

Expression and Stability of Foreign Epitopes Introduced into 3A Nonstructural Protein of Foot-and-Mouth Disease Virus

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Abstract

Foot-and-mouth disease virus (FMDV) is an aphthovirus that belongs to the *Picornaviridae* family and causes one of the most important animal diseases worldwide. The capacity of other picornaviruses to express foreign antigens has been extensively reported, however, little is known about FMDV. To explore the potential of FMDV as a viral vector, an 11-amino-acid (aa) HSV epitope and an 8 aa FLAG epitope were introduced into the C-terminal different regions of 3A protein of FMDV full-length infectious cDNA clone. Recombinant viruses expressing the HSV or FLAG epitope were successfully rescued after transfection of both modified constructs. Immunofluorescence assay, Western blot and sequence analysis showed that the recombinant viruses stably maintained the foreign epitopes even after 11 serial passages in BHK-21 cells. The 3A-tagged viruses shared similar plaque phenotypes and replication kinetics to those of the parental virus. In addition, mice experimentally infected with the epitope-tagged viruses could induce tag-specific antibodies. Our results demonstrate that FMDV can be used effectively as a viral vector for the delivery of foreign tags.

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Introduction

Foot-and-mouth disease virus (FMDV) is the causative agent of foot-and-mouth disease (FMD), a highly contagious and economically important disease of cloven-hoofed animals, including cattle, swine, goats, sheep and other species of wild ruminants. The virus belongs to the *Aphthovirus* genus within the family *Picornaviridae* and has a single-stranded positive-sense RNA genome approximately 8.5 kb in length. The 1300-nt 5' untranslated region (5' UTR) is followed by a single long open reading frame (ORF), the 3' untranslated region (3' UTR), and Poly (A) tail. The ORF encodes four structural proteins (VP1, VP2, VP3, and VP4) and several precursors, as well as a total of nine mature nonstructural proteins. Each of these nonstructural proteins is involved in multiple functions needed for RNA genome replication and particle formation in infected cells [1,2].

FMDV can be differentiated from other picornaviruses by a longer 3A protein (153 aa instead of 87 aa for poliovirus) and three copies of 3B. Although 3A has been found to be physically associated with intracellular membranes that proliferate in picornaviruses infected cells [3,4,5,6], the functions of nonstructural protein 3A in the life cycle of FMDV is less well understood. However, alterations in 3A protein, such as point mutations and deletions, were linked to altered host specificity, adaptation, attenuation and virulence of FMDV [7,8,9,10,11,12].

The potential for infectious cDNA clones to act as foreign gene expression vectors has been extensively explored for both negative-

stranded RNA viruses and positive-stranded RNA viruses in recent years. A number of recombinant RNA viruses expressing various foreign genes were stably maintained during serial passages, indicating the suitability of these viruses for the development of viral vectors [13,14,15]. For example, the green fluorescent protein (GFP) gene was inserted between the fusion (F) and hemagglutinin-neuraminidase (HN) genes of Newcastle disease virus (NDV), leading to an infectious particle maintained stably for at least five passages in embryonated eggs [16]. A recombinant measles virus (MV) containing the hepatitis B virus (HBV) small surface antigen (HBsAg) was uniformly expressed after 10 serial passages [17]. The full-length PRRSV clone containing a foreign sequence coding for a total of 31 amino acids produced an infectious progeny virus, and this virus retained its infectivity and genetic stability during four passages of examination [18]. Considerable efforts also have gone into developing a variety of recombinant picornaviruses (including enterovirus [19,20], cardiovirus [21,22], rhinovirus [23] and hepatovirus [24]) that present heterologous immunogens on their surfaces. In particular, poliovirus (PV) has been reported extensively in this regard [25,26,27,28,29], and it has been shown that PV can serve as an effective vector for expressing foreign proteins. For FMDV, more recently, the possibility for generation of recombinant FMDVs carrying foreign epitopes has been shown by insertion of foreign tags between the inter-AUG regions [30]. However, to date, no studies have further reported the feasibility of FMDV as a viral vector for expressing foreign antigens.

Table 1. Primers used for the construction of genetically modified full-length clones of FMDV.

Primer	Sequence ^a (5'→3')	Nucleotide Position
HN-1F	CAAGAAGTGATTGAGCGGGT	5274–5293
HN-1R	<i>CTTATCGTCATCGTCCTTATAGTCTGGCGCTCTTGCGCGCTT</i>	5683–5702
HN-2F	<i>GACTATAAGGACGATGACGATAAGGACGCGGCTCTTGACGATGC</i>	5613–5632
HN-2R	GCGTTGTTGATCACGCCGAC	6324–6343
HN-3F	<i>CAGCCGGAACCTGGCTCCGGAAGACCCGGAAGACGTGCCCGGAGGGAACAA</i>	5736–5755
HN-3R	<i>GTCTCCGGGCTTCCGGAGCCAGTTCGGCTGTTGCTCGGTGGGGATCT</i>	5683–5702
HN-4F	AGGTGTCGAGTACCCCGATT	5308–5327
HN-4R	GTTCCCTTCTCATTCTCGC	6297–6316

^aNucleotides encoding the FLAG and HSV tags are marked with italic type.
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In the present study, an 8-aa FLAG epitope (DYKDDDDK) and an 11-aa HSV epitope (QPELAPEDPED) derived from herpes simplex virus (HSV) glycoprotein D were introduced into the C-terminal different regions of 3A protein of an FMDV full-length infectious cDNA clone to construct recombinant viruses. We demonstrated that infectious virus expressing FLAG or HSV tag protein could be produced and the 3A-tagged viruses shared similar plaque phenotypes and replication kinetics to those of the parental virus. Furthermore, the FLAG and HSV epitopes were stably maintained and expressed even after 11 serial passages in BHK-21 cells. In addition, FLAG/HSV-specific antibodies could be induced after inoculation the 3A-tagged viruses in mice. These observations indicate that FMDV can be used effectively as a viral vector for the delivery of foreign epitope tags.

Materials and Methods

Cells and Virus

Baby hamster kidney cells (BHK-21) [31] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. BSR-T7/5 cells [32], which stably express T7 RNA polymerase, were provided by K. K. Conzelmann (Max-von-Pettenkofer Institut, Munich, Germany) and were grown in Glasgow minimal essential medium (GMEM) supplemented with 10% FCS and 1 mg/ml G418. All cells were grown at 37°C in a humidified chamber containing 5% CO₂. The parental virus (r-HN) used in this study was recovered from BSR-T7/5 cells transfected with *Not* I-linearized plasmid pOFS, which contained the complete FMDV O/HN/CHA/93 genoe. The properties of this virus have been described previously [33]. The virus was titrated on BHK-21 cells by calculating the 50% tissue culture infectious dose per ml (TCID₅₀/ml).

Antibodies

Anti-FMDV 3A protein mouse monoclonal antibodies were generated as described previously [34]. Mouse monoclonal anti-HSV antibody was purchased from Novagen and mouse monoclonal anti-FLAG antibody was purchased from Sigma. Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody used in immunofluorescence and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody used in western blot analyses were from Sigma.

Introduction of Foreign Tags into the FMDV Full-length Infectious cDNA Clone

Plasmid pOFS for recovery of r-HN has been described previously [33] and this clone was used as the genomic backbone for the generation of mutant full-length plasmids. To construct recombinant FMDVs that allow the cloning of foreign genes in frame to the C-terminal region of FMDV 3A, overlap extension PCR was used. Briefly, two independent cDNA fragments containing FLAG nucleotide sequences were amplified using HN-1F/HN-1R and HN-2F/HN-2R primer pairs (Table 1). PCR amplifications were performed using PrimeSTAR HS DNA Polymerase (Takara). Cycling conditions for both PCRs were as follows: 94°C for 1 min followed by 30 cycles of 98°C for 20 s, 68°C for 1 min, and then 72°C for 8 min. These fragments were combined and subsequently amplified using the flanking primers HN-1F and HN-2R (Table 1). Reaction conditions included an initial denaturation at 94°C for 1 min, followed by 30 cycles of 98°C for 20 s, 68°C for 1.5 min, and a final step at 72°C for 8 min. Then, the fused fragment was digested with *Bgl* II and *Nru* I restriction enzymes and cloned into the *Bgl* II- and *Nru* I-digested fragment of pOFS to yield plasmid pOFS-FLAG. Similarly, plasmid pOFS-HSV was constructed using the HN-1F/HN-3R and HN-3F/HN-2R primer pairs (Table 1). Standard cloning procedures were performed as described by Sambrook et al. [35]. All constructs were verified by nucleotide sequencing to ensure that no other mutations had occurred during the cloning process.

Transfection and Recovery of Infectious Recombinant Viruses

Plasmids pOFS-FLAG and pOFS-HSV were linearized with *Not* I restriction site located downstream of the FMDV poly(A) sequence, and then purified using a QIAquick PCR Purification Kit (Qiagen). Confluent BSR-T7/5 cell monolayers (4–6 × 10⁶ in a six-well plate) were transfected with linearized plasmid DNAs using LipofectamineTM 2000 (Invitrogen), according to the manufacturer's protocol. After 5 h of incubation at 37°C, the supernatants were removed and replaced with GMEM supplemented with 4% tryptose phosphate broth and 10% FBS, and the cells were further incubated at 37°C. The BSR-T7/5 cell monolayers showed cytopathic effects (CPE) at 2 days post-transfection and the virus-containing supernatants were collected and titrated on BHK-21 cells.

Immunofluorescence Assays (IFA)

BHK-21 cells (2 × 10⁵) were seeded in a six-well plate, incubated for 12 h, and then mock-infected or infected with r-HN or the

transfected supernatants at a multiplicity of infection (MOI) of 1. After 6 hours post-infection (hpi), the cells were washed three times with PBS and fixed with 4% paraformaldehyde (diluted in PBS) for 20 min at room temperature. The cells were washed three times and permeabilized with PBS containing 0.5% Triton X-100 (PBS-T) for 20 min. Thereafter, the cells were washed three times and blocked with blocking buffer (PBS, 10% bovine serum albumin) for 1 h. After further washing with PBS-T, the cells were immunostained with primary antibody (mouse anti-HSV tag mAb (1:2000) or mouse anti FLAG-tag mAb (1:1000) or 3 A mAb (1:100)) for 1 h. The cells were rinsed with PBS-T and incubated with secondary antibody FITC-conjugated goat anti-mouse IgG antibody (1:100) for 1 h. The cells were washed again with PBS-T and visualized using an Olympus BX40 fluorescence microscope.

Western Blot Analysis

Confluent BHK-21 cells were mock-infected or infected with r-HN or the transfected supernatants at a MOI of 1. After 12 hpi, cells were washed with PBS, scraped, collected by low-speed centrifugation, and lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 25 mg of aprotinin per ml) for 30 min on ice. The cell lysates were clarified by centrifugation at 7,000×g for 10 min and the supernatants were mixed with Laemmli sample buffer (Qiagen) and boiled for 6 min before electrophoresis. The boiled samples were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were transferred to PVDF membranes (Sigma). Then, the membranes were blocked by incubation in PBS containing 5% nonfat milk for overnight at 4°C. After washing four times with PBS-T, the membranes were incubated with mouse anti-HSV tag mAb (1:2,000) or mouse anti-FLAG tag mAb (1:1000), respectively. Following four washes in PBS-T, the membranes were incubated with HRP-conjugated goat anti-mouse antibody (1:1000). Following four additional washes with PBS-T, the proteins were visualized after further incubation with diaminobenzidine (DAB).

Identification of Recombinant Viruses by RT-PCR

Viral RNAs were extracted from the transfected supernatants using a QIAamp Viral RNA Mini kit (Qiagen) and were used as template for RT-PCR analysis of the 3A gene of the recombinant viruses. RTs were performed with reverse transcriptase XL (AMV) at 42°C for 60 min, using HN-2R primer. After the reactions were completed, the enzyme was inactivated by incubation at 100°C for 3 min. PCRs were carried out with primer HN-1F and HN-2R. PCR reaction conditions included an initial denaturation at 94°C for 1 min, followed by 30 cycles of 98°C for 20 s, 68°C for 1.5 min, and a final step at 72°C for 8 min. The resulting PCR fragments of 1050 bp were purified and sequenced to confirm that the rescued viruses were the expected recombinants.

The transfected supernatants were serially passaged in BHK-21 11 times to determine the stability of the introduced modifications. Briefly, confluent BHK-21 cell monolayers ($4-6 \times 10^6$ in 100-mm-diameter dishes) were infected with the transfected supernatants at a MOI of 1. After 1 hpi, the inoculums were removed and cells were washed with PBS to remove unattached viruses. Then, 10 ml of DMEM supplemented with 2% FCS and antibiotics, was added to the dishes and incubated at 37°C in 5% CO₂. The supernatants were collected until about 95% of the cells showed CPE (about 8–12 h). Each supernatant was serially passaged for further experiment. Viral RNAs were extracted from viral suspensions of the 4th, 8th and 11th passages and were subjected to RT-PCR analysis as described above. The stability of the recombinant

viruses (p11) expressing foreign epitopes was further confirmed by IFA and Western blot analysis.

Growth Curve Analysis and Plaque Morphology

One-step growth curves were performed for each virus in BHK-21 cells. Briefly, BHK-21 cell monolayers in 6-well plates were infected with r-HN or recombinant viruses at a MOI of 1 and incubated at 37°C in 5% CO₂. After 1 h adsorption at 37°C, supernatants were removed and cell monolayers were washed twice with phosphate-buffered saline (PBS, pH 7.4). Thereafter, 2-ml DMEM containing 2% FBS were added, and all monolayers were incubated at 37°C. At indicated times post-infection, cells were harvested and viruses were released from the cells by one freeze/thaw cycle, and the titer of the clarified supernatant was determined by TCID₅₀ as described above. Plaque morphologies of the recombinant viruses and the parental virus were compared by plaque assay on BHK-21 cells as described previously [36].

Infection of Mice

Six-week-old female Kunming white mice (n=22) were purchased from the Laboratory Animal Center of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All mice were randomly divided into four groups. Groups 1 to 3 of 6 animals each were intraperitoneally inoculated with 1 ml cell culture medium containing 10⁶ TCID₅₀ of each the 3A-tagged viruses or the parental virus. Group 4 with 4 animals were mock infected with PBS. The mice were bled retroorbitally at 28 day post-inoculation and serums were collected for analysis of anti-tag and anti-3ABC antibodies.

Detection of Anti-tag Antibodies in Mice

Antibody responses to foreign tags were analyzed by testing each serum sample in an indirect enzyme-linked immunosorbent assays (ELISAs). Briefly, ELISA plates were coated with 3×FLAG or 3×HSV peptide antigen (Sigma) at a concentration of 2 µg/ml diluted in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed four times with PBS-T, and then blocked in PBS with 5% non-fat dry milk for 1 h. The plates were washed four times with PBS-T, and then 100 µl of diluted serum samples (1:20 dilution in blocking buffer) were added. Serum samples were incubated for 1 h at 37°C, and then the plates were washed four times with PBS-T. A HRP-conjugated goat anti-mouse immunoglobulin G (1:100 dilution in blocking buffer) was then added and incubated for 1 h at 37°C. After washing four times with PBS-T, the plates were incubated with O-Phenylendiaminedihydrochloride and stopped by adding 2 M H₂SO₄. Plates were read at a wavelength of 450 nm using an ELISA microplate reader. Samples were considered to be positive for tag antibodies if the ratios of the OD value of infected sera to mean negative control sera were greater than 2.1. The anti-3ABC antibody in each serum sample was also detected using a 3ABC-ELISA kit as previously described [37]. The sample value ≥ 0.2 was considered to be positive for 3ABC antibodies [37].

All animal studies were approved by the Review Board of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permission number: SYXK-GAN-2004-0005). All animals used in the present study were humanely bred during the experiment and euthanasia was carried out at the end of the experiment.

Results

Construction and Recovery of Recombinant Viruses

Previous studies showed that PV can tolerate small insertions in the 3A gene to generate infectious viruses [38,39]. It was not clear whether lethality was due to introduction foreign epitope tags into the 3A gene of FMDV. To investigate the potential of FMDV as a viral vector to express small tags, we introduced epitope sequences into different regions of 3A protein of an FMDV full-length infectious cDNA clone. As shown in Figure 1, two mutant plasmids pOFS-FLAG and pOFS-HSV were constructed. Plasmid pOFS-FLAG encodes the full-length genome of FMDV with a substitution of FLAG-tag sequence (DYKDDDDK) for native amino acid sequences (SVDDSLDD) of 85–92 aa of 3A protein. Plasmid pOFS-HSV encodes the full-length genome of FMDV with a substitution of HSV-tag sequence (QPELAPEDPED) for native amino acid sequences (KTCDDVNTEPV) of 133–143 aa of 3A protein. Viable viruses, named FMDV-FLAG and FMDV-HSV, were rescued when the corresponding plasmids were transfected into BSR-T7/5 cells. The results indicated that the C-terminal portion of FMDV 3A could tolerate small substitutions without effecting generation of viable viruses. Transfection supernatants were collected and serially passaged in BHK-21 cells for subsequent experiments. The characterization and titers of rescued viruses are summarized in Table 2.

Analysis of Foreign Tag Expression in Cell Cultures

To determine whether the recombinant viruses express the foreign tag proteins, BHK-21 cells were mock-infected or infected with r-HN or the transfected supernatants at a MOI of 1. At 6 hpi, the cells were fixed and incubated with mouse anti-3A and anti-HSV or anti-FLAG mAbs, followed by immunostaining with FITC-conjugated goat anti-mouse IgG. The results showed that the anti-HSV mAb readily detected viral antigens in FMDV-HSV-infected cells but not in mock-infected or rHN-infected controls (Fig. 2A). Similarly, the FLAG tag protein of FMDV-FLAG was detected by anti-FLAG mAb, whereas no signal was seen in mock-infected or rHN-infected controls (Fig. 2B). In contrast, the anti-3A mAb readily detected viral antigens in r-HN, FMDV-FLAG, or FMDV-HSV-infected cells but not in mock-infected cells (Fig. 2A and Fig. 2B). To further confirm the expression of tag proteins by the recombinant viruses, mock, r-HN or 3A-tagged virus-infected BHK-21 cell lysates were harvested at 12 hpi, and cytoplasmic proteins were resolved by SDS-PAGE and analyzed by Western blotting. As expected, all 3A-containing

bands were detected in recombinant virus-infected cell lysates with anti-tag antibodies, but not in mock-infected cells. As shown in Figure 3A and Figure 3B, the fastest migrating band represents degraded-3A [12] and the remaining four bands correspond to 3A, 3AB, 3ABB and 3ABBB. Furthermore, 3A-containing protein bands were also clearly detected in FMDV-HSV, FMDV-FLAG or r-HN-infected cell lysates using anti-3A mAb, but not in mock-infected cells (Fig. 3C). The degraded-3A was not visible in this assay. Taken together, these results suggested that the tag genes were stably maintained and expressed during a large number of virus generations in cell culture.

RT-PCR Analysis of Recombinant Viruses

To confirm the rescued viruses were the correct recombinants, viral RNAs were extracted from the transfected supernatants and analyzed by RT-PCR using primers HN-1F and HN-2R. As expected, the fragments of 1050 bp were obtained from the transfected supernatants. In contrast, PCR of the same viral RNA without RT failed to produce any product (data not shown), indicating that the PCR products originated from viral RNA but not from the transfected plasmids. The amplicons were sequenced to confirm the rescued viruses contained expected modification. To determine the genetic stability of the recombinant viruses *in vitro*, the transfected supernatants were serially passaged in BHK-21 cells 11 times, and the presence of the tag genes in the recombinant viruses from 4th, 8th and 11th passage was analyzed by RT-PCR and sequencing. The results showed that the sequence identities of the tag genes were preserved (Fig. 4A and Fig. 4B). At the same time, IFA and Western blot analysis of the recombinant viruses (p11) also provided positive identification of the HSV and FLAG tags. Take together, these results suggested that FMDV can stably sustain the insertion of small foreign tags in 3A protein even after 11 serial passages in BHK-21 cells.

Growth Characteristics of the Recombinant Viruses

The effects of the tag introductions into 3A protein of FMDV on the growth characteristics were compared with those of the parental virus. As shown in Figure 5A, three viruses produced mixed plaques (containing large, medium and small plaques) and there were no significant differences in the plaque size and morphology between the 3A tagged viruses and the parental virus. The single-step growth kinetics of the mutant viruses and the parental virus in BHK-21 cells were also very similar (Fig.5B), though the titers of FMDV-HSV were slightly higher than parental virus and FMDV-FLAG at 4 h, 8 h and 12 h. These

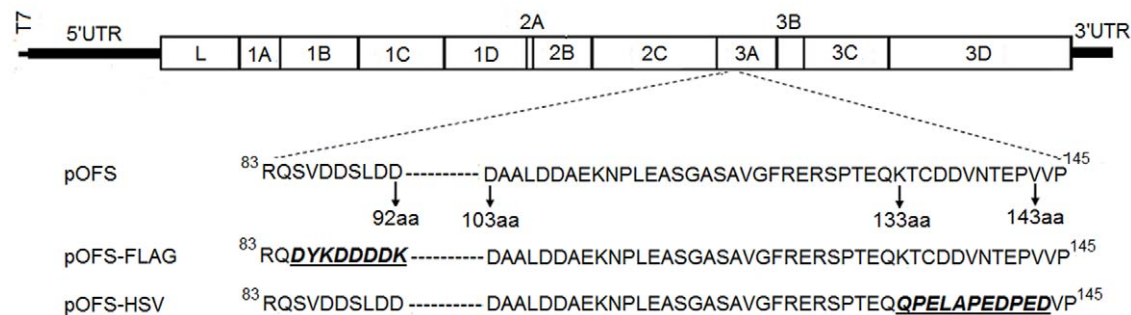


Figure 1. Schematic diagram of the FMDV genome and plasmids used in this study. The FMDV full-length genome cDNAs in these plasmids derived from the infectious cDNA clone, pOFS, were preceded by a synthetic T7 RNA polymerase promoter. The mutant plasmid pOFS-FLAG was produced by substituting native amino acid of the 85–92 region of the 3A protein for FLAG tag sequence and pOFS-HSV was produced by substituting native amino acids of the 133–143 region of the 3A protein for HSV tag sequence. Amino acid sequences of the tag are underlined. UTR: non-coding region. (–) indicates a naturally absent sequence.
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Table 2. Characterization of the recombinant viruses obtained by reverse genetics.

Construct ^a	Time of appearing CPE ^b	4th passage		8th passage		11th passage	
		Retained insert ^c	Virus titer ^d (TCID ₅₀ /ml)	Retained insert ^c	Virus titer ^d (TCID ₅₀ /ml)	Retained insert ^c	Virus titer ^d (TCID ₅₀ /ml)
pOFS-HSV	48 h	yes	4 × 10 ⁶	yes	5.6 × 10 ⁷	yes	5 × 10 ⁷
pOFS-FLAG	48 h	yes	2.7 × 10 ⁶	yes	3.2 × 10 ⁷	yes	3.4 × 10 ⁷
pOFS	48 h	ND ^e	2.3 × 10 ⁶	ND ^e	3.4 × 10 ⁷	ND ^e	3 × 10 ⁷

^aThree independent plasmid transfections were performed for each construct followed by passaging in BHK-21 cells.

^bTime (h) when CPE was detected after transfection.

^cThe tag inserts were determined by RT-PCR and sequencing analysis.

^dThe virus titer were determined on BHK-21 cells by calculating the 50% tissue culture infectious dose per ml (TCID₅₀/ml).

^eND: not done.

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results indicated that introduction of the foreign epitopes into the 3A genome of FMDV did not significantly influence the virus growth characteristics in cell culture.

Detection of Anti-tag Antibodies in Mice

To determine the presence of antibodies specific for the introduced HSV or FLAG epitopes, groups of six-week-old Kunming mice were intraperitoneally inoculated with r-HN, FMDV-HSV, or FMDV-FLAG. Four weeks after inoculation,

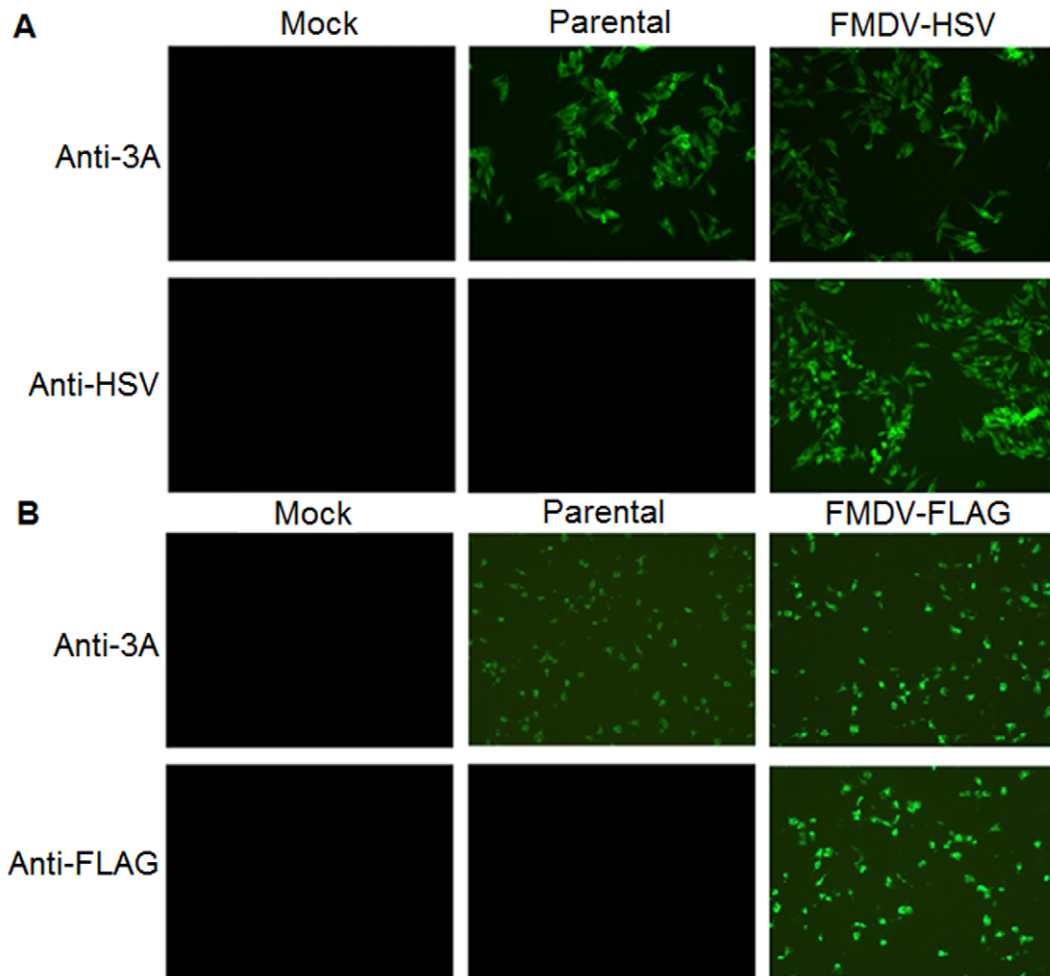


Figure 2. Immunofluorescence analysis of the expression of epitope tags in recombinant viruses. Confluent BHK-21 cells were mock-infected or infected with r-HN or the transfected supernatants at a MOI of 1, incubated for 6 h, fixed and probed with anti-3A and anti-HSV (A) or anti-FLAG mAb (B), followed by incubation with FITC-conjugated secondary antibody. The cells were visualized under an Olympus BX40 fluorescence microscope. Magnification, ×10.

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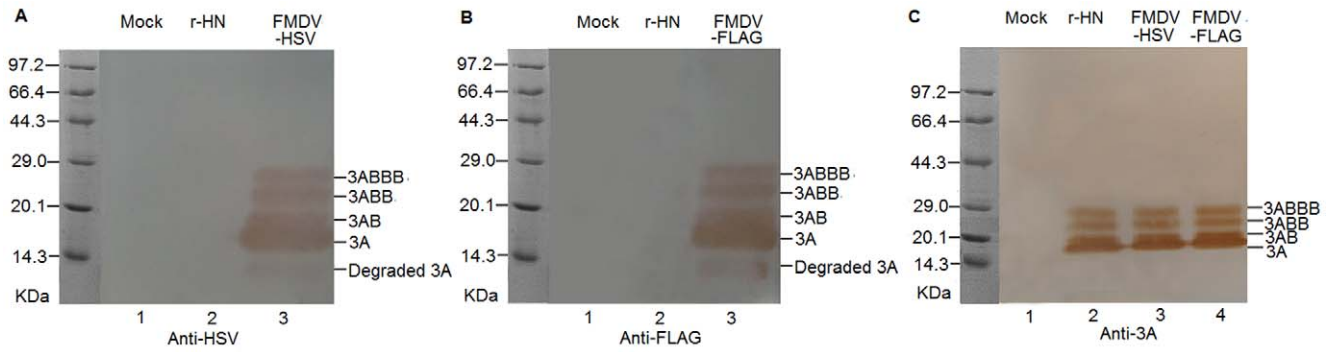


Figure 3. Western blot analysis of the expression of epitope tags in recombinant viruses. BHK-21 cells were mock-infected or infected with r-HN or the transfected supernatants at a MOI of 1 and incubated at 37°C. Infected cell extracts were prepared at 12 h post-infection. Proteins were separated on a 12% SDS-PAGE, blotted, and probed with anti-HSV(A), anti-FLAG (B) or anti-3A mAb (C). The numbers on the left represent molecular mass standards in kilodaltons (kDa).
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blood samples were analyzed with ELISA. The results showed that two mice in each group infected with FMDV-HSV or FMDV-FLAG viruses developed anti-tag antibodies and anti-3ABC antibodies (Table 3), and the antibody titers of anti-tag and anti-3ABC were highly correlated. Two mice infected with parental virus only produced anti-3ABC antibodies (Table 3). These results indicated that the 3A-tagged recombinant viruses or the parental virus could only replicate in some mice after inoculation. The reason for this phenomenon is unknown. The serological results showed that 3A-tagged viruses were able to induce tag specific antibody responses by 28 dpi that was distinguishable from the antibody response induced by the parental virus.

Discussion

The development of viral vectors is a relatively novel and promising approach to study virus replication, gene function, virus pathogenesis and to develop marker vaccines as well as gene therapy. However, identifying acceptable sites in the viral genome for insertion of foreign sequences to generate viable viruses can be technically very challenging. Genetic comparisons of the 3A proteins of different FMDV isolates revealed that the first half of the 3A coding region, which encodes an N-terminal hydrophilic domain, is highly conserved, however, the latter half of the 3A coding region (the C-terminal domain) is substantially variable in sequence [40,41] and can naturally incorporate different deletions (including residues 85–102, 93–102 and 133–143 deletions) [8,9,12,41]. The 93–102 deletion in 3A was responsible for its attenuation in bovines but remain highly pathogenic in pigs [8,42].

Furthermore, previous study showed that FMDV expressing a full-length 3A can tolerate artificial deletion (93–102 aa) and did not affect the ability of virus replication *in vitro* [43]. Based on these properties, we reasoned that the regions of 85–102 and 133–143 of 3A are non-essential regions for FMDV replication and are suitable targets for manipulation to insert foreign epitopes. In the case of O/HN/CHA/93, the deletion occurs at positions 93 to 102 aa of the 3A protein [44]. Therefore, in this study, two regions (85–92 and 133–143) of 3A were chosen for individual epitope tag insertions to study the ability of FMDV expressing foreign epitope tags.

A major obstacle common to the proposed RNA virus expression vector is their inherent genetic instability. Especially, the viruses expressing some large foreign proteins were readily lost parts or the entirety during recombinant virus replication in cell culture [26,45,46]. This tendency may be simply explained by the deleterious effect of the insertion of foreign sequences on virus replication efficiency, triggering adaptation to a faster growing phenotype. In view of these complications, in this study, we used an 8 aa FLAG epitope and an 11 aa HSV epitope to replace the native amino acid sequences of 85–92 and 133–143 regions of the 3A protein respectively, which did not increase the genome length. In addition, HSV and FLAG epitope tags were selected because they are well characterized and are recognized by commercial monoclonal antibodies.

In the present study, we explored the potential utility of FMDV to carry foreign epitopes with the aim of developing a safe and efficient viral vector platform. Using previously established FMDV reverse genetics system [33], FLAG and HSV tags were

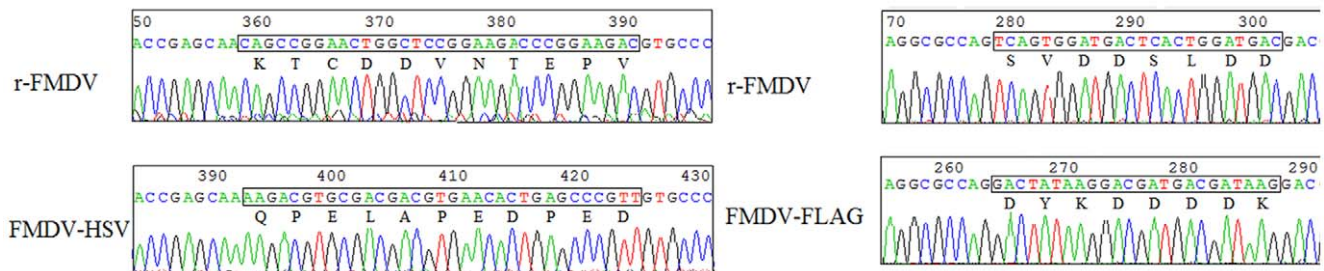


Figure 4. Electropherogram of the partial 3A gene of the parental virus in comparison with the modified 3A gene of the recombinant viruses. The modified 3A genes derived from cell-culture passage 11 (P11) of FMDV-HSV (A) and FMDV-FLAG (B) were sequenced. Amino acids are shown as the single-letter code and the nucleotide sequences of tags are boxed.
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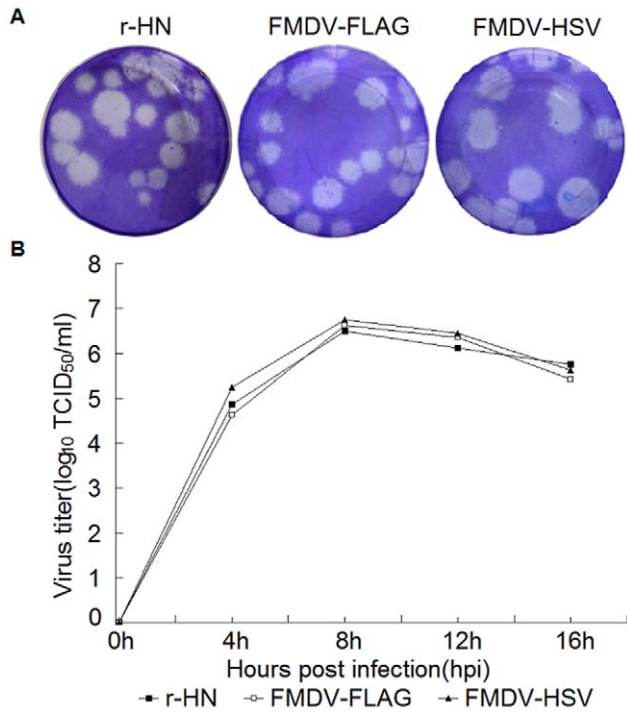


Figure 5. Plaque morphology and growth curves of the parental virus and the 3A-tagged viruses in BHK-21 cells. (A) Plaque morphology of the parental virus and the 3A-tagged viruses in BHK-21 cells. Viruses were plaque-assayed under tragacanth gum on BHK-21 cells and cells were stained at 48 h hpi. Plaques from appropriate dilutions are shown. (B) Growth curves of the parental virus and the 3A tagged viruses in BHK-21 cells. BHK-21 cells were infected with the parental virus or the 3A-tagged viruses at a MOI of 1. At 0, 4, 8, 12 and 16 hpi, cells and supernatants were harvested and virus titers were determined by TCID₅₀/ml on BHK-21 cells. The values of the viral titer represent the average obtained from triplicate experiments.
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introduced into the 85–92 and 133–143 regions of the 3A protein respectively. As expected, two viable viruses were recovered after transfection of two modified constructs into BSR/T7 cells, suggesting that FMDV 3A could tolerate introduction of the foreign epitopes into its C terminus and retain functionality in FMDV replication. IFA, Western blot and sequence analysis showed that the 3A-tagged viruses stably maintained the foreign epitopes even after 11 serial passages in BHK-21. Comparison of the recombinant viruses expressing foreign epitopes and the parental virus revealed that the viruses behaved similarly in cell culture with regards to plaque morphology and growth kinetics. This result is consistent with previous report that the insertion of foreign epitopes in 3A protein of PV, did not affect recombinant PVs growth properties and characteristics [39].

The 3A-tagged viruses experimentally infected Kunming mice, no clinical signs were observed in the virus-infected mice and control mice during the entire experimental process. After four weeks post-infection, some mice inoculated with 3A-tagged viruses could induce both anti-tag and anti-3ABC antibodies, however, some mice infected with the parental virus only produced anti-3ABC antibody. The serological results showed that 3A-tagged viruses were able to induce specific antibody response that was distinguishable from the antibody response induced by the parental virus. Therefore, the 3A-tagged viruses could potentially

Table 3. The OD₄₅₀ value of anti-tag and anti-3ABC antibodies in mice infected with the 3A-tagged viruses and the parental virus.

Virus	Animal No.	3ABC	HSV	FLAG
FMDV-HSV	M1	1.495(+)	1.665(+)	/
	M2	0.267(-)	0.282(-)	/
	M3	1.565(+)	1.623(+)	/
	M4	0.288(-)	0.308(+)	/
	M5	0.271(-)	0.295(-)	/
	M6	0.316(-)	0.365(+)	/
FMDV-FLAG	M7	1.622(+)	/	1.517(+)
	M8	0.248(+)	/	0.272(-)
	M9	0.127(-)	/	0.134(-)
	M10	0.309(-)	/	0.320(-)
	M11	1.679(+)	/	1.868(+)
	M12	0.255(-)	/	0.295(-)
r-HN	M13	1.695(+)	0.265(-)	0.237(-)
	M14	0.267(-)	0.282(-)	0.203(-)
	M15	1.565(+)	0.223(-)	0.166(-)
	M16	0.288(-)	0.308(-)	0.240(-)
	M17	0.271(-)	0.295(-)	0.272(-)
	M18	0.316(-)	0.365(-)	0.248(-)
Mean Neg. Control(n=4)		0.308	0.272	0.255
Mean Pos. Control		1.445	/	/
Cutoff value		0.525	0.571	0.536

The cutoff value of tag antibodies = 2.1 × mean negative control. The cutoff value of 3ABC antibodies was calculated as previously described [37]. +, represents antibody positive; -, represent antibody negative.
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serve as marker vaccine to allow the serologic differentiation of vaccinated animals from naturally infected ones.

Recently, the other advances in protein purification [47], intracellular location [48], examination of the role of the viral protein in the virus life cycle [48], study of interactions of virus protein with viral and cellular protein in infected cells [39] as well as investigation of virus structure and morphogenesis [49] often rely on the small tag-expressing viruses. To date, the functions of nonstructural protein 3A in the life cycle of FMDV, subcellular distribution of FMDV 3A protein containing 93–102 aa deletion in pig and bovine as well as the interactions of FMDV protein 3A with other viral and cellular factors in infected cells are poorly understood. Therefore, this work would represent useful tools to monitor subcellular localization and dynamics during FMDV infection and to determine the interactions of 3A protein with other viral and cellular factors during virus replication in infected cells.

In conclusion, this is the first report describing introduction of foreign epitope tags into FMDV protein 3A and generation of recombinant viruses that stably express small epitope tags. Further study is in progress to investigate the maximum size of foreign sequences that the 3A genome of FMDV can tolerate without losing infectivity.

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Author Contributions

Conceived and designed the experiments: PHL HY ZXL. Performed the experiments: XWB YMC CHH. Analyzed the data: ZJL. Contributed reagents/materials/analysis tools: PS. Wrote the paper: PHL.

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