Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue2-fulltext-10 *SHORT COMMUNICATION*

Sheep 7SK promoter for short hairpin RNA expression

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Received October 4, 2011 / Accepted January 27, 2012
Published online: March 15, 2012
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Abstract

Background: Gene silencing mediated by small interfering RNA (siRNA) has become a powerful biological tool for the regulation of gene expression. For the synthesis of siRNA by vector-based expression systems, several mammalian small nuclear RNA (snRNA) promoters have been cloned and shown different transcriptional efficiencies. **Results:** In this study, we identified a sheep 7SK snRNA (s7SK) promoter based on the highly conserved polymerase III promoter elements. Promoter activity was measured by promoter-driven shRNA expression to suppress expression of an exogenous reporter gene and endogenous sheep gene. **Conclusions:** The knock down assay demonstrated that the s7SK induced more stronger inhibition effect than human U6 and H1 promoters. The use of this native sheep 7SK promoter for shRNA expressionis an important component for development of RNAi-based gene therapy and production of transgenic animals in sheep species.

Keywords: 7SK promoter, RNAi, sheep, shRNA

INTRODUCTION

RNA interference (RNAi) is an important mechanism of gene expression regulation in various species (Meister and Tuschl, 2004). The RNAi pathway involves the processing of double-stranded RNAs (dsRNAs) into 21-23 nucleotide duplexes to initiate gene knockdown (Fire et al. 1998). Now there are three ways to introduce dsRNAs: Chemical synthesis, enzymatic synthesis and shRNA expression vectors. The last way is cost-efficient and useful for long-term knockdown of target genes. Vector-based expression systems often utilize RNA polymerase III (pol III) promoters, including U6, H1 and 7SK, to drive shRNA expression. The typical type III promoter includes a proximal sequence element (PSE) and a TATA box element and the enhancer region consisting of an octamer motif (OCT) and an SPH element (Schramm and Hernandez, 2002).

Recently, various U6 and 7SK promoters for shRNA expression were reported in several species including chicken (Kudo and Sutou, 2005; Wise et al. 2007), bovine (Lambeth et al. 2005; Lambeth et al. 2006) and fish (Zenke and Kim, 2008). Moreover, researchers have identified that human and bovine 7SK promoters induced more efficient shRNA expression and RNAi activity than corresponding U6 promoters (Koper-Emde et al. 2004; Lambeth et al. 2006). Here, we have identify a sheep 7SK promoter and analyzed its potential use as shRNA expression vectors.

MATERIALS AND METHODS

Cloning of sheep 7SK promoter

All oligonucleotide sequences used in this study are summarized in Table 1. The sheep 7SK promoter (s7SK) was identified by using blast in the real sheep genome browser web site (http://www.livestockgenomics.csiro.au/sheep/) with bovine 7SK promoter sequence as the input. Sheep genomic DNA was isolated from muscle using TIANamp Genomic DNA kit (Tiangen Biotech, China) and was used as PCR template. The s7SK was amplified by PCR with primer pairs s7SK-F and s7SK-R (Table 1). PCR products were subcloned into pMD18-T (TaKaRa Biotech, Dalian) and sequenced.

Table 1. Oligonucleotides sequences.

Name	Sequence
s7SK-F	CAGGATGGGGAAGGAGCGTGAGA
s7SK-R	GGCGATCAATGGGGTGACAGATGTC
s7SK-shEGFP	GTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAA CTTCAGGGTCAGCGAGGTACCCAGGCGGCGCA
hU6-F	TGAAGATCTGGGCAGGAAGAGG
hU6-shEGFP	GTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAA CTTCAGGGTCAGCGGATCCTCGTCCTTTCCACAAG
hH1-F	TCACGACGTTGTAAAACGAC
hH1-shEGFP	GTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAA CTTCAGGGTCAGCCGAGTGGTCTCATACAGAACTT
s7SK-shMSTN	GTTCCAAAAAATTTTCTTGCACTTCTGCTATCTCTTGAATAGCAGA AGTGCAAGAAAAGAGGTACCCAGGCGGCGCA
hU6-shMSTN	GTTCCAAAAAATTTTCTTGCACTTCTGCTATCTCTTGAATAGCAGA AGTGCAAGAAAAGGATCCTCGTCCTTTCCACAAG
hH1-shMSTN	GTTCCAAAAAATTTTCTTGCACTTCTGCTATCTCTTGAATAGCAGA AGTGCAAGAAAACGAGTGGTCTCATACAGAACTT
hU6-shScrambled	GTTCCAAAAAAAATAAGTCGCAGCAGTACAATCTCTTGAATTGTAC TGCTGCGACTTATGGATCCTCGTCCTTTCCACAAG

Construction of shRNA plasmids

The sheep 7SK, human U6 (hU6) and human H1 (hH1) were used as templates to generate shRNA expression cassettes targeting an exogenous EGFP gene and endogenous myostatin (MSTN) gene using a one-step PCR method (Lambeth et al. 2005). shRNA expression cassettes were amplified with primers s7SK-F and s7SK-shEGFP for 7SK-shEGFP, hU6-F and hU6-shEGFP for hU6-shEGFP, hH1-F and hH1-shEGFP for hH1-shEGFP, s7SK-F and s7SK-shMSTN for s7SK-shMSTN, hU6-F and hU6-shMSTN for hU6-shMSTN, hH1-F and hH1-shMSTN for hH1-shMSTN (Table 1). All PCR products were ligated into pMD18-T to construct shRNA expression vectors targeting EGFP or MSTN. siRNA target sequence for EGFP and MSTN was previously reported (Kim and Rossi, 2003; Jain et al. 2010).

Cell culture and transfection

Sheep fetal fibroblast cells were isolated from 45-day old sheep fetuses. The tissue pieces were implanted into the 25 ml tissue culture flask in Dulbacco's Modified Eagels Medium (DMEM) (Gibco)

supplemented with 10% fetal bovine serum (Gibco) in humidified atmosphere containing 5% CO_2 at 37ºC.

For targeting EGFP, 1.0 x 10^5 cells per well were seeded in 24-well plate (Costar) the day before transfection and cultured in fresh DMEM without antibiotics to achieve 90% confluency. Co-transfection with 0.5 µg plasmid DNA (shRNA expression vectors and or pEGFP-N1), was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

For targeting sheep MSTN, 2.0 x 10^5 cells per well were seeded in 12-well plate and cultured in fresh DMEM without antibiotics to achieve 80-90% confluence on the day of transfection. Cells were then transfected with 1.6 µg /well of each anti-myostatin shRNA expression vectors using Lipofectamine 2000. Non transfected and scrambled shRNA transfected cells were used as controls.

		SPH				
Consensus		**YYWCCCRNMATSCMYYRCR**			**ATTTGCAT**	
Sheep 7SK	-266	TGTCACCAATAGAGACGGC	-246	-244	ATTTAGCAT	-227
Bovine 7SK	-266	TTTCACAAATAGAGACGGC	-246	-244	ATTTAGCAT	-227
Human 7SK	-196	TATCCAGAATGCCTTGCAG	-216	-237	ATTTAGCAT	-220

		PSE			TATA	
Consensus		**STSACCGTGWSTGTRAAR(0-3)TG**				
Sheep 7SK	-66	GTCGACATATGCGTAAAGATC	-45	-19	TTATATA	-25
Bovine 7SK	-66	GTCGACATATCCTTAAAGACA	-45	-18	TTTATATA	-25
Human 7SK	-67	CTTGACCTAAGTGTAAAGTTG	-44	-18	TTTATATA	-25

Fig. 1 The sequences alignment of conserved elements from sheep, bovine and human 7SK promoters. PSE, OCT, TATA-box and SPH sequence in sheep 7SK promoter were shown. Matches to the consensus sequence were not underlined. The numbers showed position of each element relative to the transcription start site (+1).

EGFP knockdown assays

EGFP expression level was monitored using an Olympus fluorescent microscope and quantified by flow cytometry at 48 hrs post-transfection. After fluorescence microscopic observation, cells were trypsinized, washed and resuspended in PBSA for flow cytometer analysis (Becton Dickinson). Data analysis was performed using CELLQuest software.

Real-time RT-PCR analysis

MSTN expression level was monitored by Real-time RT-PCR. Total RNA was isolated 48 hrs post-transfection using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using random hexamer primers and Superscript First-Strand Synthesis System (Invitrogen). The following primers were used for myostatin amplification (MSTN-F 5'-ATC CGATCTCTGAAACTTGACAT-3' and MSTN-R 5'-AGTCCTTCTTCTCCTGGT TCTG -3') and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GAPDH-F 5'-GGCGCCAAGAGGGTCAT-3' and GAPDH-R 5'-GTGGTTCACGCCCATCAC A-3'). Real-time PCR (Stratagene MX3000P) was carried out using SYBR Green (TaKaRa Biotech, Dalian) following the manufacturer's protocol. The PCR thermal cycle reactions consisted of denaturation at 95ºC for 5 min followed by 45 cycles at 95ºC for 15 sec, 56ºC for 15 sec and 72ºC for 10 sec. Cycle threshold (Ct) values were normalized to

GAPDH, and comparative quantification of myostatin mRNA was done by the ΔΔCt method (Livak and Schmittgen, 2001).

Statistical analysis

All the experiments were done in triplicates and repeated three times. Statistical analyses were performed with One-way Analysis of Variance (ANOVA) and Tukey's multiple comparisons tests. The results were presented as the mean ± SEM. All statistical tests were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Identification of a sheep 7SK promoter

As a result of blast search of the real sheep genome library, one loci with high identity with bovine 7SK snRNA sequence was identified. Sequence analysis revealed that the locus featured 7SK snRNA sequence with upstream pol III promoter elements including the TATA-box, PSE, SPH and OCT domains (Figure 1). The predicted sheep 7SK promoter was amplified and verified by sequencing and deposited in GenBank (HQ727729).

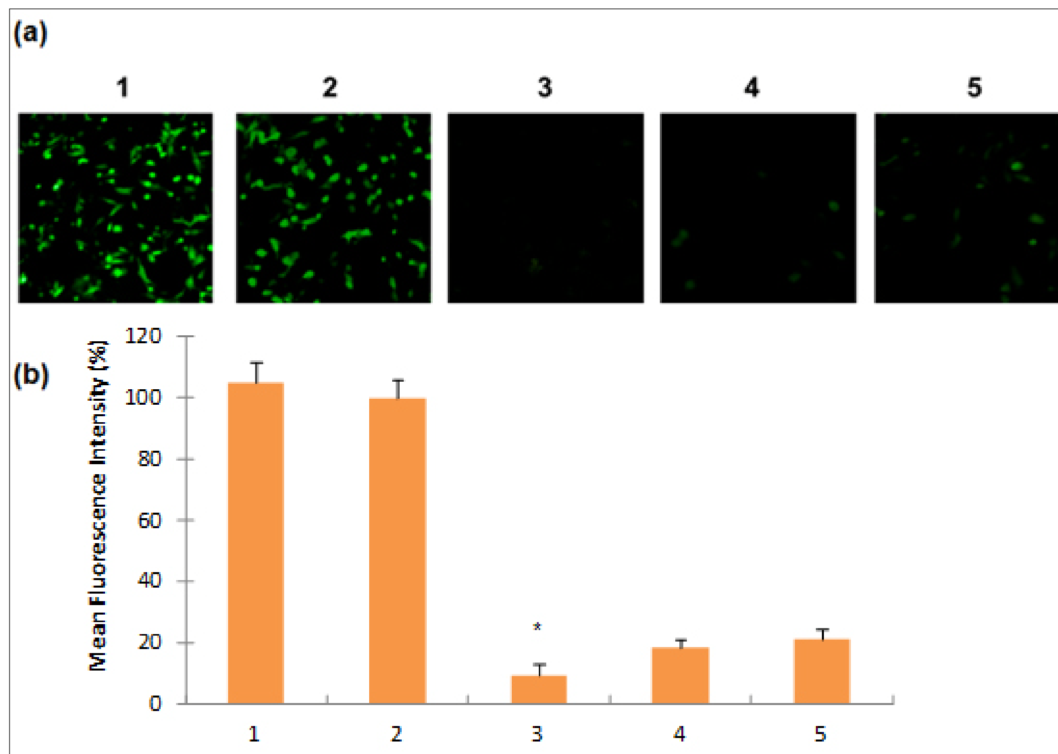

Fig. 2 Suppression of EGFP expression by shRNA driven from sheep 7SK, human U6 and H1 promoters. (1) transfection of pEGFP-N1; (2) cotransfection of pEGFP-N1 and phU6-shScrambled; (3) cotransfection of pEGFP-N1 and ps7SK-shEGFP; (4) cotransfection of pEGFP-N1 and phU6-shEGFP. (5) cotransfection of pEGFP-N1 and phH1-shEGFP. (a) Representative fluorescence images of sheep fibroblast cells at 48 hrs after transfection. (b) Mean Fluorescence Intensity (MFI) of EGFP measured by flow cytometry. MFI of each group was shown as percentages of pEGFP-N1 and phU6-shScrambled cotransfection group. Each value represented mean of triplicate experiments with standard deviation. *($P < 0.05$) denote differences between (3) and (4), or (3) and (5).

Knockdown of EGFP gene expression

To examine the function of the sheep 7SK promoter, a sheep 7SK-driven shEGFP expression plasmid was constructed. The shEGFP expression plasmid and pEGFP-N1 were cotransfected into sheep fibroblast cells for EGFP knockdown assays. EGFP expression level in sheep cells was confirmed by fluorescence microscope (Figure 2a) and quantified by flow cytometry (Figure 2b). The s7SK (91% ± 3.7%), hU6 (82% ± 3.1%) and hH1 (79% ± 3.2%) driven shEGFP induced effective suppression of EGFP expression, whereas the control vector (phU6-shScrambled) did not ($P < 0.01$) (Figure 2b). Moreover, the s7SK-mediated suppression was significantly greater than human U6 and H1 promoters ($P < 0.05$) (Figure 2b). These results clearly showed that the sheep 7SK promoter could efficiently induce RNAi in sheep cells.

Knockdown of sheep MSTN gene expression

To further validate the function of the sheep 7SK promoter for RNAi, shRNA expression vectors targeting the sheep MSTN gene were generated. Sheep fetal fibroblast cells were transfected with shMSTN expression vectors for evaluating silencing of myostatin by real-time polymerase chain reaction. As showed in Figure 3, the s7SK-mediated suppression resulted in 92% ± 3.9% silencing of MSTN gene, which was significantly greater than hH1-mediated suppression (78% ± 4.9%) ($P < 0.05$), but not significantly different to hU6-mediated suppression (83% ± 6.2%) ($P > 0.05$).

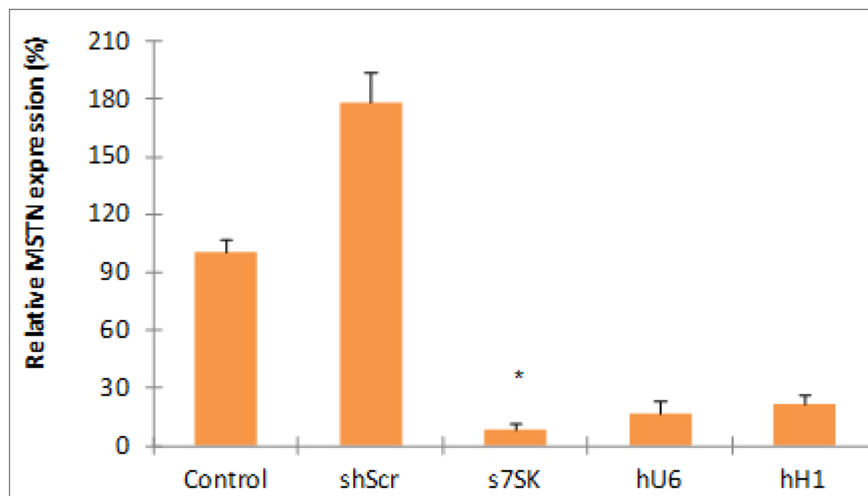

Fig. 3 Suppression of sheep MSTN expression in sheep cells. The level of myostatin mRNA expression was determined by real-time RT-PCR 48 hrs post-transfection and normalized to GAPDH mRNA. Control, nontransfection group; shScr, phU6-shScrambled transfection group; s7SK, ps7SK-shMSTN transfection group; hU6, phU6-shMSTN transfection group; hH1, phH1-shMSTN transfection group. *($P < 0.05$) denote differences between s7SK and hH1.

In conclusion, we have identified a functional sheep 7SK promoter that can trigger stronger RNAi effect compared with the human U6 and H1 promoter. The sheep 7SK promoter may be used as an efficient alternative to U6 or CMV-based shRNA expression systems in sheep RNAi-based research. Our findings contribute towards development of sheep-specific RNAi-based therapeutics and production of transgenic animals in sheep species.

Financial support: Grants from the 973 Program (2010CB530200) and Projects of Cultivating New Varieties by Transgenic Technology (2009ZX08005-003B).

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How to reference this article:

NI, W.; HU, S.; HAZI, W.; WANG, Y.; QIAO, J.; YAN, R. and CHEN, C. (2012). Sheep 7SK promoter for short hairpin RNA expression. *Electronic Journal of Biotechnology*, vol. 15, no. 2, p. 1-6. http://dx.doi.org/10.2225/vol15-issue2-fulltext-10

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