Construction of the Recombinant Feline Herpesvirus Type 1 Deleted Thymidine Kinase Gene

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ABSTRACT. We constructed a recombinant virus containing defined deletion within thymidine kinase (TK) gene from feline herpesvirus type 1 (FHV-1) C7301, which was isolated in Japan, by standard rescue procedures with thymidine arabinoside (araT) selection. The araT resistant recombinant virus was designated as C7301dTK. Southern blot analysis, together with polymerase chain reaction, revealed a deletion with the expected size, based on agarose gel electrophoresis, of the TK gene region in the C7301dTK. Growth kinetics of the C7301dTK in CRFK cells was similar to that of the parent C7301 that possesses TK activity. However, plaques produced by the C7301dTK were significantly smaller than those of the parent virus. This report might be applicable for the development of a new genetically engineered FHV-1 vaccine.—**KEY WORDS:** feline herpesvirus type 1, recombinant virus, thymidine kinase.

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Feline herpesvirus type 1 (FHV-1) is one of the most important pathogens in cats causing primarily an upper respiratory tract disease, known as feline viral rhinotracheitis (FVR). In particular, infections of newborn or debilitated cats with FHV-1 result in a severe generalized disease with a high mortality rate [32].

Many FHV-1 strains have been isolated from various parts of the world and antigenically compared by several serological tests. The results revealed that the FHV-1 isolates are antigenically indistinguishable from each other [1, 6, 20, 29, 41, 42]. In restriction endonuclease cleavage patterns of viral DNAs, slight shifts in the electrophoretic mobility of a few fragments were observed, but no remarkable change was detected [15, 16]. These data suggested a homogeneous population of FHV-1, compared with other α -herpesviruses. However, it is evident that less virulent strains of FHV-1 exist in field and these strains have been selected or developed to be used as vaccines without clear biological markers of the attenuated strain [2–4, 9–11, 31, 33, 37–39, 45].

Recently, we compared 9 FHV-1 strains including the vaccine strain of F2 [3], the prototype strain of C27 [7] and Japanese field isolates of C7301 and C7805 [28] by using immunoblot and restriction endonuclease analyses, and found that a viral structural protein with a molecular weight of 36 kDa was absent in the vaccine strain and the cleavage pattern of genome DNA with a restriction enzyme, *Mlu*I, was different between field isolates and a vaccine strain [19]. Further, we reported that 78 FHV-1 field isolates were divided into 3 genotypes by using *Mlu*I [26] and additionally into 4 types by immunoblot analysis [27]. Although the lack of the 36 kDa protein and the different *Mlu*I cleavage pattern might be used as markers

for the vaccine F2 strain, their relation to the virulence or attenuation of FHV-1 has not been studied in detail. Therefore, by deletion of the gene involved in pathogenicity, a live vaccine for FVR with a clear genetic marker to distinguish from wild strains is needed to be developed.

Thymidine kinase (TK), encoded in the genome of many herpesviruses, is not essential for virus growth in actively dividing tissue culture cells [13]. However, the TK synthesized by α -herpesviruses appears to facilitate the growth of virus in non-dividing cells [13]. Many recent investigations have demonstrated that the TK-defective mutants of herpes simplex virus (HSV) [13], pseudorabies virus (PRV) [21, 43], bovine herpesvirus-1 (BHV-1) [22], and equine herpesvirus-1 (EHV-1) [40] are remarkably attenuated for their natural hosts or mice and appear to be less readily reactivated from neurons [12]. Such mutants have been proposed as the basis for vaccine production [21].

Nunberg *et al.* [30] first identified the TK gene of American FHV-1 strain, UC-D, with 1029 base pairs (bp) long encoding the predicted FHV-1 TK protein of 343 amino acids, and constructed a recombinant FHV-1 deleted *Eco*RV-*Hind*III fragment of the TK gene showing TK[−] phenotype. Cole *et al.* [5] have reported the development of the recombinant FHV-1 expressing the envelope (*env*) or the *gag* protein of feline leukemia virus (FeLV), in which these expressing cassettes, utilizing the human cytomegalovirus immediate-early promoter, have been inserted within the FHV-1 TK gene. Wardley *et al.* [44] have vaccinated cats first with the recombinant FHV-1 expressing *env* and *gag* proteins and later with *gag/env* particles purified from insect cells that were coinfecting with two recombinant baculoviruses expressing *env* or *gag* protein, and achieved 100% protection against FeLV challenge. Beside these characteristics, the recombinant FHV-1 deleted TK gene had not been fully characterized *in vitro* or *in vivo*.

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In this report, as an initial step for development of recombinant vaccine of FHV-1 with a genetic marker against FVR, we sequenced the TK gene and constructed a recombinant FHV-1 deleted a 450 bp *EcoRV-SmaI* fragment of the gene, using a Japanese isolate of C7301 strain and characterized the biological properties of the recombinant virus *in vitro*.

MATERIALS AND METHODS

Viruses, cells and media: The C7301 strain of FHV-1, which was isolated from a cat with respiratory diseases in 1973 in Japan [28], was used as the parent strain for construction of the recombinant FHV-1. The parent and recombinant viruses were grown in Crandell feline kidney (CRFK) cells [8] in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical, Missouri, U.S.A.) supplemented with 8% heat inactivated fetal calf serum (FCS) and antibiotics. After infection, cells were maintained in the same medium without FCS.

DNA sequencing: We previously cloned a 6.6 kilobase pair (kbp) *EcoRI* fragment, carried out a mapping, and sequenced a 3746 bp *SacI* fragment in the fragment [25]. The TK gene of FHV-1 C7301 strain was reported to be located immediately upstream of gH homologous gene [25]. To sequence the TK gene of the strain, we subcloned a 1.8 kbp *BamHI-HindIII* fragment, 0.5 kbp *SacI-EcoRV* fragment and a 0.8 kbp *HindIII-EcoRI* fragment (Fig. 1B) into plasmid Bluescript (pBluescript) SK+, respectively. DNA sequencing was performed using the Dye primer cycle sequencing method supplied by Applied Biosystems (ABI) (Foster City, CA) and then analyzed with a model 370A ABI autosequencer.

Construction of a transfer vector: We cloned a 5.5 kbp *BamHI-SalI* fragment including the TK gene and 5' half

sequence of the gH homologous gene from the 19 kbp *SalI* A fragment (Fig. 1A) [34] of FHV-1 C7301 into plasmid pUC19, and carried out a mapping of the 5.5 kbp fragment (Fig. 1B). The plasmid was designated as pTK-SB and used for introduction of a transfer vector. First, after the pTK-SB was digested with *EcoRV* and *SalI*, the 3.3 kbp *EcoRV-SalI* fragment was isolated by agarose gel electrophoresis. The 3.3 kbp fragment was subcloned into *EcoRV-SalI* site of a pBluescript KS-, and the plasmid was designated as pTK (Ev-Sa). Next, by digesting the pTK-SB with *BamHI* and *SmaI*, the 1.7 kbp *BamHI-SmaI* fragment was isolated and subcloned into *BamHI-SmaI* site of the pTK (Ev-Sa). The plasmid was designated as pTK-SB Δ SE (Fig. 1C) and used as a transfer vector.

Extraction of viral DNA from infected cells: Monolayers of CRFK cells were inoculated with C7301 strain or the recombinant virus at a multiplicity of infection (MOI) of 3 plaque-forming units (PFU) per cell. When cytopathic effects (CPEs) were advanced, the cells were washed once with phosphate-buffered saline (PBS), lysed in 1% SDS, 0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl and 1 mM EDTA, and then treated with 1 mg/ml of pronase E at 37°C overnight. The viral DNA was extracted with phenol and precipitated by ethanol according to the method described by Hirai *et al.* [17] and dialyzed three times in distilled water.

Construction and selection of recombinant FHV-1: The viral DNA of C7301 strain was cotransfected with transfer vector pTK-SB Δ SE into CRFK cells by the calcium phosphate precipitation method [14]. After the propagated viruses were harvested from the transfected cultures by freezing and thawing, the recombinant viruses were selected on CRFK cells in the presence of 100 μ g/ml of thymidine arabinoside (araT) that inhibits the replication of the parent virus with TK⁺ phenotype as described by Nunberg *et al.* [30]. The selected viruses were plaque-purified three times described as below.

Southern blot analysis: After DNA extracted from virus-infected cells was digested with either *SalI* or *EcoRI*, the fragments were separated by electrophoresis on 0.5% agarose gels and transferred to nylon membranes (Biodyne, Pall Biosupport, NY, U.S.A.) according to the manufacturer's instructions. A 5.5 kbp *BamHI-SalI* fragment including the TK coding region (Fig. 1B) was labeled with [³²P] α -CTP using a nick-translation kit (Boehringer Mannheim, Germany) and used as a probe. Hybridization was done in hybridization solution [50% formamide, 0.6 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.02 M EDTA, 0.5% SDS and 100 μ g/ml denatured salmon sperm DNA] at 42°C overnight. The membrane was washed three times in 0.2 $\times$ SSC (0.3 M NaCl, 0.03 M trisodium citrate dihydrate) containing 0.1% SDS for 30 min at 42°C, and then exposed to a X-ray film at -70°C.

Amplification of TK gene regions by polymerase chain reaction (PCR): The sequences for the primers were derived from our study and the published sequence of the FHV-1 TK gene [30]. The nucleotide sequences of the

Fig. 1. Construction of the transfer vector. A, *SalI* restriction endonuclease map of FHV-1 genome [34]. UL represents the long unique region of the genome, and Us, IRs, and TRs represent the short unique, inverted, and terminal repeat regions, respectively, of the short component. B, Restriction endonuclease map of the *BamHI-SalI* fragment of pTK-SB containing the TK gene. The arrow indicates the direction and location of TK transcription. The fragment was used as a probe in southern blot analysis. C, Restriction endonuclease map of the *BamHI-SalI* fragment of pTK-SB Δ SE deleted the TK gene. The black quadrangle indicates 12 bp nucleotides of multi-cloning site of a pBluescript KS- inserted into the deleted site. B, *BamHI*; Ev, *EcoRV*; Sc, *SacI*; Sm, *SmaI*; Hm, *HindIII*; Ei, *EcoRI*; Sa, *SalI*

primers used were 5'-GAACCATCCCCGTTTCAGA-AT-3' (TK-up) at position 11-30 and 5'-CATTCATCAG-GGGTTCCTTC-3' (TK-down) at position 932-913 in the FHV-1 TK gene [30] with a target sequence of 922 bp. PCR [35] was carried out in 0.5 ml microfuge tubes in a total volume of 50 μ l. One microgram of virus DNA was added to the reaction mixture containing 1 μ M of each primer, 200 μ M of each dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, and 1 U of AmpliTaq DNA polymerase (Perkin Elmer, Connecticut, U.S.A.). All reaction samples were overlaid with mineral oil (Sigma Chemical). Subsequently, 25 cycles of denaturation (94°C; 1 min), annealing (60°C; 1 min), and extension (72°C; 2 min) were performed. After amplification, the PCR product was analyzed on 2% agarose gel.

Immunoblot analysis and indirect immunofluorescence assay (IFA): Immunoblot analysis and IFA were performed as described previously [19, 24].

Time course of viral growth: Monolayers of CRFK cells were prepared on wells in 6-well plate and inoculated with viral samples at a MOI of 3 PFU/cell. After adsorption for 90 min, the monolayers were washed three times in DMEM and incubated at 37°C in 2 ml of maintenance medium per well. At various intervals after inoculation, the infected cells together with culture fluids were scraped off with rubber policeman and centrifuged at a low speed to sediment the infected cells. The culture supernatants were used as the extracellular samples. After the pellets were washed twice with DMEM and then suspended in 2 ml of maintenance medium, the suspensions were subjected to three cycles of freeze-thaw treatment and centrifuged at 4,000 rpm for 10 min to remove the debris. The resulting supernatants were used as the intracellular samples. Both intra- and extra-cellular samples were titrated for viral infectivity (log TCID₅₀ per 50 μ l).

Plaque assay: For plaque assay, 0.2 ml each of virus samples diluted in DMEM was inoculated onto CRFK cell monolayers in 6-well plate. After adsorption for 90 min, excess inoculum was removed, and 2 ml of DMEM containing 2% FCS and 0.8% agarose was added to each well. The plates were incubated at 37°C for 3 or 7 days, and then stained with 0.75% crystal violet in phosphate-buffered formalin. The Student's *t* test was employed to compare the means of plaque sizes (*n*=20) of the parent strain and the recombinant virus. Results were considered statistically significant only if the comparison gave a *P* value of < 0.005.

RESULTS

Sequence of TK gene of FHV-1 C7301 strain: Full sequence of FHV-1 TK gene has been determined in American strain, UC-D [30]. Here we determined TK gene sequence of a Japanese isolate C7301 strain. No difference in nucleotide sequence of the TK gene was observed between these two strains (data not shown), indicating the extreme homogeneity of these FHV-1

genes.

Construction of the recombinant FHV-1 deleted TK gene: To construct the recombinant virus deleted TK gene, we cotransfected C7301 DNA with a transfer vector pFTK-SB Δ SE into CRFK cells and harvested the transfectant. TK deficient mutants were selected from transfectant in the presence of araT, which inhibits the replication of the virus with TK⁺ phenotype [36]. One of these araT resistant viruses was plaque-purified three times and designated as C7301dTK.

To examine whether C7301dTK has the intended deletion in the TK gene, we compared TK gene regions of the recombinant with that of the parent using Southern blot analysis. The size of 19 kbp *Sal*I fragment observed in parent C7301 DNA, which contains the TK gene, reduced slightly in C7301dTK (Fig. 2A). On the other hand, the size of larger *Eco*RI fragment of C7301dTK corresponding to the 6.6 kbp fragment of C7301 was 5.7 kbp (Fig. 2B). The size difference detected between them is due to the 438 bp deletion induced between the *Eco*RV and *Sma*I sites and to a new 424 bp fragment generated by an additional *Eco*RI site in 12 nucleotides of a pBluescript KS- inserted between the *Eco*RV and *Sma*I sites (Fig. 1C). Although the 424 bp fragment was too small to be detected in the analysis, the 3.6 kbp fragments of both strain, which locate immediately upstream of the TK gene, were observed as expected (Fig. 2B).

The sequence reduction of C7301dTK was also confirmed by PCR method (Fig. 3). The TK gene of C7301

Fig. 2. Southern blot analysis. Viral DNAs of C7301 (lane 1) and C7301dTK (lane 2) were digested with restriction endonuclease *Sal*I (A) or *Eco*RI (B), separated by electrophoresis, transferred to nylon membranes and hybridized as described in the text. The probe used for hybridization was shown in Fig. 1B. The sizes of hybridizing bands are indicated by big arrows, and the right small arrows in Fig. 2A mean the slight reduction in C7301dTK.

Fig. 3. PCR amplification of FHV-1 TK gene. Viral DNAs of C7301 (lane 1) and C7301d/TK (lane 2) were subjected to PCR amplification, using TK-up and TK-down primers as described in the text. The sizes of PCR products are indicated by arrows.

was properly deleted 450 bp sequence and inserted 12 bp nucleotides into the deleted site by cotransfection with transfer vector pFTK-SB Δ SE.

These data suggested that the intended deletion was introduced in the TK gene of the parent virus, expecting the loss of TK activity.

Additionally, to confirm that there is no detectable change of other immunogenic proteins in C7301d/TK, we carried out immunoblot analysis using a polyvalent cat serum against C7301 strain and IFA using monoclonal antibodies (MAbs) 22F4, 17C11, and 41G4, which recognize gp143/108, gp113, and gp60, respectively [18]. These MAbs are associated with virus-neutralizing epitopes on the virus. No difference between C7301 and C7301d/TK was detected in their reactivity (data not shown).

In vitro growth properties of the recombinant FHV-1 C7301d/TK: Next, we compared the growth properties of C7301d/TK with those of parent C7301, based on the growth kinetics in CRFK cells. We inoculated CRFK cells with the viruses and determined the amount of intra- and extra-cellular viruses produced at 0, 6, 12, 24, 48 and 72 hr post-inoculation. Figures 4A and 4B show the time course of intracellular and extracellular viral growth, respectively. The virus titers of intra- and extra-cellular samples from CRFK cells inoculated with the C7301d/TK or the parent C7301 increased rapidly and reached the maximum at 24 and 48 hr post-inoculation, respectively. The growth patterns of both viruses in intra- and extra-cellular phases were almost identical.

We also inoculated CRFK cells with these viruses at a low MOI and compared the plaque sizes of the C7301d/TK and the parent C7301 at 3 and 7 days

Fig. 4. Time course of viral growth in CRFK cells, inoculated with the parent C7301 strain (○ or □) and the recombinant C7301d/TK (● or ■) at a MOI of 3 PFU/cell. Infectivity titers of intracellular virus (A) and extracellular virus (B) are expressed as log TCID₅₀ per 50 μ l.

Table 1. Plaque sizes of parent strain and the recombinant virus in CRFK cells

Strain	Days after inoculation	
	4	7
C7301	0.60 $\pm$ 0.17 ^{a)}	1.41 $\pm$ 0.17
C7301d/TK	0.50 $\pm$ 0.09 ^{b)}	1.16 $\pm$ 0.13

a) Mean $\pm$ Standard deviation (n=20) of plaque size (mm)

b) $P < 0.005$ as determined by *t*-test

post-inoculation. The mean plaque size (n=20) of C7301d/TK was smaller than that of the parent C7301 strain and the difference in plaque size of each virus was significant statistically ($p < 0.005$) (Table 1).

DISCUSSION

In this study, we constructed successfully a recombinant FHV-1, C7301d/TK, which has a 450 bp deletion within the region encoding TK and has no detectable change in other immunogenic proteins. The results suggest that the deleted TK gene of C7301d/TK can be used not only as a genetic marker to distinguish from wild strains, which possess the TK⁺ phenotype, but also as insertion site for foreign immunogenic genes to develop the recombinant vaccines for cats.

Inactivation of the TK gene has been shown to reduce the virulence and reactivation of α -herpesviruses [12, 13, 21, 22, 40, 43]. The TK⁻ mutants of the viruses were proposed as potential agents of immunoprophylaxis or as a basis for the development of recombinant vaccines expressing foreign immunogens [5, 21, 23].

Nunberg *et al.* [30] constructed an araT resistant recombinant FHV-1 (FHV-113) and confirmed that the FHV-113 is deficient in TK enzymatic activity. Since the nucleotide sequence of TK gene was exactly same in both FHV-1 UC-D and C7301 strains and the deleted region in C7301dTK perfectly contains the *EcoRV*–*HindIII* region deleted by Nunberg *et al.* [30], it is reasonable to assume that the araT resistant C7301dTK is deficient in the TK enzymatic activity. This assumption is supported by the fact that araT treatment provides stringent selection against the replication of TK⁺ FHV-1 [36].

Here, we found no significant difference in growth patterns between the C7301dTK and parent C7301 by analysis of the time course experiments. The result showed that the TK gene of C7301 is nonessential for virus growth in actively dividing tissue culture cells as observed in other herpesviruses [13]. However, the plaque size of the C7301dTK was slightly but significantly smaller than that of the parent C7301. We also constructed and characterized a recombinant F2 vaccine strain deleted TK gene by using a same transfer vector and by the same method used in the present studies. However we obtained similar results as C7301dTK (data not shown). The deletion of the TK gene might influence the viral replication in CRFK cells inoculated with the recombinant virus at a low MOI or the viral spread by means of direct cell-to-cell transmission *in vitro*. Further studies will be required to resolve the above subjects.

Additionally, the transfer vector pFTK-SB Δ SE has a unique *Sma*I site at the end of the deletion. It may be an effective site where foreign blunted DNA fragments can be inserted into the plasmid. Therefore the recombinant C7301dTK may be useful as a vector for the expression of foreign genes in animal cells and for the development of attenuated, polyvalent live-virus vaccines.

Although loss of TK activity was associated with attenuation in other herpesviruses [12, 13, 21, 22, 40, 43], the replication of the recombinant FHV-1 deleted the TK gene *in vivo* has not been reported in detail. As a next step, we are carrying out some studies *in vivo* to assess the pathogenicity of the C7301dTK. If the strain is sufficiently attenuated as a result of the deletion in TK gene, this virus may constitute a new and safe vaccine strain with a genetic marker for FVR.

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