Genomics & Informatics Vol. 5(1) 6-9, March 2007

Construction of an RNase P Ribozyme Library System for Functional Genomics Applications

Sun Woo Hong[1], Hyo jei Choi[2], Young hoon Lee[2] and Dong-ki Lee[1,*]

[1]Department of Chemistry and BK School of Molecular Science, Pohang University of Science and Technology, Pohang 790-784, Korea, [2]Department of Chemistry, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

Abstract

An RNase P ribozyme library has been developed as a tool for functional genomics studies. Each clone of this library contains a random 18-mer and the sequence of M1 RNA, the catalytic subunit of RNase P. Repression of target gene expression is thus achieved by the complementary binding of mRNA to the random guide sequence and the successive target cleavage via M1 RNA. Cellular expression of the ribozyme expression was confirmed, and EGFP mRNA was used as a model to demonstrate that the RNase P ribozyme expression system can inhibit the target gene expression. The constructed RNase P ribozyme library has a complexity of 1.4×10^7. This novel library system should become a useful in functional genomics, to identify novel gene functions in mammalian cells.

Keywords: RNase P, ribozyme library, EGFP, functional genomics

Introduction

Identification of novel gene functions is one of the major goals of biology in the post-genome era. A number of approaches have been developed to achieve rapid, high-throughput gene annotation with the phenotype-based functional genomics approach using libraries of genome-regulating molecules being the most established. In this strategy, expression of each component of genome-regulating molecules from the library inhibits target gene expression, and the resulting phenotype is screened in a high-throughput manner. This strategy successfully identified genes that are associated with specific phenotypes such as tumorigenesis or metastasis (Beger *et al*., 2001; Lee *et al*., 2004; Li *et al*., 2000; Welch *et al*., 2000; Suyama *et al*., 2003; Suyama *et al*., 2004). To date, phenotype- based functional genomics methods have utilized libraries of various genome-regulating biomolecules such as artificial transcription factors, ribozymes, or small interfering RNAs (Lee *et al*., 2003; Akashi *et al*., 2005).

Escherichia coli RNase P nucleoprotein complex consists of a catalytic RNA subunit (M1 RNA) and a protein subunit (C5 protein). It is known that M1 RNA is solely responsible for the catalytic activity of the ribozyme complex (Kole *et al*., 1981). This catalytic activity of M1 RNA can be used for targeted degradation of RNAs *in vivo*. It was reported that the expression of hybrid RNA that consists of M1 RNA and a guide sequence which is complementary to the target mRNA efficiently represses target gene expression. The hybrid RNA binds the cellular target mRNA via its guide sequence and the catalytic M1 RNA cleaves the target. As a result, target specific gene expression inhibition is accomplished (Raj *et al*., 2003). However, a library-based phenotypic screening based upon the RNase P ribozyme has not yet been reported.

In this study, an RNase P ribozyme library was constructed which can be utilized as a tool for phenotype-based functional genomics studies in mammalian cells. The ribozyme library harbors random 18-mers as the guide sequence. Therefore, when the library is introduced into the cell, the ribozyme RNA expressed from each clone of the library binds a target mRNA complementary to the guide sequence, and successive mRNA cleavage can occur. After phenotypic screening, the plasmid encoding the ribozymes responsible for the phenotype can be retrieved and sequenced. By analyzing the nucleotide sequence of the guide sequence, the identity of mRNA targeted by the ribozyme can be revealed.

Construction of an RNase P ribozyme expression system

The first step to build an RNase P-based ribozyme library was development of a vector system for RNase P ribozyme expression. To this end, a plasmid named pSUK4 was made which can direct ribozyme synthesis in mammalian cells. pSUK4 is derived from the pSUPER plasmid, a vector optimized for short RNA expression in mammalian cells. It

*Corresponding author: E-mail dlee@postech.edu
Tel +82-54-279-2124, Fax +82-54-279-9985
Accepted 27 Feb 2007

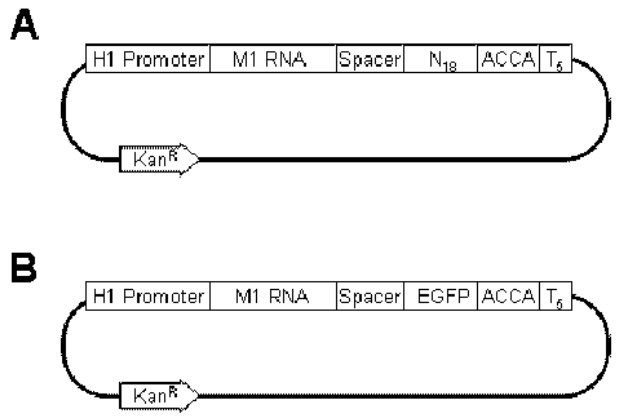

Fig. 1. Schematic drawing of an RNase P ribozyme expression vector. The hybrid RNA, which consists of M1RNA, spacer, and a guide sequence, is expressed by Pol III-driven HI-RNA promoter. The ACCA sequence is required for the M1 RNA activity, and the five consecutive thymidines (T5) are a termination signal of Pol III. (A) RNase P library vector. (B) EGFP targeting vector, pS/M1-EGFP.

uses the RNA polymerase III-driven H1 promoter for the expression of downstream RNA sequences (Brummelkamp *et al.*, 2002). The selection marker for ampicillin of pSUPER was replaced with kanamycin to facilitate the retrieval of ribozyme-encoding plasmid in the actual phenotype screening process, especially when reporter plasmid containing the ampicillin resistance marker is co-transfected with the ribozyme vector. The cloning strategy of the RNase P ribozyme unit is as follows. First, PCR was performed using an upstream primer including the *Bgl* II restriction site and the 5' end of the M1 RNA sequence and a downstream primer including the 3' end of the spacer sequence, the 18-mer guide sequence, and the *Sal* I restriction site. We used M1GS plasmid as a template which contains the M1 RNA coding sequence and the spacer sequence (5'-GAAGCTTGACCTGCAGGCATGCAAGCTTGGCGTAA TCATGGTCATAGCTGT -3', Trang *et al.*, 2003). (See below) Then the synthesized PCR product was cloned into the pSUK4 vector by using the *Bgl* II and the *Sal* I enzyme. The schematic diagram of the constructed RNase P ribozyme expression vector is shown in Fig. 1.

Validation of RNase P ribozyme expression system

To validate efficient suppression of a target gene using our RNase P ribozyme expression system, we designed and tested an EGFP (Enhanced Green Fluorescence Protein)-targeting RNase P ribozyme. First, we inspected the secondary structure of the EGFP mRNA predicted from the mFOLD program, and chose a region which was

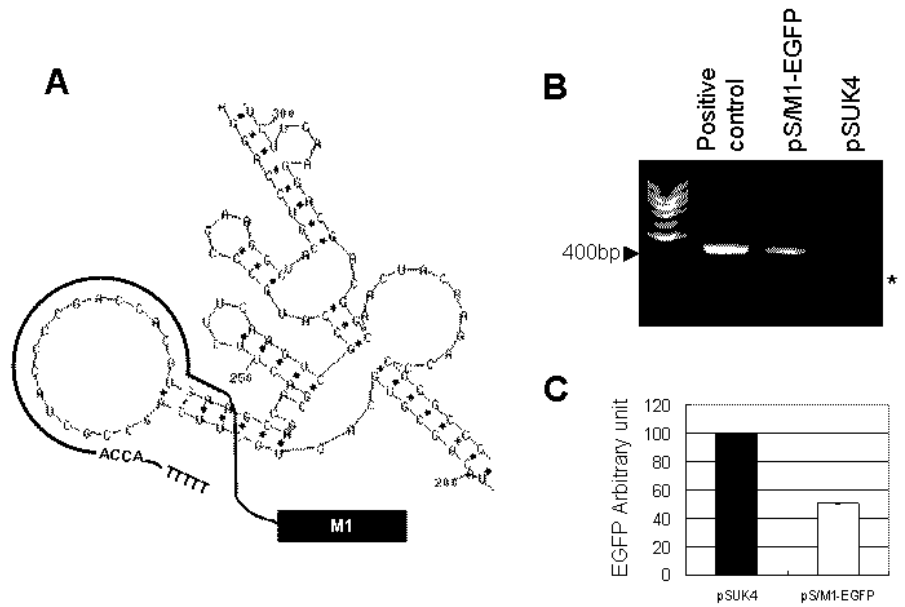

Fig. 2. Validation of RNase P ribozyme expression system. (A) Predicted secondary structure of EGFP mRNA target region. Secondary structure of EGFP RNA was predicted from the mFOLD program. M1-EGFP ribozyme RNA is expected to bind the selected target region as presented. (B) Expression of the RNase P ribozyme RNA. pS/M1-EGFP or empty pSUK4 vector was introduced into HEK293 cells and M1-EGFP RNA expression was detected by RT-PCR by using M1 RNA sequence specific primers. Marked by asterisk (*) is a non-specific band. (C) Reduction of EGFP mRNA level by the M1-EGFP ribozyme. EGFP mRNA level was quantified 48 hrs after pS/M1-EGFP or pSUK4 transfection. Values are means ±SD of duplicate experiment.

expected to be the most accessible (Fig 2A). We then designed a downstream primer EGFPDN (5'-CGCGC GGTCGACAAAAATGGTGCTACCCCGACCACATG AACAGCTATGACCATGATTACGCCAAGCTTGCAT-3'), which contains a guide sequence that can bind the target region of the EGFP mRNA. Next, we performed PCR using the M1UP primer (M1UP, 5'-CGCGCGAGATCTGA AGCTGACCAGACAGTCGC-3') and the EGFPDN primer, and the PCR product was inserted into pSUK4. The constructed vector, pS/M1-EGFP, was co-transfected with the EGFP expression vector into HEK293 cells. 48 hours after transfection, total RNA was extracted using the Trizol reagent (Invitrogen). The expression of the M1-EGFP RNA molecule was confirmed by RT-PCR (Fig. 2B), and the EGFP mRNA expression level was determined by quantitative real-time PCR analysis. We observed that the expression of EGFP mRNA was reduced to ~50% of the control experiment (Fig. 2C). Therefore, the pSUK4-based RNase P ribozyme expression system can repress target gene expression in mammalian cells.

Construction of an RNase P ribozyme library

To construct an RNase P ribozyme library with randomized guide sequence, we used a primer set of the upstream primer M1UP and a downstream primer Rd18DN (5'-CGCGCGGTCGACAAAAATGGTNNNNNN NNNNN NNNNNNNACAGCTATGACCATGATTACG CAAGCTT GCAT-3'). The Rd18DN primer has a random 18-mer guide sequence instead of a specific sequence. After ligation of these PCR products with the pSUK4 vector, the ligation mixture was transformed into *E. coli* by electroporation. A total of about 1.4×10^7 independent colonies was obtained, and plasmid DNA was prepared from the mixture of these colonies. Finally, we checked the random 18-mer sequence from 15 randomly picked colonies and found no bias in the nucleotide composition (Table 1).

In summary, we have developed a novel genomics tool that utilizes the catalytic subunit of RNase P. In this system, the M1 RNA sequence and a random guide sequence are expressed as a single RNA molecule. Via the recognition of target mRNA by the random guide sequence followed by mRNA cleavage by the catalytic M1 RNA, gene expression is blocked in a sequence-specific manner. Using EGFP mRNA as a model target, efficient target gene repression by RNase P ribozyme expression system was confirmed. Finally, we built an RNase P ribozyme library with randomness and complexity suitable for functional genomics studies. This library system should become a useful addition to the existing libraries for genome regulation (Lee *et al.*, 2003; Akashi *et al.*, 2005) for phenotype-based functional genomics studies, eventually to uncover novel gene functions.

Table 1. Sequencing result of the randomized guide sequence of the RNase P ribozyme library

	A	T	C	G	Sum
AGACAGCGCGTCCGGCT	3	2	6	6	17
TCGCTTTTCTGACCATTC	2	8	6	2	18
GCTAGTTCACAGCATTGT	4	6	4	4	18
CCGACCGGGTTACAGCTC	3	3	7	5	18
TTCGCTTAGAACATGGCC	4	5	5	4	18
GGGTGCGTTGCCTACTCC	1	5	6	6	18
AAGCGTATGACTGCAGTT	5	5	3	5	18
TGCCGGCCTTCACCGCCT	1	4	9	4	18
TGGACGTCACCTCGTGGC	2	4	6	6	18
AAGGCAGATAAAATATTT	9	5	1	3	18
TTTACCTGAGAGTCATAA	6	6	3	3	18
AATAATGAAAATCCTACC	9	4	4	1	18
CAAACCCAGTGTATTAGT	6	5	4	3	18
ATTCTGAATACCAAGACT	7	5	4	2	18
ATGCCAGATTTGTCTACC	4	6	5	3	18
Sum	66	73	73	57	269

Acknowledgement

This work was supported by a Korea Research Foundation Grant funded by the Korean Government (KRF-2005-003-C00114) to D.-k. L.

References

Akashi, H., Matsumoto, S., and Taira K. (2005). Gene discovery by ribozyme and siRNA libraries. *Nat. Rev. Mol. Cell. Biol.* 6, 413-422.

Beger, C. *et al*. (2001). Identification of Id4 as a regulator of BRCA1 expression by using a ribozyme-library-based inverse genomics approach. *Proc. Natl. Acad. Sci. USA* 98, 130-135.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550-553.

Kole, R. and Altman, S. (1981). Properties of purified ribonuclease P from *Escherichia coli*. *Biochemistry* 20, 1902-1906.

Lee, D.K., Seol, W., and Kim, J.S. (2003). Custom DNA-binding proteins and artificial transcription factors. *Curr.*

Top. Med. Chem. 3, 645-657.

Lee, D.K., Kim, Y., Kim, J.S., and Seol, W. (2004). Induction and characterization of a taxol-resistance phenotype with transiently expressed artificial transcription activator libraries. *Nucleic Acids Res.* 32, e116.

Li, Q. X., Robbins, J. M., Welch, P. J., Wong-Staal, F., and Barber, J. R. (2000). A novel functional genomics approach identifies mTERT as a suppressor of fibroblast transformation. *Nucleic Acids Res.* 28, 2605-2612.

Raj, S.M. and Liu, F. (2003). Engineering of RNase P ribozyme for gene-targeting applications. *Gene* 14, 59-69

Suyama, E., Kawasaki, H., Nakajima, M., and Taira, K. (2003). Identification of genes involved in cell invasion by using a library of randomized hybrid ribozymes. *Proc. Natl. Acad. Sci. USA* 100, 5616-5621.

Suyama, E. *et al.* (2004). Identification of metastasis-related genes in a mouse model using a library of randomized ribozymes. *J. Biol. Chem.* 279, 38083-38086.

Trang, P., Kim, K., Zhu, J., and Liu, F. (2003). Expression of an RNase P ribozyme against the mRNA encoding human cytomegalovirus protease inhibits viral capsid protein processing and growth. *J. Mol. Biol.* 328, 1123-1135.

Welch, P. J. *et al.* (2000). Identification and validation of a gene involved in anchorage-independent cell growth control using a library of randomized hairpin ribozymes. *Genomics* 66, 274-283.