Biochemical and Biophysical Research Communications 373 (2008) 48–52

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Selection of mRNA 5′-untranslated region sequence with high translation efficiency through ribosome display

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ARTICLE INFO

Article history:
Received 20 May 2008
Available online 9 June 2008

Keywords:
5′-Untranslated region
Ribosome display
In vitro translation

ABSTRACT

The 5′-untranslated region (5′-UTR) of mRNAs functions as a translation enhancer, promoting translation efficiency. Many in vitro translation systems exhibit a reduced efficiency in protein translation due to decreased translation initiation. The use of a 5′-UTR sequence with high translation efficiency greatly enhances protein production in these systems. In this study, we have developed an in vitro selection system that favors 5′-UTRs with high translation efficiency using a ribosome display technique. A 5′-UTR random library, comprised of 5′-UTRs tagged with a His-tag and *Renilla* luciferase (R-luc) fusion, were in vitro translated in rabbit reticulocytes. By limiting the translation period, only mRNAs with high translation efficiency were translated. During translation, mRNA, ribosome and translated R-luc with His-tag formed ternary complexes. They were collected with translated His-tag using Ni-particles. Extracted mRNA from ternary complex was amplified using RT-PCR and sequenced. Finally, 5′-UTR with high translation efficiency was obtained from random 5′-UTR library.

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In vitro translation systems provide a powerful tool for protein research. These systems commonly use lysates from *Escherichia coli*, wheat germ or rabbit reticulocytes, which include essential molecules necessary for translation with the exception of template RNA. Compared to in vivo expression methods, in vitro translation systems can express proteins rapidly. This is suitable for a myriad of applications including the synthesis of biologically important proteins. However, the amount of protein produced may be limited by many factors [1–3], including starvation of substrate molecules and degradation of template RNA. Two methods, continuous-flow and continuous-exchange, overcome these problems by supplying substrates continuously and removing by-products inhibit translation efficiency [4]. However, these methods extend the reaction time to increase the amount of synthetic protein and require excessive amounts of reagents as well as specific equipment. For these reasons, we have created an alternative in vitro translation method to increase protein synthesis by utilizing the 5′-UTR of mRNA.

In living cells, the 5′-UTR of mRNA regulates translational efficiency [5]. To increase protein synthesis of eukaryotic in vitro translation, a translation enhancer sequence is frequently added to the RNA templates. For example, the 5′-leader sequence (omega) of tobacco mosaic virus (TMV) has been incorporated into the wheat germ in vitro translation system [6–8]. Addition of this sequence increases the translational expression of reporter genes by 2- to 10-fold. Similarly, the Kozak consensus sequence (ACCAAUGG) in addition to translation enhancer sequences, such as the 5′-UTR of *Xenopus* β-globin, are essential for efficient in vitro translation initiation in rabbit reticulocyte lysate, the most widely used in vitro translation system [9–11]. Addition of these translational enhancer sequences leads to an increase in protein expression by 10- to 300-fold as compared to other endogenous UTR sequences.

A method to select sequences with high translation efficiency has been recently developed using the eukaryotic in vitro translation system [12,13]. In these studies, the translation efficiency is defined as the number of recruited ribosomes to an RNA template, which affects the amount of polyribosomal RNAs that are fractionated by sucrose density gradient centrifugation. Repetition of this polysome-mediated selection cycle enriches for higher translation efficiency RNAs. From a random library of mRNA 5′-UTRs, several sequences that are as efficient as the omega sequence from TMV have been identified by polysome-mediated selection in a wheat embryo in vitro translation system [12]. Additional studies show similar results [13].

In this study, we have developed a novel selection method by using ribosome display, a technique by which the ternary ribosomal complexes, consisting of mRNA, ribosome, and nascent proteins, are formed in vitro. These ternary complexes are selected by the activity of the translated proteins. This method has been successfully utilized by our laboratory to identify and subsequently study the function of various proteins, such as enzymes and

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0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.05.173

DNA-binding proteins [14–16]. The strategy of our selection system is shown in Fig. 1. Our modified method differs from the general ribosome display method in that the translation time is shortened. This favors the identification of 5′-UTRs with higher translation efficiency. In order to determine the effectiveness of this modified method, we tested whether 5′-UTRs with high translation efficiency sequences could be selected from mixtures containing competitive sequences. In addition, we utilized this method to select 5′-UTRs with high translation efficiency sequences from a random 5′-UTR library.

Materials and methods

Construction of plasmids and random library. The plasmids, pBS-UTK68-6HisRLuc (UTK) and pBS-UTK26-BS42-6HisRLuc (UTK-BS), were constructed as follows. *Renilla* luciferase (R-luc) fragment was amplified by PCR from pRL-CMV vector (TOYO-ink) using primer sets 6HisRluc_fwd (AAAAGATCTAAACCATGGAACATCATCACCA TCATCACAGCGCTGGTGGAATGACTTCGAAAGTTTATG) and Rluc_rev (TTTTCCTTTTGCGGCCGCTAGTACTGTTCATTTTTGAGAAC). Amplified Rluc fragment with His-tag was digested with NotI and BglII, and then inserted into pBS5LAlucPS which has constructed in our laboratory previously. To construct pBS-UTK68-6HisRluc, resulting plasmid pBS-6HisRluc was digested with BglII and Aor51 HI, followed by insertion of synthesized fragment (UTK68_fwd; GATCTA ATACGACTCACTATAGGGAATACAAGCTTGCTTGTTCTTTTTGCAGAAG CTCAGAATAAACGCTCAACTTTGGCAGATCTAC and UTK68_rev; CAT GGTAGATCTGCCAAAGTTGAGCGTTTATTCTGAGCTTCTGCAAAAAGA ACAAGCAAGCTTGTATTCCCTATAGTGAGTCGTATTA). UTK68 sequence was chimeric sequence of 5′-UTR of *Xenopus* β-globin mRNA and optimal initiation sequence [11]. As same as pBS-UTK68-6HisRluc, pBS-BS-6HisRluc was constructed by insertion of synthesized BS fragment (BS68_fwd; GATCTAATACGACTCACTATAGGGCG AATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTG ATATCGAATTCCTC and BS68_rev; CATGGAGGAATTCGATATCAAGC TTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCC TATAGTGAGTCGTATTA). BS68 sequence was derived from pBluescript II sk(+) located under the T7 promoter. To construct pBS-UTK26-BS42-6HisRLuc (UTK-BS), pBS-BS-6HisRluc was digested with BglII and XhoI followed by insertion of synthesized oligonucleotide which encode upstream of UTK (26 bp) and T7 promoter (BS-UTR-s; GATCTAATACGACTCACTATAGGGAATACAAGCTTGCTTG TTCTCCC and BS-UTR-a; TCGAGGGAGAACAAGCAAGCTTGTATTCCC TATAGTGAGTCGTATTA).

Random library construction. Random library was constructed by PCR using primer sets UTR-rand 2 (AGATCTAATACGACTCACTATAG GGAATACAAGCTTGCTTGTTCTNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNACCATGGAACATCATCAC) and Rev2 Xba (GGCCGCTCTAGAACTGTTCATTTTTGAGAACTCGC). UTR-rand 2 was including 42 bp of random sequence between T7 promoter and His-tag sequences. For PCR reaction, pBS-UTK68-6HisRluc was used as a template. After construction, sequences of random library were analyzed. Random library fragments were cloned into pBluscript II after digestion with HindIII and XhoI. Finally, each sequence was analyzed by using CEQ2000XL (Beckman Coulter).

In vitro transcription. For the first cycle of the competitive experiment, the constructed plasmids were linearized by digestion with ScaI which is inserted before the stop codon. For additional cycles of the competitive experiment and all cycles of the random library experiment, 1 μg of amplified cDNA fragments was used. Template RNA was transcribed from these DNA templates. mRNA transcripts were produced in vitro using the MEGAscript T7 kit (Ambion) in the presence of m7G(5′)ppp(5′)G (CAP) as described by the manufacturer. RNA transcripts were purified by phenol/chloroform extraction and ethanol precipitation and quantified using UV spectroscopy.

In vitro translation and selection cycles. In vitro translation was performed using a Flexi rabbit reticulocyte lysate system (Promega). 1.5–3.0 μg of template RNA was added to rabbit reticulocyte lysates containing 50 mM KCl, 2 mM DTT, 20 μM Complete Amino Acid Mixture (promega), and 0.8 U/μl RNasin Ribonuclease inhibitor (Promega). A total volume of 30 μl from this reaction mixture was incubated for 12–60 min at 30 °C. To stop the translation reac-

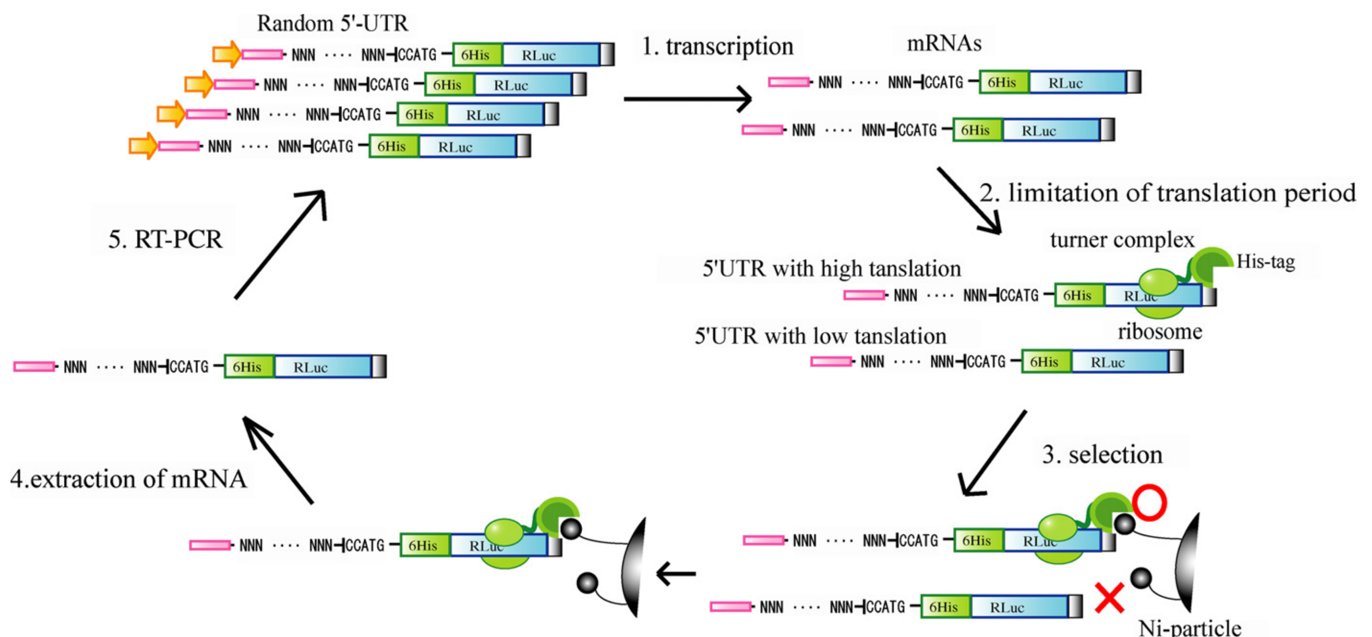

Fig. 1. Schematic drawing of the selection system using ribosome display. (1) A random 5′-UTR DNA library was used, 5′-UTR sequences followed by a His-tag and the *Renilla* luciferase gene were transcribed to mRNA. (2) Transcribed mRNAs were translated in rabbit reticulosyte lysates. Ternary complexes, containing ribosomes, translated protein, and mRNA progenitors, were made. Translation periods were limited to select for 5′-UTR sequences with high translation activity. (3) Ternary complexes expressing the His-tag were selected by Ni-particles. (4) mRNAs were extracted from selected ternary complexes. (5) Selected mRNAs were pooled and used as templates for RT-PCR.

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