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Characterization of the swine U6 promoter for short hairpin RNA expression and its application to inhibition of virus replication

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ABSTRACT

Expression of short hairpin RNAs (shRNAs) by the RNA polymerase type III U6 promoter is an effective and widely used strategy for RNA interference (RNAi) which is a sequence-specific gene silencing mechanism. The U6 promoters from human, mouse, and swine were cloned, respectively for constructing various shRNA expression vectors. The transcription efficiency of each U6 promoter was analyzed for its activity to drive expression of shRNA targeting enhanced green fluorescent protein (EGFP) mRNA in different mammalian cells. All three U6 promoters were functional and the swine U6 promoter demonstrated the most efficient knockdown of EGFP synthesis in all these three species of cell lines including porcine kidney (PK-15), human embryonic kidney (HEK293T), and mouse fibroblast (LM) cells. Furthermore, the antiviral effect of shRNA targeting the classical swine fever virus (CSFV) NS5B driven by the swine U6 promoter was confirmed by the significant reduction of virus replication.

1. Introduction

RNA interference (RNAi) is a natural cellular process by which RNA duplexes known as short interfering RNA (siRNA) can reduce gene expression through mRNA targeting (Elbashir et al., 2001; Xia et al., 2002). RNAi has become a powerful biological tool for studying gene functions and fighting against viral diseases by targeting the mRNA of viral genes (Brummelkamp et al., 2002; Hamasaki et al., 2003; Daniel-Carlier et al., 2012). RNAi-mediated silencing is commonly achieved either by transfection of synthetic siRNA duplexes or DNA or viral vectors which express siRNA as short hairpin RNAs (shRNAs). The shRNA molecule is then further processed to become siRNA by cellular ribonuclease complexes (Taxman et al., 2006).

Short interfering or short hairpin RNA synthesis systems in cells are most often driven by RNA polymerase III (pol III) promoters since the transcription efficiency of siRNA is high, resulting in uniform RNA molecules with defined 5′ and 3′ ends (Mäkinen et al., 2006; Henriksen et al., 2007). The typical pol III promoters contain a proximal sequence element (PSE), a TATA box, and a distal sequence element including an Oct-1 binding site (Jensen et al., 1998; Kunkel and Hixson, 1998). U6 promoters are most commonly used in vector-based shRNA expression systems, and the currently commercially available shRNA expression vectors contain either the human or the murine U6 promoter (Nie et al., 2010; Roelz et al., 2010). For adaption and application of shRNA expression system for RNAi in pigs, the swine U6 promoter may be expected to be more efficient in swine cells. Therefore, we constructed various shRNA expression plasmids driven by the human, mouse, and swine U6 promoter, respectively, and further analyzed their activities to drive shRNA-mediated RNAi in different mammalian cell lines. The swine U6 promoter demonstrated better ability than the others to express the shRNA targeting enhanced green fluorescent protein (EGFP) mRNA, resulting in remarkable reduction of fluorescence. Expression of shRNA targeting the classical swine fever virus (CSFV) NS5B gene in the porcine kidney cells was also conducted for confirming the antiviral effect.

2. Materials and methods

2.1. Cells and virus

Porcine kidney cell (PK-15), human embryonic kidney 293 cell (HEK 293T), and mouse fibroblast cells (LM) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 5% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. The stable EGFP-expressing PK-15 cell line was established by transfecting

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Table 1
Sequences of oligonucleotides used for cloning the U6 promoters and detecting the CSFV viral RNA by real-time PCR.

Oligonucleotide	Target gene[a]	Sequence (5′–3′ end)[b]
HU6		
Forward primer	Human U6 promoter	ATAGATCTAAGGTCGGGCAGGAA
Reverse primer		TGAATTCGGTGTTTCGTCCTTTCC
MU6		
Forward primer	Murine U6 promoter	ATAGATCTGATCCGACGCCGC
Reverse primer		TGAATTCAAACAAGGCTTTTCTCCAAG
SU6		
Forward primer	Swine U6 promoter	ATAGATCTAGGAGGACTCCAGGGAC
Reverse primer		CTGAATTCGGGTCTTCTCAGAGG
NS5B		
Forward primer	CSFV NS5B gene	TGTCAGAAGTACCCGTAATCAGTG
Reverse primer		CCGTGGCCTCGCAGAAG
GAPDH		
Forward primer	Swine GAPDH gene	GGCTGCCCAGAACATCATCC
Reverse primer		ACGCCTGCTTCACCACCTTC

[a] Target genes sequences for human U6, murine U6, and swine U6 promoters, as well as CSFV NS5B and swine GAPDH genes were referred to the GenBank accession nos. X07425.1, X06980.1, EU520423, AF352565.1, and NM_001206359.1, respectively.
[b] The sequences recognized by the restriction enzyme *Bgl*II (AGATCT) and *Eco*RI (GAATTC) are underlined.

with the reporter plasmid pEGFP-N3 (Clontech), followed by selecting the stable transformant resistant to 500 μg/ml G418 (Sigma). CSFV LPC vaccine strain was kindly provided by the Animal Health Research Institute, Council of Agriculture, Taiwan, ROC.

2.2. Construction of shRNA expression vectors driven by human, mouse, or swine U6 promoter

U6 promoters of human, mouse, and swine were cloned from HEK293T, LM, and PK-15 cells, respectively. The genomic DNA of each type of cells was extracted by QIAamp DNA Mini Kit (Qiagen), and then subjected to PCR amplification of each U6 promoter with the specific primer pair (Table 1). The *Bgl*II–*Eco*RI fragment of each U6 promoter was gel-purified and cloned into the expression vector pcDNA4/HisMax (Invitrogen) to replace its CMV promoter and generate the shRNA expression vectors driven by human U6 (pHU6), mouse U6 (pMU6), and swine U6 (pSU6) promoters, respectively. In addition, DNA fragments representing human U6 or swine U6 promoter with exchanged PSE motif were synthesized to replace the U6 promoter of pHU6 and generate plasmids pHU6pseS and pSU6pseH, respectively.

2.3. Construction of shRNA expressing plasmids targeting EGFP or CSFV NS5B gene

The target sequence specific to the EGFP or CSFV NS5B gene was selected by the Thermo siDESIGN® Center or Invitrogen BLOCK-iT™ RNAi Designer, and DNA oligonucleotides encoding the target shRNA (Table 2) were synthesized according to the methods described previously (Brummelkamp et al., 2002; Sui et al., 2002). The control shRNA (siC) comprised random sequences unrelated to the target gene or pig genome. The sense oligonucleotide was annealed with its antisense strand and then ligated to the *Eco*RI and *Xho*I sites of each shRNA expression vector to generate various shRNA-expressing plasmids targeting EGFP or CSFV NS5B driven by different species of U6 promoters (Fig. 1).

2.4. Co-transfection of reporter and shRNA expressing plasmids

Cultured cells with 50% confluence in 96-well plates were co-transfected with appropriated concentration of the reporter plasmid pEGFP-N3 and each shRNA-expressing plasmid using TurboFect™ *in vitro* Transfection Reagent (Thermo Scientific) in Opti-MEM medium (Gibco) according to the manufacturer's manual. The inhibition efficacy of shRNA-expressing plasmid was analyzed by the intensity of remaining fluorescence at 24, 48, or 72 h post transfection using TRIAD™ multimode plate reader (DYNEX) and photographed with a fluorescence microscope (Olympus).

2.5. Cell viability assays

Cultured cells with 50% confluence in 96-well plates and transfected with 0.2 μg of each shRNA-expressing plasmid, or treated with transfection reagent only or 2 mg/ml antibiotic Zeocin served as controls. After incubation for 48 h, cell proliferative responses were determined by WST-8 kit (Abnova) according to the manufacturer's manual.

2.6. Western blot assay

The transfected cell lysates were extracted using M-PER Mammalian Protein Extraction Reagent with Halt™ Protease Inhibitor Cocktail (Thermo Scientific), and equal volume of 2× sample buffer (125 mM Tris–Cl [pH 6.8], 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.25% bromophenol blue) was added. Proteins were separated by 12% SDS-PAGE and transferred by electroblotting onto PolyScreen PVDF transfer membrane (Perkin Elmer) using a semi-dry transfer cell (Bio-Rad) according to the manufacturer's manual. The membrane was then treated sequentially with phosphate buffer saline (PBS) containing 5% non-fat skim milk (blocking

Table 2
List of shRNA sequences in this study.

shRNA[a]	Sequence (5' end to 3' end)[b]
siEGFP S	AATTCGGCACAAGCTGGAGTACAATTCAAGAGATTGTACTCCAGCT
siEGFP AS	TGTGCCTTTTTC
	TCGAGAAAAAGGCACAAGCTGGAGTACAATCTCTTGAATTGTACTC
	CAGCTTGTGCCG
siCSFV S	AATTCGAGAAGAAGCCTAGAGTTATTCAAGAGATAACTCTAGGCTT
siCSFV AS	CTTCTCTTTTTC
	TCGAGAAAAAGAGAAGAAGCCTAGAGTTATCTCTTGAATAACTCT
	AGGCTTCTTCTCG
siC S	AATTCGGACAGTGGGATGGATAGGTTCAAGAGACCTATCCATCCCA
siC AS	CTGTCCTTTTTC
	TCGAGAAAAAGGACAGTGGGATGGATAGGTCTCTTGAACCTATCC
	ATCCCACTGTCCG

[a] S, sense strand; AS, antisense strand; C, control shRNA sequence.
[b] The shRNA target sequence in each target gene are underlined, and the sequences recognized by the restriction enzyme *Eco*RI (GAATTC) and *Xho*I (CTCGAG) are boxed.