Short hairpin RNA targeted to the highly conserved 2B nonstructural protein coding region inhibits replication of multiple serotypes of foot-and-mouth disease virus

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Received 14 December 2004; returned to author for revision 6 January 2005; accepted 2 March 2005

Abstract

Foot-and-mouth disease virus (FMDV) is one of the most contagious agents of animals. Recent disease outbreaks in FMD-free countries have prompted the development of new control strategies that could improve the levels of protection against this virus. We have delivered a plasmid expressing a short hairpin RNA (shRNA) directed against a highly conserved sequence in the 2B nonstructural protein coding region of FMDV RNA to porcine cells. After virus infection, these cells showed a significant reduction in the synthesis of viral RNA and proteins, as well as a decrease in virus yield when compared to control cells. The antiviral effect was sequence specific and not attributable to induction of the interferon pathway. Since FMDV is an antigenically variable virus, we examined the effectiveness of this strategy against multiple serotypes and found that expressed 2B shRNA resulted in efficient silencing of at least 4 FMDV serotypes. Thus, RNA interference may be a potential alternative control strategy to limit the spread of this highly contagious virus in livestock.

Published by Elsevier Inc.

Keywords: Foot-and-mouth disease; RNA interference; siRNA; 2B nonstructural protein coding region

Introduction

Foot-and-mouth disease (FMD) is an extremely contagious disease that affects cattle, swine and other livestock worldwide. The disease has been known for five centuries (Fracastorious, 1546) and its causative agent, foot-and-mouth disease virus (FMDV), was the first virus ascribed to an animal illness (Loeffler and Frosch, 1897). Studies over the years have led to the implementation of vaccination programs against FMDV that resulted, after 1989, in the successful eradication of FMD from Western Europe. However, recent outbreaks in Taiwan (1997) and the United Kingdom (2001), with devastating economic and social impact, have raised the awareness about the limitations of current methods to control this disease.

FMDV contains a positive-sense, single-stranded RNA genome, and is the prototype member of the Aphthovirus genus of the Picornaviridae family. The virus is antigenically variable and consists of 7 serotypes and multiple subtypes. FMDV replicates very rapidly within the infected animal and spreads among in-contact susceptible animals by aerosol or direct contact. Disease signs can appear as early as 2 days post-exposure to the virus.

The current vaccine is an inactivated whole virus preparation administered with an adjuvant (Doel, 2003). Recently, a replication-defective human adenovirus type 5 vector (Ad5) containing the capsid coding region of serotype A24 Cruzeiro and the FMDV 3C proteinase coding region has been developed and can protect both swine and cattle from homologous challenge (Moraes et al., 2002;
unpublished data). However, since vaccines are serotype specific, an effective control strategy requires constant epidemiological surveillance to identify relevant serotypes that must be included in an antigen bank. In addition, neither of the above vaccines can induce complete protection prior to 7 days post-inoculation. Early protection is required in the event of an FMD outbreak in a disease-free country to prevent virus amplification and disease spread. Chinsangaram et al. (2003) and Moraes et al. (2003) have shown that inoculation of an Ad5 vector encoding the gene for the antiviral protein, porcine interferon (IFN) alpha or a combination of Ad5s expressing IFN alpha and FMDV capsid and 3C proteinase coding regions completely protected swine when challenged from 1 to 5 days later. However, administration of the antiviral alone conferred only limited protection in cattle (Wu et al., 2003). Thus, the development of new rapid-acting antiviral strategies effective against all serotypes and all FMDV-susceptible species is imperative.

RNA interference (RNAi) is a natural process by which double-stranded RNA directs sequence specific post-transcriptional gene silencing (Fire, 1999; Ham mond et al., 2001; Sharp, 2001). Specific inhibition of endogenous or pathogen mRNA by RNAi can be triggered by the introduction of 21–23 nucleotide (nt) duplexes of RNA (siRNAs) or by transcription of DNA precursors into short hairpin RNAs (shRNAs) homologous to target sequences (Brummelkamp et al., 2002; Elbashir et al., 2001; Paddison et al., 2002). Over the past years, several laboratories have used this technology to attenuate viral infection in cell culture (Coburn and Cullen, 2002; Ge et al., 2003; Gitlin et al., 2002; Jacque et al., 2002; Phipps et al., 2004; Seo et al., 2003). Furthermore, studies in animals injected with plasmids encoding shRNAs directed against hepatitis B virus have raised expectations about the use of RNAi as an antiviral strategy (McCaffrey et al., 2003).

Recently, Chen et al. (2004) and Kahana et al. (2004) have demonstrated, in cell culture, that siRNAs can be effective against the FMDV strain to which they were designed. Furthermore, Chen et al. (2004) have extended this approach to a suckling mouse model demonstrating decreased susceptibility to viral infection. However, to be an effective antiviral strategy against FMDV, this approach must address the antigenic variability of the virus.

Here, we demonstrate that an siRNA directed against a conserved sequence within the coding region of viral nonstructural (NS) protein 2B of FMDV is capable of inhibiting virus replication in infected porcine cells (IBRS-2) by approximately 97–98%. The expression of viral RNA and proteins was significantly reduced. Stimulation of the IFN pathway was ruled out as the source for inhibition. Importantly this 2B-specific siRNA reduced virus yield in cells infected with 4 different FMDV serotypes.

**Results**

**shRNAs trigger the silencing of conserved FMDV genome sequences**

To determine if RNAi could silence specific FMDV sequences, we constructed several plasmids expressing candidate shRNAs and tested them against target sequences cloned into a firefly luciferase reporter plasmid. Based on the alignment of multiple sequences corresponding to different FMDV serotypes, seven highly conserved regions along the viral genome were selected (Fig. 1A). Three of them mapped to the 5′ untranslated region (5′UTR), that is, CRE, IRES1 and IRES2, two to the 2B coding region, that is, 2B1 and 2B2, one to 2C and one to 2D. As a control, a non-specific shRNA, directed against green fluorescent protein mRNA (GFP) was used. As reported previously (Sui et al., 2002) and confirmed in our experiments, GFPshRNA does not appear to have off-target effects. The expressed hairpins produce 21 nt siRNAs with 19 bases of homology to FMDV and 3′ overhang U’s (Fig. 1B). The cloned target sequences each contained about 500–700 base pairs of the FMDV A12 genome fused to the firefly luciferase gene (Fig. 1C). HEK-293 cells were transiently

![Fig. 1. Schematic description of the approach followed to identify targets for RNAi in FMDV. (A) Map of FMDV genome; viral proteins are indicated in the boxed area. Horizontal bars indicate approximate sequence locations targeted by RNAi. (B) DNA sequence of oligonucleotides designed to anneal and clone into the U6 promoter-containing expression vector. Also shown is the expected hairpin formed after transcription by RNA polymerase III. (C) Representation of plasmids cotransfected to assess silencing of FMDV sequences in vitro.](http://example.com/fig1.png)
transfected with a mixture of the corresponding pair of plasmids (shRNA/reporter) and 24 h later luciferase activity was measured. When we measured the ratio of luciferase activity for each combination (FMDV specific-shRNA/GFPshRNA), different levels of silencing were observed (Fig. 2). One hairpin, 2B1, produced about 90% of specific silencing. Other hairpins, IRES1, IRES2 and 3D displayed significant levels of silencing (about 50%) but not as high as 2B1. Some hairpins, CRE, 2B2 and 2C, resulted in no inhibition or an increase of luciferase activity. The ability of 2B1shRNA to induce specific silencing suggested that FMDV sequences could be targeted by RNAi.

**2B1 shRNA induces potent inhibition of viral replication in swine cells**

To examine whether shRNAs could inhibit viral replication in cell culture, all plasmids expressing specific shRNAs, control GFPshRNA or vector control were individually transfected in IBRS-2 cells, and 48 h later, the cells were infected with 100 pfu of FMDV A12-IC (infectious clone), and incubated overnight. As an additional control, non-transfected cells were treated identically. As shown in Fig. 3, the virus yield from cells transfected with vector control or plasmid expressing GFPshRNA was similar (100%—approximately 10⁵ pfu/ml). The same results were obtained with non-transfected cells (data not shown). Consistent with the results obtained in HEK-293 cells, IBRS-2 cells transfected with 2B1shRNA displayed a reduction of approximately 100-fold in virus titer (97%—1.3 × 10³ pfu/ml). Cells transfected with IRES2shRNA inhibited virus growth by approximately 50%. Other hairpins resulted in little or no inhibition or an increase in virus titer.

Since 2B1shRNA induced the highest level of inhibition it was used for further analysis.

**2B1 shRNA sustains inhibition of viral replication in swine cells**

We examined the duration of the inhibition caused by 2B1shRNA. Forty-eight hours after transfection, IBRS-2 cells were challenged with FMDV A12-IC and samples were collected at 24, 48, 72 and 96 h post-infection (hpi). As shown in Fig. 4, 2B1shRNA resulted in a reduction of virus yield of approximately 100-fold, an effect that persisted for at least 48 hpi. In the control experiments (vector or GFP), the virus reached maximum titer at 48 h and then it decreased as a result of the lysis of the cell monolayer. The same effect was observed for the 2B1shRNA-transfected cells but with a delay of approximately 24 h. By 72 h, this
sample reached almost the same maximum titer as the controls and then it decreased.

**RNAi mediated by plasmids expressing shRNAs does not induce known pathways of the IFN response in swine cells**

FMDV can induce IFN mRNA in several cell types and in the natural host (Brown et al., 2000; Chinsangaram et al., 1999, 2001; S. Botton, unpublished data). Furthermore, we have shown that the IFN activated genes, double-stranded-RNA-activated protein kinase (PKR) and 2'-5' oligoadenylate synthetase (OAS)/RNase L have an important role in the inhibition of FMDV replication (Chinsangaram et al., 2001). To rule out the possibility that the inhibition of FMDV replication by 2B1shRNA was due to activation of the IFN pathway, we measured the expression of PKR and OAS as well as Mx1, another IFN activated gene, by quantitative RT-PCR. As seen in Fig. 5, similar low level variations in the amounts of PKR, OAS and Mx1 were observed in cells transfected with vector control as well as with non-specific and specific shRNAs. Induction of these genes was confirmed by treatment of the cells with 100 U of porcine IFNβ and FMDV infection (data not shown). Thus, the inhibitory effect of 2B1shRNA was not mediated by the activation of the IFN pathway.

**2B1shRNA inhibits viral RNA and protein expression in swine cells**

To examine if shRNA specifically affected viral RNA and protein expression, transfected IBRS-2 cells were infected with A12-IC at a high multiplicity (MOI = 5) to ensure that all cells would be infected. When about 90% of the cells displayed cytopathic effects (6 hpi) total RNA and protein were extracted for northern and western blot analyses. Fig. 6A shows that the level of viral RNA is markedly reduced (about 70%) in the presence of 2B1shRNA when compared to the levels of RNA in cells transfected with either vector control or GFPshRNA. Similar amounts of viral RNA were observed in cells transfected with either of these two vectors. FMDV mRNA is synthesized as a polycistronic message, therefore cleavage at a specific location results in degradation of the full length molecule.
In parallel, as seen in Fig. 6B, in the 2B\textsubscript{1}shRNA transfected cells, the levels of 2B and other viral protein products, VP0, VP1 and VP3, were significantly reduced (more than 80%) as compared to vector or GFPshRNA transfected cells, without inhibiting the synthesis of host proteins (tubulin-\(\alpha\)). These results indicated that RNAi specifically targeted FMDV RNA and as a consequence, there was a decrease in the level of viral proteins.

2B\textsubscript{1}shRNA inhibits replication of multiple serotypes of FMDV

One of the concerns in identifying siRNAs to target FMDV is the antigenic variability of the virus. Alignment of 120 full-length FMDV sequences revealed that 2B is one of the most conserved sequences across all isolates representing the 7 FMDV serotypes (Carrillo et al., in press). To test whether 2B\textsubscript{1}shRNA, designed on the basis of the sequence for FMDV A12-IC, was able to inhibit the replication of other serotypes, we infected 2B\textsubscript{1}shRNA transfected cells with FMDV serotypes Asia 1, O1 Campos (O1C), O Taiwan (O/TAW/2/99), South African Territories type 2 (SAT2) and C3 Resende (C3R). As seen in Fig. 7A, 2B\textsubscript{1}shRNA inhibited the growth of 4 FMDV serotypes; however, the degree of inhibition varied according to the serotype. As we previously demonstrated (Fig. 3) greater than 97% inhibition was observed for A12-IC whereas 92% was observed for O1C which contains an identical target region. Between 74 and 88% inhibition was detected for Asia 1, SAT2, and O/TAW/2/99 and less than 2% inhibition was observed for C3R. Statistical analysis indicated that, with the exception of C3R, the specific inhibition for all the other serotypes was highly significant (\(P < 0.01\)). In addition, differences in the degree of inhibition between serotypes A12-IC, O1C and Asia1 were not significant (\(P > 0.05\)). Sequence analyses of the viruses used in our experiments showed...
nucleotide differences between 2B\textsubscript{1}shRNA and some of the targets (Fig. 7B). Nonetheless, 2B\textsubscript{1}shRNA was effective in silencing multiple FMDV serotypes.

Discussion

In recent years, RNAi has developed into a powerful tool to down-regulate the expression of endogenous as well as exogenous or parasitic sequences of the cell (McManus and Sharp, 2002; Morris et al., 2004). Several recent studies have demonstrated that RNAi can potentially be used as a therapeutic or prophylactic mechanism against viruses (Joost Haasnoot et al., 2003). However, to be effective against RNA viruses such as FMDV, which are highly antigenically variable, the siRNAs must be targeted to conserved regions of the viral genome. Here, we demonstrated that the replication of different FMDV serotypes in porcine cells can be inhibited by a shRNA designed against a region that is highly conserved among multiple viral genomes.

Initially, we tested the feasibility of silencing FMDV sequences, using HEK-293 cells, a cell line known to be efficient for RNAi (Elbashir et al., 2001). We showed that several regions across the FMDV A12 viral genome could be targeted by RNAi. In particular, 2B\textsubscript{1}shRNA resulted in a significant reduction of reporter gene activity. Consistent with these results, when we tested all the shRNAs against FMDV in porcine cells, 2B\textsubscript{1}shRNA caused approximately a 100-fold decrease in virus yield indicating that RNAi could effectively target the FMDV genome in swine cells. Analyses of viral RNA and protein levels led us to conclude that in swine cells RNAi induces a potent repression of gene expression by degrading the cognate mRNA.

We also observed an inhibitory effect for IRES\textsubscript{2}shRNA, but it was only approximately 2-fold in both assays. In this regard, there are differing reports in the literature concerning a role of the RNA structure in or near the target region of an siRNA in the efficiency of interference. Studies with poliovirus, another member of the Picornaviridae family, have suggested that the IRES region of the 5' UTR might not be accessible for RNAi (Gitlin et al., 2005; Saleh et al., 2004). Furthermore, recent studies on HIV-1 have revealed that this virus can escape from RNAi by evolving an alternative resistant structure in its RNA genome around an siRNA-targeted region (Westerhout et al., 2005). In contrast, for hepatitis C, RNAi targeting of the IRES region has proven successful (Yokota et al., 2003). Based on these results, it seems difficult, with our current knowledge, to predict whether or not a region of highly structured RNA on a viral target may impose a limitation on the effectiveness of RNAi.

Neither of the other shRNAs that were effective in the reporter assay, IRES\textsubscript{1} and 3D, resulted in efficient inhibition of viral growth in swine cells. Differences in the accessibility of the FMDV RNA when the target is cloned into a reporter plasmid or present as part of the complete viral genome may explain this result. Alternatively, the degree of inhibition induced by these shRNAs in the reporter assay may not have been sufficient to halt virus replication since newly formed viral particles appear as early as 4 hpi and furthermore, under genetic pressure, escape resistant mutants arise (Domingo et al., 2003). Only some sequences of the viral genome may withstand this level of challenge. This conclusion may also explain the results of the study examining the duration of the inhibitory effect of 2B\textsubscript{1}shRNA after transfection. Although inhibition extended to 48 h post-challenge, the virus yield eventually reached the level found in control transfected cells. Because cell transfection is not completely efficient, virus replication can occur in non-transfected cells. In addition, the emergence of viral
escape mutants may take over the culture masking any inhibitory effect.

One of the mechanisms by which mammalian cells can clear virus infection is through the activation of the IFN pathway (Sen, 2001). We and others have shown that FMDV is very sensitive to IFN (Ahl and Rump, 1976; Chinsangaram et al., 2001). We ruled out the activation of this pathway after treatment with FMDV-specific shRNAs, by measuring the expression of the IFN activated genes PKR, OAS and Mx1. The absence of induction of these genes suggested that the inhibition of viral growth in swine cells was due to RNAi, a process that remains active even in fibroblasts derived from PKR−/− mice (Gitlin et al., 2002; Sledz et al., 2003). Nonetheless, it is possible, that RNAi may activate a different set of genes involved in viral clearance.

2B1siRNA targets the highly conserved 2B coding region of the FMDV genome. Alignment of the full length sequences of 120 FMDV isolates, revealed that the sequence of 2B1shRNA is identical in at least 86 of the common euasian viruses. This conservation probably indicates the inability of this region to tolerate mutations. Although persistence of the virus in the field and the quasispecies nature of the RNA genome have given rise to new variants, the 2B coding region has remained the most conserved across all serotypes and subtypes of FMDV (Carrillo et al., in press). The identification of a sequence within 2B highly susceptible to RNAi is significant since one of the limitations in the use of this approach as a disease control strategy is the incidence of escape mutants resistant to silencing. We observed that 2B1shRNA resulted in growth inhibition of FMDV Asia 1, SAT2 and O/TAW/2/99, despite the presence of sequence mismatches. Previous reports have indicated that sequence mismatches can be tolerated causing efficient silencing by translational repression rather than RNA degradation (Jackson et al., 2003; Saxena et al., 2003). Other studies, however, indicate that a single mismatch between a viral RNA target and an siRNA completely prevents silencing (Gitlin et al., 2002). More recently, it has been shown that the location of a mismatch can affect the degree of silencing (Gitlin et al., 2005; Phipps et al., 2004). For FMDV C3R, a single base mismatch in the 2B1 target region was sufficient to abolish silencing. Perhaps the location of the sequence difference, at nucleotide position 10 from the 5′ end of the siRNA, is crucial for RNAi.

Recently, analyses of escape mutants from poliovirus revealed that this position and the nature of the sequence change may determine the efficacy of RNAi to cause viral inhibition (Gitlin et al., 2005).

Our experiments, as well as those of others (Chen et al., 2004; Kahana et al., 2004), support a proof-of-concept that siRNA can inhibit FMDV replication, but some significant issues remain to be addressed. The first is the method of delivery of the siRNA to an adult organism. Previous studies in mice report that sequences of HBV can be targeted by RNAi after hydrodynamic transfection of DNA expressing shRNAs against HBV (McCaffrey et al., 2003). In addition, injection of DNA expressing shRNA against FMDV increased the survival of suckling mice challenged with the virus (Chen et al., 2004). However, these reports have limitations. The first introduces the viral genome by transfection which differs from the events in a natural viral infection. The second, using a mouse model for FMDV, requires a very high dose to induce protection (50–100 µg of DNA/mouse/~3 g). Considering that the most important hosts for FMDV are cattle and swine the question arises about the dose requirement for effectiveness in these species. Initial reports indicate that retroviral (Barton and Medzhitov, 2002; Devroe and Silver, 2004) or adenoviral (Shen et al., 2003; Xia et al., 2002; Zhang et al., 2004) vectors are able to express shRNAs and induce silencing in targeted cells. This method of delivery should be more efficient in targeting and maintaining the RNA silencing state in tissues or whole organisms. An alternative approach that seems promising is the cloning of a transgenic animal that would display specific viral immunity by expression of shRNAs. This technology has proven successful for silencing of endogenous genes (Kunath et al., 2003).

The second issue of concern is the selection of viral escape mutants. As discussed earlier, although we have identified a conserved region of the viral genome sensitive to RNAi, resistant mutants may arise. In fact, this might have been the case when we examined the duration of the shRNA inhibitory effect. Combination treatment with multiple siRNAs targeted to conserved regions may prove useful in avoiding this problem. A large and careful screen for putative candidates should be performed, challenging with full length viral genomes. The success of this approach with poliovirus is encouraging (Gitlin et al., 2005).

Lastly, the potential of negative effects caused by off-target function of shRNAs may compromise their utility (Sledz et al., 2003). These problems should be addressed for each particular set of shRNAs to be used. Nevertheless, the demonstration that the replication of multiple FMDV serotypes can be attenuated by a unique conserved siRNA highlights the potential of using RNAi as a new virus control strategy.

Materials and methods

Cells and viruses

Human embryonic kidney 293 cells (HEK-293, ATCC CRL1573) were used to assay for luciferase reporter gene
activity. Swine kidney IBRS-2 cells (Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center) were used for expression of shRNAs and challenge with multiple serotypes of FMDV. Baby hamster kidney cells clone 21 (BHK-21, clone 13, ATCC CCL-10) were used for determination of all virus titers. All cells were cultured in Eagle’s minimal essential medium (EMEM, Life Technologies, Rockville, MD) supplemented with either 10% fetal bovine serum (FBS), amino acids and antibiotics (HEK-293 and IBRS-2) or 10% bovine calf serum (BCS), 10% tryptophan phosphate, amino acids and antibiotics (BHK-21).

FMDV A12-IC was derived from full length infectious clone pRMC35 (Rieder et al., 1993). FMDV Asia 1, FMDV O1C, FMDV SAT2, FMDV O/TAW/2/99 and FMDV C3R were obtained from FADDL or Dr. Peter Mason, Agricultural Research Service, Plum Island.

Design and cloning of plasmid expressing short hairpin RNAs

siRNAs were produced in mammalian cells by expression of short hairpins from a U6 promoter containing plasmid (pUBX101BbsI). This plasmid is a derivative of pSilencer 1.0-U6 (Ambion, Austin, TX) to which a BbsI site was added by linker ligation. Two 59-nt long annealed oligonucleotides with overhang ends were cloned into BbsI/EcoRI sites of pUBX101BbsI. The oligonucleotides contained a 4 nt overhang to create a BbsI site (GTTT) plus an added G followed by a 19-nt sense siRNA sequence, a 9-nt loop (TTCAAGAGA), a 19-nt reverse complementary antisense siRNA sequence, an added C and 6 Ts as RNA polymerase III terminator. The resulting RNA transcripts were expected to fold back and form a stem–loop structure with a 19 base pair region homologous to the target sequences of the FMDV genome. Conserved target sequences were selected from FMDV A12-IC based on the alignment of sequences of 7 serotypes of FMDV using Clone Manager Suite software FMDV A12-IC target sequences of approximately 500–700 bases at the unique XbaI site of pGL3-Control (Promega, Madison, WI) downstream of the firefly luciferase (FLuc) gene. The target for the CRE element was nt 13–716, the target for both IRES elements was nt 716–1230 (the 5'UTR encompasses nt 1–1116 and includes the CRE element nt 621–678 and the IRES nt 581–1116), the target for 2B and 2C was nt 3940–4617 (2B encompasses nt 3979–4440 and 2C nt 4441–5394), and the target for 3D was nt 7550–8100 (3D encompasses nt 6706–8118). All of the hairpin sequences were contained within their respective targets. These sequences were amplified by PCR using pRMCS5 as template and the primer-pairs: for the 5'UTR 5'GGATCTAGAGCTAGTGC-TCACCCCCTAGC/5'GGATCTAGACCTCTGCACTGTGCG for 2B/2C 5'GGATCTAGACTTACCTCAAGTGGGCAG/5'GGATCTAGAGCGTTGAGGTCCCCTTGC; and for 3D 5'GGATCTAGACCAGCAGTCCGTGTTGGCG and 5'GGATCTAGAGCTGTTCCCCTTGC.

Construction of reporter plasmids

Reporter plasmids were constructed by cloning FMDV A12-IC target sequences of approximately 500–700 bases at the unique XbaI site of pGL3-Control (Promega, Madison, WI) downstream of the firefly luciferase (FLuc) gene. The target for the CRE element was nt 13–716, the target for both IRES elements was nt 716–1230 (the 5'UTR encompasses nt 1–1116 and includes the CRE element nt 621–678 and the IRES nt 581–1116), the target for 2B and 2C was nt 3940–4617 (2B encompasses nt 3979–4440 and 2C nt 4441–5394), and the target for 3D was nt 7550–8100 (3D encompasses nt 6706–8118). All of the hairpin sequences were contained within their respective targets. These sequences were amplified by PCR using pRMCS5 as template and the primer-pairs: for the 5'UTR 5'GGATCTAGAGCTAGTGC-TCACCCCCTAGC/5'GGATCTAGACCTCTGCACTGTGCG for 2B/2C 5'GGATCTAGACTTACCTCAAGTGGGCAG/5'GGATCTAGAGCGTTGAGGTCCCCTTGC; and for 3D 5'GGATCTAGACCAGCAGTCCGTGTTGGCG and 5'GGATCTAGAGCTGTTCCCCTTGC.

Cell transfections and reporter plasmid analyses

HEK-293 cells were co-transfected with plasmids expressing FMDV shRNAs and derivatives of the reporter plasmid pGL3-Control (Promega, Madison, WI) containing FMDV target sequences using Lipofectamine 2000 (Invtrogen, Carlsbad, CA). Briefly, 24-well plates were seeded 24 h before transfection with 5 × 10^4 cells/well in OptiMEM-I (Life Technologies, Rockville, MD). Plasmids expressing FMDV shRNAs and reporter plasmids were co-transfected at a molar ratio of 30:1 (shRNA/target). Luciferase activity was assayed 24 h post-transfection using a Luciferase assay kit (Promega, Madison, WI). Luciferase values were normalized for specific/control activity and reported as the average of three independent experiments.

Cell transfection and viral challenge assays

IBRS-2 cells were seeded in 6-well plates with 5 × 10^5 cells/well in OptiMEM-I 24 h before transfection. The cells were transfected with 5 μg of plasmids expressing shRNAs. Four hours later 10% FBS was added to the cells without removing the transfection mixture. After 16 h, the serum-containing media was replaced by fresh OptiMEM-I and a second plasmid transfection was performed under identical conditions. Twenty four hours later, cells were challenged with FMDV at MOI of 10^-4 and the infection was allowed to proceed for the indicated times. Viral titers were determined on BHK-21 cells. For analyses of viral RNA and protein, transfected cells were challenged with FMDV at MOI of 5 and the samples were collected 6 hpi.

Quantitative RT-PCR

Twenty four hours after transfection or treatment with porcine IFNβ, total RNA was isolated from cell cultures using an RNeasy mini kit (Qiagen, Valencia, CA). Samples
were treated with DNase I (Sigma, St Louis, MO) and after heat inactivation of the enzyme, cDNA was synthesized using M-MLV-RT (Invitrogen, Carlsbad, CA). The relative levels of PKR, OAS and Mx1 mRNAs were measured using AmpliTaq gold (Applied Biosystems, Foster City, CA) in an ABI PRISM 7700 sequence detection system. Primers and probe were designed using Primer express software (Applied Biosystems, Foster City, CA). TAgMan probe labeled with a fluorescent dye was used in this assay. Primer and probe sequences are listed in Table 1. 18s rRNA was used as an internal standard control and was amplified for each sample. Primers and probe used for 18s rRNA were from the control reagent kit (Applied Biosystems, Foster City, CA).

Northern and Western blot analyses

Total RNA was extracted from cell cultures using a RNeasy mini kit (Qiagen, Valencia, CA) and run on a 1% formaldehyde agarose gel. RNA was transferred to a cationic charged nylon membrane Zeta-probe (Biorad, Hercules, CA), UV-crosslinked and hybridized to a digoxigenin labeled FMDV probe derived from pRMC35 (O’Donnell et al., 2001). The signal was detected by chemiluminescence using a DIG Luminescent Detection kit (Roche Applied Science, Indianapolis, IN). For western analysis protein extracts were prepared from cell cultures in lysis buffer (10 mM Tris–HCl pH = 8.0, 150 mM NaCl, 15 mM MgCl2, 1% NP-40, 1% sodium deoxycholate, 1 mM PMSF) for 10 min on ice. After clarification, proteins were resolved by SDS-PAGE using the Nu-PAGE pre-cast gel system (Invitrogen, Carlsbad, CA) followed by transfer to PVDF membranes. Viral proteins were detected with a mixture of rabbit polyclonal antibodies raised against viral proteins 2B, VP1, VP2 and VP3 and cellular tubulin-α was detected with a commercially available monoclonal antibody (Lab Vision, Freemont, CA) using the ECL detection system (Amersham, Piscataway, NJ) according to the manufacturer’s directions. RNA and proteins were quantitated using Alphaimager 2200 with AlphaEase software (AlphaInnoteck, San Leandro, CA) and normalizing with respect to the ribosomal RNA bands for the Northern blot or the tubulin-α band for the Western blot.

Accession numbers for viruses cited in this paper: A12-A12/UK/119/32- (L11360; M10975); Asia 1/IND/63/72 (AY593795), O1/Campos/BRA/58 (AJ320488), O/TAW/2/99 (AJ539136), SAT2 (AJ251473), C3 Resende (AY593807).

Acknowledgments

The authors would like to thank Marla J. Koster and Mario C. S. Brum for technical support, Consuelo Carrillo for sharing information of unpublished results, and Jose Barrera for help with the statistical analysis.

References


Table 1

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<td>porcine PKR-994T&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5’CCAGGTGTGTCGAAGAT3’</td>
</tr>
</tbody>
</table>

* NCBI GenBank accession code.
<sup>b</sup> Forward primer.
<sup>c</sup> Reverse primer.
<sup>d</sup> Probe.


