Modification of the internal ribosome entry site element impairs the growth of foot-and-mouth disease virus in porcine-derived cells

Chao Sun,† Decheng Yang,† Rongyuan Gao, Te Liang, Haiwei Wang, Guohui Zhou and Li Yu

Division of Livestock Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, PR China

The 5′ untranslated region (5′ UTR) of foot-and-mouth disease virus (FMDV) contains an internal ribosome entry site (IRES) that facilitates translation initiation of the viral ORF in a 5′ (m7GpppN) cap-independent manner. IRES elements are responsible for the virulence phenotypes of several enteroviruses. Here, we constructed a chimeric virus in which the IRES of FMDV was completely replaced with that of bovine rhinitis B virus (BRBV) in an infectious clone of serotype O FMDV. The resulting IRES-replaced virus, FMDV(BRBV), replicated as efficiently as WT FMDV in hamster-derived BHK-21 cells, but was restricted for growth in porcine-derived IBRS-2, PK-15 and SK-6 cells, which are susceptible to WT FMDV. To identify the genetic determinants of FMDV underlying this altered cell tropism, a series of IRES-chimeric viruses were constructed in which each domain of the FMDV IRES was replaced with its counterpart from the BRBV IRES. The replication kinetics of these chimeric viruses in different cell lines revealed that the growth restriction phenotype in porcine-derived cells was produced after the replacement of domain 3 or 4 in the FMDV IRES. Furthermore, the change in FMDV cell tropism due to IRES replacement in porcine-derived cells was mainly attributed to a decline in cell-specific IRES translation initiation efficiency. These findings demonstrate that IRES domains 3 and 4 of FMDV are novel cell-specific cis-elements for viral replication in vitro and suggest that IRES-mediated translation determines the species specificity of FMDV infection in vivo.

INTRODUCTION

Foot-and-mouth disease (FMD) is an extremely contagious viral disease of cloven-hoofed animals that has an unusually broad host range, infecting pigs, cattle, sheep and goats (Grubman & Baxt, 2004). The disease is distributed worldwide and has a major negative economic impact not only on livestock health and production but also on international trade. Disease outbreaks occur frequently on almost every continent, and outbreaks in previously FMD-free countries often have devastating economic consequences (Uddowla et al., 2012). The aetiological agent, FMD virus (FMDV), is a member of the genus Aphthovirus in the family Picornaviridae. There are three other species in the genus Aphthovirus, Bovine rhinitis A virus (BRAV), Bovine rhinitis B virus (BRBV) and Equine rhinitis A virus (ERAV). In a recent study, Bovine rhinitis A virus was reported to be genetically closer to Foot-and-mouth disease virus than to Equine rhinitis A virus on the basis of phylogenetic characterization and full-genome analysis (Hollister et al., 2008). The FMDV genome consists of a single-stranded positive-sense RNA with a length of approximately 8500 bases, which encodes a single ORF flanked by 5′ and 3′ untranslated regions (UTRs) and a 3′ polyadenylated tail (Serrano et al., 2006). In contrast to host cellular mRNAs, the FMDV mRNA lacks a 5′ cap structure and its translation is controlled by an internal ribosomal entry site (IRES) located within the 5′UTR.

Picornavirus IRES elements are highly structured RNA elements that compose an unusually large segment of the viral genome RNA (Wimmer et al., 1993). These IRES elements are classified into five types based on sequence and structural homology: type I IRES of the enterovirus group with poliovirus as prototype, type II IRES of the cardiovirus/aphthovirus group with encephalomyocarditis virus (EMCV) and FMDV as prototypes type III IRES of hepatitis A virus (HAV), and type IV IRES of porcine teschovirus (PTV) (Borman et al., 1997; Brown et al., 1991; Jackson et al., 1994; Pisarev et al., 2004). Sweeney et al. (2012) and colleagues recently identified a fifth IRES class among members of the Kobuvirus, Salivirus and Oscivirus genera. The nucleotide sequence identity among IRES elements of the same type is only moderate, but their predicted secondary structure is highly conserved.
The IRES of FMDV belongs to type II, which also includes IRESes of EMCV, Theiler’s murine encephalomyelitis virus (TMEV), equine rhinitis A virus (ERAV) and bovine rhinitis B virus (BRBV) (Hinton et al., 2000; Jackson et al., 1994). The FMDV IRES consists of 450 nt and folds into multiple stem–loops that are organized into four domains (Fig. 1). These domains interact with cellular binding proteins such as eukaryotic initiation factors (eIFs) and IRES trans-acting factors (ITAFs), which play crucial roles in IRES-directed translation (Hellen & Sarnow, 2001; Lunde et al., 2007; Martínez-Salas et al., 2001, 2013). Although numerous studies of IRES function have shed some light on the mechanism of translational initiation in IRES elements, most of these investigations were performed in cell-free systems and the function of IRESes in viral pathogenesis in vivo remains obscure.

Like other RNA viruses, FMDV exhibits a high potential for variation and adaptation, as reflected by its antigenic diversity, broad host range and capacity to produce persistent infections in both host animals and cell culture (Domingo et al., 1990; Sobrino et al., 2001). Little is known about the genetic determinants of FMDV host range and virulence. In the 3A protein, the amino acid substitution Q44R is sufficient to exacerbate the virulence of FMDV strain C-S8c1 adapted to guinea pigs (Núñez et al., 2001). Deletions in 3A have been associated with FMDV attenuation in cattle and with the porcinephilic phenotype of O Taiwan/97 (Knowles et al., 2001). This evidence implicates FMDV 3A in virus host range and virulence, similar to the 3A proteins of other picornaviruses (Graff et al., 1994; Lama et al., 1998). Beyond the 3A protein, limited information is available on the determinants of host range and virulence for FMDV.

The primary determinant of picornaviral species tissue tropism is the presence or absence of the viral receptor on the cell surface (Lin & Shih, 2014; Schneider-Schaulies, 2000). However, several results suggest that the IRES may also be a determinant of viral tropism (Borman et al., 1993; del Angel et al., 1989; Meerovitch et al., 1993). In this study, we constructed a series of viruses in which the complete FMDV IRES or one of its domains was replaced with the corresponding sequence from BRBV in an infectious clone of serotype O FMDV. Our results showed that domain 3 or 4 of the FMDV IRES determines the growth restriction phenotype of FMDV in porcine-derived cells. Most importantly, our studies indicate that the change in FMDV cell tropism due to IRES substitution is mainly attributable to a decrease in translation initiation efficiency of the IRES. Thus, IRES-mediated cell specificity represents a potential pathogenesis determinant of FMDV.

RESULTS

Replacement of the FMDV IRES with the entire BRBV IRES impairs FMDV replication in a cell-specific manner

The RNA structure of the IRES region of FMDV was defined previously (Fernández-Miragall et al., 2009;
Based on the secondary structure of the EMCV IRES, we modelled the secondary structures of the FMDV O/YS/CHA/05 and BRBV EC11 IRESes using Mfold version 3.2 as described previously (Duke et al., 1992; Pilipenko et al., 1989), which indicated similar secondary structures for the FMDV and BRBV IRESes (data not shown). Transposition of the human rhinovirus type 2 (HRV2) IRES element into poliovirus (PV) type 1 (Mahoney) resulted in a viable virus chimera, PV1(RIPO) (Gromeier et al., 1996). Similarly, an IRES-chimeric FMDV mutant was constructed by replacing the FMDV IRES with the BRBV IRES (Fig. 1) and named FMDV(BRBV). The size and morphology of FMDV(BRBV) plaques in hamster-derived cells (BHK-21) and porcine-derived cells (IBRS-2, PK-15 and SK-6) were evaluated. As shown in Fig. 2(a), the plaque sizes of FMDV(BRBV) and WT FMDV [FMDV(WT)] did not differ in hamster-derived BHK-21 cells, but FMDV(BRBV) formed much smaller plaques than did FMDV(WT) in porcine-derived IBRS-2, PK-15 and SK-6 cells.

To further determine whether IRES substitution influences the replicating ability of FMDV in porcine-derived cells, the in vitro replication kinetics of FMDV(BRBV) and FMDV(WT) were evaluated in cell lines originating from different species. As shown in Fig. 2(b), both viruses replicated with similar kinetics in hamster-derived BHK-21 cells, whereas the replication of FMDV(BRBV) in porcine-derived cells (IBRS-2, PK-15 and SK-6) was 10- to 100-fold lower compared with that of FMDV(WT) throughout the virus growth cycle. These results indicate that replacement of the FMDV IRES with the entire BRBV IRES impairs FMDV replication in a cell-specific manner.

**Replacement of IRES domain 3 or 4 with the corresponding domain of BRBV impairs the replication of FMDV in porcine-derived cells**

To localize the portion of the IRES responsible for the growth restriction phenotype of FMDV in porcine-derived cells, five IRES-chimeric FMDV mutants were constructed in which each domain of the FMDV IRES (from 2 to M) was replaced with its counterpart from BRBV (Fig. 1). The growth kinetics of these IRES-domain-chimeric FMDV mutants were compared to those of FMDV(WT) and FMDV(BRBV); WT FMDV [FMDV(WT)] was used as a negative control, and the entire IRES-replaced FMDV [FMDV(BRBV)] was used as a positive control. As shown in Fig. 3(a, b), the chimeras FMDV(R2), FMDV(R5) and FMDV(RM) exhibited growth profiles very similar to that of FMDV(WT) in both BHK-21 and IBRS-2 cells. By contrast, the chimeras FMDV(R3) and

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**Fig. 2.** Comparison of the replication abilities of FMDV(BRBV) and FMDV(WT) in several cell lines. BHK-21, IBRS-2, PK-15 and SK-6 cells were infected with FMDV(BRBV) or FMDV(WT) at an m.o.i. of 0.05. The viruses produced were harvested at different times, and virus titres were determined as TCID<sub>50</sub> ml<sup>-1</sup> in BHK-21 cells. Plaque phenotypes (a) and growth curves (b) of FMDV(BRBV) and FMDV(WT) are shown. Error bars represent s.d. (n=3).
FMDV(R4), in which domain 3 or 4 of the FMDV IRES was replaced with the corresponding domain of BRBV (Fig. 1), exhibited a significant decrease in FMDV replication in porcine-derived IBRS-2 cells, and even showed a 10-fold lower titre compared with FMDV(BRBV) in which the complete IRES of FMDV was replaced (Fig. 3b).

To study the combined effect of domain 3 and 4 replacement, we constructed a domains 3- and 4-replaced recombinant virus, FMDV(R3-4), and its converse recombinant virus, FMDV(R2,5-M) (Fig. 1). However, repeated attempts to select viable virus FMDV(R3-4) were unsuccessful, suggesting that the combined replacement of FMDV domains 3 and 4 with their BRBV IRES counterparts is lethal for FMDV replication. By contrast, the converse recombinant virus, FMDV(R2,5-M), in which domains 3 and 4 of BRBV IRES were replaced with those of FMDV (Fig. 1), was easily recoverable and retained efficient growth in BHK-21 and IBRS-2 cells. These results are consistent with the virus plaque phenotypes (Fig. 3c, d), indicating that the replacement of IRES domain 3 or 4 with the corresponding domain of BRBV IRES severely impairs replication of FMDV in porcine-derived cells.

**Defective translation and replication of FMDV mutants in which IRES domain 3 or 4 has been replaced in porcine-derived cells**

The IRES-replaced or -chimeric FMDV mutants, including FMDV(BRBV), FMDV(R3) and FMDV(R4), were serially passaged at low m.o.i. in both BHK-21 and IBRS-2 cells to assess their replication stability during cell culture passage; FMDV(WT) was used as a control. At each passage, the supernatant was used to determine the infectious virus titre using the TCID₅₀ assay, detect viral RNA replication by reverse transcription PCR (RT-PCR), and analyse viral protein expression by Western blot.

After five passages in BHK-21 cells, all IRES-chimeric FMDV mutants displayed a titre of ~6 log TCID₅₀ ml⁻¹.
at 12 h post-infection, very similar to that of FMDV(WT) (Fig. 4a). By contrast, in IBRS-2 cells, as shown in Fig. 4(a), the titre of FMDV(BRBV) declined from 5.33 log TCID50 ml\(^{-1}\) in passage 1 to 3.08 log TCID50 ml\(^{-1}\) in passage 3, and was less than 2 log TCID50 ml\(^{-1}\) from passage 4 (1.42 log TCID50 ml\(^{-1}\)) to passage 5 (1.08 log TCID50 ml\(^{-1}\)). The growth profiles of FMDV(R3) and FMDV(R4) were similar, declining from 5.33 log TCID50 ml\(^{-1}\) in passage 1 to 4 log TCID50 ml\(^{-1}\) in passage 2, and less than 2 log TCID50 ml\(^{-1}\) from passage 3 (1.33 log TCID50 ml\(^{-1}\)) to passage 5 (1.08 log TCID50 ml\(^{-1}\)).

For viral RNA detection, as shown in Fig. 4(b), all IRES-replaced and -chimeric viruses as well as FMDV(WT) were detected at high concentrations in passages 1 to 5 of BHK-21 cells. However, in IBRS-2 cells, the viral RNA of FMDV(BRBV) was detected only in passages 1 to 3, along with weakening of the RT-PCR signal; similarly, no viral RNA was detected for FMDV(R3) or FMDV(R4) after the first two passages (Fig. 4b). Taken together, these results imply that the mutants FMDV(BRBV), FMDV(R3) and FMDV(R4) cannot replicate efficiently in porcine-derived IBRS-2 cells. To further support this conclusion, the kinetics of viral gene expression were detected by Western blot analysis of FMDV VP2 accumulation in cells infected with these mutants. As shown in Fig. 4(c), VP2 protein was expressed as efficiently in BHK-21 cells infected with FMDV(BRBV), FMDV(R3) or FMDV(R4) as in those infected with FMDV(WT). However, in IBRS-2 cells, as shown in Fig. 4(c), high VP2 protein expression occurred through passages 1 to 5 only for FMDV(WT); for FMDV(BRBV), VP2 protein expression was detected only in passages 1 to 3, along with weakening of the signal; and for FMDV(R3) and FMDV(R4), no VP2 protein was detected after the first two passages. These results are consistent with the detection of infectious virus and viral RNA (Fig. 4a, b), confirming that the translation and replication of FMDV(BRBV), FMDV(R3) and FMDV(R4) are defective in porcine-derived cells.

![Fig. 4. Growth stability of the IRES domain 3- and domain 4-chimeric FMDV mutants in BHK-21 and IBRS-2 cells. Five serial passages were performed in BHK-21 and IBRS-2 cells infected with FMDV(BRBV), FMDV(R3) or FMDV(R4) at an initial m.o.i. of 0.05; FMDV(WT) was used as a control. Supernatants were analysed using a TCID50 assay for infectious virus titre (a), RT-PCR detection for viral RNA replication (b) and Western blot analysis for VP2 protein expression (c). Error bars in (a) represent so (n=3).](http://jgv.microbiologyresearch.org)
The replication defect of FMDV with a replaced IRES domain 3 or 4 in porcine-derived cells is due to decreased efficiency of IRES-mediated translational initiation

After cell receptor integrin-mediated internalization, the released FMDV genome RNA serves as the mRNA for the translation of a single polypeptide, followed by proteolytic processing and genome replication (Grubman & Baxt, 2004; Palmenberg, 1990). To investigate the IRES-directed translation of viral protein, a critical step in viral FMDV replication, we constructed four luciferase replicons, RepFMDV(BRBV), RepFMDV(R3), RepFMDV(R4) and RepFMDV(WT), which contain the respective IRES elements in the same context as in their parent viruses.

The capacity of these luciferase replicons for IRES-mediated translation was assessed by transfecting BHK-21 and IBRS-2 cells with in vitro-transcribed replicon RNA. To differentiate the luciferase signal from translation of input viral RNA and from the translation of newly replicated RNA, one portion of the transfected cells received 2 mM guanidine hydrochloride (GnHCl), a potent inhibitor of RNA replication that has no toxic effects on cellular processes or viral translation (Caliguiri & Tamm, 1968; Jacobson & Baltimore, 1968; Loddo et al., 1962). In BHK-21 cells, no significant differences in directing internal translation initiation were observed among the IREses of FMDV(BRBV), FMDV(R3), FMDV(R4) and FMDV(WT). However, in IBRS-2 cells, all IRES-replaced or chimeric replicons [FMDV(BRBV), FMDV(R3) and FMDV(R4)] showed lower luciferase activity compared with the FMDV(WT) IRES. Most strikingly, the FMDV(R3) and FMDV(R4) IREses generated a 100-fold reduction luciferase activity compared with the FMDV(WT) IRES (Fig. 5). This finding is consistent with the growth characteristics of the corresponding IRES-replaced or -chimeric FMDV mutants in BHK-21 and IBRS-2 cells, demonstrating that the IRES-mediated translation of FMDV(BRBV), FMDV(R3) and FMDV(R4) is defective in porcine-derived cells. These data confirm that the change in cell tropism of RepFMDV(BRBV), RepFMDV(R3) and RepFMDV(R4) was mainly due to the decreased cell-specific translation initiation efficiency of the replaced or chimeric IRES.

DISCUSSION

Picornaviruses, a large family of human and animal pathogens, cause a bewildering array of disease syndromes. One of the major determinants of picornaviral species and tissue tropism is the presence or absence of the viral receptor on the cell surface (Holland, 1961). Virion stability and cell-internal restriction of replication play equally important roles in the outcome of infection (Gromeier et al., 1996). Among the picornaviruses, FMDV is noted for its broad host range, yet the underlying biological mechanisms that contribute to the phenotypes observed have remained elusive. In this study, we report for the first time to our knowledge that the higher-order structure of the IRES element determines FMDV replication in a cell-specific manner.

We first constructed a chimeric virus in which the IRES of FMDV was precisely replaced with that of BRBV. Such changes could have abolished the translational function of the FMDV IRES. The secondary structure of this chimeric IRES was therefore predicted using Mfold software (version 3.2) to ensure folding into the correct stem–loop structures. This recombinant FMDV genome with IRES replacement was expected to produce a viable virus chimera because (i) the viruses FMDV and BRBV are phylogenetically relatively closely related and belong to same genus, Aphthovirus (Hollister et al., 2008; Lauber et al., 2001).
& Gorbalenya, 2012); and (ii) despite the low level of nucleotide sequence similarity (58.8 %) of the IRES elements from these two viruses, the secondary structures of the IRES elements are very similar and are therefore both classified as type II IRESes (Holllister et al., 2008). The chimeric virus FMDV(BRBV) rescued from the IRES-replaced FMDV genome exhibited an impaired growth phenotype in the three porcine-derived cell types tested but retained efficient growth in the hamster-derived BHK-21 cells (Fig. 2). These results indicate that the IRES substitution in the 5’UTR influences the replication ability of FMDV in a cell-specific manner. This important finding prompted us to characterize the role of the functional domain(s) of the IRES in the cell-specific replication of FMDV.

We subsequently determined that replacement of only domain 3 or 4 of FMDV IRES with the corresponding domain of BRBV impaired replication of FMDV in porcine-derived cells (Fig. 3b, d), indicating that domain 3 or 4 is responsible for cell-specific FMDV replication. Domain 3, the largest and central domain in the FMDV IRES, acts as a scaffold structure that holds the IRES domains together and contributes to the stability of the entire IRES (Drew & Belsham, 1994; Ramos & Martinez-Salas, 1999; Roberts & Belsham, 1997). Thus, small changes in domain 3 may induce reorganization of the IRES structure and have important consequences for IRES function (Martinez-Salas & Fernández-Miragall, 2004). Domain 4, which is organized into two hairpin-loops with A-rich internal bulges, is responsible for the interaction with the translation initiation factor eIF4G, an essential step in FMDV IRES-dependent translation initiation (López de Quinto & Martínez-Salas, 2000; Pilipenko et al., 2000; Yu et al., 2011a). Thus, as demonstrated in this study, the replacement of FMDV IRES domain 3 or domain 4 mainly modulates the translation efficiency of FMDV mutants, presumably by altering IRES stability and the recruitment of cellular factors, eIFs and/or ITAFs.

Similar to this finding in FMDV, the growth restriction phenotype of coxsackievirus B3 (CBV3) in primary murine cell cultures was localized to IRES domain 2 within the Travis strain of echovirus 12 (ECV12) (Bradrick et al., 2001). In addition, the exchange of PV IRES domains 5 and 6 with their HRV2 counterparts was sufficient to reduce the growth kinetics of PV1(RIPO) in SK-N-MC cells (Gromeier et al., 1999). For the attenuated PV vaccine strains, all single point mutations identified in the IRESees of the three Sabin strains were mapped to domain 5 (Gromeier et al., 1999). In combination with these published data derived from other IRES-chimeric picornaviruses, our results for FMDV indicate that different domains regulate picornaviral IRES function mainly by altering IRES-mediated translation.

The present study also indicates that inefficient translation is the major cause of the replication block of the IRES-replaced or chimeric viruses FMDV(BRBV), FMDV(R3) and FMDV(R4) in porcine-derived cells (Fig. 5). Actually, we had also developed bicistronic reporter constructs to analyze IRES activity (data not shown). These results showed that, in BHK-21 cells, the IRES activity of FMDV(BRBV), FMDV(R3) and FMDV(R4) was not significantly different from that of FMDV(WT). However, in IBRS-2 cells compared to FMDV(WT), FMDV(BRBV) and FMDV(R3) exhibited reduction in IRES activity. Unexpectedly, the IRES activity of FMDV(R4) was found to be about 1.4-fold greater than that of FMDV(WT). This finding is not completely consistent with the growth characteristics of these mutants in BHK-21 and IBRS-2 cells. Thus, the data from the bicistronic reporter system only reflect IRES activity to some extent, because bicistronic reporter constructs include only the IRES element and thus do not take into account other genomic elements that may be involved in the modulation of translation, such as the 3’UTR (Kok et al., 2012). Consequently, to authentically assess the capacity for viral IRES-mediated translation, we subsequently constructed replicons in which only the P1 region of FMDV was replaced with the Rluc gene (Fig. 5). In our experiments, these replicons more closely reflect the conditions encountered by their infectious virus counterparts.

Although our studies confirmed that inefficient translation is the major block to FMDV(BRBV), FMDV(R3) and FMDV(R4) proliferation in porcine-derived cells, the exact mechanism of the altered IRES translational efficiency of these IRES-replaced or -chimeric viruses remains to be determined. The picornavirus IRES mediates translation through its interaction with host cell RNA-binding proteins such as eIFs and ITAFs. In vitro studies have identified different cell factors that bind to the different IRESees for viral translation initiation (Borman et al., 1993; del Angel et al., 1989; Meervorch et al., 1993). As a consequence of their role as regulators of IRES activity, cell factors can mediate the cell type specificity of viral infection and consequently determine viral spread (Pacheco & Martinez-Salas, 2010; Pilipenko et al., 2001). Double-stranded RNA-binding protein 76 (DRBP76) inhibits its translation and propagation of the PV chimera PV1(RIPO) in neuronal cells by associating specifically with the HRV2 IRES (Merrill et al., 2006; Merrill & Gromeier, 2006). Central nervous system (CNS)-specific attenuation of the PV vaccine strain Sabin 3 is caused by reduced binding of polypyrimidinetract-binding protein (PTB) to the PV Sabin3 IRES (Guest et al., 2004). Based on these results, we speculate that cell-specific factors that reduce the binding of domain 3 or 4 of the BRBV IRES may be responsible for the cell-specific growth restriction of the IRES-replaced FMDV(BRBV) and IRES-chimeric FMDV(R3) and FMDV(R4) in porcine-derived cells. Thus, the mechanism underlying the altered IRES translational efficiency of these IRES-replaced or -chimeric viruses and their functional interaction with swine eIFs and ITAFs will be examined in future studies.
Alternatively, the BRBV IRES domains 3 and/or 4 may have an impact on the viral growth phenotype by negatively affecting FMDV genomic RNA replication specifically within the porcine-derived cells utilized. Indeed, the FMDV cre, a cis-acting replication element, is located within the 5'UTR (domain 1 just before the IRES). It could be that different interactions between the FMDV core element and BRBV IRES domains 3 and/or 4 affect RNA replication efficiency in the hybrid constructs. Thus, further study is required to determine the exact role of the BRBV IRES domains 3 and/or 4 in FMDV genomic RNA synthesis.

In summary, our studies have confirmed the importance of the IRES in the cell-specific replication of FMDV. Furthermore, we have identified IRES domain 3 or 4 of FMDV as a novel cell-specific cis-element for viral replication in vitro. Based on previous reports of IRES-chimeric attenuated picornaviruses, our findings suggest that IRES-mediated translation determines the species specificity of FMDV infection and pathogenesis in vivo. Therefore, the testing of FMDV(BRBV), FMDV(R3) and FMDV(R4) in a mouse model and natural hosts is under way to evaluate their host tropism and virulence.

**METHODS**

**Cells, viruses and antibodies.** Baby hamster kidney cells (BHK-21) and porcine kidney cells (IBRS-2, PK-15 and SK-6) were propagated in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco) and 2 mM L-glutamine. Cells were maintained at 37°C in 5% CO₂. O/Y/S/CHA/05 (GenBank accession number: HM008917), the WT strain of FMDV serotype O used in our study, was generated from the infectious cDNA clone pYS (Yang et al., 2009). The mAb 4B2, which recognizes a conserved linear epitope on the VP2 protein of FMDV, was prepared in our laboratory as described previously (Yu et al., 2011b).

**Construction of FMDV/BRBV IRES chimeras.** The IRES chimeras were constructed using a BglII-NheI cloning cassette to exchange the cognate IRES of FMDV(WT) with heterologous IRES elements of varying composition. The genetic structure of the cloning cassette in the plasmid pYS was as follows: BglII-T7 promoter, 5'UTR, L protein, part VP4-NheI (1925 nt). The primers used to generate the composite IRES elements recombining the stem-loops of BRBV (GenBank accession number: NC_010354.1) with those of FMDV(WT) are listed in Table 1.

To construct pFMDV(BRBV), primer pairs 1-2, 5-6 and 3-4 were used to generate PCR fragments of the FMDV 5’ terminus (nt 1 to 667), the BRBV IRES from domain 2 to the M region, and FMDV L-VP4 (nt 1113 to 1925), respectively. The resulting PCR products were used as templates to amplify overlapping products using primer pairs 1-4. For pFMDV(R2), primer pair 1-7 was used to generate a PCR fragment containing the FMDV 5’terminus and BRBV IRES domain 2 from pFMDV(BRBV); this fragment was ligated to the PCR fragment corresponding to the FMDV IRES from domain 3 to M, and FMDV L-VP4 obtained from pYS using primer pair 8-4. For pFMDV(R3), primer pair 1-11 was used to generate a PCR fragment encompassing the 5’ terminus and domain 2 of the FMDV IRES; this fragment was ligated to the PCR fragment obtained from pFMDV(BRBV) using primer pairs 3-9 corresponding to BRBV IRES domain 3 and the PCR product corresponding to the FMDV IRES domains from 4 to M and FMDV L-VP4 obtained from the plasmid pYS using primers 12-4. For pFMDV(R4), primer pairs 1-15, 13-14 and 16-4 were used to generate PCR fragments encompassing the FMDV 5’terminus and IRES domain 2 to 3, BRBV IRES domain 4, and FMDV IRES domain 5 to FMDV L-VP4, respectively. The resulting PCR products were used as templates to amplify overlapping products using primer pairs 1-4. For pFMDV(R5), primer pair 1-17 was used to generate a PCR fragment containing FMDV 5’terminus to IRES domain 5 from pYS; this fragment was ligated to the PCR fragment corresponding to BRBV IRES domain M and FMDV L-VP4 obtained from pFMDV(BRBV) using primer pair 20-4. For pFMDV(R3-4), primer pairs 1-11, 9-14 and 16-4 were used to generate PCR fragments encompassing the FMDV 5’terminus to IRES domain 2, BRBV IRES domain 3 to 4, and FMDV domain 5 to FMDV L-VP4, respectively. The resulting PCR products were used as templates to amplify overlapping products using primer pair 1-4. Finally, pFMDV(R2,5-M) was constructed by amplifying the FMDV 5’terminus and BRBV IRES domain 2, FMDV IRES domain 3 to 4, and BRBV IRES domain 5 to FMDV L-VP4 using primer pairs 1-7, 8-17 and 18-4, respectively, with subsequent fusion using primers 1 and 4. The overlapping PCR products were inserted into the pYS vector. The secondary structure of each chimeric IRES construct was predicted using Mfold software (version 3.2), which revealed that the overall IRES structure predicted for both FMDV and BRBV was not affected by the intergeneric exchange of intact stem-loop domains or subfragments described in this study. The portal for the mfold web server is http://www.bioinfo.rpi.edu/applications/mfold.

**Construction of the FMDV replicon.** The FMDV replicon was generated by replacing the P1 region of the virus construct with the Renilla luciferase (Rluc) gene using the following strategy. Briefly, the Rluc gene was amplified by PCR from the vector pRL-TK (Promega) using a forward-sense oligonucleotide containing two codons of VP4, followed by 10 additional Lpro codons and the first six codons of the Rluc coding region, and an antisense oligonucleotide containing the entire sequence of the 2A protein, a single Gln codon from the C terminus of 1D and seven C-terminal codons of Rluc. A fragment containing the entire 5'UTR sequence and the Lpro was amplified using primers 1 and 21 (Table 1) to create a fragment containing the T7 promoter with a BglII site at the 5’ end. Next, a fragment containing the entire 2ABC and part of 3A was amplified using primers 22 and 23 (Table 1) to create a fragment containing an EcoRI site at the 3’ end. Finally, overlap PCR was performed using primers 1 and 23 and the three PCR products. The resulting product was digested with BglII and EcoRI and inserted into the corresponding sites in the infectious cDNA clone pYS. The resulting FMDV Rluc replicon was named RepFMDV(WT). The RepFMDV(BRBV), RepFMDV(R3) and RepFMDV(R4) constructs were prepared using a similar strategy.

**In vitro transcription and transfection.** Plasmids were linearized by digestion with EcoRV, and transcripts were generated using the Ribonucleic Acid Production Systems-T7 kit (Promega). After transcription, the reaction mixture was treated with 1 U of RNase A (Qiagen). The supernatant of the transfected cells was used to transfect the BHK-21 cells. Viral RNA was extracted from supernatant of infected cells using TRIzol reagent (Qiagen). RNA samples from infected cells were reverse-transcribed and amplified. The primer pairs used were designed to amplify the entire 3A region of the virus construct.

**Growth curves.** To determine viral replication kinetics, growth experiments in BHK-21, IBRS-2, PK-15 and SK-6 cells were performed.
as follows. Briefly, cell monolayers in 6-well tissue culture plates were washed with PBS and inoculated with the virus to be tested at a m.o.i. of 0.05. The plates were incubated for 1 h at 37 °C. The cells were then washed three times with PBS to remove unbound virus particles and covered with DMEM supplemented with 2 % FBS. The infected cells were incubated at 37 °C and harvested at different times. The plates were subjected to three consecutive freeze–thaw cycles, and the viral titres of the supernatants were determined by TCID₅₀ assay as described previously (Pizzi, 1950). Mean values and standard deviations were calculated from three independent experiments.

**Plaque assay.** Plaque characterizations of virus were conducted in BHK-21, IBRS-2, PK-15 and SK-6 cells. Briefly, confluent cells in 6-well plates were inoculated for 1 h at 37 °C with virus serially diluted in DMEM, washed once with PBS, and overlaid with DMEM supplemented with 1 % low-melting-point methyl cellulose and 2 % FBS. The infected cells were incubated at 37 °C and harvested at different times. The same blot was reused, and the same steps were repeated to detect actin using anti-actin mouse mAb (Sigma) as the primary antibody and Dylight 800-labelled anti-mouse IgG antibody as the secondary antibody.

**Statistical analysis.** All statistical analyses were performed using the two-tailed Student’s t-test available in the software GraphPad Prism. A P value of < 0.05 was considered statistically significant.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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**Table 1. Primers used in this study**

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**Luciferase assay.** The in vitro-transcribed replicon RNA was transfected into monolayers of BHK-21 and IBRS-2 cells using Effectene Transfection Reagent (Qiagen). At 12 h post-transfection, the growth medium was removed from the dishes and the cells were washed gently with 2 ml PBS. Luciferase activity was measured in cell lysates using a Renilla Luciferase Assay System kit (Promega) and a microplate reader (Bio-Tek). The viral translation was assayed by incubating the transfected cells in the presence or absence of 2 mM GnHCl, a potent inhibitor of FMDV replication.

**Western blot analysis.** For immunoblot analysis, lysates of BHK-21 and IBRS-2 cells collected at 24 h post-infection were separated by SDS-PAGE (12 % acrylamide). Following electrotransfer to PVDF membranes, the membranes were blocked with 5 % skimmed milk in 0.1 % Tween-20 PBS for 2 h. The membranes were then probed with mAb 4B2 at a 1 : 200 dilution overnight at 4 °C. The membranes were washed three times and incubated with Dylight 800-labelled anti-mouse IgG antibody for 1 h at room temperature. After three washes, specific proteins were detected using an Odyssey infrared imaging system (LiCor BioSciences). The same blot was reused, and the same steps were repeated to detect actin using anti-actin mouse mAb (Sigma) as the primary antibody and Dylight 800-labelled anti-mouse IgG antibody as the secondary antibody.


