Analysis of the N-Terminal Polypeptide of the Capsid Precursor Protein and the ORF3 Product of Feline Calicivirus

Yukinobu TOHYA, Hidetoshi SHINCHI*, Yuichi MATSUURA, Ken MAEDA[1)], Sinryo ISHIGURO[2)], Masami MOCHIZUKI[2,3)] and Takaaki SUGIMURA

Department of Veterinary Microbiology, Faculty of Agriculture, Kagoshima University, 21–24 Korimoto 1, Kagoshima 890–0065, [1)]Department of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, and [2)]Tsukuba Central Laboratories and [3)]Laboratory of Clinical Microbiology, Kyoritsu Shoji Corporation, 1–12–4 Kudankita, Chiyoda-ku, Tokyo 102–0073, Japan

(Received 21 April 1999/Accepted 1 June 1999)

ABSTRACT. The N-terminal unique polypeptide region of the capsid precursor protein of feline calicivirus (FCV) and the protein encoded by ORF3 of FCV were expressed as fusion proteins with glutathione S-transferase to analyze the expressed products in FCV-infected cells. Immunoblot analysis using a serum from a cat experimentally infected with FCV indicated relatively high immunogenicity of the N-terminal polypeptide in FCV-infected cats, as compared with the ORF3 protein. Specific antisera were prepared by immunization to mice with the fused proteins and used in immunoblot analysis. A 14 kD product corresponding to the N-terminal polypeptide and a 10 kD polypeptide of the ORF3 product were identified in the FCV-infected cells but not detected in the purified particles. No neutralization activity against FCV was detected in these antisera. The proteins identified as polypeptides of 14 kD and 10 kD in this study may have functions as non-structural proteins.—KEY WORDS: capsid precursor protein, expression, feline calicivirus, glutathione S-transferase, immunogenicity.

J. Vet. Med. Sci. 61(9): 1043–1047, 1999

Feline calicivirus (FCV) is a major cause of upper respiratory diseases in cats [5]. FCV possesses a single stranded RNA genome of approximately 7.7 kilobases (kb) containing three open reading frames (ORF) [2]. ORF1 was suggested to encode the non-structural proteins including an RNA-dependent RNA polymerase and a cysteine proteinase [8]. ORF2 encodes the capsid precursor protein [10, 18] and ORF3 encodes a small protein of which function is unknown [6]. The capsid precursor protein with a molecular mass of 76 kilodaltons (kD) is processed to capsid protein (62 kD) immediately after translation [1]. Although a virus-encoded proteinase is known to cleave the capsid precursor protein between the amino acid positions 124 and 125 [16], the existence of an N-terminal polypeptide of the precursor protein has not been shown.

In this study, we subcloned the precursor unique region (PUR), 5'-end 372 nucleotides (nt) of ORF2, which encodes an N-terminal and unique polypeptide of the capsid precursor protein, and the ORF3 (318 nt) into expression plasmid vectors to analyze the expressed products in FCV-infected cells.

MATERIALS AND METHODS

Virus and cells: Crandell feline kidney (CRFK) cells were grown in Dulbecco's modified Eagle's minimun essential medium supplemented with 8% fetal calf serum and antibiotics. CsCl-purification of FCV was performed essentially as described for canine calicivirus (CaCV) previously [13]. The capsid precursor protein was prepared from cells infected with the FCV F4 strain [17] under an elevated growth temperature as described by Carter [1] with a modification described by Shin *et al.* [15].

Construction of expression plasmids: The PUR, 5'-end 372 nt of ORF2, was amplified by polymerase chain reaction (PCR) using primers PrU5'+E (5'GGAATTCATGTGCT-CAACCTGCGCT3') and PrU3'+X (5'GACTCGAGCTCC-AATCGGAAGAGCG3') and the plasmid pMCV-II [15] as the template. Primer PrU5'+E corresponded to nt 1–18 of the FCV F4 ORF2 with an *Eco*RI restriction enzyme site upstream from the first ATG codon. Antisence primer PrU3'+X was complementary to nt 356–372 of the ORF2 and contained an *Xho*I restriction site. The amplified DNA fragments were digested with *Eco*RI and *Xho*I and ligated into a glutathione S-transferase (GST) gene fusion vector pGEX-5X-1 (Pharmacia Biotech) and the resulting plasmid was designated pGEX-PUR. The deduced size of the product of pGEX-PUR is 40 kD. DNA fragments which contained ORF3 were generated by PCR with primers 7655R (5'GCCCAAAGGGATACATGATC3') and 7317F (5'GAG CATGAATTCAATTCTTGGTTTGATT3') and with the plasmid pFCV 119 [18] as the template. Primer 7655R was complementary to the 20 nt just downstream from the end of the ORF3 and primer 7317F corresponded to nt 1–24 of the ORF3 with an additional 4 nt to adjust its GC/AT ratio. The amplified ORF3 fragments was once inserted in pUC119 and excised by the digestion with *Eco*RI and *Sal*I. The digested fragments were cloned into another

* PRESENT ADDRESS: SHINCHI, H., Kyoto Biken Laboratories, 24–16 Makishima-cho, Uji, Kyoto 611–0041, Japan.

GST gene fusion vector, pGEX-5X-3 and the resulting plasmid was designated pGEX-ORF3, which should produce a 38 kD fusion protein.

Expression in E. coli: The transformed *E. coli* cells with the plasmids described above were grown to plateau by overnight incubation at 37°C in 2xYT medium. The cultures were diluted tenfold in prewarmed medium and incubated with shaking at 37°C. One hr after the incubation, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and the incubation was continued for an additional 3 hr. The cells were collected by centrifugation, washed twice in chilled phosphate-buffered saline (PBS) and resuspend in PBS.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis: The suspension of the IPTG-induced *E. coli* cells or FCV-infected CRFK cells was mixed with an equal volume of 2x SDS-loading buffer (0.1 M Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) and boiled for 5 min. The samples were separated by SDS-PAGE using 12% or 15% gels and either stained with Coomassie Brilliant Blue or transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were first incubated with the appropriate sera and then reacted with peroxidase conjugates. The bands were visualized by the addition of a diaminobenzidine-hydrogen peroxide substrate.

Preparation of mouse antisera: The bands of the GST fusion proteins were visualized with Coomassie Brilliant Blue after SDS-PAGE using a 12% gel and cut out as gel slices. The fusion proteins were eluted from the gel slices using an electro-eluter (Model 422, Bio-Rad) according to the manufacture's instruction. The eluted proteins were inoculated to ddy mice with complete Freund's adjuvant. Additional injections of the proteins with incomplete Freund's adjuvant were given to the mice four weeks after the first injections. The mice were sacrificed by cardiac bleeding under an anesthetized condition two weeks after the second injections. Anti-capsid protein serum was prepared by immunizing mice with the capsid protein of CsCl-purified FCV which was separated by SDS-PAGE and electro-elution as described above.

Experimental cat infection: A specific-pathogen-free cat was used for experimental infection. The cat was inoculated intraocularly, intranasally and orally with 1.0×10^8 plaque forming units of the FCV F4 strain. Blood samples were collected at 0, 2, 5, 8, 12, 15, 22, 28 and 30 days after infection.

RESULTS

The expressions of the GST fusion proteins by the vectors of pGEX-PUR and pGEX-ORF3 were investigated by estimating the sizes of the resulting proteins relative to native GST in SDS-PAGE analysis using a 12% gel (Fig. 1). After Coomassie Blue staining, the lanes corresponding to the *E. coli* transformed with the plasmids showed major bands of fusion proteins with GST (26 kD, lanes 2 and 5) of expected sizes, 40 kD for the GST-PUR fusion protein (lane 3) and 38 kD for the GST-ORF3 fusion protein (lane 6), respectively, indicating translational continuities between the GST and the viral genes targeted in this study.

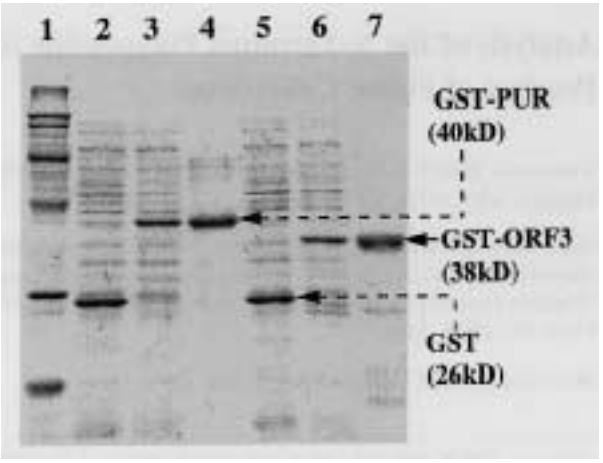

Fig. 1. SDS-PAGE analysis of the expressed proteins in *E. coli* using a 12% gel. Lanes: 1, molecular weight markers; 2, pGEX-5X-1-transformed *E. coli*; 3, pGEX-PUR-transformed *E. coli*; 4, Electro-eluted PUR-GST fusion protein; 5, pGEX-5X-3-transformed *E. coli*; 6, pGEX-ORF3-transformed *E. coli*; 7, Electro-eluted ORF3-GST fusion protein.

The proteins separated by the SDS-PAGE were transferred onto a PVDF membrane and analyzed by immunoblotting using serum samples of a cat experimentally infected with FCV to investigate the antigenic properties of the fusion proteins. The results shown in Fig. 2 demonstrate that the cat serum 30 days after FCV infection strongly reacted with the GST-PUR fusion protein including the N-terminal polypeptide of the capsid precursor protein. However, the reactivity with the GST-ORF3 fusion protein was not apparent in this analysis. The N-terminal polypeptide seemed to have relatively high immunogenicity in FCV-infected cats, as compared with the ORF3 product. The immunogenicity of the N-terminal polypeptide was confirmed by the immunoblot analysis using serum samples serially collected from the cat experimentally infected with FCV (Fig. 3). Although some degree of background reaction of the sera was observed, positive reactivity was first recognized at 12 days after infection and the intensity of the fusion protein band increased after the seroconversion.

The fused proteins were purified by SDS-PAGE followed by electro-elution and the purity of the eluted proteins was examined by SDS-PAGE (Fig. 1., lanes 4 and 7). Specific antisera were prepared by immunization of mice with the purified proteins and used in immunoblot analysis to detect and identify the proteins in FCV-infected cells and in CsCl-purified virus particles (Fig. 4). A 14 kD product corresponding to the N-terminal polypeptide and a 10 kD polypeptide of the ORF3 product were identified in the

FCV-infected cells (lanes 2 and 6), although some difference between intensities of the bands were observed. As expected, in the infected cells with an elevated temperature treatment, an additional band of the capsid precursor protein

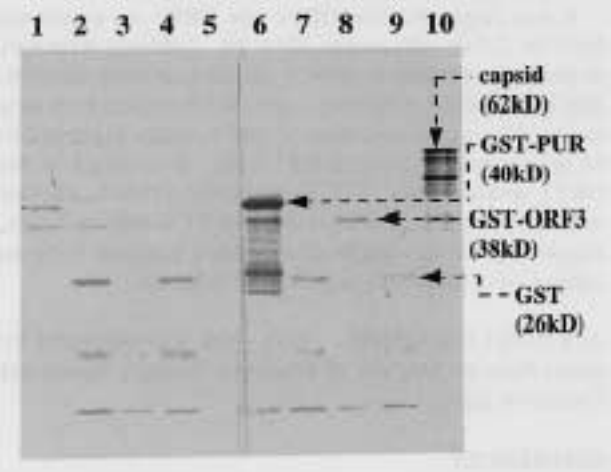

Fig. 2. Reactivities of FCV-infected cat sera to PUR- and ORF3-GST fusion proteins in immunoblot analysis. *E. coli* transformed with the plasmids (lanes 1 and 6, pGEX-PUR; lanes 2 and 7, pGEX-5X-1; lanes 3 and 8, pGEX-ORF3; lanes 4 and 9, pGEX-5X-3) and purified FCV particles (lanes 5 and 10) were solubilized and electrophoresed in 12 % polyacrylamide gel. The resolved proteins were transferred to a membrane and immunoblotted with serum samples of a cat before (lanes 1 to 5) and after (lanes 6 to 10) FCV infection.

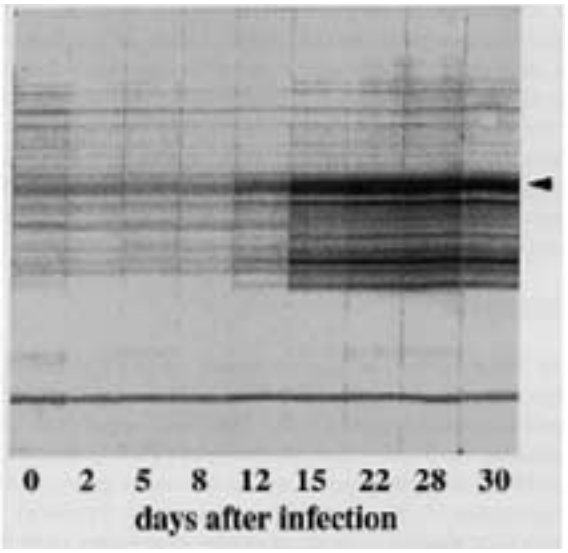

Fig. 3. Reactivity of the PUR-GST fusion protein with serum samples collected from a cat infected with FCV in immunoblot analysis. The PUR-GST fusion protein was resolved by SDS-PAGE (12% gel) and transferred to a membrane, which was sliced and reacted with feline serum samples serially collected from a cat experimentally infected with FCV. The arrow head indicates the position of the PUR-GST fusion protein (40 kD).

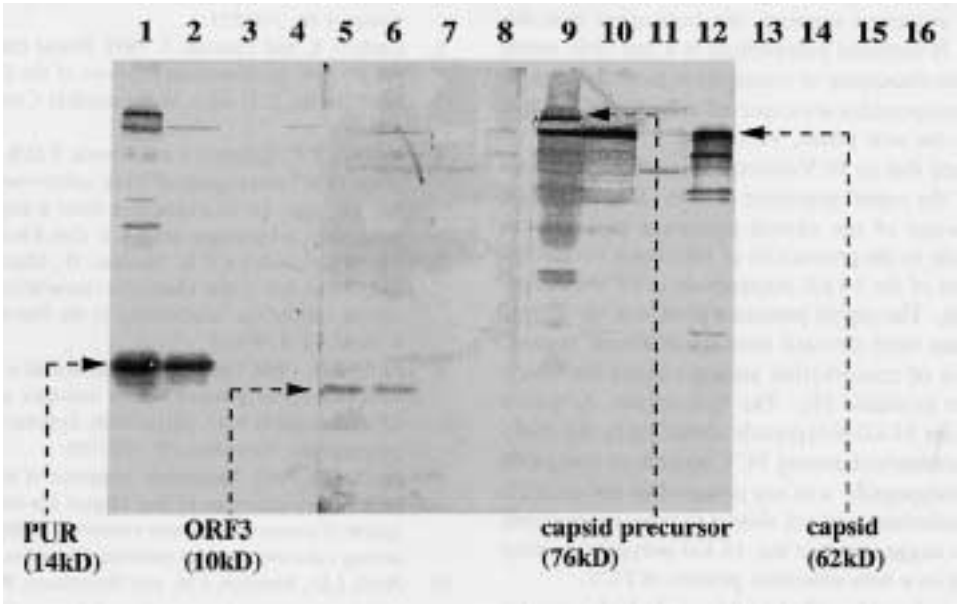

Fig. 4. Immunoblot analysis of the proteins translated from ORFs 2 and 3 of FCV. FCV-infected CRFK cells treated with an elevated temperature (lanes 1, 5, 9 and 13), the infected cells without the treatment (lanes 2, 6, 10 and 14), mock-infected cells (lanes 3, 7, 11 and 15) and CsCl-purified FCV (lanes 4, 8, 12 and 16) were solubilized and electrophoresed in a 15 % polyacrylamide gel. The resolved proteins transferred to membranes were reacted with mouse antisera specific to the PUR-GST fusion protein (lanes 1 to 4), to the ORF3-GST fusion protein (lanes 5 to 8), and to the capsid protein (lanes 9 to 12), and non-immunized mouse serum (lanes 13 to 16).

(76 kD) was recognized by the antisera to the GST-PUR fusion protein and to the capsid protein (lanes 1 and 9). On the other hand, no obvious difference was detected by the antiserum specific to the GST-ORF3 fusion protein between the infected cells with and without the treatment (lanes 5 and 6). No protein band was shown in the purified particles by the antisera to the fusion proteins (lenes 4 and 8) and no neutralization activity against FCV was detected in these antisera (data not shown), suggesting that the proteins detected in this study may have functions as non-structural proteins.

DISCUSSION

In the members of *Caliciviridae*, only FCV and San Miguel sea lion virus (SMSV) have been known to produce capsid precursor protein [1, 4]. They have larger ORF2, by containing the 5'-end region designated as PUR in this study, than ORF2 or corresponding region for capsid gene of other caliciviruses [7, 9]. Recently, a new genus, *Vesivirus*, of which type species is *swine vesicular exanthema virus* has been proposed by the *Caliciviridae* study group in the international committee on Taxonomy of Viruses [11]. Sequence analysis suggested FCV and SMSV would be classified in the genus with newly analyzed CaCV, which also has a large ORF2 encoding a capsid precursor protein [12]. In this study, the N-terminal part of the precursor protein encoded in the PUR was identified as a 14 kD polypeptide in FCV-infected cells and the polypeptide was shown to be produced by cleavage of the capsid precursor protein. This evidence supports the indication that the removal of the N-terminal polypeptide is a one-time event and not a process consisting of consecutive proteolytic steps [16]. Similar polypeptides are expected to be found in other caliciviruses in the new genus, Vesivirus.

It was reported that an FCV-encoded proteinase mediates the cleavage of the capsid precursor protein [16]. Although complete cleavage of the capsid precursor protein was suggested critical in the production of infectious virions [1, 16], the function of the 14 kD polypeptide in FCV-infected cells is unknown. The capsid precursor protein of the animal caliciviruses has been divided into six different regions based on degree of conservation among viruses for which sequences were available [9]. The first region, A, which corresponds to the 14 kD polypeptide identified in this study was relatively conserved among FCV sequences compared [14]. As the polypeptide was not detected in the purified FCV and the antiserum was not shown to have neutralizing activity, it was suggested that the 14 kD polypeptide may have a function as a non-structural protein of FCV.

The ORF3 product identified in this study had a similar molecular mass of 10 kD to that of 8 kD reported by Herbert *et al.* [6]. ORF3 as a small ORF found in the extreme 3' end of the genome is common to all caliciviruses [3]. The protein encoded by ORF3 was thought to have a nucleic acid binding function because of the number of conserved basic amino acid residues [10]. Although we could not detect the ORF3 product in the purified FCV, the ORF2 (corresponding to ORF3 of FCV) product of rabbit hemorragic disease virus was detected as a minor structural protein of approximately 10 kD [19]. Further analysis will be necessary to find the function of the product of FCV.

It was suggested that ORF2 and ORF3 are expressed from the 2.4 kb subgenomic RNA [6]. Antibody responses to the fusion proteins in an FCV-infected cat were different. The low antibody response to the ORF3 product may be a result of the expression level of ORF3, which suggested to be approximately 10% of ORF2 [6]. In contrast to the ORF3 product, the GST-PUR fusion protein showed relatively high immunogenicity in FCV-infected cats, suggesting that the fusion protein has a potential to be an antigen for a diagnostic test of FCV infection.

ACKNOWLEDGMENTS. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Carter, M.J. 1989. Feline calicivirus protein synthesis investigated by western blotting. *Arch.Virol.* 108: 69–79.
2. Carter, M.J., Milton, I.D., Meanger, J., Bennett, M., Gaskell, R.M. and Turner, P.C. 1992. The complete nucleotide sequence of a feline calicivirus. *Virology* 190: 443–448.
3. Clarke, I.N. and Lambden, P.R. 1997. The molecular biology of caliciviruses. *J. Gen.Virol.* 78: 291–301.
4. Fretz, M. and Schaffer, F.L. 1978. Calicivirus proteins in infected cells: evidence for a capsid polypeptide precursor. *Virology* 89: 318–321.
5. Gaskell, R. and Dawson, S. 1998. Feline respiratory disease. pp. 97–106. *In*: Infectious Diseases of the Dog and Cat, 2nd ed. (Greene, C.E. ed.), W.B.Saunders Company, Philadelphia.
6. Herbert, T.P., Brierley, I. and Brown, T.D.K. 1996. Detection of the ORF3 polypeptide of feline calicivirus in infected cells and evidence for its expression from a single, functionally bicistronic, subgenomic mRNA. *J. Gen.Virol.* 77: 123–127.
7. Liu, B.L., Lambden, P.R., Gunther, H., Otto, P., Elschner, M. and Clarke, I.N. 1999. Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. *J. Virol.* 73: 819–825.
8. Neill, J.D. 1990. Nucleotide sequence of a region of the feline calicivirus genome which encodes picornavirus-like RNA-dependent RNA polymerase, cysteine protease and 2C polypeptides. *Virus Res.* 17: 145–160.
9. Neill, J.D. 1992. Nucleotide sequence of the capsid protein gene of two serotypes of San Miguel sea lion virus: identification of conserved and non-conserved amino acid sequences among calicivirus capsid proteins. *Virus Res.* 24: 211–222.
10. Neill, J.D., Reardon, I.M. and Heinrikson, R.L. 1991. Nucleotide sequence and expression of the capsid protein gene of feline calicivirus. *J. Virol.* 65: 5440–5447.
11. Pringle, C.R. 1998. Virus Taxonomy - San Diego 1998. *Arch. Virol.* 143: 1449–1459.
12. Roerink, F., Hashimoto, M., Tohya, Y. and Mochizuki, M. 1999. Organization of the canine calicivirus genome from the RNA polymerase gene to the poly (A) tail. *J. Gen. Virol.* 80: 929–935.

13. San Gabriel, M.C.S., Tohya, Y., Sugimura, T., Shimizu, T., Ishiguro, S. and Mochizuki, M. 1997. Identification of canine calicivirus capsid protein and its immunoreactivity in western blotting. *J. Vet. Med. Sci.* 59: 97–101.
14. Seal, B.S., Ridpath, J.K. and Mengeling, W.L. 1993. Analysis of feline calicivirus capsid protein genes: identification of variable antigenic determinant regions of the protein. *J. Gen. Virol.* 74: 2519–2524.
15. Shin, Y.-S., Tohya, Y., Oshikamo, R., Kawaguchi, Y., Tomonaga, K., Miyazawa, T., Kai, C. and Mikami, T. 1993. Antigenic analysis of feline calicivirus capsid precursor protein and its deleted polypeptides produced in a mammalian cDNA expression system. *Virus Res.* 30: 17–26.
16. Sosnovtsev, S.V., Sosnovtseva, S.A. and Green, K.Y. 1998. Cleavage of the feline calicivirus capsid precursor is mediated by a virus-encoded proteinase. *J. Virol.* 72: 3051–3059.
17. Takahashi, E., Konishi, S. and Ogata, M. 1971. Studies on cytopathogenic viruses from cats with respiratory infections. II. Characterization of feline picornaviruses. *Jpn. J. Vet. Sci.* 33: 81–87.
18. Tohya, Y., Taniguchi, Y., Takahashi, E., Utagawa, E., Takeda, N., Miyamura, K., Yamazaki, S. and Mikami, T. 1991. Sequence analysis of the 3'-end of feline calicivirus genome. *Virology* 183: 810–814.
19. Wirblich, C., Thiel, H.-J. and Meyers, G. 1996. Genetic map of the calicivirus rabbit hemorrhagic disease virus as deduced from *in vitro* translation studies. *J. Virol.* 70: 7974–7983.