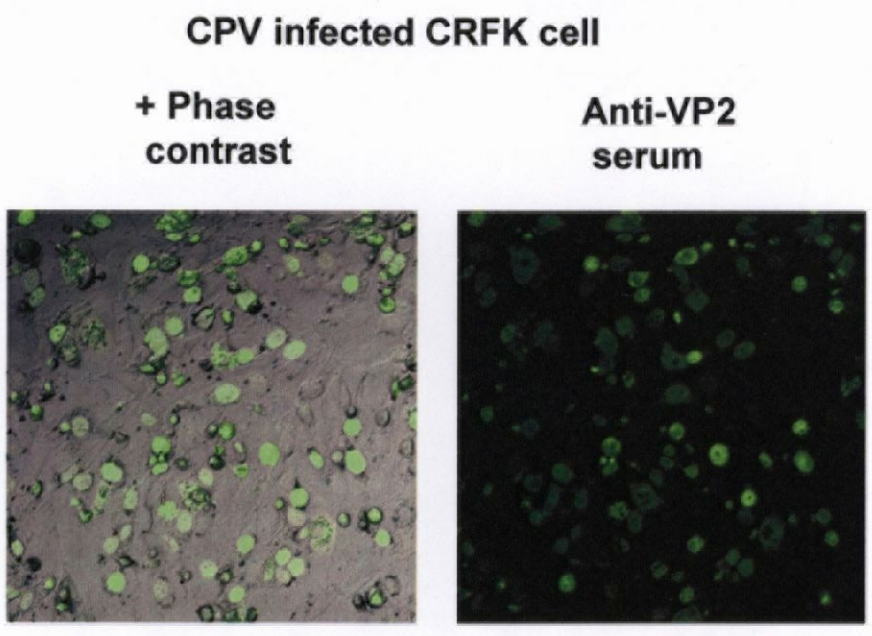
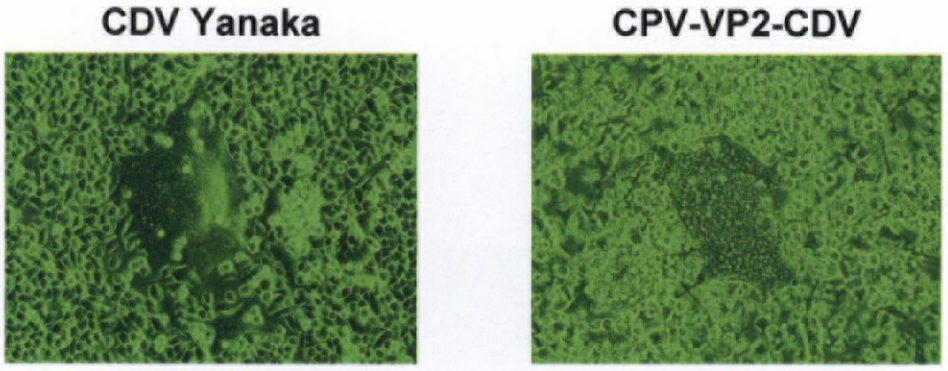
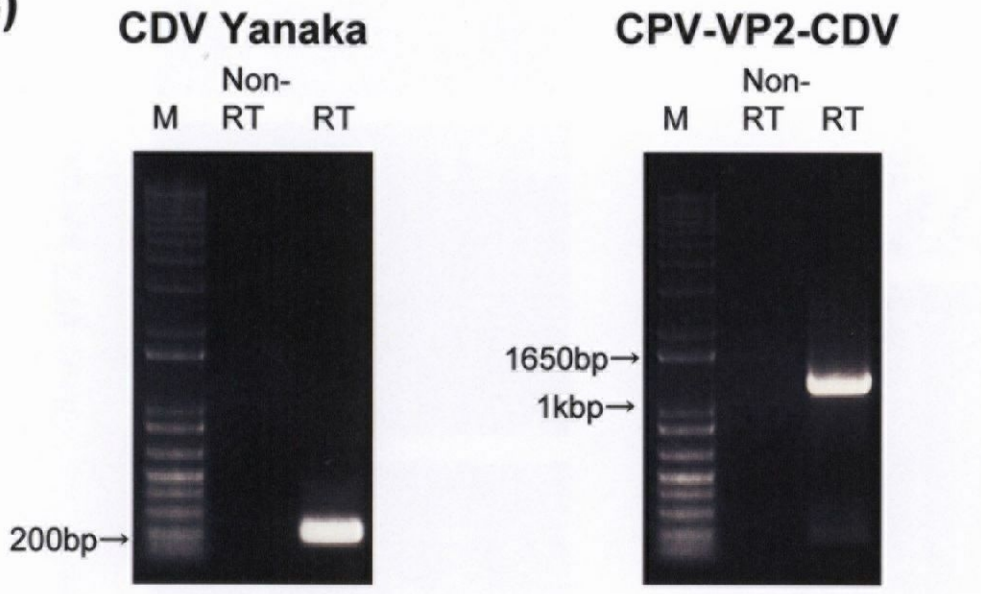

CPV infected CRFK cell
+ Phase contrast
Anti-VP2 serum

Fig. 2-2.3

(A)


CDV Yanaka
CPV-VP2-CDV

(B)


CDV Yanaka
Non-
M RT RT
200bp→
target 204 bp
CPV-VP2-CDV
Non-
M RT RT
1650bp→
1kbp→
target 1.2 kbp

(C)

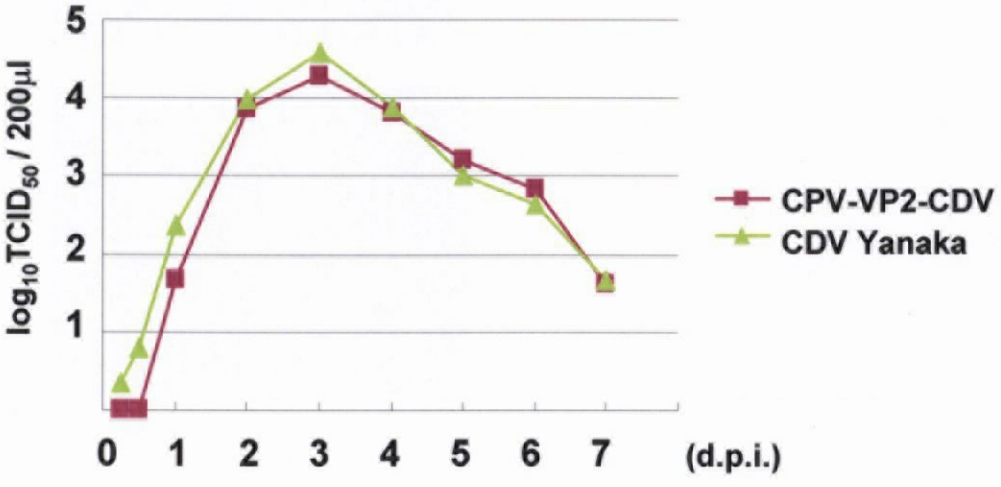

log10TCID50 / 200µl
5
4
3
2
1
0 1 2 3 4 5 6 7
(d.p.i.)
CPV-VP2-CDV
CDV Yanaka

Fig. 2-2.4

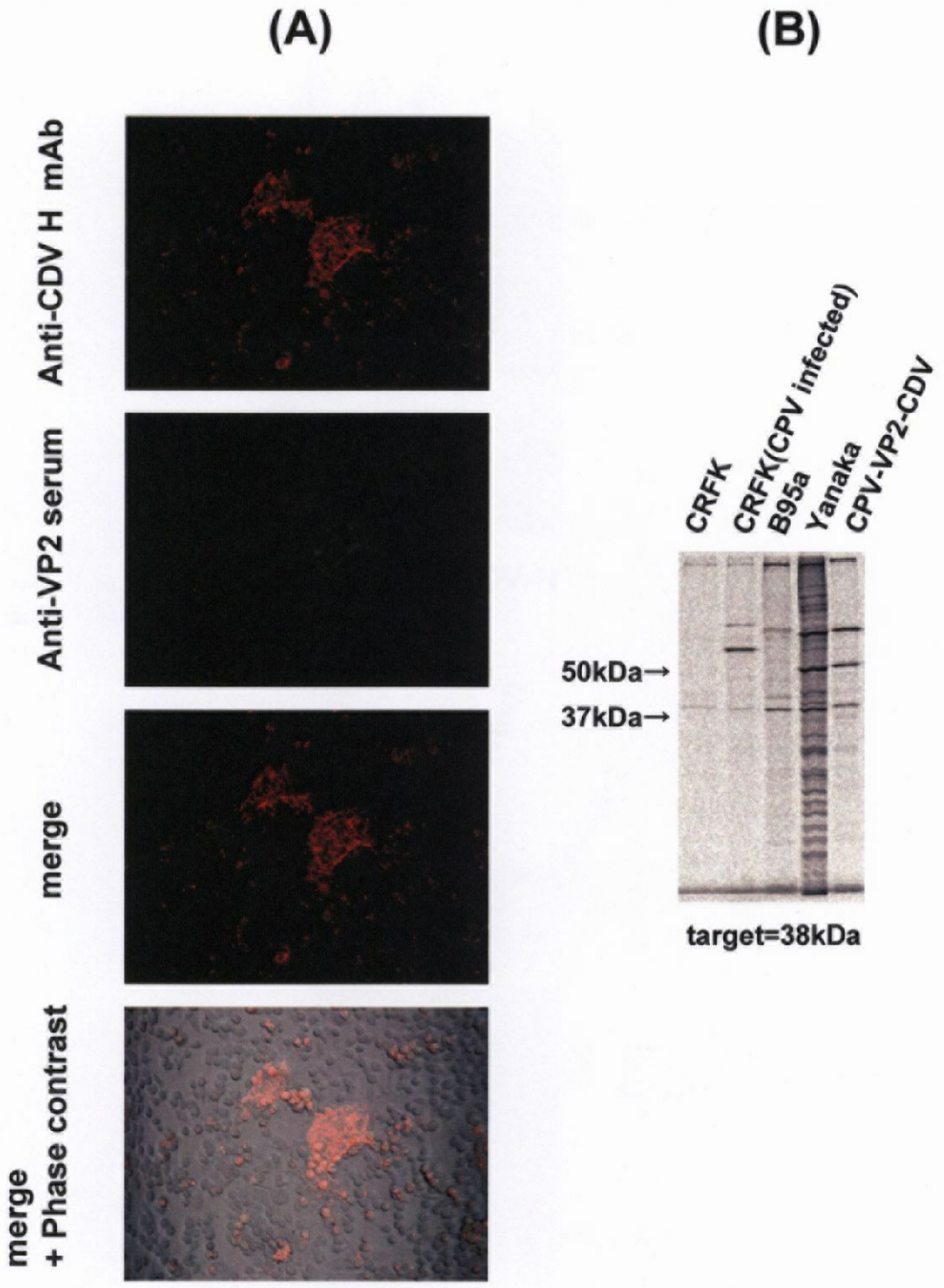

(A)
(B)
Anti-CDV H mAb
Anti-VP2 serum
merge
merge
+ Phase contrast
CRFK
CRFK(CPV infected)
B95a
Yanaka
CPV-VP2-CDV
50kDa→
37kDa→
target=38kDa

Fig. 2-2.5

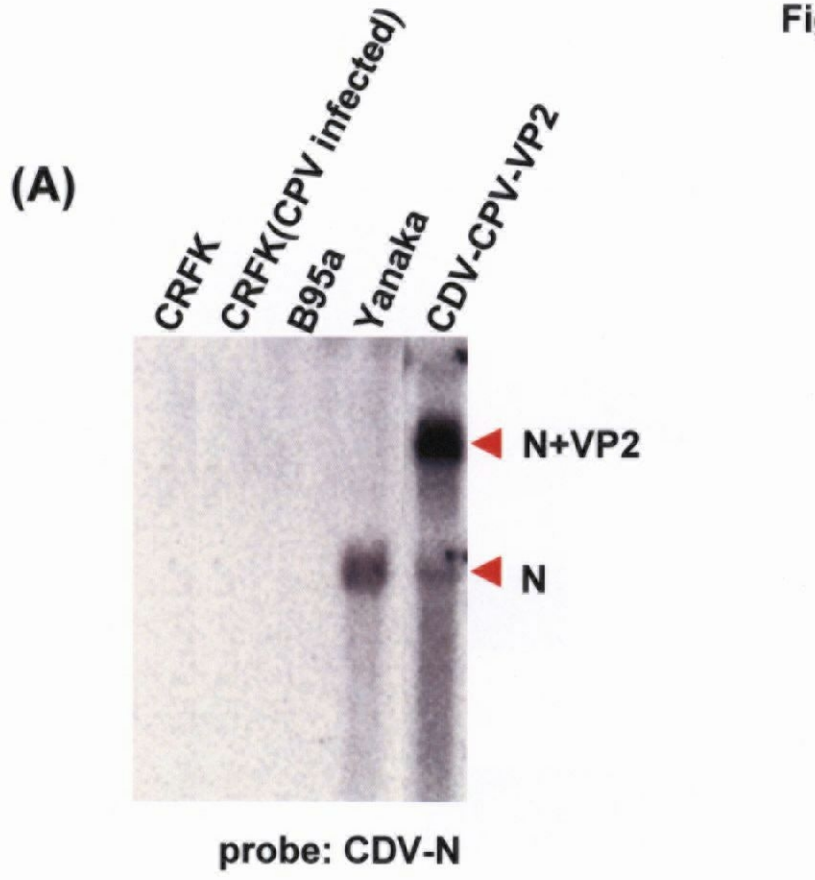
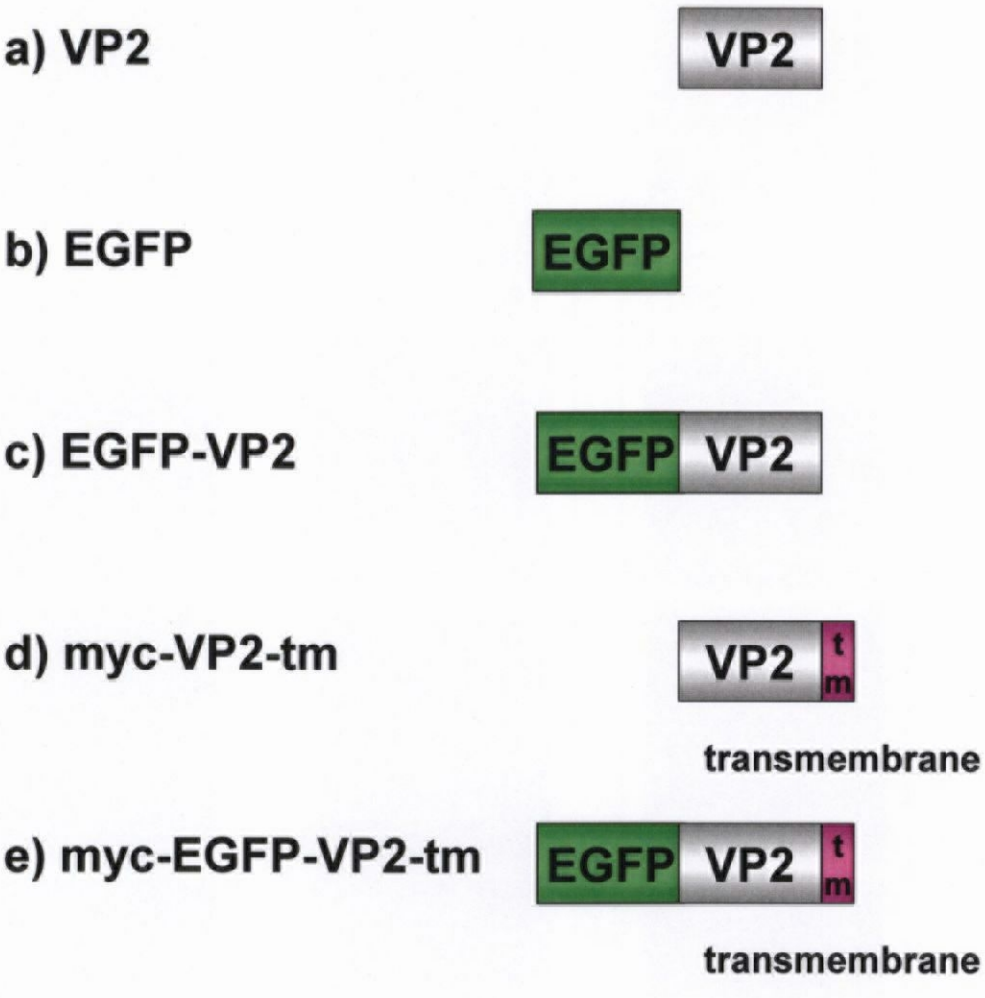

(A)
CRFK
CRFK(CPV infected)
B95a
Yanaka
CDV-CPV-VP2
N+VP2
N
probe: CDV-N
(B)
CRFK
CRFK(CPV infected)
B95a
yanaka
CDV-CPV-VP2
VP2(Full length)
N+VP2
VP2
probe: CPV-VP2

Fig. 2-2.6 (A)


a) VP2
VP2
b) EGFP
EGFP
c) EGFP-VP2
EGFP
VP2
d) myc-VP2-tm
VP2
t
m
transmembrane
e) myc-EGFP-VP2-tm
EGFP
VP2
t
m
transmembrane

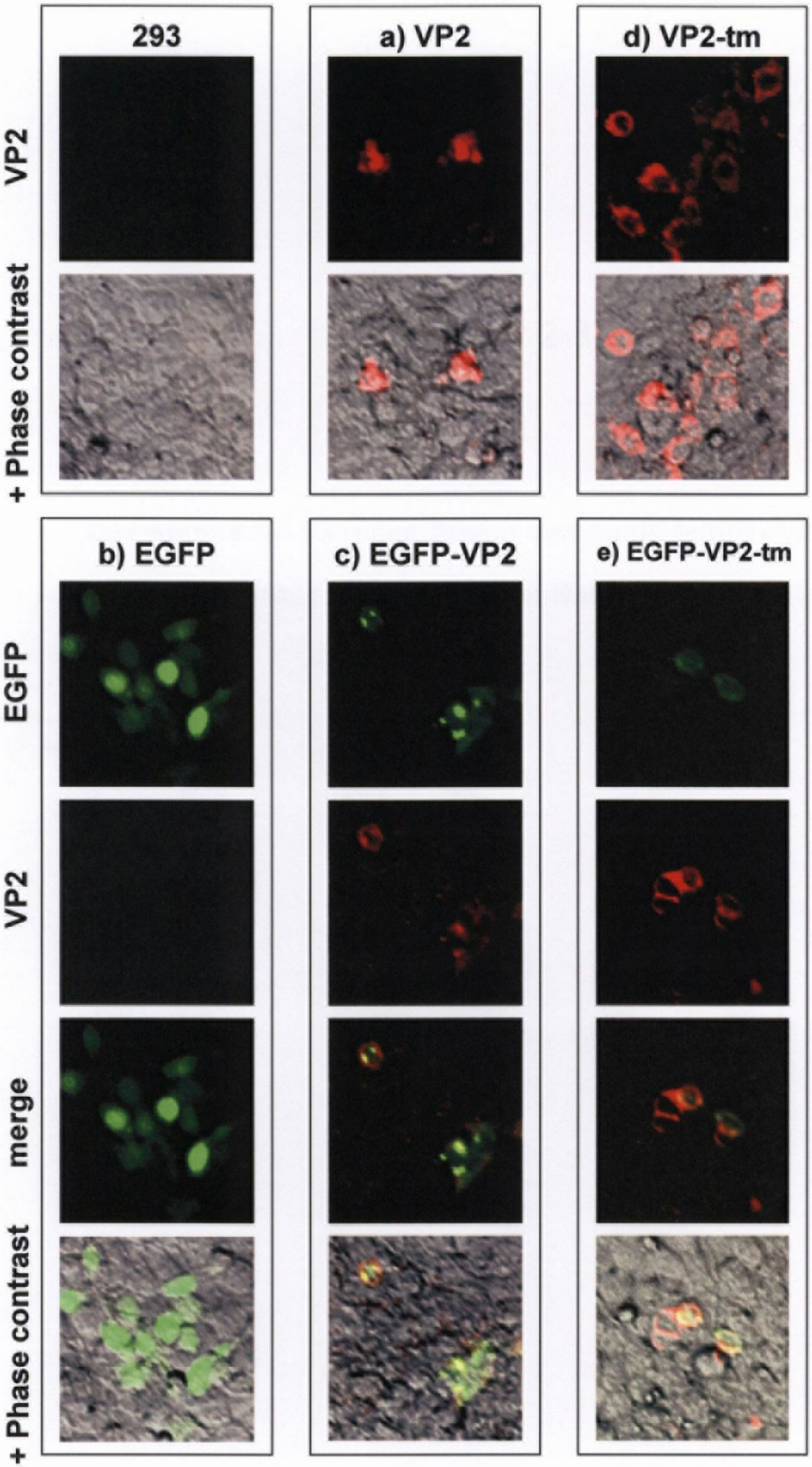

293
a) VP2
d) VP2-tm
VP2
+ Phase contrast
b) EGFP
c) EGFP-VP2
e) EGFP-VP2-tm
EGFP
VP2
merge
+ Phase contrast

Fig. 2-2.6 (B)

CHAPTER 2-3

Development of a recombinant canine distemper virus expressing G protein of Rabies virus

SUMMARY

In chapter 2-3, the author generated recombinant CDVs expressing the RaV G protein and investigated the characteristic, designated CDV-RaV-G. The size of CPE caused by CDV-RaV-G was significantly smaller than the parental strain CDV-Yanaka. The virus growth of CDV-RaV-G was delayed three day compared to CDV-Yanaka but showed a similar maximum titer. RaV G protein was obviously expressed in B95a cells infected with CDV-RaV-G. Northern blot analysis showed that RaV-G mRNA was expressed as almost monocistronic mRNA. Additionally, CDV-RaV-G acquired the susceptivety of infection to BHK-21 cells, one of RaV host cells.

INTRODUCTION

Rabies virus belongs to the order Mononegavirales, viruses with a nonsegmented, negative-stranded RNA genomes as well as CDV. Rabies is a preventable viral disease of mammals most often transmitted through the bite of a rabid animal. 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]. Currently, improved inactivated virus vaccines prepared from rabies virus and controlled onset of rabies in domestic dogs.

Rhabdoviruses generally encode five proteins, designated G (glycoprotein), N (nucleoprotein), P or NS (phosphoprotein), M (matrix protein), and L (RNA-dependent RNA transcriptase). The G protein is the primary surface antigen capable of inducing and reacting with virus-neutralizing antibodies, and it is associated with receptor activities. The G protein represents the only antigen that induces virus-neutralizing antibodies and is able to confer immunity against a lethal challenge infection [Cox et al., 1977]. The G protein is one of the antigens that induce CTL responses [MacFarlan et al., 1984]. A vaccinia-rabies glycoprotein (V-RG) vaccine was the first recombinant rabies vaccine to be constructed, field tested, and considered for regulation in Europe and North America for wildlife rabies control [Brochier et al., 1991;

Fearneyhough et al., 1998; Hanlon et al., 1998; Robbins et al., 1998; Roscoe et al., 1998].

In this chapter 2-3, the author generated recombinant CDVs expressing the RaV G protein and investigated the characteristic of these viruses.

MATERIALS AND METHODS

Cells, viruses and genes

For 293 cells, B95a cells and MVA-T7 were described in previous chapter. BHK-21 cells (a baby hamster kidney cell line) were maintained in Dullbecco's modified Eagle's medium (DMEM) (Sigma) containing 5% fetal calf serum (FCS). The rescued virus was grown on B95a cells. The open reading frame of G protein of the RaV HL strain was a kind gift from Dr. Minamoto, University of Gifu.

Plasmid constructions and virus rescue

The author planed to generate recombinant CDV expressing a full length of G protein (1575 bp, 525 aa) in RaV [GenBank Accession No. AB009663]. Infectious plasmids and virus rescue were described previously [Fujita, 2002]. The DNA fragment including RaV-G was amplified by PCR using following primers, G-F-in; 5'-CTTAGGACCCAGGTAGTCCAACAATGGTTCCGCAAGCTCTTTTG -3' and G-R; 5'- GGCCGGCCTCACAGTCCAGTCTCA -3'. The DNA fragment was attached with *FseI* recognition sequence and CDV transcription unit at 5' ends of the above primers (rCDV-F-out; 5'-GGCCGGCCTCTAAACTCATTATAAAAAACTTAGGACCCAGGTAG -3') (*FseI*

site underlined). Amplified cDNA fragment was subcloned into TA cloning vector pCR2.1™ (Invitrogen) and these sequences were confirmed. The plasmids were inserted into a cDNA clone, pCDV coding the genome of the Yanaka strain and obtained cDNA clones were applied for the virus rescue as mentioned in chapter 2-2. After the appearance of cytopathic effect (CPE), cells and supernatants were harvested and stored at -80°C. For confirmation of rescued CDV, RT-PCR was performed using primers amplified between N and P sequence and the obtained PCR fragments were determined.

Indirect immunofluorescence staining

CDV infected B95a cells or BHK-21 cells were prepared. The cells were fixed by formaldehyde and then washed in PBS. The cells were consecutively processed with the first antibody. The polyclonal anti-CDV dog sera (diluted at 1:500 in PBS), mAb anti-rabies (Abcam; diluted at 1:1000) and mAb anti-CDV-H (d-7: diluted at 1:1000) were used as first antibody. After washed by PBS as well, the cells were reacted with the secondary antibody. The goat anti-dog antibody-fluorescent isothiocyanate (diluted at 1:1000), goat anti-mouse antibody-sulforhodamine (Alexa Flour 568; diluted at 1:1000) and goat anti-mouse antibody-FITC (Alexa Flour 488; diluted at 1:1000) were used as secondary antibody. After washing in PBS, imaging analysis was performed with confocal microscopy.

Radioimmunoprecipitation assay

The details were described in chapter 2-2. Virus-infected cells or mock-infected cells were radiolabelled and lysed. The lysate was incubated with mixture of the mAb anti-rabies with protein A beads (Amersham biosciences), and washed in PBS. The immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography.

Northern blot analysis

The details were described in chapter 2-2. Total cellular RNA of infected or mock-infected B95a cells was separated in agarose/formaldehyde gel, and subsequently transferred to Hybond-N+. The membrane was hybridized with ^{32}P-dCTP labeled specific probes for RaV-G or CDV-N gene, and subsequently detected.

Virus titration

Multistep growth kinetics was analyzed by B95a cells infected with CDV-RaV-G or CDV-Yanaka at an moi of 0.004 in 12-well plates. The cells infected with CDV-RaV-G were immediately supplied for virus titration assay without freezing and thawing and the released virus was determined by

endpoint dilution. The titration assay was performed as described in other chapters.

RESULTS

Rescue of recombinant CDV expressing antigens derived from RaV

The author attempted to generate recombinant viruses expressing RaV-G protein using CDV as a viral vector (Fig.2-3.1). RaV membrane glycoprotein G [GenBank Accession No. AB009663] was known to be an immunological target for RaV protection. The plasmid was applied for our standard CDV reverse genetics system [Fujita, 2002]. At two weeks after overlay of B95a cells on 293 cells, the typical cytopathic effect (CPE) was observed in B95a cells transfected with the infectious clone (Fig. 2-3.2). The virus was designated CDV-RaV-G. The size of CPE caused by CDV-RaV-G was significantly smaller than the parental strain CDV-Yanaka. Foreign gene insertion was confirmed by RT-PCR using set of specific primers, which encoded the downstream of N gene and upstream of P gene. The amplified DNA fragment was shown in expected size and consistent with sequence (Fig. 2-3.1).

Growth kinetics of the recombinant CDVs

For virus storage, CDV expanded in B95a cells were normally prepared by standard freezing and thawing method followed by sonication. The preparation of CDV-RaV-G, however, exhibited quiet lower titer of less than 10^2

$TCID_{50}$/ml than the parental strain CDV-Yanaka (approximately 10^5 $TCID_{50}$/ml). Therefore, culture medium with B95a cells infected with CDV-RaV-G was immediately supplied for virus titration assay without freezing and thawing and the titer was simultaneously determined. CDV-RaV-G or CDV-Yanaka was inoculated with B95a at an moi of 0.004 and these growth kinetics were compared (Fig. 2-3.2). The virus growth of CDV-RaV-G was delayed 2-3 days compared to CDV-Yanaka but showed similar maximum titer (10^4-10^5/200μl).

Expression of foreign antigen in the recombinant CDV infected cells

To confirm the expression of virus antigen RaV-G, B95a cells infected were analyzed by immunofluorescence staining and immunoprecipitation. RaV-G protein was obviously expressed in B95a cells infected with CDV-RaV-G (Fig. 2-3.3), while the expression of corresponding antigen was not found in CDV-CPV-VP2 (See the chapter 2-2). Northern blot analysis showed that almost monocistronic RaV-G mRNA was expressed in CDV-RaV-G infected cells. Thus these results demonstrate that RaV-G protein was expressed in CDV-RaV-G infected cells.

CDV-RaV-G infectivity in RaV-susceptive cells

RaV is also a non-segmented single stranded RNA virus and comparatively relative to CDV. RaV-G has a function of attachment and fusion

to host cells and binds to carbohydrates, phospholipids, and gangliosides and to protein receptors including the nicotinic acetylcholine receptor [Broughan and Wunner, 1995]. Thus it was considered that RaV-G may be incorporated into CDV virion and CDV-RaV-G can possess an additional infectivity in RaV host cell lines. BHK-21 cells, baby hamster kidney cells, are known to be highly susceptive to RaV [Broughan and Wunner, 1995] and CDV-EGFP showed quite low infectivity of BHK-21 cells [Fujita, 2002]. Thus, the author investigated the infectivity of BHK-21 cells with CDV-RaV-G, compared with the parental CDV-Yanaka (Fig. 2-3.5). Since, RaV generally does not cause a specific syncytium in cell culture and CDV-RaV-G infected BHK-21 cells have morphologically no difference from CDV Yanaka infected cells, the cells at 7 days post infection were stained by mAb against CDV-H. CDV-RaV-G infected cells were obviously increased compared with CDV-Yanaka infected cells, indicating that RaV-G was sorted at the surface of virus particle and the virus acquired an additional infectivity of RaV susceptive cells.

DISCUSSION

In this chapter, recombinant CDV, CDV-RaV-G was successfully rescued, and produced detectable amount of foreign antigen G in the infected B95a cells, while CDV-CPV-VP2 could not produce VP2 protein in the infected cells. These results can contribute to the development of polyvalent CDV vaccines and a new type of viral vectors for gene transfer approaches.

The growth characteristic of CDV-RaV-G was investigated and showed slower kinetics than the parent CDV-Yanaka, indicating that replication of the recombinant CDV was apparently repressed. The reason of the reduced viral growth was not fully understood. The additional transcription unit and/or the additional genome length itself are known to affect the replication of the recombinant viruses. EGFP-CDV and Luc-CDV (0.8kb for EGFP and 1.7kb for luciferase) showed slightly slower kinetics but similar maximum titers [Fujita, 2002]. In sendai virus, the recombinant virus with 3.2 kbp foreign gene was able to be rescued and grew [Sakai et al., 1999]. RaV-G gene was 1.8 kbp, and these recombinant CDVs were predicted to have similar characters to the parental strain. The cells solely expressing G by expression vector transfection survived normally [Burger et al., 1991]. Therefore, when the antigens from other infectious pathogens are coexpressed in the same cells, CDV replication may be interfered.

CDV-RaV-G obtained infectivity in host cells of RaV, BHK-21 cells. RaV-G has a function of attachment and fusion to host cells and binds to carbohydrates, phospholipids, and gangliosides that are generally produced at the cell surface [Broughan and Wunner, 1995]. In mononegavirus, addition or modification of glycoprotein necessary for host cell attachment were known to obtain infectivity to cells. Recombinant VSV replaced glycoproteins with measles virus, showed infectivity to only MV-susceptive cells [Tatsuo et al., 2000]. Recombinant MV retargeted to tumor antigens, CD38 or epidermal growth factor receptor entered to cells through their respective targeted receptors in vitro and in vivo [Peng et al., 2003]. RaV-G may be incorporated into CDV virion in the same manner. Since it is possible that CDV-RaV-G can infect neuron, mediated by nicotinic acetylcholine receptor which is a neuronal receptor for RaV, it may be useful for analysis of CDV neurovirulence using mice.

FIGURE LEGENDS

Figure 2-3.1 Establishment of infectious cDNA clones for CDV-RaV-G

(A) The original infectious cDNA clone was based on the Yanaka strain of CDV described previously [Fujita, 2002]. The foreign antigen cDNA, Rav-G, was attached at the downstream of a transcriptional unit of CDV and inserted at *FseI* site located between N and P genes of the infectious clone. The cDNA clone was applied for the reverse genetics system [Fujita, 2002]. The rescued virus was designated as CDV-RaV-G. (B) To confirm of foreign gene insertion in the recombinant CDV, RT-PCR was performed using a pair of specific PCR primers coding downstream of N and upstream of P sequence. The amplified DNA was detected in expected size.

Figure 2-3.2 Characteristics of the recombinant CDV

(A) Comparison of the recombinant CDV infected B95a cells. B95a cells were infected with CDV-RaV-G and incubated for 2-3days. Syncytium formation was observed by light microscope. The size of syncytia caused by CDV-RaV-G was significantly smaller than the parental strain CDV-Yanaka. (B) B95a cells were infected with CDV-RaV-G or CDV-Yanaka at an moi of 0.004 and serially collected. The released virus titers were determined. The virus growth of CDV-RaV-G was delayed compared to CDV-Yanaka.

Figure 2-3.3 Immunofluorescence staining and immunoprecipitation of RaV-G in the recombinant CDV infected cells

(A) The virus infected B95a cells were stained by immunofluorescence. The polyclonal anti-CDV dog sera and mAb anti-rabies as the first antibody mixture, and the goat anti-dog antibody-FITC and goat anti-mouse antibody-sulforhodamine as the secondly antibody mixture were used. (B) The infected cells were radiolabeled for 4 hrs and then lysed by RIPA buffer. The cell extracts reacted with anti-rabies monoclonal antibody and were separated by SDS-PAGE.

Figure 2-3.4 Northern blot analysis of RaV-G mRNA in the recombinant CDV infected cells

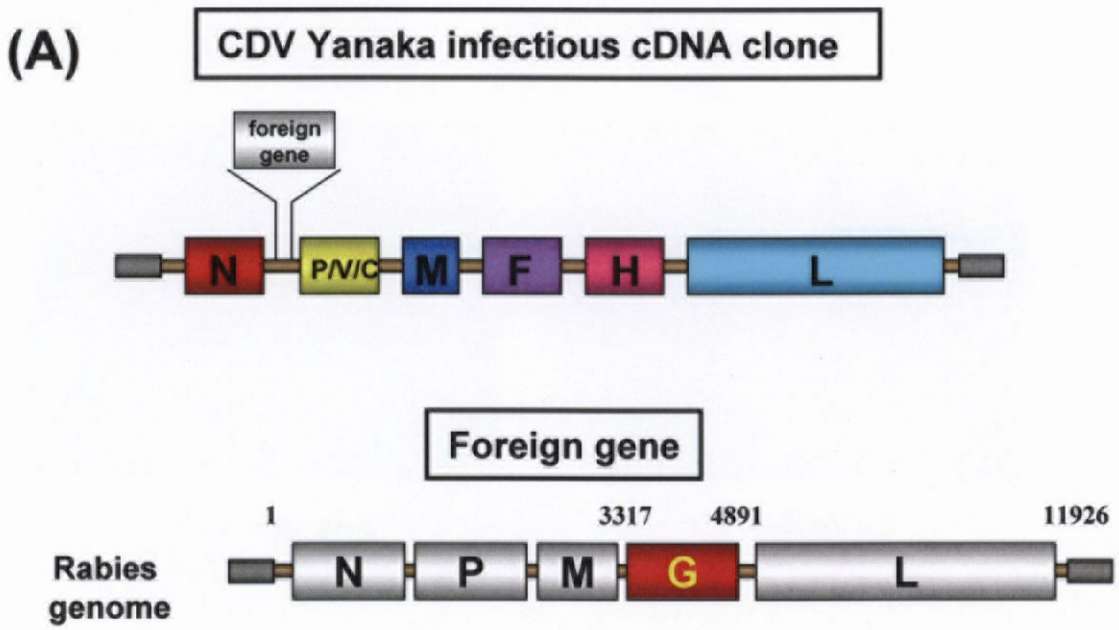
Total RNA from CDV-RaV-G, Yanaka infected or mock-infected B95a cells were analyzed by northern blotting with probes encoding RaV-G or CDV-N. RaV-G was detected as both monocistronic- (arrows at lower) and bicistronic mRNA (arrows at upper).

Figure 2-3.5 Infection of BHK-21 cells with CDV-RaV-G.

BHK-21 cells, host cells of RaV, was infected with CDV-Yanaka and CDV-RaV-G. (A) Comparison of infectivity. (B) Immunofluorescence staining of

CDV infected BHK-21 cells. The mAb anti-CDV-H as the first antibody, and the goat anti-mouse antibody-FITC as the secondly antibody were used.

Fig. 2-3.1


(A)
CDV Yanaka infectious cDNA clone
foreign gene
N
P/V/C
M
F
H
L
Foreign gene
1
3317
4891
11926
Rabies genome
N
P
M
G
L

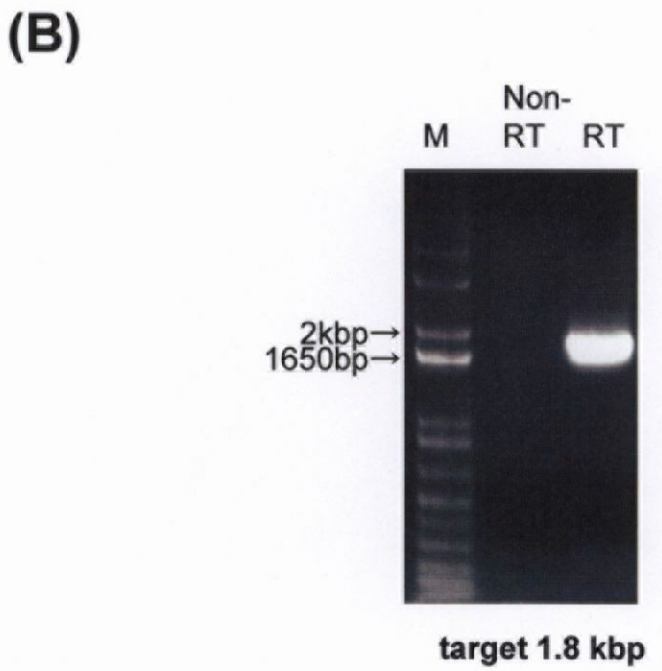
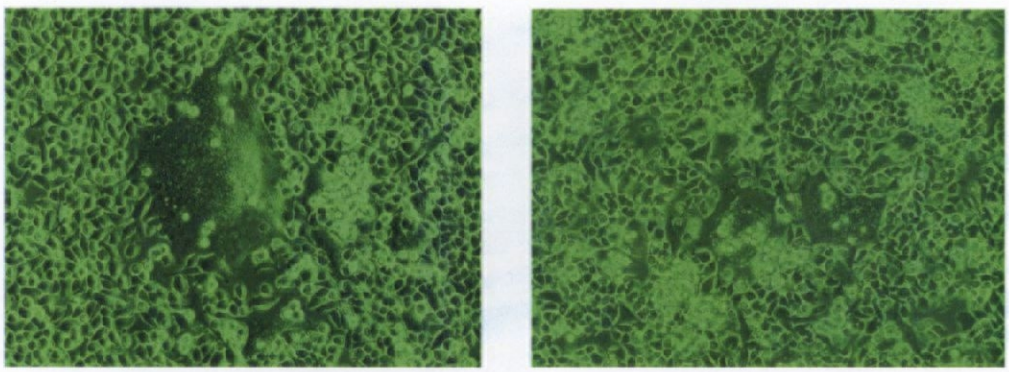

(B)
M
Non-RT
RT
2kbp→
1650bp→
target 1.8 kbp

Fig. 2-3.2

(A)



CDV Yanaka　　CDV-RaV-G

(B)

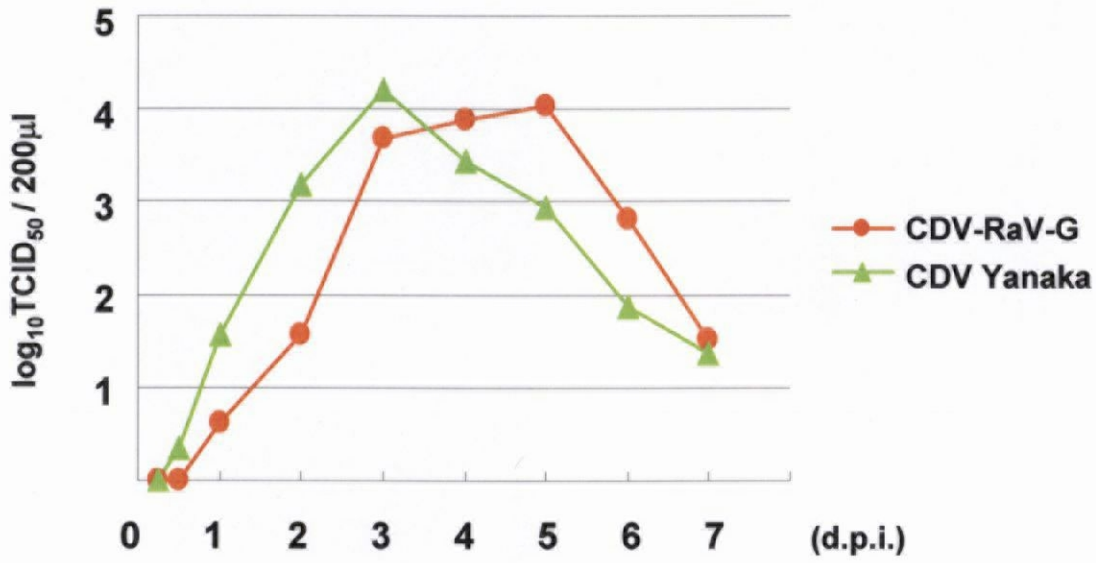


Fig. 2-3.3

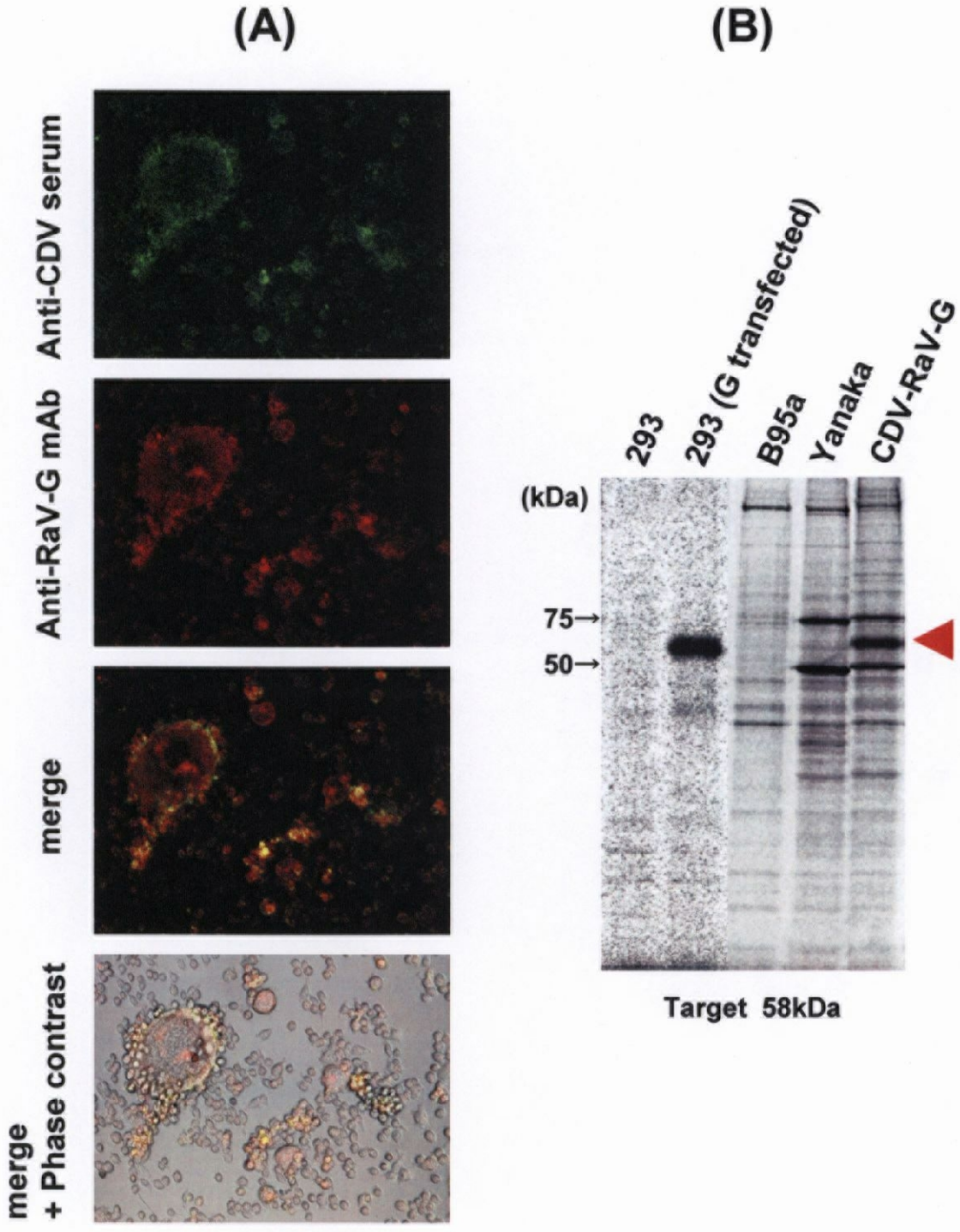

(A)
Anti-CDV serum
Anti-RaV-G mAb
merge
merge
+ Phase contrast
(B)
293
293 (G transfected)
B95a
Yanaka
CDV-RaV-G
(kDa)
75→
50→
Target 58kDa

Fig. 2-3.4

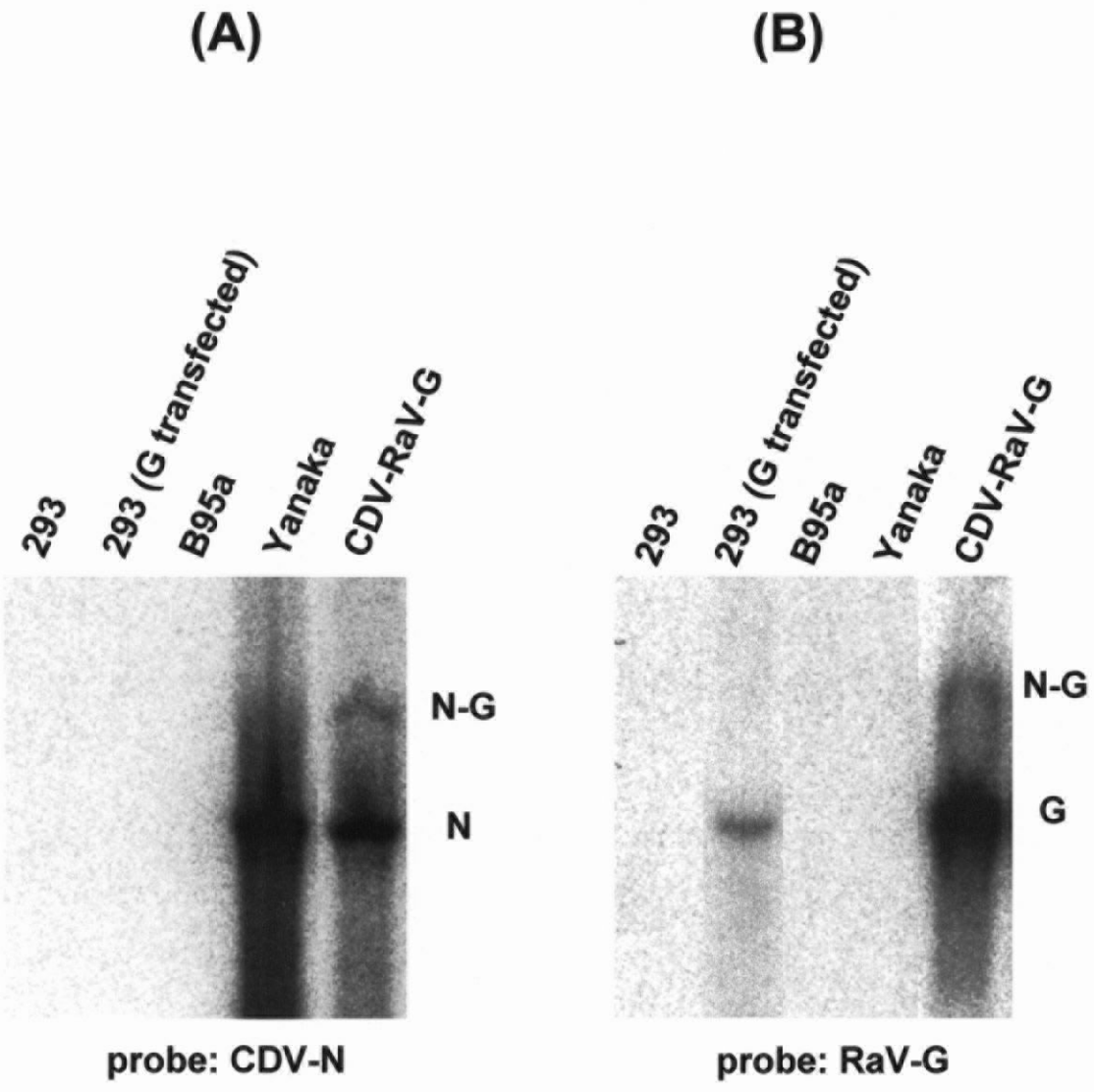
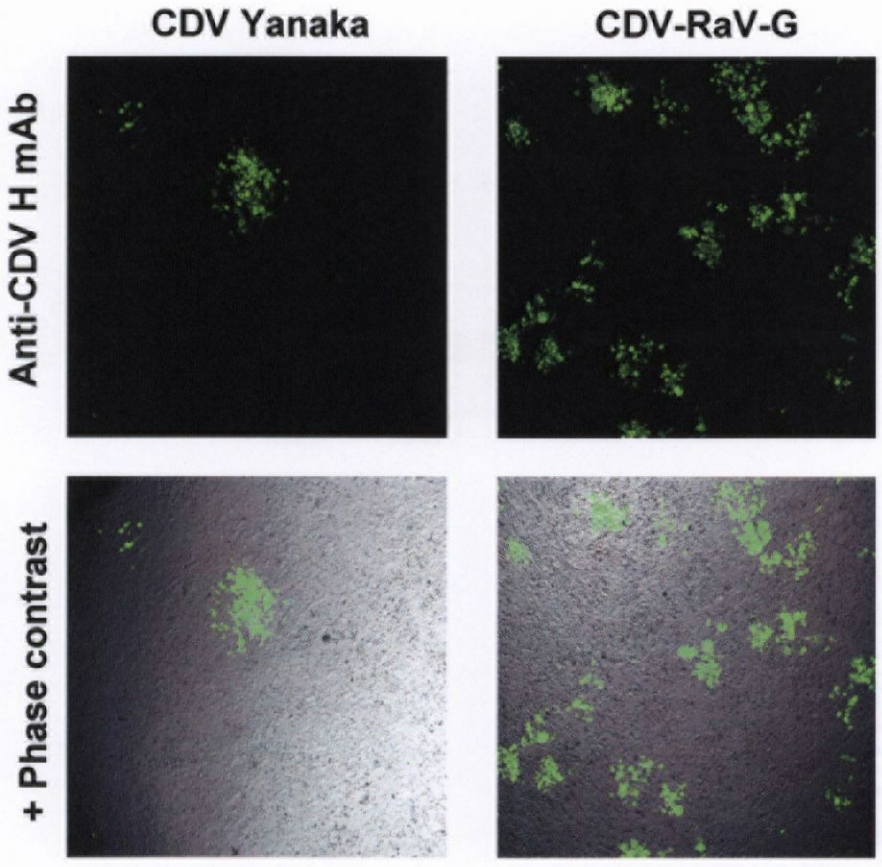
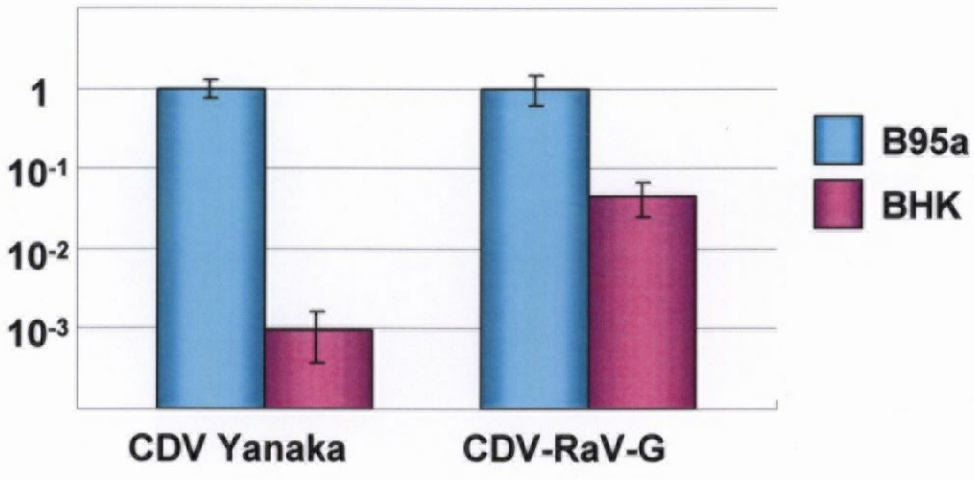

(A)
(B)
293
293 (G transfected)
B95a
Yanaka
CDV-RaV-G
N-G
N
G
probe: CDV-N
probe: RaV-G

Fig. 2-3.5

(A)


CDV Yanaka
CDV-RaV-G
Anti-CDV H mAb
+ Phase contrast

(B)


1
10-1
10-2
10-3
B95a
BHK
CDV Yanaka
CDV-RaV-G

CONCLUSION

Canine contagious virus diseases, in particular, canine distemper, parvovirosis and rabies are two of the most important diseases for serious health threats to susceptible dogs. With respect to their causative agents, canine distemper virus (CDV), canine parvovirus (CPV) and rabies virus (RaV), the author performed basic studies, which are 1) to understand the mechanism of a persistence of CDV in lymphocytes, 2) to understand what types of CPV are prevalent in Japan and 3) to analyze CDV-based recombinant viruses containing CPV-VP2 gene or RaV-G gene.

In chapter 1, using CDV-EGFP as a parental virus which allow us to observe the infected cells easily under the confocal microscopy, the author newly established a noncytopathic CDV strain (CDV-EGFP-BP2) that caused persistent infection in B95a cells. The 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 the parental CDV. Analysis using stably SLAM-expressing cells, however, showed that CDV-EGFP-BP2 induced CPE although the fusogenicity was reduced 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 implicated in the interaction with F protein.

In chapter 2-1, the author genetically analyzed the VP2 genes of canine parvovirus isolated in 1999 and 2000 from 7 domestic dogs in Japan. 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.

In chapter 2-2, the author generated a recombinant CDV harboring the gene of the CPV-2 VP2 protein and investigated the characteristics of the recombinant virus designated as CDV-CPV-VP2. The size of CPE and growth kinetics caused by CDV-CPV-VP2 was as similar as the parental CDV-Yanaka strain. Northern blot analysis showed that VP2 mRNA was detected as a large amount of bicistronic mRNA and little monocistronic VP2 mRNA. By immunoprecipitation assay and immunofluorescence staining, CDV-CPV-VP2 produced no detectable VP2 due to the inadequate N-VP2 mRNA caused by read-through. Therefore, additional infectious cDNA clones that possessed DNA fragment of EGFP-VP2, VP2-transmembran domain (tm) or EGFP-VP2-tm were constructed. However, these clones did not produce recombinant CDV, suggesting that VP2 may prevent virus propagation and host cell survival.

In chapter 2-3, the author generated a recombinant CDV expressing the rabies G protein and investigated the characteristics of the recombinant virus designated as CDV-RaV-G. Compared to CDV-Yanaka strain, the size of CPE was significantly smaller and the virus growth was delayed two days caused by CDV-RaV-G. By immunoprecipitation assay and immunofluorescence staining, RaV-G protein was obviously expressed in B95a cells infected with CDV-RaV-G. Additionally, CDV-RaV-G acquired the susceptivety of infection to BHK-21 cells, RaV host cells. The results in chapter 2-2 and 2-3 could contribute to the development of polyvalent CDV vaccines and of a new type of viral vectors for gene transfer.

These findings described in chapter 1 were invaluable for understanding the mechanism of persistence of CDV in lymphocytes. The findings in chapter 2-1 were also invaluable for knowledge of recent prevalent CPV in Japan. The findings in chapter 2-2 and 2-3 provided new information of application for the reverse genetics system to generate gene expression for vaccine and gene delivery vectors for therapy based on CDV. Thus, the studies in the thesis would provide valuable basic knowledges for canine contagious virus diseases.

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論文の内容の要旨

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論文題目

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）

≪副題；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）≫

イヌジステンパー、イヌパルボウイルス感染症、狂犬病は、イヌのウイルス性感染症の中でも最も重篤な症状を引き起こす感染症に含まれる。本研究では、これらの原因ウイルスであるイヌジステンパーウイルス(CDV)、イヌパルボウイルス(CPV)、狂犬病ウイルス(RaV) に着目し、CDVリバースジェネティクス法を用いることによりCDV持続感染機構の解析と組換え多価ワクチン開発を主題に基礎的研究を行った。

CDVはパラミクソウイルス科モービリウイルス属に属し、麻疹ウイルスや牛疫ウイルス、小反芻獣疫ウイルスと近縁のウイルスである。他のモービリウイルスと同様に高病原性ウイルスで、発熱、胃腸炎、肺炎、結膜炎、脳脊髄炎など全身感染を引き起こし、子犬では高い致死率を示す。感染伝播は主に飛沫感染、直接接触、ウイルス汚染物質との接触による。ウイルスは主に上部気道より感染し、マクロファージにより近接するリンパ節に到達してそこで増殖する。増殖したウイルスはリンパ節を通して広がり、次いでウイルス血症を呈し全身に広がり発症に至る。自然感染からの回復によって終生免疫を獲得すると考えられている。

CDVが属する一本鎖マイナス鎖RNAウイルス（モノネガウイルス）では、長い間

組換えウイルスを作出することができなかった。しかし、1994年に狂犬病ウイルスで初めて組換えウイルス作出系（リバースジェネティクス系）が開発され、著者らの研究グループも1999年に世界で初めてCDVリバースジェネティクス系の開発に成功した。これにより構成遺伝子の欠失や交換、変異の導入や外来遺伝子の挿入が可能となり、遺伝子解析の比較だけではわからなかった病原性の発現機序や宿主特異性等の解明に新たな解決方法をもたらした。また、細胞性免疫を誘導する特性を生かしたウイルスベクターの開発やその応用も盛んに進められている。

CPV はパルボウイルス科パルボウイルス亜科パルボウイルス属に属する。1970年代に初めてイヌへの感染が報告されて以来、世界中に急速に拡大した。最初の流行はCPV-2 型であったが、1979 年には新しい CPV-2a 型が出現しわずか一年で流行の主流は 2a 型に置き換わった。2a 型は宿主域も変化しており、ネコへの感染も見られた。その後 1984 年には CPV-2b 型、2000 年代初頭には CPV-2c 型という新たな抗原型が出現した。現在ワクチンにより概ね流行はコントロールされているが、比較的短期間にウイルスの抗原変異が起こることから新型株の出現が予想されている。

狂犬病ウイルス(RaV)はCDV と同じモノネガウイルス群のラブドウイルス科に属する。感染動物からの咬傷により感染し、中枢神経系に伝播し脳症を起こして死亡する。古くから知られた感染症で、現在不活化ワクチンの接種によって飼育犬への感染は抑えられているが、主に野生動物や非飼育犬からの感染により現在も年間の死亡者数が数万人に達する。

本研究では、CDVの持続感染機構の解明に有用なツールとして、蛍光蛋白EGFP発現組換えCDVを用いて、リンパ球系細胞における新たなCDV持続感染株を樹立し持続感染機構の解析を行った（第1章）。次に、近年日本で分離されたCPVの系統樹解析を行った。（第2-1章）。また、CDVをウイルスベクターとしたイヌに対する多価ワクチン開発の基礎研究として、CPVの主要抗原VP2（第2-2章）またはRaVの主要抗原G（第2-3章）を組み込んだ組換えCDVの作出と解析を行った。

第 1 章：GFP発現CDV持続感染株の樹立と性状解析

CDV は細胞障害性をもたらすと共に持続感染能を有する。しかしながら、持続感染の成立機構や機序は明らかになっていない。本章では、著者らの研究グループが CDV リバースジェネティクス法により作出した、蛍光蛋白 EGFP 発現組換え CDV (CDV-EGFP) を用い、リンパ球由来の B95a 細胞に対する新たな持続感染株(CDV-EGFP-BP2)を樹立しその性状解析を行った。樹立した CDV-EGFP-BP2 は、元株と同様のウイルス増殖と細胞外へ

のウイルス粒子放出を示したが、感染細胞に細胞融合性巨細胞を形成せず、非感染細胞と同等の細胞生存率を示した。CDV-EGFP-BP2 について、細胞侵入の役割を担う膜蛋白 H と F の発現様式を比較したところ、感染細胞での両膜蛋白の mRNA 量、蛋白量、細胞表面での局在は元株と相違なかった。持続感染株の膜蛋白の塩基配列を決定して元株と比較したところ、H と M 蛋白にそれぞれ一ヶ所ずつアミノ酸変異があった。また、リンパ球でのレセプターである SLAM を導入した細胞を用いた実験では、CDV-EGFP-BP2 は元株よりも細胞融合能が減少していた。細胞融合には膜蛋白 H と F の相互作用が必要であることから、H のアミノ酸置換は F との相互作用の減少をもたらし、持続感染を引き起こしている可能性が考えられた。

第２章－１：イヌパルボウイルスVP2遺伝子の系統樹解析

CPVは幼若イヌで嘔吐、下痢、白血球減少などを伴う重篤な感染症を起こし、比較的短期間で抗原変異を繰り返し宿主域の変化も見られる。本章では、1999年と2000年の国内CPV感染7症例よりCPVを分離し、カプシドの大半を占め宿主域決定や中和抗体誘導に関連するVP2蛋白をコードする遺伝子の塩基配列を決定した。その結果、７株中３株がタイプ2a、残りの４株がタイプ2bの遺伝子型を持ち、系統樹解析の結果、近年の日本や台湾の野外株クラスターに属することが示唆された。いずれも初期流行株の抗原型を元にした現在のCPVワクチン株とは異なるクラスターを形成しており、将来のワクチン開発に有用な知見を与えた。

第２章－２：CPV抗原VP2遺伝子組換えCDVの開発

本章では、CDVをベクターとした多価ワクチン開発の基礎的研究として、CDVリバースジェネティクス法により、イヌにおいて重要なウイルス性病原体であるCPVのカプシド蛋白VP2を組み込んだ組換えCDVの作出と解析を行った。得られた組換えウイルスCDV-CPV-VP2は、元株Yanaka株と比べウイルス増殖速度と細胞融合性巨細胞の形状に顕著な差異は認められなかった。しかし、特異抗体を用いて蛍光免疫染色と免疫沈降法で外来蛋白VP2の発現を調べたところ感染B95a細胞でVP2の発現は確認できなかった。ノーザンブロット法でmRNA発現を調べたところ、N-VP2間でリードスルーが高頻度で起こりVP2を翻訳しないbicistronicなmRNAが主に転写されていることがわかった。原因としてVP2遺伝子内にリードスルーを誘発する配列が存在するか、VP2蛋白が発現しない組換えCDVが

選択的に得られた可能性が考えられたため、VP2遺伝子をEGFP遺伝子の下流に配置し転写調節領域から離した遺伝子(EGFP-VP2)や、VP2蛋白が核に集積しないよう膜局在シグナルを付加した遺伝子(EGFP-VP2-tm)を構築し発現プラスミドに組み込んだ。各遺伝子を細胞に導入したところ、EGFP-VP2-tmは膜に局在を示し、EGFP-VP2では多くの導入細胞で浮遊がみられ細胞死が起こる傾向にあった。これら遺伝子断片をCDV cDNAプラスミドに組み込み、CDVリバースジェネティックス法を試みたが、組換えCDVの作出に至らなかった。以上の結果から、VP2 の発現がウイルスの増殖や宿主細胞に影響を及ぼすためにCPV-VP2発現組換えCDVが作出できなかったと考えられた。

第２章－３：RaV抗原Gを発現した組換えCDVの開発

本章では、前章と同様CDVリバースジェネティクス法によりRaVの抗原遺伝子Gを組み込んだ組換えCDV(CDV-RaV-G)の作出と解析を行った。得られたCDV-RaV-Gは元株Yanaka株と比較して増殖の遅れと小型の細胞融合が観察された。特異抗体を用いた蛍光免疫染色と免疫沈降法で感染B95a細胞での外来蛋白の発現を調べたところ、G蛋白の発現が認められた。また、RaV感受性細胞(BHK-21細胞)に対して感染能を獲得していることが明らかになった。本章では、多価ワクチンとして有望なCDV-RaV-Gを得ることができた。前章の結果も含めこれらの成果は今後の組換えCDVの作出に寄与する重要な知見と考えられた。

本研究において、第１章では、GFP発現CDV持続感染株を新たに樹立し、持続感染の成立機構とその機序を示唆することができた。第２章－１では、近年わが国でのCPV感染症についての系統樹解析により疫学的傾向を示すことができた。さらに第２章－２では、CPVの抗原遺伝子VP2について、第２章－３では、RaVの抗原遺伝子Gについて組換えCDVの作出と解析を行い、今後の組換えCDV多価ワクチンの開発につながる有用な重要な知見を得ることができた。本研究により、CDVの持続感染機構の基礎的研究およびCDVベクターを用いた組換えワクチンの開発における有用な知見を与えることができたと考える。