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Down-regulation of viral replication by lentiviral-mediated expression of short-hairpin RNAs against vesicular stomatitis virus ribonuclear complex genes

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ABSTRACT

Vesicular stomatitis virus (VSV) causes great economic impact to livestock industry and is a prototype for studying non-segmented negative-stranded RNA (NSNR) viruses. In this study, we evaluated the antiviral potential of unique short-hairpin RNA (shRNA) targeting genes that form the ribonuclear protein (RNP) complex of VSV serotype Indiana (VSIV). We used lentiviral vectors to construct cell lines that stably expressed one of seven shRNAs targeting the RNP genes of VSIV, namely nucleocapsid (N), phosphoprotein (P), or polymerase (L). We reported two N-shRNA sequences targeting the 5′ or 3′ end of N that significantly reduced N, P, and L viral transcripts ($p < 0.001$), reduced viral protein expression, and reduced the viral particles shed in Vero cells ($p < 0.01$). When we analyzed the sequence diversity in the target region of this N-shRNA from two field isolates, we detected a single base substitution outside the seed region. We also reported five other shRNA sequences targeting components of the viral RNA that significantly reduce N, P, and L viral transcripts ($p < 0.001$) but failed to efficiently impair viral replication. The differences in the efficiency of the shRNAs tested were not due to mismatches within the target region in the genome of VSIV. Although partial silencing of viral transcripts by single shRNAs impaired but did not block VSIV replication, the combination of the shRNAs identified here into a multiple shRNA vector may result in inhibition of viral replication. These data contribute to ongoing development of RNAi-based technologies to combat viral diseases.

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1. Introduction

Vesicular stomatitis virus (VSV) belongs to the *Rhabdoviridae* family, order Mononegavirales (Wertz et al., 1998; Lyles and Rupprecht, 2007). The viral infection is characterized by vesicles that produce to ulcerative lesions in mouth, teats, and/or coronary bands of cattle, horses, and pigs (Martinez et al., 2003). The disease represents great economic impact (Letchworth et al., 1999; Rodriguez, 2002; Howerth et al., 2006) particularly in endemic countries. Clinical manifestations of VSV infection are indistinguishable from those of foot and mouth disease (FMD) (Martinez et al., 2003; Rodriguez, 2002) but unlike FMDV, outbreaks of VSV are periodically reported in United States and other countries in Central and South America. Therefore, efforts to rapidly control VSV outbreaks are relevant to both limit the impact of the viral infection on the livestock industry due to quarantines as wells as the impact on animal health.

VSV is also considered a model for studying non-segmented negative-stranded RNA (NSNR) viruses (Stillman et al., 1995), a group of many significant pathogens of humans, animals, plants, and insects (Wertz et al., 1998; Lyles and Rupprecht, 2007). VSV encodes five genes, in the following sequential order from a single polymerase entry site: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and polymerase (L) (Lyles and Rupprecht, 2007; Flanagan et al., 2001; Whelan and Wertz, 2002; Clarke et al., 2007). The location of the genes in the genome (Fig. 1A) constitutes a conserved strategy for transcriptional attenuation and gene regulation, thus relative molar ratios of the N, P, and L are crucial for optimal RNA replication (Wertz et al., 1998; Flanagan et al., 2001). The ribonuclear complex (RNP), which functions as the transcription and replication unit, is composed by the L, P, and N proteins (Rubio et al., 1980). N protein is the most conserved and abundant viral protein expressed in infected cells (Rodriguez et al., 2002). It wraps the negative-strand genome RNA along its full-length to protect it from nuclease-mediated degradation (Li and Pattnaik, 1999). The phosphoprotein mediates the binding of L protein to the N protein–RNA complex and it functions as an essential transcription factor for the viral polymerase (Li and Pattnaik, 1999; Bitko and Barik, 2001). The large (L) protein is the major subunit of the multifunctional RNA dependent-RNA polymerase (RdRP) which performs the genome replication and mRNA processing (Fu, 2005; Rahmeh et al., 2010).

RNA interference (RNAi) is a versatile tool to induce sequence-specific post-transcriptional silencing of gene expression (Fire

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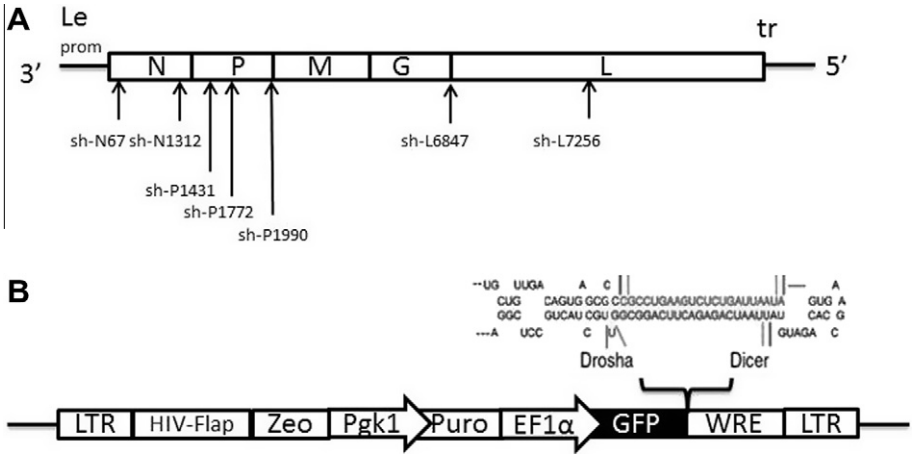

Fig. 1. Schematic diagrams of the genome organization of the viruses used in this study. (A) Vesicular stomatitis virus genome including 3′ leader sequences, promoter, and 5′ trailer sequence. Viral structural genes in their respective order of transcription: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and polymerase (L). Labels located below the 3′–5′ vRNA represent the name and target positions in the viral genome of the shRNAs used in this study. (B) Lentiviral unidirectional promoter construct used to transduce cell lines. HIV FLAP directs nuclear import of the construct, zeomycin (Zeo) is a bacterial selectable marker, Pgk1 denotes one promoter, puromycin is the eukaryotic selection cassette, EF1A is the promoter that drives the expression of the shRNA; WRE represents the Woodchuck regulatory element. The inset between the GFP and WRE ORFs exemplifies the sequence and structure of a shRNA including the processing sites for Dicer and Drosha enzymes during RNAi pathway.

et al., 1998; Grimm and Kay, 2007; de Fougerolles et al., 2007). The idea behind RNAi-based antiviral therapy is to activate RNAi machinery that targets specific viral transcripts inducing selective gene silencing of indispensable viral genes and ultimately leads to the reduction of viral titers in infected cells (Lopez-Fraga et al., 2008). The application of RNAi-based therapeutics has shown real promise in enhancing our ability to defend agriculture animal resources against viral disease (Lopez-Fraga et al., 2008; Shah and Schaffer, 2011). Successes in applying RNAi-based antiviral therapies in poultry (Chen et al., 2007, 2009; Hu et al., 2002; Sui et al., 2009) highlight the rationality of exploring its use in livestock species (Wise et al., 2008).

Barik (2004) reported the use of siRNA targeting G, P, and L genes to temporary target VSV genes (Barik, 2004). Otsuka et al. (2007) demonstrated the activation of the host RNAi pathway for targeting specific VSV genes and observed an increased VSV replication in mouse cells deficient of Dicer presumably ascribable to the lack of activation of endogenous RNAi (Otsuka et al., 2007). The purpose of this study was to test the silencing potency of different shRNA targeting the genes comprising the RNP complex of VSV. We utilized an established lentiviral vector system for delivery and stable expression of shRNA into target cell lines (Golding and Mann, 2011). We also aimed to evaluate the utility of this approach to produce an RNAi-induced antiviral effect in cultured mammalian cells before implementing *in vivo* systems.

2. Material and methods

2.1. Cell lines and viruses

Baby hamster kidney (BHK-21), Vero, and Mardin-Darby Bovine Kidney (MDBK) cell lines were used for the experiments. Cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin 10 μg/ml streptomycin and 0.25 μg/ml amphotericin B (GIBCO, Carlsbad, CA). A VSV strain serotype Indiana was kindly provided by Dr. Judith Ball (Texas A&M, USA). While two wild-type VSIV variants were isolated from a tissue collection of the Laboratory of Virology, School of Veterinary Medicine (Universidad Nacional, Costa Rica). Standard methods for viral isolation were employed (Wilson et al., 2009). Frozen bovine mucosa tissues with lesions compatible to VSV infection were the initial samples for viral isolation. In all cases, the serotype was confirmed by seroneutralization assay.

2.2. Challenge assays

Cells were trypsinized, counted, and seeded in duplicates into 24-well plates overnight prior to infection. Viral infections were carried out at MOI = 0.01 or 0.10 in FBS-free medium for 1 h. Supernantants were collected at 12 or 24 hpi. Viral supernatants were analyzed by microtitration in MDBK cells using standard methods for TCID50 determination. The titer was calculated using the method of Reed and Muench (Condit, 2007).

2.3. Design of shRNA

Sequences within N, P, or L genes of the VSIV were chosen after alignment of several published sequences using an online computer algorithm (RNAi codex) (Olson et al., 2006). Particular attention was paid to nucleotide sequences described in the literature as either being essential (Grdzelishvili et al., 2005), highly conserved (Ribeiro et al., 2008), or as locations where viral protein–protein interactions take place (Rodriguez et al., 2002). In this report position of shRNAs in the VSIV genome are given according to GenBank accession No. J02428.

2.4. Lentiviral constructs expressing shRNAs and generation of transgenic cell lines

A lentiviral shRNA-mir library (Silva et al., 2005) was used to clone each shRNA into the PEG unidirectional lentiviral construct (Golding and Mann, 2011) (Fig. 1A). Each shRNA was cloned using the PCR-based strategy described previously (Silva et al., 2005). Restriction enzyme analysis and DNA sequencing confirmed correct insertion and integrity of shRNAs. Self-inactivating (SIN) HIV-based recombinant lentiviral vectors were harvested after co-transfection of 293T cells with plasmids expressing the shRNA cassettes, VSV-G and the packaging construct (Miyoshi et al., 1998). These recombinant lentiviruses were used to transduce the Vero and BHK cells. Transgene expression was confirmed at 48 h post-infection by green fluorescent protein (GFP) expression, immediately followed by drug selection using puromycin. GFP expression in at least 90% of cell population was confirmed by flow