Journal of Virological Methods 207 (2014) 178–181

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Development of a novel single step reverse genetics system for feline calicivirus

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Article history:
Received 5 April 2014
Received in revised form 1 July 2014
Accepted 4 July 2014
Available online 12 July 2014

Keywords:
Positive sense, single-stranded RNA virus
Reverse genetics
Elongation factor-1 alpha promoter

A B S T R A C T

The reverse genetics system is a useful tool to generate infectious virus. Feline calicivirus (FCV), a member of the genus *Vesivirus* in the family *Caliciviridae*, has a positive sense, single-stranded RNA genome. Two reverse genetics systems have been established for FCV; however, these methods need multi-steps to produce progeny infectious virus. In this study, a novel plasmid-based single step reverse genetics system for FCV has been developed. The plasmid carries FCV F4 strain genomic sequence with an introduced silent mutation. In addition, at the 5′- and 3′-end, a human elongation factor-1α promoter and a *cis*-acting hepatitis delta virus ribozyme following poly-A, were added, respectively. When the plasmid was transfected into Crandell-Rees feline kidney cells, progeny FCV was generated. The reverse genetics system-derived FCV (rFCV) showed similar growth kinetics and antigenic characteristics and had identical genomic terminals to those of the original FCV F4 strain. The presence of the introduced silent mutation in the rFCV genomic cDNA supported that the progeny virus was originated from the plasmid. This novel FCV reverse genetics system is simple and can be used to evaluate the functions of the viral genome, proteins, and phenotypic characterization of FCV strains in the future.

Feline calicivirus (FCV), a member of the genus *Vesivirus* in the family *Caliciviridae*, is a veterinary pathogen and causes respiratory and systemic infections in cats (Radford et al., 2009). The FCV genome is a positive sense, single-stranded RNA molecule of approximately 7.7 kb excluding the poly-A tail (Carter et al., 1992). FCV is one of the well studied caliciviruses, because of their ability to grow in cultured cells (Green, 2007).

Two types of reverse genetics systems have been established for FCV and were used for introducing mutations or deleting genes, to investigate gene effect on viral growth (Mitra et al., 2004; Sosnovtsev et al., 1998, 2002, 2005) and antigenicity (Neill et al., 2000), and to insert heterologous genes into the viral genome (Abente et al., 2010; Thumfart and Meyers, 2002). The first reverse genetics system for FCV has been achieved by transfecting FCV susceptible cells with in vitro transcribed and capped FCV RNA molecules (Sosnovtsev and Green, 1995). In the second system, FCV susceptible cells were first infected with genetically modified vaccinia virus expressing the T7 polymerase, then were transfected with plasmid harboring FCV full-length cDNA under T7 promoter (Sosnovtsev et al., 1997). However, these two systems need multi-steps, and the latter method depends on a genetically modified vaccinia virus (Fig. 1).

Recently, a plasmid-based reverse genetics system using human cytomegalovirus promoter has been reported for rabbit hemorrhagic disease virus, which belongs to *Lagovirus* genus within *Caliciviridae* (Liu et al., 2008).

In this study, a simpler plasmid-based FCV reverse genetics system using human elongation factor-1α (EF-1α) promoter (Kim et al., 1990; Uetsuki et al., 1989) has been established (Fig. 1). The pKS435 vector, a derivative of pKS336 vector, which harbors human EF-1α promoter (generously provided from Dr. Koji Sakai, National Institute of Infectious Diseases, AIDS Research Center) (Arita et al., 2006; Saijo et al., 2002) was used as a backbone of the infectious FCV plasmid. A FCV F4 strain sequence (7681 nt in length: GenBank ID: D31836) with a single silent mutation (T to C) at the nucleotide position 4048, and a 30 nt poly A and a *cis*-acting hepatitis delta virus (HDV) ribozyme sequence (Oka et al., 2007; Wrzesinski et al., 2001) at the 3′ end of the FCV genomic cDNA were cloned into

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http://dx.doi.org/10.1016/j.jviromet.2014.07.004
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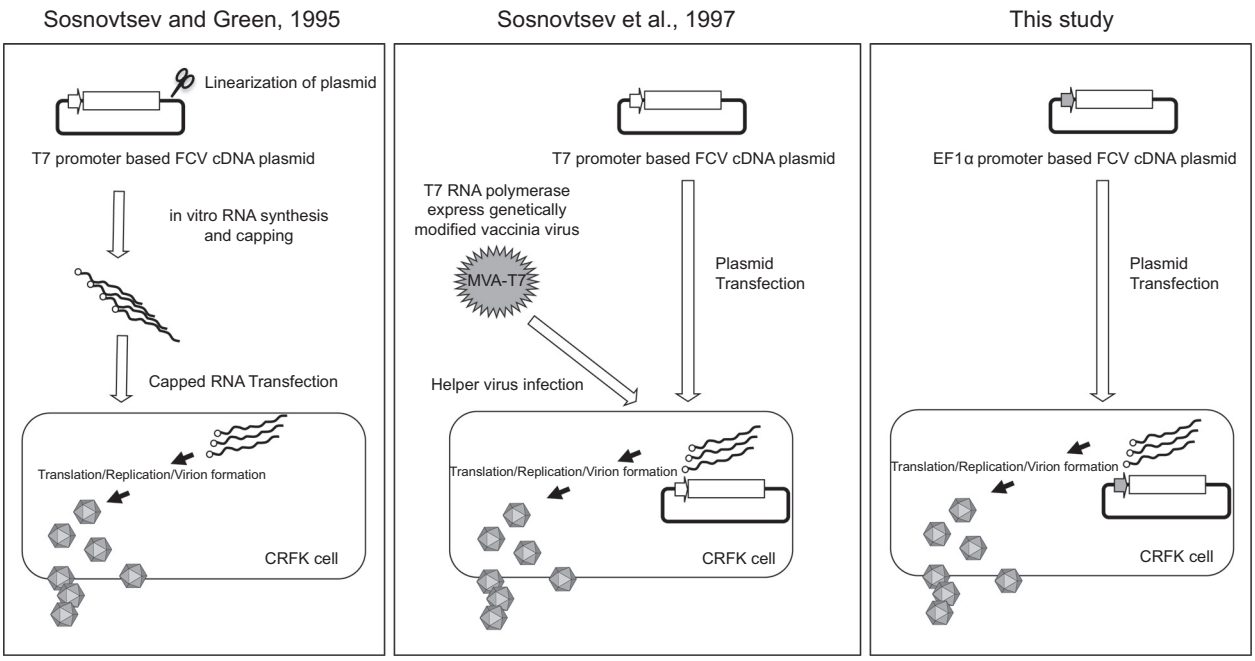

Fig. 1. *Comparison of the newly developed FCV reverse genetics system with previous methodologies.* The first reverse genetics system for FCV (Sosnovtsev and Green, 1995) needs plasmid linearization, T7 promoter driven in vitro RNA transcription and capping, and then RNA transfection into FCV susceptible cells (CRFK cells) for the production of progeny FCV. In the second system (Sosnovtsev et al., 1997), CRFK cells were first infected with genetically modified vaccinia virus expressing the T7 polymerase (MVA-T7), then were transfected with plasmid harboring FCV full-length cDNA under T7 promoter. The newly developed system could recover progeny FCV simply transfected CRFK cells with plasmid harboring FCV full-length cDNA under human elongation factor-1α (EF-1α) promoter.

the pKS435 vector. The obtained plasmid designated as "infectious full-length FCV F4 clone" was schematically illustrated in Fig. 2.

Crandell-Rees feline kidney (CRFK) cells obtained from the Japanese Collection of Research Bioresources were cultured in the growth medium (Eagle's minimal essential medium [MEM: Sigma-Aldrich] supplemented with 5% heat-inactivated fetal bovine serum [Invitrogen] and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin [Invitrogen]).

The infectious full-length FCV F4 clone was transfected into semi-confluent CRFK cells (~80%) grown in a six-well plate (BD Falcon) using Effectene Transfection Reagent (Qiagen). Briefly, one μg of plasmid DNA (10 μL in volume) was mixed with 90 μL of EC buffer and 3.2 μL of enhancer and incubated at room temperature for 10 min. Then, 10 μL of Effectene was added and further incubated at room temperature for 10 min. Finally 600 μL of growth medium was added to form the transfection mixture. The cell culture medium in the well was replaced with fresh growth medium (2 mL/well) and then all the transfection mixture was added to the well. The cells were incubated at 37 °C in 5% CO_2 for an appropriate period. The collected cell culture supernatant was centrifuged (10,000 × *g*, 1 h at 4 °C), and stored at −80 °C until use. The 50% tissue-culture infectious dose ($TCID_{50}$) in the supernatant was determined as described previously (Oka et al., 2011). Cytopathic effect (CPE) appeared at 3 days after plasmid transfection, and the viral titer at 4 days after transfection were reached to 5.42–5.94 $\log_{10}$ $TCID_{50}$/50 μL from three independent experiments (data not shown).

To compare the viral growth kinetics between the rFCV F4 (progeny FCV recovered from the plasmid) and the FCV F4 (FCV F4 strain virus stock) (Oka et al., 2011), virus was inoculated into confluent CRFK cells grown in a six-well plate (approximately 10^6 cells/well) at an approximate multiplicity of infection of 0.0001. After adsorption for 2 h, the inoculum was removed and the cells were washed once with PBS without Ca^{2+} and Mg^{2+} [PBS (−)], which was then replaced with 2 mL of the medium. The cell culture supernatant (0.5 mL) was collected from each well at 0, 5, 10, 25, 35, and 48 h post-inoculation (hpi), and an equivalent volume of the medium was added to the well immediately after collecting the cell culture supernatant at each time-point to maintain a constant media volume (2 mL). Cell debris was removed by centrifugation at 10,000 × *g* for 1 h at 4 °C, and the supernatant was stored at −80 °C until use. As shown in Fig. 3, the growth kinetics between the rFCV

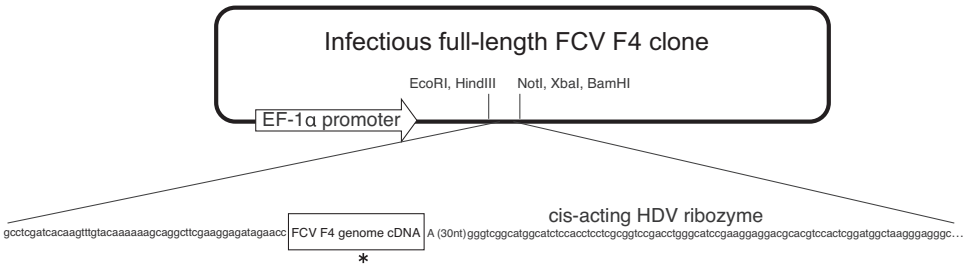

Fig. 2. *Schematic diagram of the infectious full-length FCV F4 clone.* A FCV F4 strain sequence (7681 nt in length: GenBank ID: D31836) with a single silent mutation (T to C) at the nucleotide position 4048, 30 nt-poly-A tail, and *cis*-acting hepatitis delta virus ribozyme sequence, were cloned into a pKS435 vector. The introduced silent mutation is indicated by an asterisk. Both up and down stream sequences of cloned FCV F4 cDNA are indicated. The human elongation factor-1α (EF-1α) promoter and EF-1α intron and exon region (identical to nt 1–1192 of pKS336 vector [Genbank ID: AF403737]), in the expression vector are schematically indicated. The total size of the infectious full-length FCV F4 clone is approximately 13-kb.