Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 352 (2007) 372–377
BBRC
www.elsevier.com/locate/ybbrc

Nascent chain, mRNA, and ribosome complexes generated by a pure translation system

Tomoaki Matsuura [a,d], Hayato Yanagida [b], Junya Ushioda [c], Itaru Urabe [a], Tetsuya Yomo [b,c,e,*]

[a] *Department of Biotechnology, Graduate School of Engineering, Osaka University, Japan*
[b] *Graduate School of Frontier Biosciences, Osaka University, Japan*
[c] *Department of Bioinformatics Engineering, Graduate School of Information Science and Technology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-9871, Japan*
[d] *Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), Japan*
[e] *Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency (JST), Japan*

Received 1 November 2006
Available online 17 November 2006

Abstract

Ribosome display is based on the concept that ternary complexes consisting of a nascent chain, ribosome, and mRNA can be generated, thereby establishing the linkage between genotype and phenotype that is essential for evolutionary experiments. With cell extract-based *in vitro* translation systems, it has been shown that ternary complexes can be generated by omitting the termination codon from the constructs, which can be stabilized at low temperature in the presence of high Mg^{2+} concentrations. Using an *Escherichia coli*-based reconstituted *in vitro* translation system (PURE system), in which all components necessary for the translation reaction were highly purified and reconstituted, ternary complexes could be generated equally well with a variety of sequences at the 3′ end of the RNA, even those with a termination codon. Moreover, the generated complexes were stable at temperatures between 4 and 50 °C, and are thus highly stable in contrast to previous assumptions.
© 2006 Elsevier Inc. All rights reserved.

Keywords: *In vitro* translation; Ribosome display; Directed evolution; PURE system

Ribosome display is based on the generation of ternary complexes consisting of a nascent chain, ribosome, and mRNA, thereby establishing the linkage between genotype and phenotype necessary for evolutionary experiments [1]. Various *in vitro* translation systems, including those derived from *Escherichia coli* [2], rabbit reticulocytes [3], and wheatgerm [4], have been used for ribosome display experiments. Using these systems, it has been shown that ternary complexes can be generated by omitting the termination codon from constructs that can be stabilized at low temperature in the presence of high concentrations of Mg^{2+}.

Here, we investigated the differences in quantity and stability of the ribosome complexes generated using an *E. coli*-based reconstituted *in vitro* translation system (PURE system) [5], in which all components necessary for translation are highly purified and reconstituted to carry out the reaction. All the components as well as their concentrations in this system have been identified, and it is therefore possible to exclude, add, or optimize the concentrations of components to verify their effects on the translation system and production of ternary complexes (see the accompanying paper for examples with displaying antibodies [6]). This provides an opportunity to observe the pure properties of the translation machinery under conditions of very low RNase activity and in the absence of the ribosome rescue machinery.

* Corresponding author. Fax: +81 6 6879 7428.
E-mail address: yomo@ist.osaka-u.ac.jp (T. Yomo).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.050

We found that ribosome display experiments could be performed equally well with a variety of sequences at the 3′ end of the RNA, even those with a termination codon. Moreover, the presence of release factors had no influence on the performance of the experiments. The ternary complexes prepared with the PURE system are stable at temperatures between 4 and 50 °C, and are thus highly stable in contrast to previous assumptions. These finding expand the usage of ribosome display for altering and evolving protein functions.

Materials and methods

DNA and RNA preparation. The plasmid pRD-ww, part of which is shown schematically in Fig. 1, is a derivative of pQE30 (Qiagen, Hilden, Germany). pRD-ww was constructed by cloning the gene encoding the ww domain of human Yes protein-associated protein [7] between the *Nco*I and *Bam*HI sites of pRD-n1n2 [8]. pRD-zif268 was prepared by replacing the ww domain of the plasmid pRD-ww with the gene encoding DNA binding protein zif268 [9] using *Nco*I and *Bam*HI sites.

DNA used for all *in vitro* translation reactions was prepared by PCR using the high proofreading polymerase PYRObest polymerase (Takara Bio, Tokyo, Japan). RNA was prepared essentially as described previously [8]. Details regarding the primers used for preparation of each DNA construct and the methods used for RNA preparation are given in Supplementary data.

In vitro translation. All *in vitro* translation reactions were carried out using a reconstituted *in vitro* translation system (PURE system: Post Genome Institute, Tokyo, Japan) developed by Shimizu et al. [5]. Unless otherwise stated, translation was carried out using PURE system classic-II, which includes all components reported previously [5]. When RFs were absent, RF1 and RF2 were omitted from the translation reaction, and when present RF1 and RF2 were added to final concentrations of 10 and 20 μg/ml, respectively. All protein synthesis reactions using the reconstituted *in vitro* translation system were carried out at 37 °C with 0.5 μg of the PCR product or 2 μg of RNA in a reaction volume of 50 μL.

For real-time detection of the protein synthesis reaction, 5 μM FlAsH (Invitrogen, San Diego, CA) [10], fluorescein derivative with two As(III) substituents that fluoresces only after the arsenics binds to the tetracysteine tag (CCPGCC), and 0.5 μM ROX (Invitrogen), a glycine conjugate of 5-carboxy-X-rhodamine, and succinimidyl ester were added to the *in vitro* translation system. Note that FlAsH was first mixed with an equal volume of 1 M dithiothreitol (DTT) and incubated for 15 min at 4 °C prior to addition to the *in vitro* translation system. ROX was used as an internal dye to normalize the differences in fluorescence intensity among the wells. Real-time detection was carried out using a real-time PCR system (Mx3005P; Stratagene, La Jolla, CA).

For labeling of the total synthesized products, 0.5 μL BODIPY-labeled Lys-tRNA (Promega, Madison, WI) was added to a 50-μL volume of the *in vitro* translation system. The samples were treated according to the manufacturer's instructions prior to SDS–PAGE, and fluorescence images of the gel were obtained using a Typhoon9210 (Amersham Biosciences, Piscataway, NJ).

Ribosome display. *In vitro* translation was carried out as described in the previous section. After a 30-min incubation at 37 °C, the reaction was stopped by adding 400 μL of ice-cold wbkt buffer (50 mM Tris–OAc, pH 7.4, 150 mM NaCl, 250 mM KCl, 50 mM MgOAc, and 0.1% Tween 20) supplemented with 0.5% BSA (Sigma, St. Louis, MO), and 2.5 mg/mL heparin (Sigma) to the 50-μL translation reaction mixture, and then placed immediately at 4 °C. The following panning procedures were carried out using an automatic magnetic bead separation apparatus (KingFisher; Thermo Electron Corporation, Waltham, MA) at the temperatures described in the text.

Recovery of the ribosome complexes *via* the ww domain was carried out by adding 0.5 μM biotinylated PY ligand (biotin-EYPPYPPPPYPSG) to the terminated reaction mix. Biotinylated PY ligand was prepared by labeling the N-terminal amine of the PY ligand with Sulfo-NHS-SS-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions, and purified by HPLC. Hence, PY-ligand can be cleaved from the biotin moiety under reducing conditions. After shaking for 1 h, aliquots of 50 μL of streptavidin-coated magnetic beads (Dynal, Oslo, Norway) were added and shaken for a further 30 min. Subsequently, the beads were washed five times with wbkt buffer, with 1 and 5 min of shaking between the first two and between the last three washes, respectively. Finally, RNA was recovered by shaking the beads in wbkt buffer supplemented with 50 mM DTT, which cleaves the S—S bond between the biotin moiety and PY ligand (see below), for 30 min.

Recovery of the ribosome complexes *via* the zif268 DNA binding protein was carried out essentially as with the ww domain, except that the ligand used was 0.5 μM biotinylated double-stranded DNA (prepared by annealing the oligonucleotide 5′-biotin-GCGGGCTATAGCGTGGGCGTACGAATT with its complementary strand; zif268 recognition sequence is underlined), termed T2 DNA, which is known to bind zif268 [9]. In addition to the difference in ligand, the wbkt buffer was replaced with wbt buffer (50 mM Tris–OAc, pH 7.4, 150 mM NaCl, 5 mM MgOAc, 0.1% Tween 20, 50 μM $ZnCl_2$, and 5 mM DTT). Elution of the RNA was carried out by shaking the beads in elution buffer (50 mM Tris–OAc, pH 7.4, 150 mM NaCl, 250 mM KCl, and 50 mM EDTA) [2].

RT-PCR and quantitative RT-PCR were carried out using a One-step RT-PCR kit (Qiagen) and One-step quantitative PCR kit (Invitrogen), respectively, according to the manufacturer's instructions. More details are given in the Supplementary Materials.

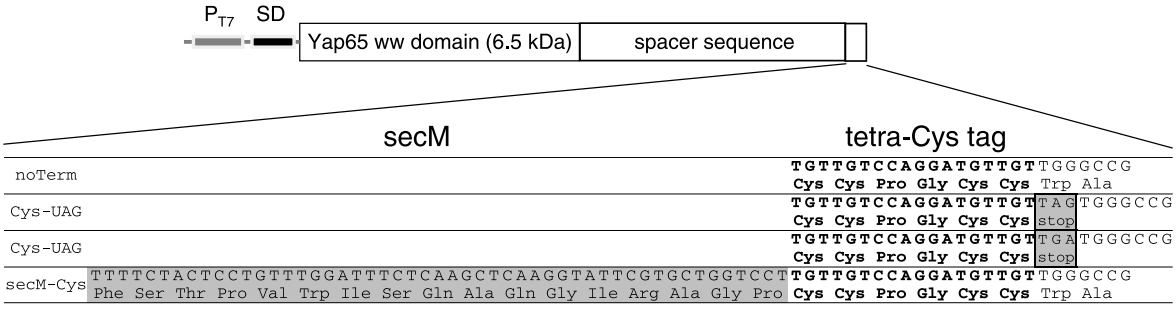

Fig. 1. Schematic representation of the constructs used for ribosome display experiments. DNA and amino acid sequences of the 3′ end are shown. All constructs carried the T7 promoter (P_{T7}) and Shine–Dalgarno sequence (SD) at the 5′ end, and the sequence encoding the Yes-associated ww domain (Yap65) followed by a spacer sequence (protein D, a phage Lambda capsid protein). A tetra-Cys tag (CCPGCC), shown in bold, was introduced at the 3′ end of the sequence. noTerm contained no termination codons in the entire sequence. Cys-UGA and Cys-UAG possessed termination codons (shown in gray), UGA and UAG, respectively, at the 3′ end of the sequence. secM-Cys encoded the secM sequence, shown in gray, upstream of the tetra-Cys tag (secM-Cys).

Download English Version:

https://daneshyari.com/en/article/10767568

Download Persian Version:

https://daneshyari.com/article/10767568

Daneshyari.com