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Generation of Constructs for DNA-Directed RNA Interference of Venezuelan Equine Encephalitis Virus Genes

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

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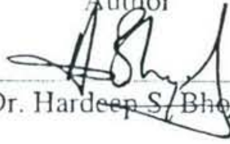

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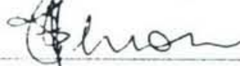

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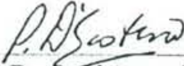

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Abstract

Venezuelan equine encephalitis (VEE) virus is an important human and veterinary pathogen with no effective treatment or prophlaxis. One strategy that has shown promise as an antiviral is a mechanism of gene silencing known as RNA interference. Although conventional RNA interference involves the use of dsRNA molecules, here we describe the generation of a panel of DNA cassettes which encode siRNA sequences. Three different VEE virus genes encoding E2 glycoprotein, nucleocapsid, and non-structural protein 4 were selected as candidates for gene silencing. Using a PCR-based approach, we report here on the selection of the VEE targets, construction of these siRNA expression cassettes, and the cloning of these cassettes into siRNA expression plasmids. These DNA plasmids, once transfected into mammalian cells, are able to express putative small interfering RNA molecules targeting specific regions of the VEE viral genome.

Résumé

Le virus de l'encéphalomyélite équine du Venezuela (EEV) est un pathogène humain et vétérinaire important pour lequel il n'existe pas de traitement ni de prophylaxie efficaces. Un mécanisme de silençage de l'expression génique connu sous le nom d'interférence ARN est une stratégie antivirale qui se montre prometteuse. L'interférence ARN traditionnelle utilise les molécules d'ARN bicaténaires mais on décrit ici la reproduction d'un panel de cassettes d'ADN qui code des séquences de petits ARNi. Trois gènes différents de virus VEE de codage de glycoprotéine E2, capside nucléide et protéine 4 non structurelle ont été sélectionnés comme candidat au silençage de la séquence. On documente ici l'utilisation de la méthode PCR pour la sélection de cibles EEV, la construction de ces cassettes d'expression de petits ARNi et le clonage de ces cassettes en plasmides d'expression de petits ARNi. Ces plasmides ADN, sont capables, une fois transfectés dans des cellules mammaliennes, d'exprimer des petites molécules putatives d'ARN interférants, en ciblant des régions spécifiques du génome viral EEV.

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Executive summary

Introduction: Venezuelan equine encephalitis virus (VEE) is one of a number of different alphaviruses, which can cause encephalitis in humans and animals. VEE is an important human and veterinary pathogen with no effective treatment or prophylaxis. Outbreaks of this virus over the last century, the re-emergence of this virus, and the potential use of this virus as a biological weapon, all support a need for further research into specific antiviral strategies. One such strategy is RNA interference. RNA interference involves the targeted silencing of a gene using short double-stranded RNA molecules. It is a ubiquitous mechanism found in eukaryotic cells to suppress genes expression. These RNA molecules, known as short interfering RNAs (siRNAs), are approximately 21-23 nucleotides in length. One strand of the siRNA duplex combines with an RNA endonuclease in the cell and, subsequently, directs the cleavage of complementary mRNA. This degradation of mRNA results in gene silencing or knockdown.The key to targeted silencing is the use of siRNAs that are complementary to the region on the gene of interest.

Results: Since sequence information for the VEE virus genome is published, appropriate target sequences can be identified, and small interfering molecules with complementary sequences can be designed. We have selected 2 different regions on each of 3 different VEE genes for targeted silencing. These three genes encode the E2 glycoprotein which is critical for virus attachment to the host cell, the nucleocapsid which is important in virus budding, and the non-structural protein 4 which is an enzyme required for replication. It is hypothesized that targeting essential virus genes, either individually or simultaneously, will lead to knockdown or silencing of the genes, and subsequent inhibition of virus replication. This paper describes the PCR-based approach used to generate DNA cassettes that encode siRNA molecules. With a commercially available system combined with specifically designed oligonucleotides for our genes of interest, we report here on the generation of a panel of siRNA expression cassettes. Once generated, these siRNA expression cassettes were cloned into siRNA expression vectors and the correct structure of these cassettes was confirmed by sequencing

Significance: This DNA-directed RNA interference approach offers advantages over traditional dsRNA approaches, such as increased stability of the constructs and easy re-generation of DNA plasmids in bacteria. In addition, DNA sequences are more cost effective to synthesize than RNA sequences.

Future Plans: The siRNA expression vectors will be tested for their ability to knockdown three essential VEE genes and, subsequently, their ability to inhibit viral replication.

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Sommaire

Introduction: Le virus de l'encéphalomyélite équine du Venezuela (EEV) appartient à un certain nombre d'arbovirus différents pouvant causer l'encéphalite chez les humains ou les animaux. L'EEV est un pathogène humain et vétérinaire important pour lequel il n'existe pas de traitement ou de prophylaxie efficaces. Des épidémies de ce virus durant le siècle dernier, la réémergence de ce virus et son utilisation potentielle comme arme biologique prêtent à penser qu'il existe un besoin d'approfondir la recherche dans le domaine spécifique des stratégies antivirales. Une de ces stratégies est l'interférence ARN. Cette dernière comprend le silençage ciblé d'un gène, au moyen des molécules d'ARN courts double brin. Il s'agit d'un mécanisme ubiquiste que l'on trouve dans les cellules eucaryotes pour supprimer l'expression génétique. Ces molécules ARN, connues comme ARN interférants courts (petits ARNi) sont d'une longueur d'environ 21 à 23 nucléotides. Un brin du petit ARNi duplex se lie avec une endonucléase ARN dans la cellule et dirige par la suite la division d'un ARNm complémentaire. La dégradation de cet ARNm résulte en un silençage du gène ou en un effet de choc. La clé du silençage ciblé est l'utilisation de petits ARNi qui sont complémentaires à la région du gène auquel on s'intéresse.

Résultats: L'information relative à la séquence pour le génome du virus EEV étant publiée, des séquences appropriées de cibles peuvent être identifiées et de petites molécules interférentes ayant des séquences complémentaires peuvent être ainsi conçues. Nous avons sélectionné deux régions différentes sur chacun des 3 différents gènes VEE pour le silençage ciblé. Ces trois gènes encodent la glycoprotéine E2 ce qui est crucial pour l'attachement du virus à la cellule hôte, la capside nucléide ce qui important pour l'éclosion du virus et la protéine 4 non structurelle qui est un enzyme requis pour la réplication. On soumet l'hypothèse que le ciblage de gènes essentiels de virus, soit individuel soit simultané, amène à l'effet de choc ou au silençage des gènes et ultérieurement à l'inhibition de la réplication du virus. Cet article décrit l'utilisation de la méthode PCR pour la reproduction de cassettes d'ADN qui codent les séquences de molécules de petits ARNi. On documente ici la reproduction d'un panel de cassettes d'expression de petits ARNi réalisée au moyen d'un système disponible sur le marché combiné à des oligonucléotides spécialement conçus pour les gènes qui nous intéressent. Une fois reproduites, ces cassettes d'expressions de petits ARNi ont été clonées en des vecteurs d'expression de petits ARNi et la structure correcte de ces cassettes a été confirmée par le séquençage.

La portée des résultats: Cette méthode d'ARN d'interférence dirigée par l'ADN offre des avantages par rapport aux méthodes traditionnelles d'ARN bicaténaires, tels que la stabilité accrue des gènes hybrides et la reproduction aisée de ces plasmides ADN dans des bactéries. De plus, les séquences ADN sont plus économiques à synthétiser que les séquences ARN.

Plans futurs : Les vecteurs d'expression de petits ARNi seront testés pour leur capacité à soumettre à l'effet de choc trois gènes EEV essentiels et ultérieurement pour leur capacité à inhiber leur réplication virale.

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Table of contents

Abstract i

Executive summary iii

Sommaire iv

Table of contents v

List of figures vi

Acknowledgements vii

Introduction 1

Materials and Methods 3

- Design of Hairpin siRNA Template Oligonucleotides 3
- Generation of siRNA Expression Cassette (SEC) 4
- Cloning SECs into pSECneo 5
- Screening pSECneo-siRNA Constructs 5
- Sequencing Conditions 6

Results and Discussion 7

Conclusion 11

References 13

List of symbols/abbreviations/acronyms/initialisms 15

List of figures

Figure 1. Precursor SEC Assembly 8

Figure 2. Generation of SECs 9

Figure 3. SEC Expression Cassettes 10

Figure 4. Overview 12

List of tables

Table 1. siRNA Target Sequences 3

Table 2. Hairpin siRNA Template Sequences 4

Table 3. Sequencing Primers 6

Table 4. siRNA Clones 10

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Introduction

Venezuelan equine encephalitis (VEE) virus is a member of the alphavirus family which includes western equine encephalitis (WEE) and eastern equine encephalitis (EEE) viruses, Semiliki Forest virus, and Middelburg virus, among others. VEE is a positive-sense, single-stranded, enveloped, RNA virus which functions like mRNA. VEE virus is transmitted to vertebrates through the bite of an infected mosquito. A number of outbreaks of VEE this century have involved thousands of human and equine cases [1]. The absence of an effective vaccine or therapeutic for VEE, a highly infectious virus, underscores the need for research in this area. In addition, the potential for the use of VEE as a biological weapon has renewed research into this human and veterinary pathogen [2].

One antiviral strategy is RNA interference (RNAi). Also known as post-transcriptional gene-silencing, RNAi has quickly become an important tool in modern medical research. Although the phenomenon was discovered in nematodes [3], RNAi has been shown to be an effective tool to silence genes from different viruses including HIV [4], poliovirus [5], influenza virus [6], Ebola virus [7], Coxsackievirus B3 [8], among others. RNAi involves sequence-specific gene silencing, or gene knockdown, using short dsRNA molecules. Gene specific silencing using 19-23 nucleotide small interfering RNAs (siRNAs) was first demonstrated by Elbashir and colleagues [9].

Silencing of viral genes via RNAi offers advantages to other antiviral therapeutics which rely upon the capacity to discriminate host from virus [10]. Since viruses rely on much of the host cell machinery for essential functions involved in viral replication, there are only a small number of viral targets that can be exploited for therapeutic purposes. Due to the fact that RNAi only relies on targeting very short stretches of the viral genome, many potential targets are possible, even if only a small handful of viral proteins exist. Since all eukaryotic cells contain endogenous RNAi machinery, virtually any gene sequence can be targeted specifically using siRNAs.

Three functionally essential VEE virus genes have been identified as putative targets for knockdown. These include the E2 glycoprotein, nucleocapsid (NC), and nonstructural protein 4 (nsP4). Nonstructural protein 4 is an RNA-dependent RNA polymerase, and is highly conserved among different strains [11]. The VEE virus E2 glycoprotein is a structural protein that is required for the attachment of the virus to the host cell [12]. The nucleocapsid protein is also a structural protein which has been shown to be important for viral budding [13].

Here we describe the generation of various constructs to be used for the knockdown of VEE E2, NC, and NSP4 for use in a DNA-directed RNAi approach. Two target sequences, spanning 21 nucleotides each, have been identified for each gene. These targets were selected from the viral genome of the attenuated vaccine strain of VEE [14], TC-83, based on their suitability for siRNA design. Using the Silencer Express system (Ambion Inc., Austin, Texas, USA), a PCR-based approach was employed to construct DNA cassettes that encoded a hairpin siRNA flanked by an RNA promoter. The use of these siRNA Expression Cassettes (SECs) to express siRNA in mammalian cells was first described by Castanotto and

colleagues [15]. These siRNA expression cassettes (SECs) were cloned into SEC expression vectors. Transfection of these vectors into mammalian cells will drive the expression of hairpin siRNA. Future studies will utilize these vectors to study the ability of these hairpin siRNAs to silence VEE virus genes.

Materials and Methods

Design of Hairpin siRNA Template Oligonucleotides

Target regions within the genes encoding VEE E2, nucleocapsid (NC) and nonstructural protein 4 (nsP4) were identified based on the sequence of VEE virus (GenBank Accession # L01443). Two 21-mer target sequences were selected for each gene (Table 1). Hairpin siRNA template oligonucleotides (Table 2) were designed by inputting target sequences into Ambion's web based target sequence converter (www.ambion.com/techlib/misc.SEC-converter.html). Oligonucleotides were ordered through Invitrogen (Burlington, ON) with standard desalting.

Table 1. siRNA Target Sequences

Gene	Target Sequence	Target Position (in gene)
VEE E2	AATCCTGTAGGCAGAGAACTC	394-414
	AACACCTAACGCTAGGATACC	1206-1226
VEE NC	AATTAACCCGCTCGATGGCTA	119-139
	AAGGCTATTACAGCTGGCATC	590-610
VEE nsP4	AATTGCCCGTATTGGATTCGG	656-676
	AAGACTTTGACGCTATTATAG	1067-1087

Table 2. Hairpin siRNA Template Sequences

Target	Sense Oligo	Antisense Oligo
VEE E2-T1	CTC CTA CAC AAA GAG TTC TCT GCC TAC AGG ACC GGT GTT TCG TCC TTT CCA CAA G	CGG CGA AGC TTT TTC CAA AAAATC CTG TAG GCA GAG AAC TCC TAC ACA AAG AGT
VEE E2-T2	ACC CTA CAC AAA GGT ATC CTA GCG TTA GGT GCC GGT GTT TCG TCC TTT CCA CAA G	CGG CGA AGC TTT TTC CAA AAA ACA CCT AAC GCT AGG ATA CCC TAC ACA AAG GTA
VEE NC-T1	CTA CTA CAC AAA TAG CCA TCG AGC GGG TTA ACC GGT GTT TCG TCC TTT CCA CAA G	CGG CGA AGC TTT TTC CAA AAA ATT AAC CCG CTC GAT GGC TAC TAC ACA AAT AGC
VEE NC-T2	ATC CTA CAC AAA GAT GCC AGC TGT AAT AGC CCG GTG TTT CGT CCT TTC CAC AAG	CGG CGA AGC TTT TTC CAA AAA AGG CTA TTA CAG CTG GCA TCC TAC ACA AAG ATG
VEE nsP4-T1	CGG CTA CAC AAA CCG AAT CCA ATA CGG GCA ACC GGT GTT TCG TCC TTT CCA CAA G	CGG CGA AGC TTT TTC CAA AAA ATT GCC CGT ATT GGA TTC GGC TAC ACA AAC CGA
VEE nsP4-T2	TAG CTA CAC AAA CTA TAA TAG CGT CAA AGT CCG GTG TTT CGT CCT TTC CAC AAG	CGG CGA AGC TTT TTC CAA AAA AGA CTT TGA CGC TAT TAT AGC TAC ACA AAC TAT

Generation of siRNA Expression Cassette (SEC)

SECs were generated with Ambion's Human H1 Silencer Express kit. This system generates a hairpin siRNA that is flanked by a human H1 RNA polymerase promoter at one end and an RNA terminator at the other end. Although the SECs can then be directly transfected into mammalian cells to determine the level of gene knockdown, the SECs generated here were cloned into a mammalian expression vectors for future studies as described.

All PCR's were conducted on an Eppendorf Mastercycler gradient thermocycler (Mississauga, ON) using thin wall strip tubes and KOD hot start HiFi polymerase. All PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

Precursor SEC Generation

The precursor 20 µL PCR reaction contained 1.0 µL H1 promoter element, 1.0 µL H1 promoter primer, 1.0 µL 10 µM sense oligo, 1.0 µL 10 µM antisense oligo, 2.0 µL 10 x KOD PCR buffer, 2.5 µL 2 mM dNTP mix, 1.2 µL 25 mM Mg (SO_4), 1.0 µL KOD polymerase (1u/µL), 9.3 µL H_2O. Cycling parameters for precursor SEC PCR were 94°C for 2 min (polymerase activation), followed by 25 cycles of 94°C for 25 sec, 50°C for 25 sec, 68°C for 20 sec. This was followed by a final extension at 72°C for 5 min.

SEC Generation

The 50 µL SEC PCR reaction contained 0.25 µL precursor SEC (1/100 dil), 2.00 µL H1 promoter primer, 2.00 µL Terminator primer, 5.00 µL 10 x KOD PCR buffer, 5.00 µL 2mM dNTP mix, 2.00 µL 25mM Mg (SO_4), 1.0 µL KOD polymerase (1u/µL), 32.75 µL H_2O. Cycling parameters for SEC PCR were 94°C for 2 min (polymerase activation), followed by 25 cycles of 94°C for 25 sec, 55°C for 25 sec, 68°C for 20 sec. This was followed by a final extension at 72°C for 5 min.

Cloning SECs into pSECneo

SEC PCR products were purified with a Qiaquick 8 PCR purification kit (Qiagen, Mississauga, ON) following the manufacturer's instructions. Purified PCR products were digested at 37°C for 2 hr in 2xTango buffer with 10 units each of *Eco*R I and *Hind* III (Fermentas, Burlington, ON). Restriction enzymes were heat inactivated at 65°C for 15 min.

Digested SECs were ligated with 50 ng pSECneo (10:1 molar ratio) at room temperature (~25°C) for 1 hr using a rapid ligation kit (Fermentas). Five µL of each ligation reaction was transformed into 50 µL oneShot TOP 10 F' cells (Invitrogen) or Sure cells (Stratagene, Cedar Creek, TX) following manufacturer's instructions. Fifty µL was plated onto LB agar plates supplemented with 100 µg/mL ampicillin (LBA) and incubated overnight at 37°C.

Screening pSECneo-siRNA Constructs

Colonies from each transformation plate were inoculated into 5 mL LBA broth and grown overnight at 37°C. Plasmid DNA was isolated using standard alkaline lysis methods and sequenced using M13 (-40) forward primer or pSEC reverse primer. Sequencing reactions were purified by ethanol precipitation and analyzed on a Beckman CEQ 8000 (Beckman Coulter, Mississauga, ON).

Sequencing Conditions

Dideoxy-terminator cycle sequencing (DTCS) was performed using Beckman Coulter's CEQ DTSC quick start kit. Twenty µL reactions were setup using 70 fmol template DNA and 5 pmol primer. Template DNA was first heat treated (to nick DNA) for 3 min at 86°C. Dideoxy-terminated fragments were ethanol precipitated and resuspended in formamide before being analyzed by capillary electrophoresis on a CEQ 8000.

The 20 µL sequencing reaction contained 5.0 µL Template DNA (39 ng/µL), 4.0 µL DTCS master mix (2.5x), 1.0 µL 10x Sequencing buffer, 1.0 µL 5 µM primer, 9.0 µL H_2O. Cycling parameters for sequencing were 30 cycles of 96°C for 20 sec, 50°C for 20 sec, 60°C for 3 min.

Table 3. Sequencing Primers

Primer	Sequence	Location
M13(-40)-F	GTTTTCCCAGTCACGAC	pSECneo
pSEC-R	GAGTTAGCTCACTCATTAGGC	pSECneo

Results and Discussion

A one-step PCR was used to construct precursor SECs (Figure 1). The main product of this PCR was a 160 bp assembled precursor SEC, but also contained a 130 bp band and several bands above 500 bp. The 130 bp band represents a partially assembled precursor SEC (no antisense oligo), whereas the bands above 500 bp are H1 promoter/primer products. The assembled precursor SECs served as template to generate SECs using H1 promoter and terminator primers (which contain *Eco*R I and *Hind* III sites, respectively). This reaction produced a single band at 171 bp corresponding to the full-length functional SEC amplification product (Figure 2).

Once the functional SECs were generated using PCR, they were cloned into pSECneo and the the inserts were sequenced. A total of 42 clones were sequenced. Only 11 clones contained the correct sequence (Table 4). Fourteen clones had a single base deletion, 4 clones had mismatch mutations and 13 clones had gross deletions/wrong sequence. The partial sequence of the siRNA expression cassettes is depicted in Figure 3. Factors that may have resulted in the generation of SECs containing incorrect sequences include the formation of secondary structure (inherent in the SEC sequence) as well as the run of nucleotides in the RNA polymerase terminator region, both of which may influence the polymerization during the PCR. All of the SECs contained the predicted sense, loop, and antisense sequences. They were flanked by sequences encoding the human H1 RNA promoter and a RNA polymerase terminator.

The approach used here describes the construction of DNA cassettes that will drive the expression of hairpin siRNA molecules (Figure 4). The precursor SEC was generated by PCR using a specific promoter element and promoter PCR primer. This was followed by large scale PCR production of the SEC using specific promoter and terminator primers. Although the functional SEC can be directly transfected into mammalian cells, these cassettes were cloned into siRNA expression vector plasmids. Upon transfection into mammalian cells, the plasmids bearing these cassettes, should provide the template for hairpin siRNA expression driven by the flanking RNA promoter sequence.

This strategy allows for large volume propagation of specific siRNA expression vectors in bacteria with a single SEC preparation. In addition, DNA plasmids encoding siRNAs are inherently more stable than traditional dsRNA molecules. Also, expression plasmids bear a gene for antibiotic resistance, a useful feature when screening for transfected cells.

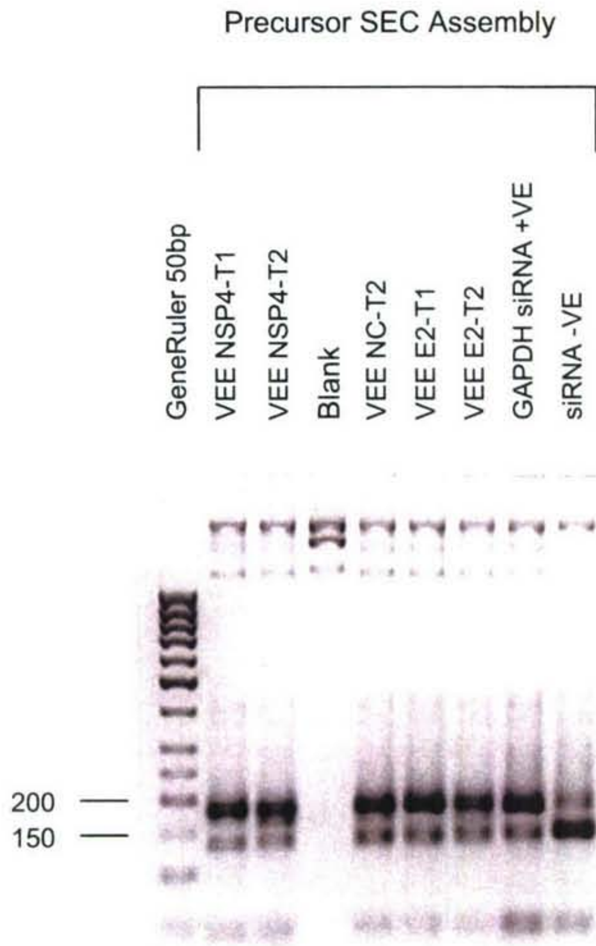

Figure 1. Precursor SEC Assembly

One step PCR was used to construct precursor r SECs. The main product of this PCR was a 160 bp assembled precursor but also contained a 130 bp band and several bands above 500 bp. The 130 bp band represents a partially assembled precursor SEC (no antisense oligo), while the bands above 500 bp are H1 promoter/primer products.

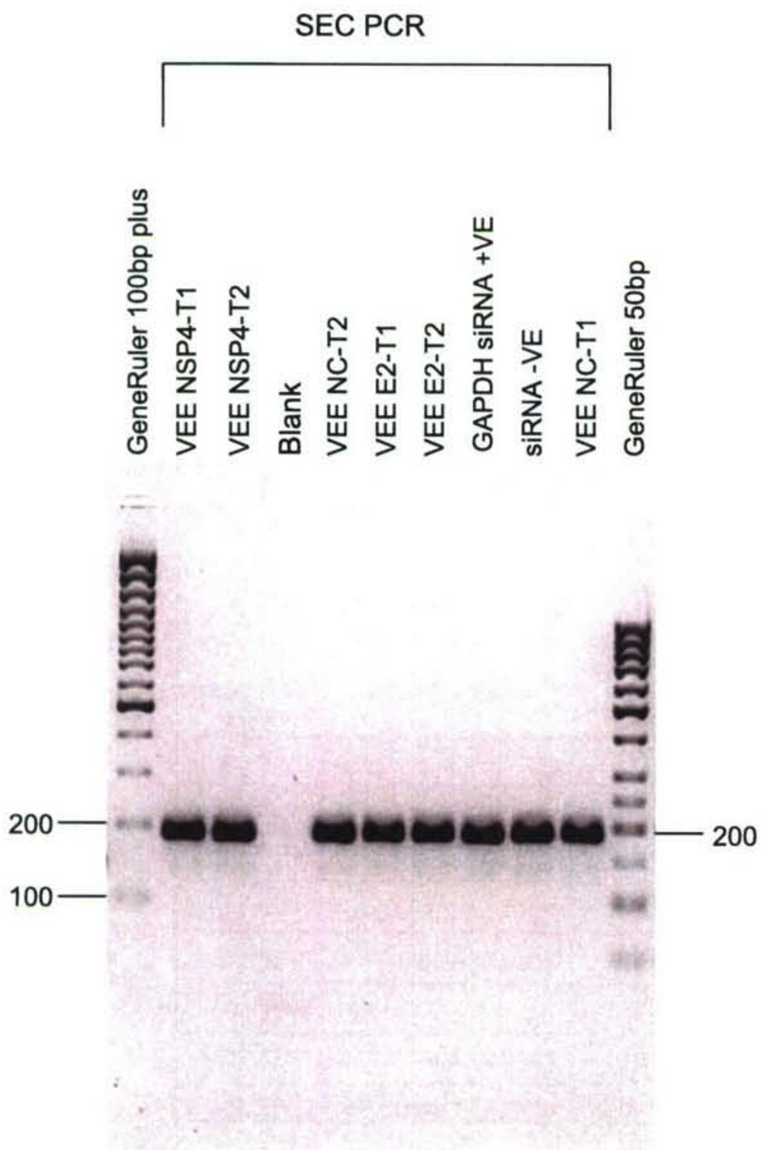

Figure 2. Generation of SECs

Assembled precursor SECs served as template to generate SECs. This reaction produced a single band at 171 bp. SECs are identical to precursor SECs except they contain EcoR I and Hind III restriction sites at their 5' and 3' terminus respectively. The functional SECs can be directly transfected into cells or cloned in an expression plasmid.

Table 4. siRNA Clones

Sequencing identified 11 clones with the correct SECs.

siRNA Target	Clone
GAPDH	GAPDH.2
Negative control	siRNA neg.1
VEE nsP4 T1	VNSP4 T1.1
VEE nsP4 T2	VNSP4 T2.1
VEE NC T1	VNC T1.4
VEE NC T2	VNCT2.10
VEE NC T2	VNC T2.12
VEE E2 T1	VE2 T1.2
VEE E2 T2	VE2 T2.1
VEE E2 T2	VE2 T2.2
Negative control	siRNA neg.2

	Human H1 RNA Promoter	Sense Strand	Loop	Antisense Strand	RNA Polymerase Terminator
VEE E2-T1	CGAAACACCG	GTCCTGTAGGCAGAGAACTC	TTTGTGTAG	GAGTTCTCTGCCTACAGGA	TTTTTTGGAAA
VEE E2-T2	CGAAACACCG	GCACCTAACGCTAGGATACC	TTTGTGTAG	GGTATCCTAGCGTTAGGTG	TTTTTTGGAAA
VEE NC-T1	CGAAACACCG	GTTAACCCGCTCGATGGCTA	TTTGTGTAG	TAGCCATCGAGCGGGTTAA	TTTTTTGGAAA
VEE NC-T2	CGAAACACCG	GGCTATTACAGCTGGCATC	TTTGTGTAG	GATGCCAGCTGTAATAGCC	TTTTTTGGAAA
VEE nsP4-T1	CGAAACACCG	GTTGCCCGTATTGGATTCGG	TTTGTGTAG	CCGAATCCAATACGGGCAA	TTTTTTGGAAA
VEE nsP4-T2	CGAAACACCG	GACTTTGACGCTATTATAG	TTTGTGTAG	CTATAATAGCGTCAAAGTC	TTTTTTGGAAA
GAPDH siRNA	CGAAACACCG	GTGGATATTGTTGCCATCA	TTTGTGTAG	TGATGGCAACAATATCCAC	TTTTTTGGAAA
siRNA Neg	CGAAACACCG	ACTACCGTTGTTATAGGTGT	ATTGTGTAA	ACACCTATAACAACGGTAG	TTTTTTGGAAA

Figure 3. SEC Expression Cassettes

The structure/sequence of each SEC that was generated is shown. Only a portion of the human H1 RNA polymerase promoter is displayed. The SEC is flanked by Eco RI and Hind III sites at the 5' and 3' terminus respectively.

Conclusion

We have outlined the successful generation of SECs for three different VEE virus genes: envelope glycoprotein E2, nucleocapsid, and nonstructural protein 4. These SECs were cloned into a mammalian expression vector. Future studies will assess the capacity of these constructs to silence VEE virus genes for each of the individual targets. The ability of siRNAs to inhibit viral replication will depend on a number of factors including the efficiency of transfection of the vector constructs into the cell, the stability of the expressed siRNA, and the accessibility of the siRNA to the viral genome. Ultimately, these constructs may be a useful tool to inhibit VEE replication and will help determine the usefulness of RNA interference as an antiviral strategy.

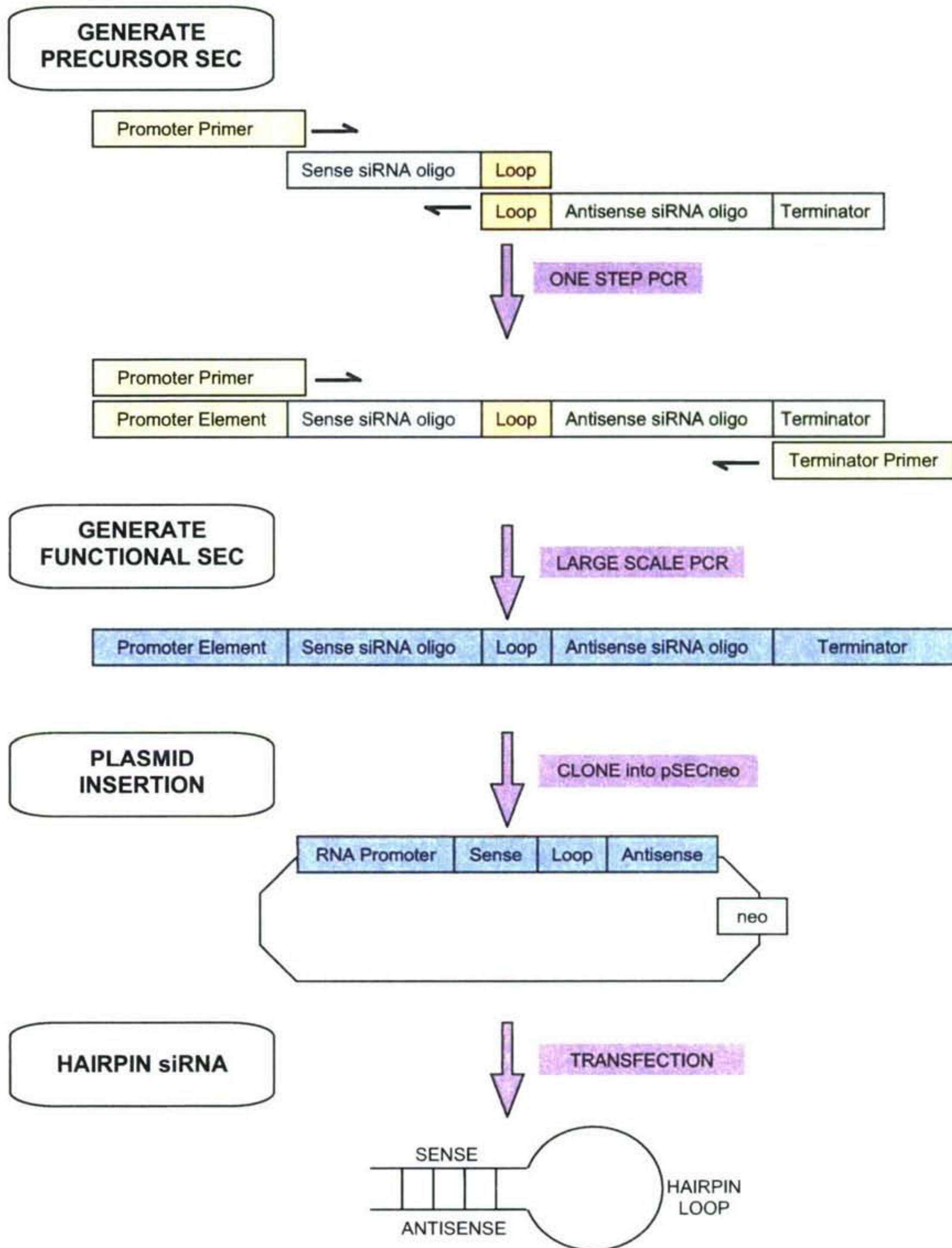

Figure 4. Overview

Overview of the generation of a functional SEC and cloning into an expression vector. Upon transfection of the SEC expression vector into cells, the expression of hairpin siRNAs are driven by an RNA promoter.

References

1. Weaver, S.C., Ferro, C., Barrera, R., Boshell, J., and Navarro, J.C. (2004). Venezuelan equine encephalitis. *Annu. Rev. Entomol.*, 49 141-174.

2. Hawley, R.J. and Eitzen, E.M., Jr. (2001). Biological weapons--a primer for microbiologists. *Annu. Rev. Microbiol.*, 55 235-253.

3. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391 (6669), 806-811.

4. Jacque, J.M., Triques, K., and Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. *Nature*, 418 (6896), 435-438.

5. Gitlin, L., Karelsky, S., and Andino, R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature*, 418 (6896), 430-434.

6. Ge, Q., McManus, M.T., Nguyen, T., Shen, C.H., Sharp, P.A., Eisen, H.N., and Chen, J. (2003). RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc. Natl. Acad. Sci. U. S. A*, 100 (5), 2718-2723.

7. Geisbert, T.W., Hensley, L.E., Kagan, E., Yu, E.Z., Geisbert, J.B., ddario-DiCaprio, K., Fritz, E.A., Jahrling, P.B., McClintock, K., Phelps, J.R., Lee, A.C., Judge, A., Jeffs, L.B., and MacLachlan, I. (2006). Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.*, 193 (12), 1650-1657.

8. Werk, D., Schubert, S., Lindig, V., Grunert, H.P., Zeichhardt, H., Erdmann, V.A., and Kurreck, J. (2005). Developing an effective RNA interference strategy against a plus-strand RNA virus: silencing of coxsackievirus B3 and its cognate coxsackievirus-adenovirus receptor. *Biol. Chem.*, 386 (9), 857-863.

9. Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.*, 15 (2), 188-200.

10. Leonard, J.N. and Schaffer, D.V. (2006). Antiviral RNAi therapy: emerging approaches for hitting a moving target. *Gene Ther.*, 13 (6), 532-540.

11. Meissner, J.D., Huang, C.Y., Pfeffer, M., and Kinney, R.M. (1999). Sequencing of prototype viruses in the Venezuelan equine encephalitis antigenic complex. *Virus Res.*, 64 (1), 43-59.

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Venezuelan equine encephalitis (VEE) virus is an important human and veterinary pathogen with no effective treatment or prophlaxis. One strategy that has shown promise as an antiviral is a mechanism of gene silencing known as RNA interference. Although conventional RNA interference involves the use of dsRNA molecules, here we describe the generation of a panel of DNA cassettes which encode siRNA sequences. Three different VEE virus genes encoding E2 glycoprotein, nucleocapsid, and non-structural protein 4 were selected as candidates for gene silencing. Using a PCR-based approach, we report here on the selection of the VEE targets, construction of these siRNA expression cassettes, and the cloning of these cassettes into siRNA expression plasmids. These DNA plasmids, once transfected into mammalian cells, are able to express putative small interfering RNA molecules targeting specific regions of the VEE viral genome.

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

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