Recovery of infectious foot-and-mouth disease virus from full-length genomic cDNA clones using an RNA polymerase I system

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The prototypic foot-and-mouth disease virus (FMDV) was shown more than a century ago to be the first filterable agent capable of causing FMD, and it has served as an important model for studying basic principles of Aphthovirus molecular biology. However, the complex structure and antigenic diversity of FMDV have posed a major obstacle to the attempts at manipulating the infectious virus by reverse genetic techniques. Here, we report the recovery of infectious FMDV from cDNAs based on an efficient in vivo RNA polymerase I (pol) transcription system. Intracellular transcription of the full-length viral genome from pol-based vectors resulted in efficient formation of infectious virus displaying a genetic marker. Compared with wild-type virus, an abundance of genomic mRNA and elevated expression levels of viral antigens were indicative of the hyperfunction throughout the life-cycle of this cDNA-derived virus at transcription, replication, and translation levels. The technology described here could be an extremely valuable molecular biology tool for studying FMDV complex infectious characteristics. It is an operating platform for studying FMDV functional genomics, molecular mechanism of pathogenicity and variation, and lays a solid foundation for the development of viral chimeras toward the prospect of a genetically engineered vaccine.

Keywords foot-and-mouth disease virus; RNA polI-based system; infectious cDNA clone

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Introduction

Foot-and-mouth disease (FMD) is an extremely contagious disease of cloven-hoofed animals, notably pigs, cattle, sheep, and goats. The disease has the potential to cause severe economic hardship, and thus FMD has been the target of international regulations designed to limit its spread [1]. The causative agent of the disease is FMD virus (FMDV), which belongs to the Aphthovirus genus of the Picornaviridae family [2]. FMDV has an icosahedral capsid comprising 60 copies, and four structural proteins (VP1[1D], VP2[1B], VP3[1C], and VP4[1A]) surrounding its genome which is a positive-stranded RNA consisting of ~8500 nt. The viral RNA contains a single lengthy open reading frame encoding the viral polyprotein which is subsequently cleaved into multiple mature proteins by virally encoded proteinases [3–5]. The 5′-terminus of the genomic RNA is covalently bound to a small viral protein termed VPg [6,7]. For controlling essential functions during the viral life-cycle, the 5′-untranslated region (5′-UTR) is a highly structured span of ~1300 nt, and composed of the S fragment, poly(C) tract, pseudoknots (PKs), cis-acting replication element (cre), and the internal ribosome entry site (IRES), in that order [8]. There is also a short 3′-UTR of ~90 nt followed by a poly(A) tail at the 3′-terminus [9].

A little is know in many aspects of FMDV, while much remains poorly understood regarding the functions of the UTRs, proteins, protein precursors in virulence, host range, and virus transmission. As an important element of proper epidemiological control, a molecular genetic analysis of FMDV is essential for determining the origin of FMD outbreaks. So the production of an infectious FMDV from full-length cDNA clones is an excellent technique for studying virus assembly and function, such as replication, maturation, and viral genome quasispecies as well as antigenicity, virulence, and pathogenesis. The first infectious clone of FMDV (O1K) was rescued by Zibert et al. [10]. Then, strains A12 [11], OH99 [12,13], A24 [14], and ZIM/7/83 [15]
were subsequently rescued. In addition, chimeric transcripts were assayed with an in vitro transcription system using T7 RNA polymerase [15,16]. While a disadvantage of a genome-modification method, such as gene recombination, mosaicism, or mutagenesis, is that heterogeneity and instability of the synthesized RNAs may hamper or interfere with replication, assembly, and release of the virus [17].

To increase the efficiency of FMDV rescue, we adopted a more efficient, convenient, and economical method, with a stable cell line constitutively expressing T7 RNA polymerase [13]. However, construction of such a cell line is complicated and, moreover, the low fidelity of T7 RNA polymerase could be a limitation to the research. In a previous study, Ward et al. [18] developed a method utilizing cellular RNA polymerase II (polII) transcription of transfected full-length genomic cDNA clones, which did not require either in vitro transcription or additional T7 RNA polymerase. However, this design of the full-length cDNA FMDV clones did not take into account the precise transcription that is critical for efficient virus rescue. Hence, we attempted to design an enhanced RNA polymerase I (polI)-based reverse genetics system for rescuing FMDV. In order to ensure precise transcription of the genome, the 5'- and 3'-termini of the FMDV GD/China/86 strain were flanked by RNA pol promoter and terminator sequences, respectively. To differentiate rescued virus from wild-type virus, we engineered a genetic marker into the full-length genomic cDNA clones. By directly transfecting a baby hamster kidney cell line (BHK-21) with the recombinant GD/China/86 viral vectors, intracellular transcription of the full-length viral genome from polI-based vectors and FMDV was efficiently rescued. The rescued virus was evaluated for virulence in suckling mice, for infectivity in plaque assays, real-time RT–PCR, and growth kinetics in BHK-21 cells. The RNA polI-based reverse genetics system described in this study was proven to be a valuable tool for understanding the molecular mechanisms of FMDV replication, pathogenesis, and for exploring the process of viral maturation toward the development of an effective vaccine.

Materials and Methods

Cell culture and virus propagation

BHK-21 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, Logan, USA) containing 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin. The GD/China/86 strain of FMDV serotype O that caused an outbreak of FMD in Guangdong Province, China, in 1986 was isolated from swine. The genome sequences were determined and the virus was propagated in BHK-21 cells as described previously [19]. After 48 h of incubation at 37°C, more than 80% of the BHK-21 cell cultures showed a cytopathic effect (CPE) and the cell suspension was submitted to three cycles of freeze and thaw. The virus suspension was clarified by centrifugation at 3028 g for 10 min and stored at −70°C in DMEM with 2% FBS.

Vectors and cloning procedures

pcDNA3.1(+) vector containing the promoter and terminator sequences of mouse RNA polI was preserved in National Food and Mouse Disease Reference Laboratory (Lanzhou, China). Genomic RNA of GD/China/86 FMDV was extracted from viral suspension with an RNAeasy mini kit (Qiagen, Hilden, Germany), and four fragments covering the complete genome were subsequently amplified by RT–PCR using specific primer pairs (Table 1). All fragments were cloned into the pGEM-T vector (Promega, Madison, USA) and the sequences were determined using fluorescent BigDye terminator sequences of mouse RNA polI was preserved in Applied Biosystems). A full-length cDNA clone of the GD/China/86 strain was assembled by the multistep strategy using unique restriction sites, which were naturally found in the viral sequence, except for BamHI. The BamHI site was introduced as a genetic marker during the construction of the recombinant plasmid to distinguish rescued virus from wild-type virus. The recombinant plasmid encoding full-length cDNA of FMDV strain GD/China/86, designated as pPfmdv3.1(+), was sequenced and analyzed.

Transfection

The recombinant plasmid pPfmdv3.1(+) was purified with an SNAPT™ MidiPrep Kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. A mixture of 10 μl Lipofectamine 2000 (Invitrogen) diluted in 50 μl of Opti-MEM® I medium (Gibco-BRL, Gaithersburg, USA) was prepared for transfection by incubating for 5 min at room temperature. Meanwhile, 4 μg of circular plasmid DNA was added to 50 μl of Opti-MEM® reduced serum medium (Gibco-BRL) without serum and incubated for 20 min. The mixtures were combined and added to 6-well plates containing cultured subconfluent monolayers of BHK-21 cells that had been washed twice with 0.5 ml of Opti-MEM® I medium.
After 6 h of incubation at 37°C, the cells were washed and the transfection media were replaced with fresh medium containing 10% FBS, then the cells were incubated for 72 h at 37°C. Transfection media without circular plasmid DNA were added to BHK-21 cells as a negative control whereas transfected viral RNA from wild-type virus GD/China/86 served as a positive control.

Characterization of the rescued virus

**Rt–PCR analysis and identification of genetic marker.** Viral RNA was extracted from supernatant using an RNeasy mini kit at 72 h post-transfection, and RT–PCR was carried out to amplify the full-length genome of the GD/China/86 strain. For the identification of the genetic marker, the 2.6 kb fragment was digested with *Bam*HII and sequenced by TaKaRa Biotech (Dalian, China).

**Indirect immunofluorescence assay.** Viral proteins were detected by indirect immunofluorescence assay (IFA) as previously described [12]. Transfected BHK-21 cell cultures were plated on 10-mm glass coverslips and fixed with cold acetone for 30 min at −20°C. The fixed cells were washed several times with PBS followed by 45 min of incubation at 37°C with hyperimmune rabbit ascitic fluid (1:100) specific for FMDV. Then, the glass coverslips were washed three times with PBS and incubated with an FITC-labeled secondary antibody (goat anti-rabbit immunoglobulin G conjugated with fluorescent isothiocyanate; 1:100) at 37°C for 30 min, then washed as described above. Finally, the cells were mounted on a slide using fluorescent mounting medium (KPL) and examined by using a fluorescence microscope equipped with a video documentation system.

**Electron microscopy.** Supernatants from virus-infected BHK-21 cell cultures were lysed by addition of 0.1% Triton X-100 and EDTA (pH 8.0) to a final concentration of 20 mM. Cell lysates were centrifuged to remove cellular debris (5821 g, 30 min, 4°C). After extraction with chloroform and sedimentation with PEG-6000 and 4% NaCl, the supernatants were ultracentrifuged at 45,000 rpm for 3 h in an SW41 rotor, at 4°C. The pelleted virions were resuspended in NET buffer (150 mM NaCl, 10 mM Tris, pH 7.5, and 2 mM EDTA). Then, 5 μl samples were absorbed onto glow-discharged, carbon-coated copper grids. Grids were washed with water, stained with 2% uranyl acetate, and air-dried. The virions were viewed in an H-7100FA electron microscope at 80 kW.

**Specific infectivity assays**

**Mouse virulence study and one-step growth curve of rescued virus.** The rescued virus had been harvested after three passages in BHK-21 cells, after infectious pPfmdv3.1(+) FMDV had achieved stable infectivity. A 10-fold dilution of rescued virus was prepared in PBS containing 1% FBS, and groups of 3-day-old mice (each group with five mice) were inoculated intraperitoneally with 0.2 ml of virus diluted in PBS. The mice were observed for 72 h after infection and the 50% lethal dose (LD50) was determined by the method of Reed and Muench [20].

The growth kinetics of rescued virus was determined on confluent BHK-21 cell cultures in 96-well plates. Subconfluent monolayers were infected with serial 10-fold dilutions of a rescued virus suspension. After incubation for 1 h at 37°C, the cells were washed with medium and cultured in DMEM with 2% FBS under the same conditions. Supernatants were sampled from the
infected cell cultures at various time points in order to calculate virus titers, expressed as the 50% tissue culture infective dose (TCID\textsubscript{50}) per milliliter.

**Real-time RT–PCR.** The change of mRNA expression with time was quantified by real-time RT–PCR. BHK-21 cell monolayers were infected for 1 h with 10-fold dilution of the rescued virus in cell culture medium at a multiplicity of infection (MOI) of 8, washed, and incubated at 37°C for 2, 4, 8, 12, 16, 20, 24, or 28 h. Virions were released by 3 cycles of freeze and thaw and their RNA were reverse transcribed with MultiScribe reverse transcriptase (TaKaRa Biotech) in a total volume of 20 μl with 5 μl total RNA using a T-d(t) of 42°C for 90 min, followed by 5 min at 95°C. The primers (up-3D: 5'-GAACACATCCTCTATACCAAG AAT-3'; low-3D: 5'-CAAATCTTTGCAAT CAACG TCAG-3') targeted locations within 3D of FMDV GD/China/86 strain. After reverse transcription, 2 μl of cDNA was added to 20 μl of SYBR Premix Ex Taq\textsuperscript{TM} PCR mix (TaKaRa Biotech), consisting of 1 pmol/μl of each primer and 12.5 μl SYBR Premix Ex Taq\textsuperscript{TM}. PCR amplification was carried out in the Rotor Gene 2000 real-time thermal cycler (Applied Biosystems) using the optimal parameters: 1 cycle of 95°C for 4 min; 50 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s. Each sample was measured in triplicate and all sample reactions were repeated three times independently to ensure the reproducibility of the results. The CT (threshold cycle) value was used to compare relative expression levels among samples [21], and relative abundances of wild-type virus or rescued virus mRNA were calculated using their ΔCT values following Equation 1:

$$\text{Relative abundance} = 2^{-\Delta \text{CT}}, \Delta \text{CT} = \text{CT}_{n} - \text{CT}_{2h} \quad (1)$$

where $n$ is incubation time except 2.

### Results

#### Construction of full-length cDNA clone of GD/China/86 strain

A full-length cDNA encoding the entire genome of the GD/China/86 strain of FMDV was flanked by unique restriction endonuclease sites as described in Fig. 1. For the poly(C) tract in the 5'-UTR, we used an SmaI cloning strategy for the inclusion of 41C residues surrounded by flanking sequence (Fig. 1), where the restriction site is located at the joint where the different length homopolymer tails are linked. Sequencing results showed that the complete genome was 8104 nt in length (excluding the poly(C) tract and poly(A) tail) (Table 2), which was preceded by the polI promoter. A terminator sequence for RNA polI transcription followed the

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**Figure 1** Strategy for the construction of the full-length cDNA clone of the GD/China/86 strain of FMDV

Fragments A, B, C, and D were generated by RT–PCR from total RNA of GD/China/86, while fragment D, used as a template, was PCR amplified with the primer pairs E-up and T-d(T) to give fragment E. Then, the full-length genomic cDNA clones were assembled as described in ‘Materials and Methods’. The recombinant contained 41C and 38A residues. Transcript RNAs were synthesized from transfected circular plasmid cDNA templates [pPfmdv3.1(+)1] by using mouse RNA polymerase I (polI) \textit{in vivo}. PK, pseudoknot; IRES, internal ribosome entry site.
38-nucleotide poly(A) tail at the 3′-terminus of the GD/China/86 genome.

**pPfmdv3.1(+) transfection**
The circular full-length plasmid pPfmdv3.1(+) was transfected into monolayer cultures of BHK-21 cells with Lipofectamine 2000 reagent. CPE similar to those produced by wild-type virus was detected at 24 h post-transfection; supernatant was collected from confluent BHK-21 cell cultures at 48 h post-transfection and over 80% cells were lysed (Fig. 2).

**Characterization of rescued virus pPfmdv3.1(+)**

*Sequencing of RT–PCR products from rescued virus.* To verify that the rescued virus was derived from the cDNA plasmid, RNAs from BHK-21 cells infected with the rescued virus were subjected to RT–PCR using specific primer pairs (see above). Sequencing revealed the presence of the BamHI genetic marker indicating that virus rescued from cells transfected with pPfmdv3.1(+) was, indeed, derived from the plasmid and not from contaminating wild-type virus (Fig. 3).

*The results of IFA.* To determine whether viral proteins were expressed in BHK-21 cell cultures transfected with circular plasmid pPfmdv3.1(+), rescued virus-infected cells were analyzed by IFA. BHK-21 cells plated on glass coverslips were analyzed for fluorescence via immunostaining at various time points following transfection [Fig. 4(A)]. There was no fluorescence detected in the negative control uninfected BHK-21 cells [Fig. 4(B)]. These results showed that viral proteins were expressed in the transfected cells.

*Electron microscopy.* To confirm the results of the transfection experiment described above, the non-enveloped, icosahedral-shaped FMDV particles with diameters of ~20–30 nm were viewed by an electron microscope (Fig. 5).

**Specific infectivity assays**

*Mice virulence studies.* Virulence of the rescued virus was evaluated after intraperitoneal injection of 3-day-old mice with a 10-fold dilution of rescued virus. The first casualty among the mice infected with the rescued virus occurred at 50 h, with the mouse exhibiting signs of hind leg paralysis at 48 h. The fate of these mice was indistinguishable from that of the wild-type virus-infected mice that showed signs of paralysis 36 h post-infection and died 16–24 h later. The LD₅₀ value

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**Table 2 The constitutions of complete FMDV genome of GD/China/86 strain**

<table>
<thead>
<tr>
<th>Genome part</th>
<th>S</th>
<th>PK</th>
<th>cre</th>
<th>IRES</th>
<th>L</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>3′-UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (nt)</td>
<td>371</td>
<td>175</td>
<td>54</td>
<td>442</td>
<td>603</td>
<td>255</td>
<td>654</td>
<td>660</td>
<td>639</td>
</tr>
</tbody>
</table>

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**Figure 2 CPE in BHK-21 cells infected with rescued virus** (A) Normal monolayer of BHK-21 cells appeared as spindle shape (negative control). (B) The supernatant harvested from circular plasmid pPfmdv3.1(+) transfected experiments and CPE were observed in rescued virus-infected BHK-21 cells, which took the form of grape-like cluster, pycnosis of nucleus at 48 h post-infection. Results implied that rescued viruses have recovered from the full-length genome cDNA clones of GD/China/86 FMDV strain in BHK-21 cell cultures, by using an RNA polymerase I system. Magnification, ×400.
also showed similar characteristics between the rescued virus and the wild-type virus (LD50 was 10^{-5.67} for the rescued virus compared with 10^{-6.3} for the wild-type virus).

**Infectivity and growth kinetics of rescued virus.** To further compare growth characteristics of the rescued virus and wild-type virus, their growth kinetics were
examined. One-step growth curves showed that virus recovered from pPfmdv3.1 (+) displayed slightly lower titers at each time point than those of the wild-type virus. Peak titers were observed at 8 and 16 h post-infection for wild-type and rescued viruses, respectively (Fig. 6).

Real-time RT–PCR. Real-time RT–PCR was used to detect mRNA levels of FMDV GD/China/86 at several different time points. The primers were designed based on the sequence from the 3D region of the virus, which is the most conserved and shows the greatest difference from the target cells’ genome. The specificity of the assay was evaluated previously [22]. The reaction specificity was tested with multiple FMDV templates [Fig. 7(A) and Table 3], and in these experiments, we examined the relative amounts of FMDV RNA [Fig. 7(B)]. Results showed that, although the translation efficiency of rescued viral infected in BHK-21 cells is lower than that of wild-type virus at 12 h post-infection, the efficiency of transcription is higher.

Fragments A, B, C, and D were generated by RT–PCR from total RNA of GD/China/86, while fragment D, used as a template, was PCR amplified with the primer pairs E-up and T-d(T) to give fragment E. Then, the full-length genomic cDNA clones were assembled as described in Materials and Methods. The recombinant contained 41C and 38 A residues. Transcript RNAs were synthesized from transfected circular plasmid cDNA templates [pPfmdv3.1 (+)] by using mouse polymerase I (polI) in vivo.

Discussion

Recovering infectious rescued viruses using a reverse genetics approach has become a powerful method to study the function of viral genes at a genomic level. The modification of viral genomes, such as gene recombination, mutation, deletion, and insertion, has greatly contributed to research of virus replication, maturation, mechanisms of molecular pathogenesis, and vaccine development. In practice, it is difficult to obtain an ideal infectious cDNA clone of FMDV. The genome of FMDV

Figure 6 One-step growth curves of rescued virus and wild-type virus BHK-21 cells were infected with rescued virus at an MOI of 8. Infected cells were harvested at several time points and the virus titers (TCID₅₀) were determined.

Figure 7 Dissociation curve analysis of PCR products and detection of relative abundance level of FMDV mRNA (A) Dissociation curve analysis of PCR products obtained after 40 cycles of amplification. (B) Detection of relative abundance level of FMDV mRNA in BHK-21 cells by real-time RT–PCR. Total RNA was extracted from supernatants (virus-infected BHK-21 cells) at 2, 4, 8, 12, 16, 20, 24, or 28 h, SYBR RT–PCR for wild-type virus and rescued virus were performed on 5 μl of total RNA from each sample. To examine the relative mRNA abundances of wild-type virus and rescued virus, the CT value at 2 h was subtracted from the CT value for other times to give a value for ΔCT = CTₙh – CT₂h. The fold change was calculated from $2^{-ΔΔCT}$, yielding the highest FMDV amounts for samples with the lowest ΔCT values. As mentioned above, the wild-type virus at 8 h and the rescued virus at 16 h displayed the highest FMDV mRNA amounts relative to the respective viruses at the time point of 2 h.
HamRz and HdvRz could have solved these problems by length genomic cDNA clones of FMDV flanked by editing-deficient RNA polymerases used to rescue infectivity. Additionally, any mutations introduced by sequence at genomic termini may have influence on viral ness, and the detrimental effects that non-native methods include their complexity, expense, laborious-

In this study, we describe an accurate in vivo preparation of FMDV strain GD/China/86 RNA from mice tissue by utilizing a eukaryotic cellular poll. The final product includes the poly(C) tract containing 41C residues and the poly(A) tail containing 38A residues. To avoid instability, these constructs were never propagated more than once and DNA amplification was performed with newly transformed bacteria. Nucleotide sequence of the rescued virus was identical to the consensus sequence of the pPfmdv3.1(+) plasmid, with the exception of the genetic marker introduced at position 6545 (A–T, silent, BamHI) of the cDNA. This mutation site was located in 3C proteinase, which was one of the most conserved regions, due to its important functions potentially. The method successfully led to the generation of a cDNA encoding a fully active FMDV that could replicate within both cell culture and intraperitoneal-injected suckling mice.

A number of detection methods for FMDV were based on its genomic RNA, including hybridization assays [37], conventional RT–PCR [38,39], and real-time RT–PCR [21,40–44]. Undoubtedly, real-time RT–PCR has advantages over other techniques: greater sensitivity, better reproducibility, reduced risk of carry-over contamination, and less time-consuming. In these experiments, the growth kinetics of rescued and wild-type viruses has advantages over other techniques: greater sensitivity, better reproducibility, reduced risk of carry-over contamination, and less time-consuming. In these experiments, the growth kinetics of rescued and wild-type viruses were analyzed by comparing the relative abundance of FMDV mRNA and the expression of antigenic proteins to the number of FMDV genomic RNA copies, as detected by real-time RT–PCR. As shown in Figs. 6 and 7(B), peak mRNA and titer counts occur concomitantly for each virus; however, the rescued virus’ response is delayed with respect to wild-type. Possible reasons for these observations are as follows: on the one hand, virus recovered from cDNA clone normally has lower fitness than wild-type virus probably due to the absence of quasispecies of the rescued virus in the positive selection propagation and encapsidation. In addition, virions assembly could be occurred in the cytoplasm, in the final step of infectious cycles. Upon acid sensitivity,
the release of FMDV particles outside of BHK-21 cells would be in the proceeding of capsid degradation and RNA dissociation, for pH value descended in cell culture medium; on the other hand, a study recently indicated that cells containing FMDV genomic RNA did not always lyse and, thus, did not release viral particles [45]. It has been suggested that there is a threshold lower limit of viral RNA copies required for a cell to lyse. Furthermore, the presence of the poly(C) tract upstream of the IRES suggests that it may also play a role in FMDV translation, genome replication, or both. It has also been postulated that PCBP facilitates a circularization of the poliovirus genome to modulate the balance between translation and RNA replication [46,47]. The lengths of the poly(C) tract and the poly(A) tail after several passages of a persistently infected BHK-21 cell line were difficult to determine; hence, they remain uncertain. Although there is a slight nucleotide sequence divergence between the wild-type virus and the rescued virus because of a few mutations located at the 5'-most terminal region (S fragment) and at the IRES, the mutations do not appear to significantly affect the secondary structure predicted for these RNA segments.

Similar to previous research results, this study may demonstrate that a polI-based vector system represents an efficient alternative strategy for the recovery of a cytoplasmic sense-strand RNA virus from cDNA. To our knowledge, there is little information available in the equivalent cDNA under the promoter of a different RNA polI-based suitable for in vitro transcription, and this is the first report of polI's ability to transcribe the 8.2-kb gene for sense-strand RNA virus. Until recently, compared with other strategies, an RNA polI-based system increases the efficiency of virus rescue by allowing for precise transcription [32].

In conclusion, an infectious clone of FMDV isolated from swine was generated using an RNA polI-based system. This study helps to develop a much broader platform for understanding the mechanisms of FMDV molecular pathology toward the development of genetically engineered FMDV vaccines.

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