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Gene Knockdown of Venezuelan Equine Encephalitis Virus E2 Glycoprotein Using DNA-Directed RNA Interference

H.S. Bhogal, L.J. McLaws and S.J. Jager
DRDC Suffield

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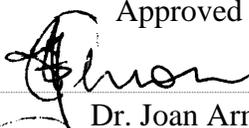DRDC Suffield

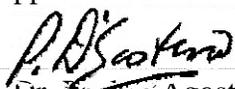## Defence R&D Canada – Suffield

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Abstract

Venezuelan equine encephalitis virus is an important veterinary and human pathogen that also has the potential to be used as a bioterrorist agent. Since there are no approved vaccines or antiviral drugs for this virus, it is prudent that antiviral strategies be developed. RNA interference, an evolutionarily conserved but only recently discovered biological phenomenon, may be an effective gene manipulation tool to combat viruses. The ability of RNA interference to silence or knockdown specific mRNA through the use of short dsRNA fragments has been an effective tool to study gene function in many systems. In this study, we demonstrated the effectiveness of RNA interference to knockdown the VEE E2 gene expressed in mammalian cells. Here, a DNA-directed approach was used to transfect Vero cells with siRNA expression vectors. We demonstrated that both target siRNAs were effective in significantly reducing the level of E2 expression based on RT-PCR analysis of mRNA levels. Furthermore, the use of these vectors demonstrates the usefulness of a vector-based approach to silencing genes. Future studies will assess the efficacy of these E2-specific siRNA expression constructs in the inhibition of the VEE virus *in vitro*.

Résumé

Le virus de l'encéphalomyélite équine du Venezuela est un pathogène humain et vétérinaire important qui a aussi le potentiel d'être utilisé comme agent de bioterrorisme. Il n'existe pas de vaccin ou de drogues antivirales qui soient approuvés contre ce virus aussi est-il prudent de développer des stratégies antivirales. L'interférence ARN, un phénomène biologique, conservé évolutivement mais découvert récemment, s'avère un outil de manipulation génique efficace à combattre les virus. La capacité de l'interférence ARN d'inactivation ou de choc d'ARNm spécifiques au moyen de fragment d'ARNds courts a été un outil efficace pour étudier la fonction génique dans beaucoup de systèmes. Cette étude démontre l'efficacité de l'interférence ARN à effectuer le choc sur le gène EEV E2 exprimé en cellules mammaliennes. On utilise ici une méthode dirigée par l'ADN pour transfecter les cellules Vero avec des vecteurs d'expression de petits ARNi. On a démontré que les deux cibles de petits ARNi sont efficaces à réduire de manière importante le niveau de l'expression E2 basée sur l'analyse RT-PCR des niveaux ARNm. De plus, l'utilisation de ces vecteurs démontre l'utilité d'une méthode d'inactivation de gène à base de vecteurs. Des études ultérieures évalueront l'efficacité de ces gènes hybrides d'expressions de petits ARNi E2-spécifiques à inhiber le virus EEV *in vitro*.

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Executive summary

Introduction: Alphaviruses are a large family of RNA viruses that can cause acute infection resulting in arthritis and encephalitis. One of the important alphaviruses is the Venezuelan equine encephalitis virus. This virus has been linked to a number of outbreaks in both North and South America and has the potential to infect both humans and animals. It is transmitted to vertebrates through the bite of an infected mosquito. In addition to natural outbreaks, this virus has the potential to be utilized as a biological weapon. Therefore, medical countermeasures are necessary to protect against both natural and deliberate spread of this virus. Although a vaccine strain of VEE exists, it has some limitations and adverse effects. Further research is required to develop an antiviral against VEE that is both safe and effective. One antiviral strategy that has shown considerable promise is RNA interference. This biological process involves the targeted silencing or knockdown of genes using short dsRNA molecules, called short interfering RNAs. These siRNAs assemble with a nuclease to form an effector complex that specifically cleaves target mRNA. The degradation of mRNA results in gene silencing.

Results: In this study, the VEE virus E2 glycoprotein, required for attachment of the virus to cells, is used as a target to determine the effectiveness of using DNA-directed RNA interference as a mechanism of gene silencing. We have previously described the generation of siRNA expression vectors that express siRNAs directed against the E2 gene. Here we demonstrate via RT-PCR that these siRNA expression constructs, targeting two different regions of the gene, reduce the level of E2 mRNA expression in a mammalian cell line when co-transfected with an E2 expression vector. The decrease in gene expression was observed to be significant with both target siRNAs.

Significance: This initial approach demonstrates that siRNAs designed against two different targets on the VEE E2 gene are effective in cleaving the E2 mRNA. These results indicate that the siRNA expression vectors can facilitate the silencing of a VEE gene.

Future Plans: Future studies will utilize these expression vectors to test their ability to inhibit the replication of VEE virus *in vitro*.

Sommaire

Introduction: Les arbovirus appartiennent à une grande famille de virus ARN qui peuvent produire des infections graves causant l'arthrite et l'encéphalite. L'un des arbovirus importants est le virus de l'encéphalomyélite équine du Venezuela. Ce virus est associé à un certain nombre d'épidémies à la fois en Amérique du nord et Amérique du sud et a le potentiel d'infecter les humains comme les animaux. Il se transmet aux vertébrés par la morsure d'un moustique infecté. En plus des épidémies naturelles, ce virus a le potentiel d'être utilisé comme arme biologique. Les contremesures médicales sont par conséquent nécessaires pour protéger contre la dissémination délibérée du virus. Il existe une souche du vaccin de l'EEV mais elle a des limites et des effets néfastes. Il faudra donc continuer la recherche pour mettre au point un antiviral contre l'EEV qui soit à la fois sécuritaire et efficace. Une stratégie antivirale ayant montré des promesses considérables est l'interférence ARN. Ce processus biologique comprend l'inactivation ou le choc des gènes au moyen de molécules courtes ARNds aussi appelées ARN interférants courts. Ces petits ARNi s'assemblent avec un nucléase pour former un effecteur complexe qui clive spécifiquement l'ARNm ciblé. La dégradation de l'ARNm résulte en l'inactivation du gène.

Résultats: Dans cette étude, la glycoprotéine E2 du virus EEV requise pour l'attachement du virus aux cellules est utilisée comme cible pour déterminer l'efficacité de l'interférence ARN dirigée par l'ADN comme mécanisme d'inactivation du gène. Nous avons décrit antérieurement la génération de vecteurs d'expression de petits ARNi qui expriment des petits ARNi dirigés contre le gène E2. Nous démontrons ici par la méthode RT-PCR que ces gènes hybrides d'expressions de petits ARNi, ciblant deux régions différentes du gène, réduisent le niveau E2 de l'expression ARNm E2 dans une ligne de cellule mammalienne quand ils sont co-transfectés avec un vecteur d'expression E2. On a observé que la diminution en expressions de gènes est importante avec les deux cibles de petits ARNi.

Portée des résultats: Cette méthode initiale démontre que les petits ARNi conçus contre deux cibles différentes sur le gène E2 de l'EEV sont efficaces à cliver l'ARNm E2. Ces résultats indiquent que les vecteurs d'expression de petits ARNi peuvent faciliter l'inactivation du gène EEV.

Plans futurs: Des études ultérieures utiliseront ces vecteurs d'expressions pour tester leur habileté à inhiber la réplication du virus EEV *in vitro*.

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Introduction

Venezuelan equine encephalitis (VEE) virus, first isolated in 1938, has been linked to a number of outbreaks in the last century affecting both equines and humans [1]. VEE virus, a member of the alphavirus family, is a plus sense RNA virus with a genome that acts as a mRNA. The viral genome is infectious as it can initiate a cycle of infection on its own without encapsulation of the viral coat. This virus is transmitted to vertebrates via the bite of an infected mosquito. Although a live attenuated VEE vaccine (TC-83) under the Investigational New Drug (IND) status exists to protect against mosquito-borne infections and aerosols, it has been associated with adverse reactions [2]. Therefore, safe and effective therapies and vaccines are required for VEE. Recently, a potential vaccine candidate (V3526) has been proposed that has shown protection in mice from lethal challenge with virus [3] as well protection in nonhuman primates [4].

The VEE viral genome encodes a number of proteins that are required for the replication and assembly of the virus. These include a series of nonstructural proteins involved in capping of viral RNAs, RNA synthesis and replication, and protein processing. In addition, there are a series of structural proteins including envelope proteins and a nucleocapsid. One structural VEE virus protein, the E2 glycoprotein, is required for the attachment of the virus to the cell and subsequent entry [5]. Therefore, this protein represents an attractive target for an antiviral strategy.

RNA interference has rapidly become a valuable laboratory tool as a method for manipulating gene expression. The phenomenon of RNAi was first described by Fire and colleagues in a study in which double stranded RNA molecules silenced gene expression in nematodes [6]. RNAi is an evolutionarily conserved process and is ubiquitous to all eukaryotic cells.

RNA interference is initiated by the processing of dsRNA molecules into siRNAs by a ribonuclease known as the Dicer enzyme [7]. These short siRNA molecules, approximately 21-23 nucleotides, were discovered to associate with a RNA endonuclease to form an effector nuclease complex [8]. This complex, known as RNA-induced silencing complex (RISC), once activated via an ATP-dependent mechanism [9], specifically targets and cleaves mRNA sequences at regions complementary to the siRNA [10,11]. An overview of the mechanism of RNAi is illustrated in Figure 1. Interestingly, the ability of RNAi to spread throughout an organism, referred to as systemic silencing, was also demonstrated during the initial discovery of the phenomenon in nematodes [6]. This systemic silencing effect, which would require that the silencing signal be transmitted from cell to cell, may be linked to the transmembrane protein SID-1 [12]. This systemic silencing effect has also been observed in plants [13]. The ability of siRNAs to systemically silence genes bodes well for the use of RNAi in the treatment of disease and infection. RNAi has shown considerable promise as an antiviral therapy both *in vitro* and *in vivo* [14].

In this preliminary study, we describe the use of a vector-based siRNA transcription system for the knockdown of VEE E2 gene. Previously, we have described the generation of these siRNA expression vectors encoding sequences homologous to a number of different VEE virus genes [15]. Expression cassettes containing sequences encoding hairpin siRNA

molecules were designed using a PCR-based approach [16]. With this system, siRNAs are generated inside the cells from a DNA template directed by an RNA promoter. Here we show that siRNAs targeting two different regions of the E2 gene reduce the level of expression of E2 mRNA.

dsRNA

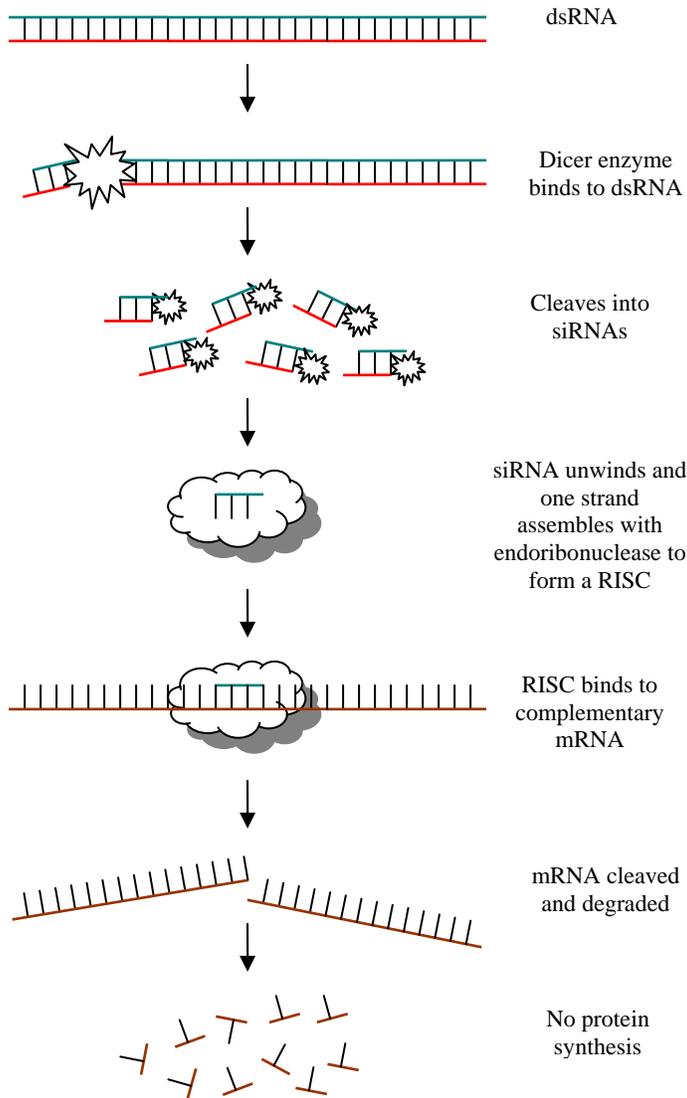Dicer enzyme binds to dsRNA

Cleaves into siRNAs

siRNA unwinds and one strand assembles with endoribonuclease to form a RISC

RISC binds to complementary mRNA

mRNA cleaved and degraded

No protein synthesis

Figure 1. Overview of RNAi mechanism.

Materials and Methods

Construction of VEE-E2 Eukaryotic Expression Vector

VEE-E2 glycoprotein was PCR amplified from pRSVE2 using primers VE2_Kozak K-5' and VE2E-3'. They contain *Kpn* I and *Eco*R I sites respectively for cloning into pVAX-1 (Invitrogen, Burlington, ON). VE2_KozakK-5' also contains a Kozak consensus sequence for optimizing eukaryotic translation. Oligonucleotides were ordered from IDT (Coralville, IA) with standard desalting (Table 1).

E2 was amplified with KOD Hot Start DNA polymerase (Novagen, Madison, WI)) on a MJ Research PTC-200 DNA engine (Bio-Rad, formerly MJ Research, Mississauga, ON). Amplification products (5 µL) were separated on a 1% agarose gel and stained with ethidium bromide. A typical 50 µL PCR reaction contained 25 ng pRSVE2 plasmid DNA, 1x KOD buffer, 1 mM MgSO$_4$, 0.2 mM dNTP mix, 0.3 µM each VE2_KozakK-5'/VE2E3' primers and 1 unit of KOD polymerase. Cycling parameters were 94°C for 2 min (*Taq* activation), followed by 30 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 5 min.

Cloning VEE-E2 into pVAX-1

The amplified product was purified with a Qiaquick PCR purification spin column (Qiagen, Mississauga, ON) according to manufacturer's recommendations. Purifed PCR product and pVAX-1 were digested at 37°C for 1.5 hours in 1x buffer B (Fermentas, Burlington, ON) with 10 units each of *Kpn* I (NEB) and *Eco*R I (Fermentas).

Digested VEE-E2 was ligated with 50 ng pVAX-1 (3:1 molar ratio) at room temperature (~25°C) for 0.5 hr using a rapid ligation kit (Fermentas). Five µL of each ligation reaction was transformed into 50 µL oneShot TOP 10 F' cells (Invitrogen) following manufacturer's instructions. Two hundred µL was plated onto LB agar plates supplemented with 50 µg/mL carbenicillin (LBA) and incubated overnight at 37°C

Screening pVAX-VE2 Constructs

Colonies were screened by PCR (as above) for correct insert size (data not shown) and positive clones inoculated into 5 mL LBA broth and grown overnight at 37°C. Plasmid DNA was isolated using standard alkaline lysis methods and sequenced using sequencing primers in Table 1. Sequencing reactions were purified by ethanol precipitation and analyzed on a Beckman CEQ 8000 (Beckman Coulter, Mississauga, ON).

VEE-E2 was successfully cloned into pVAX-1. The sequence of E2 has been modified to contain a 5' Kozak consensus sequence and a 3' stop codon. The resulting clone (pVAX-VE2.3) was used as a target in siRNA knockdown assays.

Table 1. Amplification and Sequencing Primers.

VE2_KozakK-5'and VE2E3' were used to PCR amplify VEE-E2 glycoprotein from pRSBVE2. They contain KpnI and EcoRI sites respectively for cloning into pVAX-1 (Invitrogen). VE2_KozakK-5' contains a Kozak consensus sequence for optimizing eukaryotic translation. The remainder of the primers were used for sequence analysis.

Primer	Sequence	Location
VE2_KozakK-5'	AAG GGT ACC ATG GCT TCC ACC GAG GAG CTG TTT AAG	Bases 1-21 of VEE E2
VE2E3'	TAA GAA TTC TCA GGC TCT GGC AGT GCG GGC	Bases 1284-1266 of VEE E2
VE2sA-F	CCC CCA GAA CAC GGA GTA GAG CA	Bases 424-446 of VEE E2
VE2sB-F	TAA CCA CCC GCC AAC TTG CTG ATG AG	Bases 884-909 of VEE E2
VE2sY-R	CAT AAG CTC CTC TGT TCT GTG C	Bases 493-472 of VEE E2
VE2sZ-R	GGC GGG TGG TTA GAT ATG TGG GAT TC	Bases 895-870 of VEE E2
CMV Promoter-F	CGC AAA TGG GCG GTA GGC GTG	Promoter in pVax
BGH-R	CCT CGA CTG TGC CTT CTT A	Terminator in pVax

Transfection of Vero Cells

Vero cells (ATCC #CCL-81) were used for the transfection experiments. For plate preparation, Vero cells were grown to confluence in a T150 tissue culture flask with Dulbecco's Modified Eagle Medium, 1X, low glucose (Invitrogen) supplemented with 5% Fetal Bovine Serum and 1% MEM sodium pyruvate, MEM non-essential amino acids, L-glutamine, MEM vitamin, and antibiotic-antimycotic (standard DMEM). The cells were washed 2X with Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, Oakville, ON), harvested after addition of Trypsin-EDTA (Invitrogen) and collected. An aliquot of cells was

counted using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter) and a total of 40 mL containing 1.6×10^5 cells/mL in standard DMEM was prepared. Each well of a six well plate was seeded with 3.2×10^5 cells by adding 2.0 mL of the cell suspension. The plates were rocked back and forth to mix and incubated at 37ºC, 5% CO_2, 80% relative humidity for 24 hours. The plates were observed to have approximately 90% confluent growth after 24 hours. The growth media was aspirated from each well and replaced with 2.0 mL fresh standard DMEM.

The transfection of each DNA (or combination) was performed with Lipofectamine 2000 (Invitrogen) using the manufacturer's instructions. All DNA constructs (Table 2) encoding the siRNAs were constructed as described previously [15]. The DNA or combination to be transfected were as follows: EGFP, GAPDH, siRNA, E2 (pVAX-VE2.3) + Target 1 (VE2-T1.2), E2 + Target 2 (VE2-T2.2) and E2 + negative control siRNA. For each well to be transfected, a total of 4.0 μg of test DNA (or combination) was diluted in 250 μL of standard DMEM, and mixed gently. The Lipofectamine 2000 reagent was mixed gently, and then diluted in a ratio of 10 μL Lipofectamine to 250 μL of standard DMEM for each well to be transfected. The diluted Lipofectamine was incubated at room temperature for a minimum of 5 min (no longer than 25 min). After the 5 min incubation, 260 μL diluted Lipofectamine was added to each tube of diluted DNA, mixed gently and incubated for 20 min at room temperature to allow for the formation of the complexes.

For the transfection of cells, the total volume from each tube of DNA/Lipofectamine complex was added dropwise to the six well plates that contained cells and medium. The plates were mixed gently by rocking back and forth, and then incubated at 37ºC, 5% CO_2, 80% relative humidity. Plates were incubated for either 24 hours or for 48 hours.

Transfection efficiency was visualized using a Nikon Eclipse E600 microscope (Nikon Canada Ltd, Mississauga, ON) equipped with a Nikon Y-FL EPI-Fluorescence attachment. A FITC filter block was used to detect the EGFP emission. Digital photographs were taken using a Nikon Digital Still Camera DXM 1200 and Nikon ACT-1 Version 2.20 software.

RNA Isolation of Transfected Cells

Transfected Vero cells were lysed directly in the wells of the six-well plate they were transfected in using a TRIzol method. The standard DMEM growth media was aspirated from each well and discarded. Cells were washed with 1.0 mL Dulbecco's Phosphate Buffered Saline (Sigma Aldrich) by rocking back and forth. The PBS was then aspirated and discarded. 1.0 mL TRIzol Reagent (Invitrogen) was added to each well. The plate was tipped at approximately 45º angle. The TRIzol was aspirated from the lowest part of the well, and then dispensed again at the upper part of the well so that the reagent washed over the well surface. Care was taken to avoid the creation of bubbles. This was repeated several times. The plate was then allowed to sit for one minute. The TRIzol lysed cell mixture from each well was collected into a labeled microtube and placed on ice. Microtubes were stored at -70ºC until ready for RNA isolation.

Cells previously lysed with TRIzol reagent were removed from -70ºC storage, thawed and placed on ice. RNA isolation was performed on all samples as per the manufacturer's instructions. In brief, the samples were incubated for 5 min at approximately 30ºC. Chloroform (0.2 mL) was added to each tube containing 1.0 mL TRIzol reagent. Tubes were capped securely and shaken by hand for 15 sec, then incubated for 2 to 3 min at 30ºC. Samples were centrifuged at 12,000 x g for 15 min at 4ºC. The upper aqueous phase was transferred to a fresh microtube. The RNA was precipitated from the aqueous phase by adding 0.5 mL isopropyl alcohol and incubating at 30ºC for 10 min, followed by centrifugation at 12,000 x g for 10 min at 4ºC. The supernatant was removed and the RNA pellet was washed with 1.0 mL of 75% ethanol. The sample was mixed by vortexing and centrifuged at 7,500 x g for 5 min at 4ºC. The RNA pellet was dried for approximately 5-10 min, taking care not to let the pellet dry completely. The RNA was dissolved in 20 µL RNase free water by mixing the solution through a pipette tip, then incubating for 10 min at 60ºC. Samples were stored at -70ºC.

RT-PCR of siRNA Transfected Vero Cells

The concentration of each RNA sample was determined by UV spectroscopy using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and normalized to 50 ng/µL (Table 2). All RT-PCR reactions were amplified with a OneStep RT-PCR kit (Qiagen) on a Eppendorf Master Cycler Gradient PCR machine (Eppendorf, Mississauga, ON). Amplification products (5 µL) were separated on a 2% agarose gel and stained with ethidium bromide. A typical 25 µL RT-PCR reaction contained 25 ng total RNA, 0.0625 units RNase inhibitor, 0.4 mM dNTP mix, 0.6 µM each VE2sA-F/VE2sZ-R primers and/or 0.3 µM each ACTF1/ACT-R1. Cycling parameters were $50^{o}C$ for 30 min (RT reaction), $95^{o}C$ for 15 min (*Taq* activation), followed by 30 cycles of $94^{o}C$ for 30 sec, $55^{o}C$ for 30 sec, $72^{o}C$ for 30 sec and a final extension at $72^{o}C$ for 10 min. To determine the presence of plasmid or genomic DNA contamination, RNA was sometimes added only after the *Taq* activation step (reverse transcriptase is heat labile). Contaminating DNA was removed by DNase digestion as follows: 9 µL total RNA was added to 1 µL 10x DNase Buffer (Fermentas), 1 unit DNase (Fermentas), 20 units RNase inhibitor (Fermentas) and incubated at $37^{o}C$ for 30 min. DNase was heat inactivated by adding EDTA to 2.5 mM and incubating $65^{o}C$ for 10 min.

Table 2. RNA templates for RT-PCR reactions

Plasmids were transfected or co-transfected into Vero cells as indicated. Total RNA was purified with TRIzol 24 hr or 48 hr post-transfection.

Transfected Plasmid(s)	Plasmid Function
pEGFP-N3	Transfection Control
pVAX-VE2.3	Full length VEE E2 construct
pVE2-T1.2	siRNA against VEE E2 (target 1 sequence)
pVE2-T2.2	siRNA against VEE E2 (target 2 sequence)
pGAPDH	siRNA against human GAPDH
psiRNA-ve-1	negative siRNA control, no homology in human genome
PVAX-VE2.3 psiRNA-ve-1	E2 co-transfected with negative siRNA control
PVAX-VE2.3 pVE2-T1.2	E2 co-transfected with VEE E2 (target 1 sequence)
PVAX-VE2.3 pVE2-T2.2	E2 co-transfected with VEE E2 (target 2 sequence)

Results

Amplification of E2

The E2 glycoprotein was used as a target for E2 siRNA knockdown. The E2 glycoprotein gene of VEE virus was amplified from a bacterial expression vector containing the E2 gene using E2 gene-specific primers (Figure 2). The 5' primer contained a Kozak consensus sequence for eukaryotic translation. The digested amplification product was subsequently cloned into pVAX-1, a mammalian expression vector, for expression of E2 in mammalian cells.
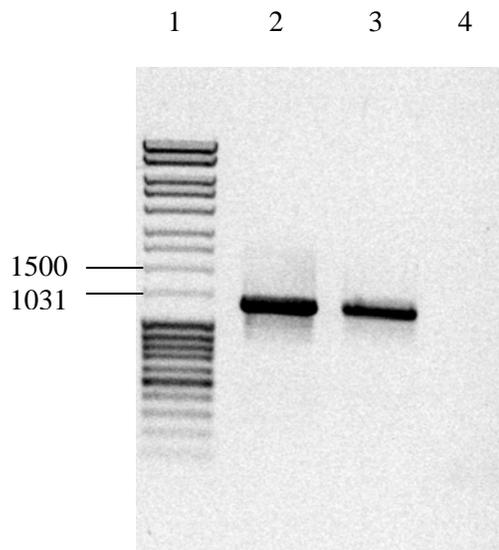

Figure 2. PCR Amplification of VEE-E2 glycoprotein.

VEE-E2 glycoprotein was PCR amplified from pRSVE2 using primers VE2_Kozak K-5' and VE2E-3'. The purified product (1296bp) was digested with Kpn I and Eco RI. Lane 1: MassRuler DNA Ladder Mix; Lane 2: Kpn I/EcoR I Digested; Lane 3: Undigested; Lane 4: Negative PCR control.

Transfection of Vero Cells

Transfection efficiency of Vero cells was determined using pEGFP, a plasmid expressing enhanced green fluorescent protein (EGFP). After 24 hours post-transfection, the EGFP transfectants revealed confluent cell growth using light microscopy (Figure 3A) and approximately 50-60% transfection using fluorescence microscopy (Figure 3B). After 48 hours post-transfection, the EGFP transfectants revealed confluent cell growth using light microscopy (Figure 3C) and approximately 75% transfection using fluorescence microscopy (Figure 3D). Therefore, the results suggest that a large percentage of cells are transfected after 24 hours and but increased transfection efficiency is observed after 48 hours.
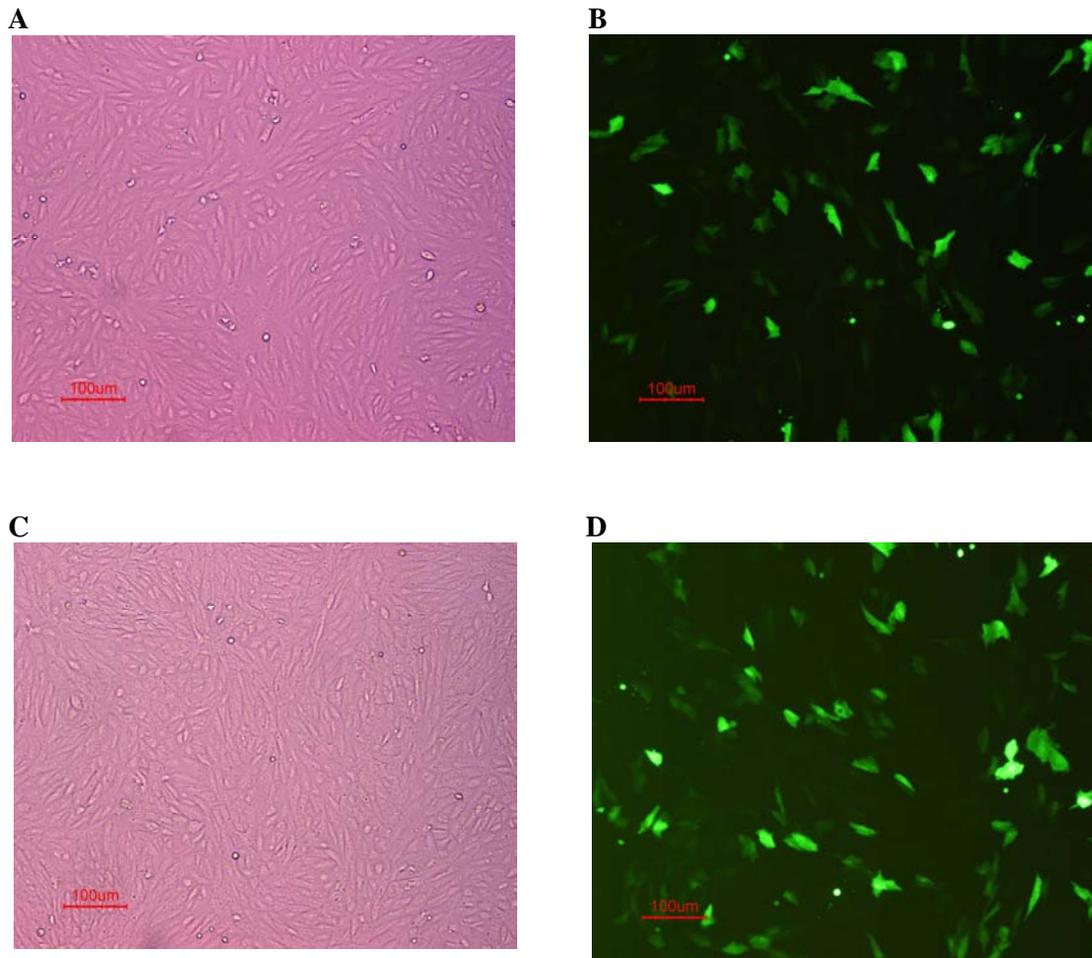

Figure 3. Transfection efficiency of pEGFP transfected cells.

Light microscopy of the confluent monolayer of cells after 24 hours (A) and 48 hours (C). Fluorescence was detected after 24 hours (B) and 48 hours (D). Photomicrographs were taken using a 10x objective.

Expression and Knockdown of VEE E2

The ability of siRNA expression vectors to knockdown E2 was assessed by co-transfecting Vero cells with pVAX-VE2.3 and with different siRNA expression vectors. Either 24 or 48 hours after transfection, cells were harvested and RNA was isolated. RT-PCR was performed to determine expression of E2 mRNA in the cells. All cells transfected with pVAX-VE2.3 expressed VEE E2 mRNA as seen by the 472 bp band in Figure 4 (lanes 5-7, 12-14, 18). Gene knockdown was not observed under initial RT-PCR conditions. Most methods used to purify RNA are contaminated with DNA to differing extents. To determine if this was a problem with TRIzol extracted RNA, RT-PCR reactions were set up and RNA was added at the start of the RT-PCR program or after the *Taq* activation step. Products detected from the first case can arise from cDNA or contaminating DNA. Products detected from the second case can only arise from contaminating DNA (the reverse transcriptase is heat-denatured during the *Taq* activation step). As seen in Figure 5, strong bands are seen at 472 bp (VEE-E2) and 353 bp (β-actin) when RNA is added at the beginning of the program. A strong band at 472 bp (and weaker band at 353 bp) is seen when RNA is added after *Taq* activation. This indicates that the RNA isolated with TRIzol is contaminated with plasmid DNA and to a lesser extent genomic DNA.

DNase digestion of the template RNA is effective for removing contaminating DNA (genomic or plasmid DNA) as demonstrated by the absence of bands when template was digested with DNase and added to reaction after *Taq* activation (Figure 6). After DNase digestion, the presence of bands corresponding to β-actin mRNA and E2 mRNA confirms the presence of these respective mRNAs in the sample. Figure 7 demonstrates that knockdown of VEE-E2 expression is occurring with both siRNA targets T1 snd T2, relative to β-actin expression. It appears that there is a low level of E2 expression, due to the presence of a faint band corresponding to E2 mRNA in both siRNA-targeted samples.
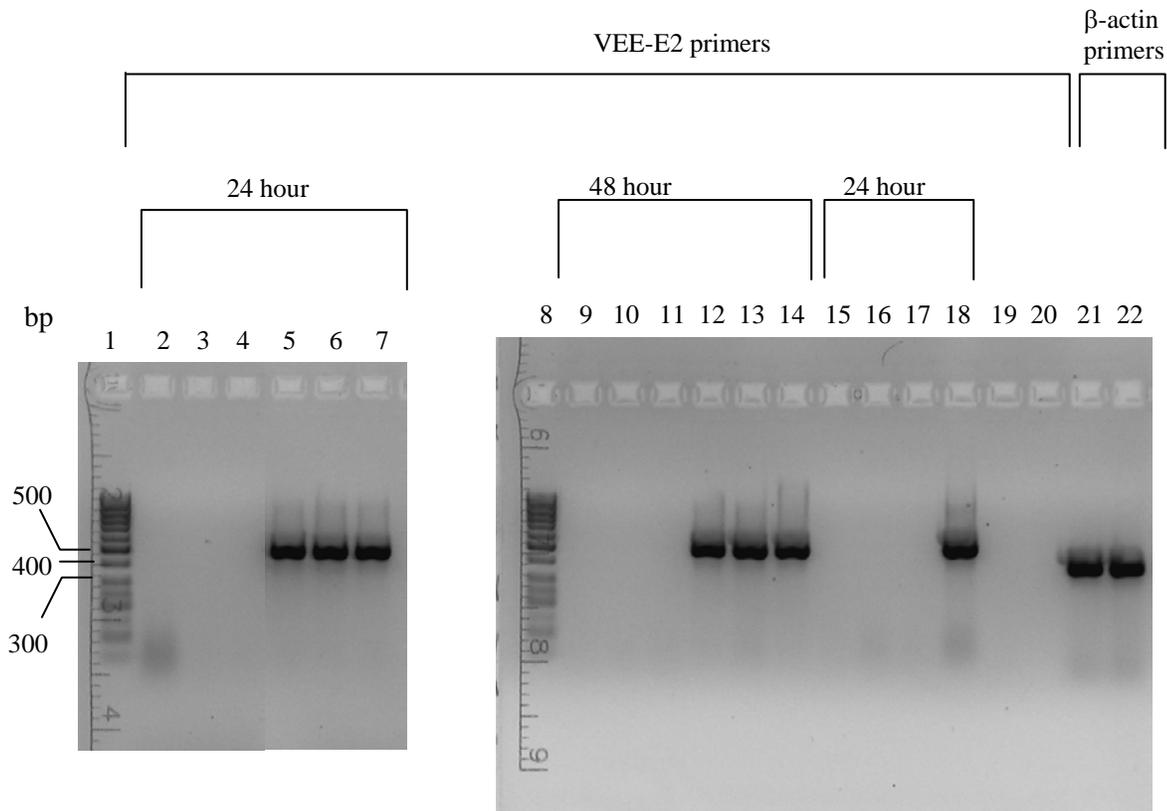
Figure 4. RT-PCR of total RNA from transfected Vero cells.

RT-PCR was performed on total RNA extracted from transfected Vero cells (Table 1). The RT-PCR reactions contained either VEE-E2 specific primers or β-actin specific primers. Template (if any) was added at the beginning of the cycling program. The expected size of VEE-E2 and β-actin PCR products are 472 bp and 353 bp respectively. Lane 1: 50 bp ladder; Lane 2: EGFP; Lane 3: GAPDH; Lane 4; siRNA-ve; Lane 5: VE2/siRNA-ve; Lane 6: VE2/T1; Lane 7: VE2/T2; Lane 8: 50 bp ladder; Lane 9: EGFP; Lane 10: GAPDH; Lane 11: siRNA-ve; Lane 12: VE2/T1; Lane 13: VE2/T2; Lane 14:VE2/siRNA-ve; Lane 15: EGFP; Lane 16: T1; Lane 17: T2; Lane 18: VE2; Lane 19: -RNA Control; Lane 20: -primer control; Lane 21:EGFP; Lane 22: E2/T1.
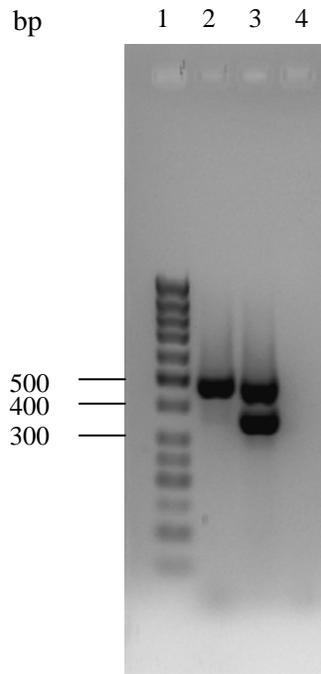
Figure 5. Template Test for Plasmid and Genomic DNA Contamination.

RT-PCR was performed on total RNA extracted from transfected vero cells (Table 1: 48hr-VE2/T1). All reactions were diplexed, containing both VEE-E2 and β-actin specific primers. RNA was added at the start of the RT-PCR cycling program (RT-PCR sample), after the Taq activation (-RT control), or absent (-RNA control). The expected size of the VEE-E2 and β-actin PCR products are 472 bp and 353 bp respectively. Lane 1: 50 bp ladder; Lane 2: -RT control; Lane 3: RT-PCR; Lane 4: -RNA control.
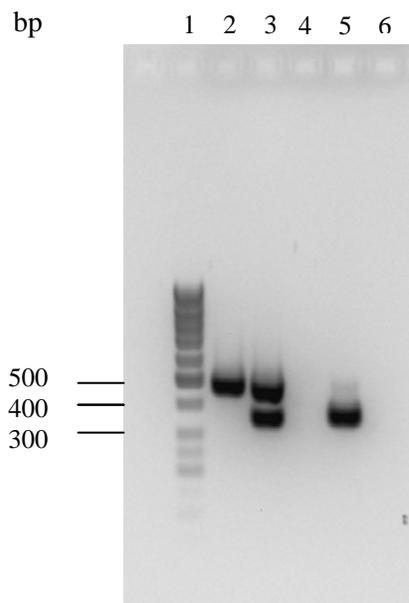
Figure 6. Removal of Contaminating DNA from RNA Template.

RT-PCR was performed on total RNA extracted from transfected Vero cells (Table 1: 48hr-VE2/T1). All reactions were diplexed, containing both VEE-E2 and β-actin specific primers. RNA template was untreated (-DNase) or DNase digested (+DNase) and was added at the start of the RT-PCR cycling program (+RT), after the Taq activation (-RT), or absent (-RNA). The expected size of the VEE-E2 and β-actin PCR products are 472 bp and 353 bp respectively. Lane 1: 50 bp ladder; Lane 2: -DNase, -RT; Lane 3: -DNase,+RT; Lane 4: +DNase, -RT; Lane 5: +DNase, +RT; Lane 6: -RNA.
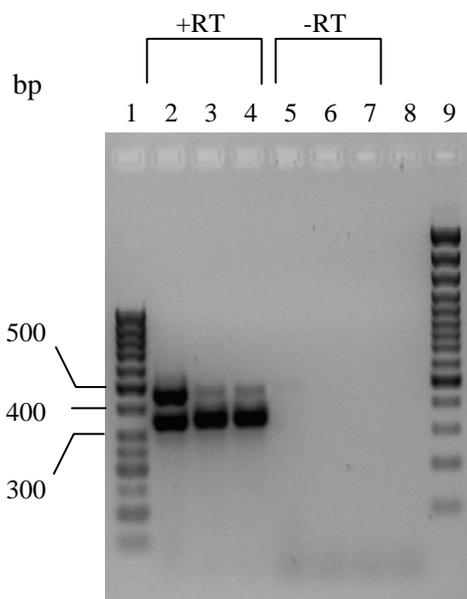
.

Figure 7. RT-PCR Using DNase Digested RNA.

RT-PCR was performed on total RNA extracted from transfected Vero cells (Table 1). All reactions were diplexed, containing both VEE-E2 and β-actin specific primers. RNA template was DNase digested and added at the start of the RT-PCR cycling program (+RT), after the Taq activation (-RT), or absent (-RNA). The expected size of the VEE-E2 and β-actin PCR products are 472 bp and 353 bp respectively. Lane 1: 50 bp ladder; Lane 2: VE2; Lane 3: VE2/T1; Lane 4: VE2/T2; Lane 5: VE2; Lane 6: VE2/T1; Lane 7: VE2/T2; Lane 8: -RNA control; Lane 9: 100 bp ladder.

Discussion

In this preliminary study, we have demonstrated the ability of DNA-directed RNA interference to knockdown a viral gene. E2 mRNA is significantly reduced using either of two different siRNA expression vectors. Each of the expressed siRNAs target distinct regions of the E2 gene. It may be useful to determine whether pooling both siRNA expression vectors (expressing T1 and T2 siRNAs) would completely abrogate the expression of E2. In addition, it may be important to test other VEE-specific siRNA vectors, targeting regions outside of the E2 gene, to confirm that the effect is indeed specific. Real-time PCR will be utilized to determine the amount of knockdown with these vector expressed siRNAs. Future studies using whole virus will be important to determine whether either of these siRNAs has an inhibitory effect on virus replication and propagation *in vitro* and *in vivo*.

RNA interference has rapidly become a valuable laboratory tool, particularly in gene function studies. The use of siRNAs to silence gene expression is advantageous, particularly when compared to a more traditional approach such as gene knockout via homologous recombination, which is time-consuming and expensive, and certain gene knockouts can be lethal. Using RNAi, large libraries of dsRNA molecules can be used to screen for genes involved in specific biological processes in different organisms. Thus, for defining gene function, high throughput studies are possible. The potential for RNAi to treat diseases extends the usefulness of this laboratory tool into the clinic. Virtually any rogue gene has the potential to be silenced using siRNA molecules. This would include genes involved in cancer, neurological disorders, and autoimmunity, as well as genes that are associated with virus and parasites during an infection. As a result of the significant impact their discovery has had on modern medical research, Andrew Fire and Craig Mello were awarded the 2006 Nobel Prize in Physiology or Medicine.

A number of issues may need to be addressed if RNAi is to be effective as a tool to combat viruses. The ability of viruses to accumulate mutations and the presence of RNAi suppressors in viruses may reduce the effectiveness of specific siRNAs. In addition, the delivery of siRNAs to virally infected cells may be inherently challenging. Notwithstanding these challenges, the ability of siRNA to manipulate gene expression has tremendous potential in the development of antiviral therapeutics.

Conclusion

Evidence of VEE E2 gene knockdown using a DNA-directed RNA interference approach has been presented in this study. It will be important to confirm these results using real-time PCR as well as to determine the level of knockdown. In addition, the ability of this E2-specific siRNA expression system to inhibit the replication of VEE virus *in vitro* will be the focus of future studies. If the silencing of the E2 gene is able to inhibit VEE virus replication, RNA interference may be a useful antiviral strategy for VEE virus as well as other viruses.

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List of symbols/abbreviations/acronyms/initialisms

EEE	Eastern equine encephalitis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PCR	Polymerase chain reaction
RNAi	RNA interference
siRNA	Small interfering RNA
VEE	Venezuelan equine encephalitis
WEE	Western equine encephalitis
LB	Luria-Bertani

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Venezuelan equine encephalitis virus is an important veterinary and human pathogen that also has the potential to be used as a bioterrorist agent. Since there are no approved vaccines or antiviral drugs for this virus, it is prudent that antiviral strategies be developed. RNA interference, an evolutionarily conserved but only recently discovered biological phenomenon, may be an effective gene manipulation tool to combat viruses. The ability of RNA interference to silence or knockdown specific mRNA through the use of short dsRNA fragments has been an effective tool to study gene function in many systems. In this study, we demonstrated the effectiveness of RNA interference to knockdown the VEE E2 gene expressed in mammalian cells. Here, a DNA-directed approach was used to transfect Vero cells with siRNA expression vectors. We demonstrated that both target siRNAs were effective in significantly reducing the level of E2 expression based on RT-PCR analysis of mRNA levels. Furthermore, the use of these vectors demonstrates the usefulness of a vector-based approach to silencing genes. Future studies will assess the efficacy of these E2-specific siRNA expression constructs in the inhibition of the VEE virus *in vitro*.

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RNA interference, siRNA, gene silencing, gene knockdown, alphavirus, Venezuelan equine encephalitis, E2 glycoprotein