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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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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.
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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;

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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; Hammond 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, $IRES_1$ and $IRES_2$, two to the 2B coding region, that is, $2B_1$ and $2B_2$, one to 2C and one to 3D. 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

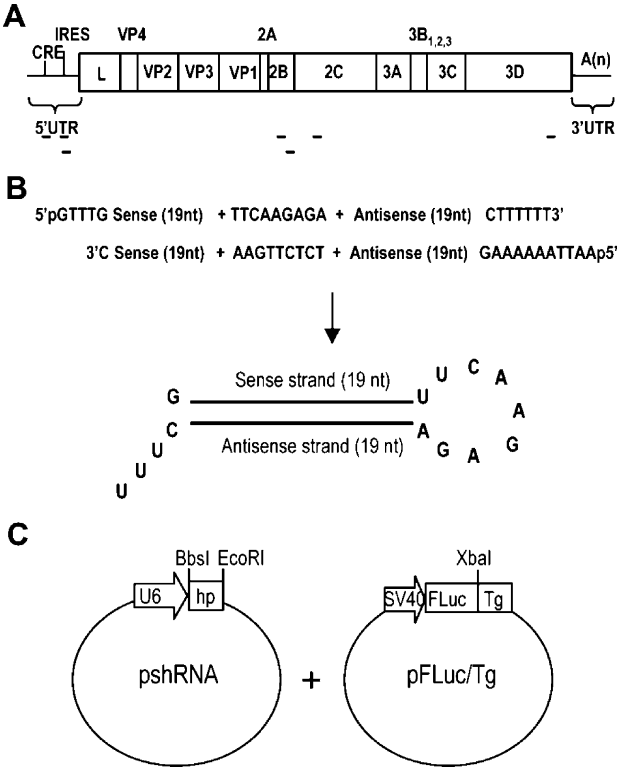

Fig. 1. Schematic description of the approach followed to identify targets for RNAi in FMDV. (A) Map of FMDV genome; viral proteins are indicated in the boxed area. Horizontal bars indicate approximate sequence locations targeted by RNAi. (B) DNA sequence of oligonucleotides designed to anneal and clone 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.