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Evolution of Translation Initiation Sequences Using In Vitro Yeast Ribosome Display

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ABSTRACT: We report a novel in vitro yeast ribosome display method based on cell-free protein synthesis (CFPS) using linear DNA templates. We demonstrate that our platform can enrich a target gene from a model library by 100-fold per round of selection. We demonstrate the utility of our approach by evolving cap-independent translation initiation (CITI) sequences, which result in a 13-fold increase in CFPS yields after four rounds of selection, and a threefold further increase by placing the beneficial short sequences in tandem. We also show that 12 of the selected CITI sequences permit precise control of gene expression in vitro over a range of up to 80-fold by enhancing translation (and not as cryptic promoters). These 12 sequences are then shown to tune protein expression in vivo, though likely due to a different mechanism. Looking forward, yeast ribosome display holds promise for evolving libraries of proteins and DNA regulatory parts for protein engineering and synthetic biology.

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KEYWORDS: ribosome display; yeast cell-free protein synthesis; cap-independent translation initiation; directed evolution; synthetic biology

Introduction

The ability to regulate gene expression is critical to bioengineering applications. Over the last few decades, many different regulatory strategies have been used to control the expression of toxic proteins in hosts, to maximize yield of recombinant proteins, and to tune the expression of key enzymes in metabolic pathways in order to balance the flux of metabolites (Farmer and Liao, 2000; Jones et al., 2000; Kim and Keasling, 2001). Recently, rapid progress in the field of synthetic biology has enabled de novo metabolic pathway design, genetic circuit construction, and artificial genome synthesis (Anesiadis et al., 2013; Bond-Watts et al., 2011; Dueber et al., 2009; Gibson et al., 2010). Several groups have also developed efficient tools to achieve accurate regulation of gene expression in *Escherichia coli* via multiplex automated genome engineering (MAGE) (Wang et al., 2009, 2012), promoter engineering (Alper et al., 2005), and ribosome-binding site (RBS) engineering (Salis et al., 2009), among others.

However, less progress has been made in regulating protein production in eukaryotic cells, mainly due to the complexity of regulatory mechanisms involved in transcription and translation. Translation initiation in eukaryotes most commonly follows a cap-dependent mechanism where the 43S preinitiation ribosomal complex is recruited to a 7-methylguanosine cap at the 5′-end of mRNA (5′ cap) via initiation factor eIF4F (Jackson et al., 2010; Sonenberg and Hinnebusch, 2009) and many other trans-factors. Alternatively, a number of native and viral genes are known to undergo cap-independent translation initiation (CITI) (Craig et al., 1992; Gallie and Walbot, 1992; Kozak, 1986; Sawasaki et al., 2000; Suzuki et al., 2006). In these cases, the 5′-end untranslated region (5′-UTR) can directly recruit initiation factors or bind to the small ribosomal subunit via an internal ribosome entry site (IRES) (Chappell et al., 2006; Fitzgerald and Semler, 2009; Iizuka et al., 1994; Macejak and Sarnow, 1991). Compared to the cap-dependent ribosome-scanning mechanism, CITI- and IRES-mediated translation provide an ideal opportunity for engineers to tune target gene expression because the RNA structure in the 5′-UTR is simpler to manipulate than the dozens of trans-factors involved in

Conflict of interest: None declared.

Abbreviations: 5′-UTR, 5′-end untranslated region; CFPS, cell-free protein synthesis; CITI, cap-independent translation initiation; GFP, green fluorescence protein; IRES, internal ribosome entry site; PRM, protein-ribosome-mRNA; RRL, rabbit reticulocyte lysate; TMV, tobacco mosaic virus; Tx/Tl, transcription and translation.

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cap-dependent translation. Previous research has shown that the 5′-UTR can act as a cis-regulatory factor with activity influenced by element length (Lin and Li, 2012), nucleotide content (Dorokhov et al., 2002), secondary structure (Xia and Holcik, 2009), and microORF content (Hinnebusch, 1997; Hood et al., 2009; Kochetov, 2008). In one example, computational models have captured the effects of the above factors to influence approximate 70% of the variability of gene expression in a certain environment in yeast (Dvir et al., 2013).

However, rapidly discovering 5′-UTRs for engineering efforts remains a challenge. A prominent bottleneck is the complexity that exists between changes at the sequence level and the resulting impacts on gene expression. For example, Crook et al. demonstrated that multicloning sites (MCSs) in expression vectors near the 5′-UTR can strongly influence gene expression (Crook et al., 2011). In another example, Zhou et al. selected tens of active IRES sequences from an 18-nt randomized 5′-UTR library; yet, most of these IRES sequences were only complementary to yeast 18S rRNA (Zhou et al., 2003). Another bottleneck includes limits imposed by the throughput and speed of constructing, validating, and prototyping large 5′-UTR libraries. Although several in vivo-based evolution strategies have been reported for selecting gene expression regulatory elements (Curran et al., 2014; Dvir et al., 2013; Redden and Alper, 2015; Zhou et al., 2003), all of these strategies are still dependent on various time-consuming manipulations including plasmid construction and cell cultivation. Moreover, the library size is limited by transformation efficiency. Such limitations motivate the need for new approaches for designing and prototyping DNA regulatory elements.

In the last decade, a technical renaissance has revitalized cell-free protein synthesis (CFPS) systems for use in high-throughput protein expression without time-consuming and laborious cloning steps (Brodel et al., 2014; Hodgman and Jewett, 2013; Hong et al., 2014a,b, 2015; Iizuka et al., 1994; Kamura et al., 2005; Kozak, 1986; Mureev et al., 2009; Sawasaki et al., 2000; Schoborg et al., 2013; Suzuki et al., 2006). In addition, recent efforts suggest the possibility of using cell-free environments for rapid prototyping (Sun et al., 2014). For example, in pioneering work, Murray, Noireaux, Lucks, Doktycz, and their colleagues have used cell-free systems to rapidly characterize DNA- and/or RNA-based genetic circuits (Chappell et al., 2015; Karig et al., 2012; Karzbrun et al., 2011; Shin and Noireaux, 2012; Takahashi et al., 2014). In another groundbreaking example, Freemont and colleagues used an *E. coli* based CFPS system to compare the activity of promoters in vitro with their in vivo functions, and enumerated a number of specific advantages that in vitro methods afford for identifying DNA sequence elements (Chappell et al., 2013). These works open the possibility of developing similar in vitro methods in eukaryotic CFPS systems based on existing platforms (Gan and Jewett, 2014; Hodgman and Jewett, 2013; Sawasaki et al., 2000, 2002; Schoborg et al., 2013; Suzuki et al., 2006) that are capable of rapid selection and characterization of eukaryotic regulatory parts.

Here, we aimed to develop a cell-free approach for library selection based on a combined transcription/translation (Tx/Tl) CFPS system in *Saccharomyces cerevisiae* (Fig. 1). The goal was to develop a yeast ribosome display system. Development and application of this technology involved three steps. First, we established and validated a yeast ribosome display method for capturing beneficial DNA regulatory sequences. We chose to use ribosome display because it is a powerful cell-free strategy for the in vitro selection of proteins and peptides from large genetic libraries. As in the ribosome display methods established in *E. coli* CFPS by Plückthun and colleagues (Hanes and Plückthun, 1997; Mattheakis et al., 1994; Zahnd et al., 2007) and in rabbit reticulocyte lysates (RRL) by He and Taussig (Douthwaite et al., 2006; He and Taussig, 1997, 2007), the ribosome stalls at the end of an experimental mRNA due to the absence of a stop codon to form a ternary protein-ribosome-mRNA (PRM) complex that can be specifically enriched and analyzed to link genotype to phenotype. While ribosome display has been mainly used to evolve proteins that can bind to a ligand (e.g., antibody fragments), we hypothesized that it could be applied to the selection of beneficial 5′-UTR sequences as well. Second, after we established a yeast ribosome display method, we applied this technique to the selection of several active CITI sequences from a randomized 15-nt library. We showed that these sequences have utility for improved protein production in CFPS by enhancing translation efficiency. Third, we selected a small library of 5′-UTR sequences and demonstrated that these sequences could permit a range of expression values. In sum, our work yielded a yeast ribosome display system based on PCR templates that is able to rapidly evolve and identify genetic elements in vitro prior to putting them into a host. Our work has implications for bioengineering, protein engineering, and synthetic biology projects.

Materials and Methods

Reagents and Buffers

Chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless designated otherwise. DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs (NEB, Ipswich, MA). T7 polymerase was prepared as described previously (Kwon and Jewett, 2015). Plasmids were extracted using Plasmid Miniprep Kit (Omega Bio-Tek, Norcross, GA). All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa) (Supplementary Table S1). Buffer 5 × B&W contains 250 mM Tris acetate (pH 7.5), 750 mM NaCl, 250 mM magnesium acetate, 2.5% (w/v) Tween20. Beads blocking buffer contains 1 × B&W buffer and 5% bovine serum albumin (Sigma). Phosphate-buffered saline (PBS) contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, and 2 mM KH_2PO4.

Cloning and Construction

The plasmid pRDV was kindly provided by Plückthun (Zahnd et al., 2007). The Ω sequence from tobacco mosaic virus was inserted in front of initial codon to replace the *E. coli* ribosome-binding sequence via the primers Omega-f and Omega-r. A 3 × FLAG tag (Sigma–Aldrich) fused in-frame to the spacer sequence *tolA* (52) (Supplementary Fig. S1) was inserted into the N-terminus of the protein-coding region to replace the 6xHis tag in pRDV via the primers Flag3-f and Flag3-r. The resulting plasmid was designated as pRDVOme-3F. In the negative control plasmid pRDVOme, the

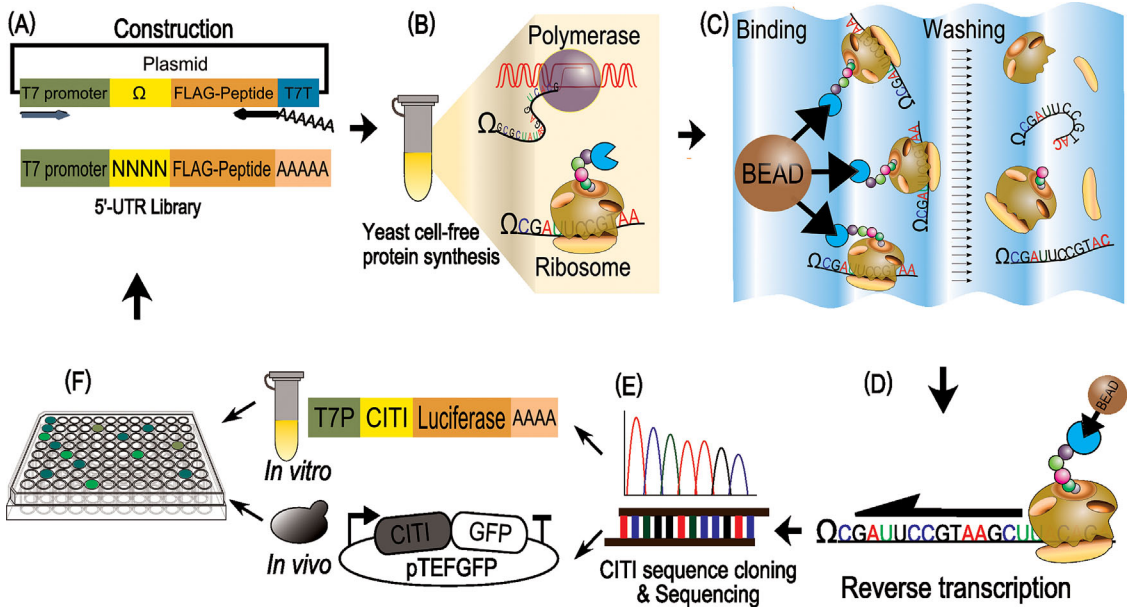

Figure 1. Schematic diagram of yeast ribosome display procedure. **A**: DNA templates are prepared for combined transcription/translation (Tx/Tl) yeast CFPS that include a T7 promoter; a cap-independent translation initiation sequence, the 3X FLAG tag, an amino acid spacer, and the T7T (T7 terminator). Linear templates for ribosome display are then prepared by amplifying the expressional cassette with a poly(A)$_{50}$ tail. **B**: DNA templates are used in a combined Tx/Tl reaction. The ribosome stalls at the end of the mRNA due to the absence of a stop codon to form protein-ribosome-mRNA (PRM) complexes. **C**: PRM complexes are captured by ANTI-FLAG® M2 Magnetic Beads. Untranslated or unbound components are washed off. **D**: The PRM complexes bound to beads are collected on magnetic beads and the mRNA is reverse transcribed into cDNA in situ. **E**: Sequence information of selected mutants is analyzed. Then, a T7 promoter and poly(A)$_{50}$ tail are added to restore full-length DNA template for the next round selection. **F**: The activity of selected mutants are identified in vivo and in vitro, respectively.

3xFLAG tag was deleted from pRDVOme-3F via the primers Nohis-f and Nohis-r, moreover, a 99-nt fragment was deleted from the *tolA* protein-coding region via the primers Del99-f and DisR-1 (Fig. 2A).

In Vitro Transcription/Translation for Yeast Cell-Free Protein Synthesis

Yeast cell-free extract was prepared according to previous publications (Gan and Jewett, 2014; Hodgman and Jewett, 2013). Tx/Tl CFPS reactions were prepared on ice from stock solutions adjusted to the following working concentrations: 25 mM HEPES-KOH pH 7.4, 120 mM potassium glutamate, 6 mM magnesium glutamate, 1.5 mM ATP, 2 mM of each GTP, CTP, and UTP, 0.1 mM of each of 20 amino acids, 25 mM creatine phosphate, 2 mM DTT, 0.27 mg/mL creatine phosphokinase (C3755-1KU, Sigma), 200 U/mL RNase Inhibitor (Qiagen), 27 μg/mL T7 RNA Polymerase, and 50% (v/v) S60 yeast extract. Reactions were performed for 120 min at 24°C unless specified otherwise.

Non-capping in vitro transcription reactions were prepared as described in previous publications (Gan and Jewett, 2014; Mureev et al., 2009). The mRNA-directed cell-free translation-only reactions were performed in 15 μL reactions at 24°C for 1 h with 20 nM template mRNA. The cell-free reaction mixture was assembled on ice from stock solutions to the following working concentrations: 25 mM HEPES-KOH pH 7.4, 120 mM potassium glutamate (unless otherwise noted), 1 mM magnesium glutamate, 1.5 mM adenosine triphosphate (ATP), 0.2 mM guanosine triphosphate (GTP), 0.1 mM of each of 20 amino acids, 25 mM creatine phosphate, 2 mM DTT, 0.27 mg/mL creatine phosphokinase (Sigma), 200 U/mL RNase Inhibitor (Qiagen), and 50% (v/v) S60 yeast extract.

Library Construction and Selection

To create a model library for method validation, the positive and negative DNA templates were amplified from plasmid pRDVOme-3F and pRDVOme, respectively, using the primers DisF-1 and RibDpA50 (Fig. 2A; Supplementary Table S1). The PCR products were purified by Cycle Pure Kit (Omega Bio-Tek, Norcross, GA), and quantified using Nanodrop 2000c spectrophotometer (Thermo scientific). Mixing the two templates with molar ratios of 1:10, 1:100, and 1:1000, respectively, created three model libraries.

The 5′-UTR plasmid library was created by randomizing the 15 nucleotides (nts) upstream of the initial FLAG-tolA-coding region as D (D: A, T, or G), with the first 24 nucleotides of Ω sequence used as a spacer for PCR amplification. By inverse PCR, the full length of the plasmid pRDVOme-3F except the wild type Ω sequence was amplified using the primers Lib3-f and Lib3-r, with the 15-nt randomized sequences introduced via the 5′-end tail of the primer. The linear PCR product was self-ligated to create the library pRDVLib, which bears the 15D-randomized sequences. (Supplementary Fig. S2A).

DNA libraries were amplified as linear templates by use of the primers DisF-1 and RibDpA50-r without a stop codon and delivered into yeast Tx/Tl CFPS. The TX/Tl CFPS reactions were stopped by adding equal volume of 1× B&W buffer and 30 μL of

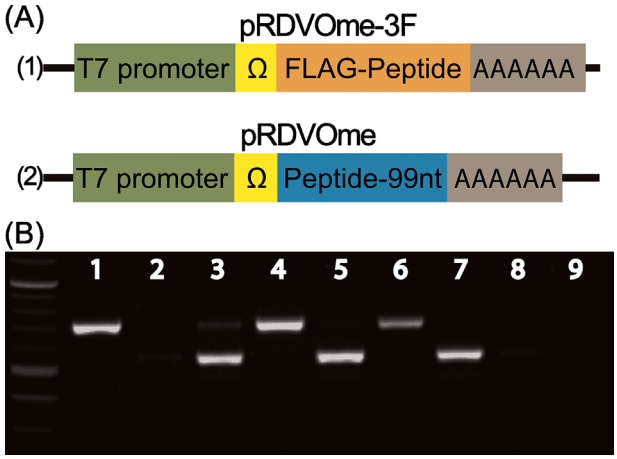

Figure 2. Model library enrichment validates yeast ribosome display. **A**: Template construction of model library. Positive template (1) contains a T7 promoter, Ω sequence, N-terminal 3XFLAG tag, a peptide-coding region, and the T7 terminator. Negative template (2) contains all positive template components except for the N-terminal 3X FLAG tag. The peptide-coding region is 99-nucleotides shorter than that in the positive template for ease of resolution on gel. **B**: Single-round enrichment rate using model libraries is shown. Mixing the positive and negative DNA template with molar ratios of 1:10, 1:100, and 1:1000 created a serial dilution of model libraries. Captured mRNA after ribosome display was converted to cDNA, which was resolved on an agarose gel. Lane 1: cDNA from only positive template after selection; lane 2: cDNA from only negative template after selection; lane 3: cDNA 1:10 library before selection; lane 4: cDNA from 1:10 library after selection; lane 5 cDNA 1:100 library before selection; lane 6: cDNA 1:100 library after selection; lane 7: cDNA 1:1000 library before selection; lane 8: cDNA from 1:1000 library after selection; lane 9: cDNA from no reverse transcription sample as a control.

ANTI-FLAG® M2 Magnetic Beads (Sigma) (washed with 200 μL of 1× PBS buffer once before use). The mixture was then rotated at 4°C for 2 h allowing the anti-FLAG antibody to capture the FLAG-tagged PRM complex. The beads were washed with 200 μL of 1× B&W buffer twice to remove non-bound PRM. Then, the beads were resuspended in total 50 μL of solution containing 1 μL of RNase-free DNaseI (NEB), 5 mM magnesium acetate, and 1× DNase buffer at 37°C for 15 min to digest DNA templates of CFPS. After that, the beads were washed twice with 1× B&W buffer and once with 200 μL of 0.5× PBS buffer. Finally, the mRNA of PRM was in situ reverse transcribed on beads and amplified directly into cDNA by use of SuperScript™ III RT/ Platinum® Taq (Invitrogen, Carlsbad, CA) via the primer RT5-f and RibD-r. The cDNA fragment was purified by gel extraction kit (Omega Bio-Tek) and restored to full-length template by adding T7 Promoter and polyA50 tail by use of the primers Lib2URF and RibD2pA50 (Supplementary Fig. S2B).

Identification of CITI Mutants

Identification of CITI mutant sequences was performed after multiple rounds of selection. Before and after the 2nd and 4th round of selection, eight single colonies were sequenced to collect mutation information (Supplementary Table S2) and the CITI sequences were cloned into pET23c vector in front of a luciferase reporter via the primers SalRT5-f and RDisR-1. Linear expression templates with and without the T7 promoter were generated from the plasmids via PCR. These templates were not capped, and hence cap-independent. The CITI activity of each mutant was characterized by the enzymatic activity of luciferase produced in yeast Tx/Tl CFPS derived from these PCR templates. Three short CITI sequences (with spacer) obtained after four rounds of selection were used to create 2-copy CITI sequences and were placed between T7 promoter and luciferase reporter. The best mutant, S4-2, was used to create a 3-copy CITI. A control plasmid, which contains only 2-copy spacer was also constructed. Linear expression templates for green fluorescent protein (GFP) were prepared by a two-step PCR method as described in our previous publication (Gan and Jewett, 2014).

Luciferase and GFP Assay

The enzymatic activity of luciferase and autoradiography were performed according to previous publications (Gan and Jewett, 2014; Hodgman and Jewett, 2013). The GFP reporter protein was assayed immediately after yeast CFPS reaction. Fifteen microliters of CFPS reaction was mixed with 85 μL 1× PBS buffer in 96-well plate. The fluorescence was read by Synergy2 (BioTek, Winooski, VT) (Excitation/emission = 488/525 nm).

In Vivo Expression Tuning via CITI Sequences

Thirteen CITI sequences (with spacer) identified in vitro were cloned into a plasmid in front of a green fluorescent protein gene controlled by the TEF promoter for in vivo analysis (Crook et al., 2011). The multicloning site was completely replaced by the CITI sequences via invert PCR. *S. cerevisiae* ATCC-MYA3666 (*MATa*, *his3-delta200*, *trpl-delta1*, *ura3-52*, *ade2-101*, *lys2*, *psi + cir°*) were cultivated in YPAD medium (20 g of Peptone (BD)/L, 10 g of yeast extract (BD, Becton, Dickinson and Company), 20 g glucose/L, and 40 μg/mL Adenine); for plates, additional 1.5% agar was also included. For yeast transformations, 50 μL of competent cell were prepared and electroporated with 100 ng purified plasmid according to manufacturer's instructions (Micropulser™ Electroporator, BIO-RAD). The cells were plated on *Ura*⁻ selective media (6.7 g of Yeast Nitrogen Base, 1.92 g of Drop-out media supplement without Uracil, and 20 g glucose per liter) and incubated for 2 days at 20°C. Single colonies were picked for further testing. Each clone was inoculated into 3 mL of synthetic media (*Uracil*-) and grown overnight to saturation. The next day, cultures were diluted 1:20 into 3 mL fresh media and grown to mid-exponential phase, when approximate 5.5×10^7 of cells of each sample were harvested. The cells were washed twice with 200 μL of PBS buffer and resuspended in 100 μL of PBS buffer in 96-well plate. Fluorescence was read by Synergy2 plate reader (BioTek) (Excitation/emission = 488/525 nm) and normalized by optical density at 600 nm.

Results

Development of a Yeast Ribosome Display

We began our investigation by developing an in vitro yeast ribosome display system (Fig. 1), where the goal was to capture PRM complexes. Initially, we tried to directly adopt the protocol from

Plückthun and colleagues (Hanes and Plückthun, 1997; Zahnd et al., 2007) that was developed for *E. coli* based ribosome display, with the exception that we did not include an anti-ssrA oligonucleotide required to prevent subunit dissociation in the bacterial system. In this approach, transcription of the mRNA template is performed separate from in vitro translation and capture. Our mRNA template harbored a sequence encoding the 3XFLAG peptide (DYKDGDYKDIDYKDDDDK) at the 5′ end followed by a spacer sequence encoding the TolA peptide devoid of a stop codon (Zahnd et al., 2007) (Fig. 2A). With this design, the FLAG peptide at the N-terminus of the polypeptide emerges from the ribosome first and is displayed for affinity purification and capture. Unfortunately, our initial experiments from mRNA templates failed. We suspect that this was due to the fact that low protein synthesis yields in cell-free translation-only reactions limited the number of selective tags available for capture.

We hypothesized that increasing protein synthesis yields by combining transcription and translation (Tx/Tl) in the same cell-free reaction could increase the number of PRM complexes. Previously, our lab demonstrated that a Tx/Tl system from PCR templates that used the Ω leader sequence produced higher protein yields than comparable systems relying on either mRNA or plasmid DNA templates in yeast CFPS (Gan and Jewett, 2014). We, therefore, investigated the ability to capture PRM complexes in a combined Tx/Tl reaction. We performed 15 μL batch-mode cell-free Tx/Tl reactions for 2 h at 24°C with 60 ng of PCR template DNA (Fig. 2A). We stopped the reactions by placing them on ice and the PRM complexes were stabilized against dissociation by adding 50 mM magnesium acetate. After a single-round of selection, the recovered cDNAs were analyzed on 2% agarose gel. Strikingly, our positive control demonstrated the ability to capture mRNA (Supplementary Fig. S3). In contrast, a negative control reaction using plasmid template that lacked the DNA sequence encoding the FLAG peptide resulted in little to no captured product. Following demonstration of FLAG peptide capture, we subsequently identified that the relative capture was greatest 45 min into the Tx/Tl reaction, rather than 2 h, which corresponds to the time in the reaction when the rate of protein synthesis reaches its peak value.

We next sought to validate and assess the selection capability of our ribosome display system with the FLAG-peptide capture method (see Materials and Methods for optimized conditions). As above, cDNA from the sample containing only positive template showed the desired band (Fig. 2B, lane 1) while the cDNA recovered from the sample containing only negative template showed a very faint band due to unspecific selection (Fig. 2B, lane 2). Next, we created three model libraries by mixing positive and negative DNA templates with the molar ratios 1:10, 1:100, and 1:1000, respectively. After a single-round of selection, the positive template was successfully enriched from model libraries 1:10 and 1:100 but not from the 1:1000 library (Fig. 2B, lanes 3–8). These data indicate that the enrichment rate of our yeast ribosome display method is at least 100-fold per round selection. Additionally, no cDNA was obtained without reverse transcription (Fig. 2B, lane 9), which confirmed that all cDNA on the gel results from reverse-transcription of selected mRNA and not from the DNA template used for cell-free protein synthesis.

Application of Yeast Ribosome Display for High-Throughput Selection of a 5′-UTR Library

With our yeast ribosome display system in hand, we next sought to use it to select for 5′-UTR sequences with enhanced yeast-based CFPS activity. A/T-rich sequences at 5′-UTR favor cap-independent translation initiation while G and C tend to work in the opposite way mainly due to the GC pairs interfering with the ribosome-scanning mechanism in eukaryotes (Kamura et al., 2005). We, therefore, created a PCR template-based library by randomizing 15 nucleotides as D (D: A, T, or G) with a diversity of 1.4×10^7 (Supplementary Fig. S2A). In addition, we included a 24-nt spacer with a specific sequence upstream of the randomized section for use as a primer-binding site to amplify recovered cDNA for multiple rounds of selection and cDNA cloning (Supplementary Fig. S2B). Importantly, because we did not use in vitro capped and purified mRNA as a template, any 5′-UTR sequence identified would be cap independent (Hodgman and Jewett, 2013). Our method contrasts to typical ribosome display libraries for protein engineering. While protein engineering methods typically use mRNA libraries that are unique for each protein target, our design approach utilizes a single mRNA species encoding a selective peptide tag and spacer sequence with variant 5′-UTR regions. Before selection, we initially tested the CFPS activity of both the randomized 5′UTR library pool with the spacer sequence and also the spacer sequence alone. The basal combined Tx/Tl activities of the library before selection were dramatically reduced by ∼90–98% as compared to the positive control Ω leader sequence, which demonstrates that our randomized sequence pool has no significant Tx/Tl activity before selection (Supplementary Fig. S4).

We applied four rounds of selection on the 5′-UTR library using yeast ribosome display. In each round of selection, 1.9 μg linear DNA template was delivered into 450 μL of yeast CFPS (∼10^5 copies per mutant) (Supplementary Fig. S2B). Eight mutants were sequenced prior to selection, after two rounds, and after four rounds of selection, respectively (Supplementary Table S2). CFPS of the pooled libraries showed a substantial increase in CITI activity with additional rounds of selection (Fig. 3). After four rounds of selection, the best mutant, S4-2, achieved a 13-fold improvement in CFPS activity as compared to the starting library pool. While all individual clones sequenced after four founds of selection had higher CFPS activity than the initial pool, we did observe that several mutants continued to have lower activities, a fact which is likely attributable to a low-level of non-specific selection.

After demonstrating the ability to use ribosome display to capture 5′-UTR sequences with enhanced activity, we next attempted to elucidate the determinants of beneficial CITI activity, (i.e., whether nucleotide content and/or secondary structure is predictive of activity). We used the Vienna RNA Website (Gruber et al., 2008) to predict the secondary structure (minimum free energy [MFE]) for each of the selected sequences (Supplementary Table S3 and S4). We did not observe an obvious correlation between sequence MFE and CITI activity (Supplementary Fig. S5A). However, the A/T content could explain variation of CITI activity to some extent ($R^2 = 0.5$) (Supplementary Fig. S5B), which is consistent with previous works (Kamura et al., 2005). To draw

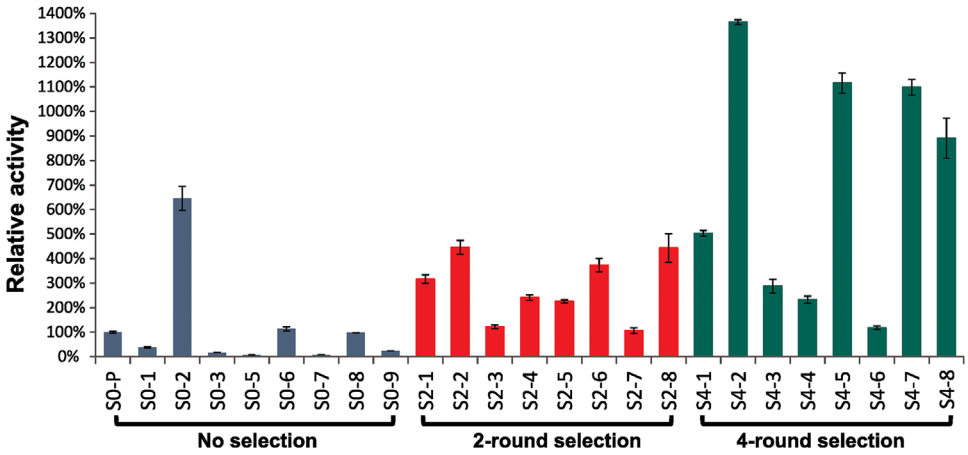

Figure 3. Yeast ribosome display is used to select several active CITI sequences from a 15-nt randomized library. Eight CITI mutants before selection (blue), after two rounds of selection (red), and after four rounds of selection (green) were validated in yeast CFPS using a luciferase reporter. The protein synthesis activity of the initial library pool (before selection performed) was counted as 100% (S0-P). The standard deviation was estimated from three independent reactions.

firmer conclusions, we plan to explore the significance of the protein expression and CITI sequence/structure in a future study.

5′-UTR Containing Tandem CITI Sequences Improves Protein Yield

It has been previously reported that tandem short CITI sequences (i.e., repeated) are able to significantly improve protein translation initiation rate (Kamura et al., 2005). We, therefore, constructed eight 2-copy CITI sequences using three beneficial mutants (S4-2, S4-5, and S4-7) and one 3-copy CITI sequence using S4-2 (Supplementary Table S3). The MFE (−6 to −3 kcal/mol) calculations of these novel 5′-UTR regions comprising repetitive CITI sequences showed that no significant secondary structures formed when using multiple CITI sequences (Supplementary Table S4). We placed the new CITI elements comprising the repeated beneficial short sequences in front of the luciferase gene and carried out yeast Tx/Tl reactions. Consistent with previous works (Kamura et al., 2005), we observed that the activities of CITI elements increased significantly in vitro upon multimerization, demonstrating the modularity of the enhancer elements. Specifically, all 2-copy CITI sequences showed improvements compared to single-copy ones (Fig. 4). In many cases, the effect on Tx/Tl was synergistic or non-additive. For example, combining S4-2/5 resulted in CITI activity that was greater than would be expected based on the individual activities of S4-2 + S4-5. Multimerization of three or more CITI elements does not appear to further increase activity, based on the fact that the three-copy S4-2 did not show further improvement over its 2-copy version (Fig. 4).

The Evolved CITI Sequences are not Cryptic Promoters

After demonstrating the evolution and activity of multiple distinct CITI sequences in vitro, we subsequently confirmed that the activity of these sequences served to increase translation initiation efficiency rather than act as cryptic promoters that simply increased the amount of mRNA present in the Tx/Tl reactions. To verify that the enhanced CITI activity was not a result of cryptic promoter activity, we carried out 15 μL batch cell-free translation-only reactions for 1 h at 24°C. These reactions were charged with 0.3 pmol in vitro transcribed and purified luciferase mRNA having a CITI element, the luciferase gene, and 50-mer poly(A) tail. By using a defined amount of mRNA as the template for cell-free translation, we were able to eliminate any differences in luciferase output that could result from transcription. We tested each of the evolved single-copy (SC) mutants: S0-1, S0-3, S2-1, S-2, S4-2, S4-5, and the artificially constructed multi-copy (MC) mutants: S4-2/2, S4-2/5, and S4-2/2/2. As a positive control, we again used the **Ω** sequence from tobacco mosaic virus. As a negative control, we placed the spacer sequence only (without the CITI elements) upstream of the luciferase gene. The protein synthesis yields of all the CITI sequences in cell-free translation only reactions (charged with the same amount of mRNA) had a similar trend to combined Tx/Tl reactions charged with DNA (Fig. 5A). In other words, CITI sequence elements that enabled high yielding Tx/Tl reactions when using DNA template, also resulted in high protein synthesis yields when only mRNA template was used. These consistent trends suggest that CITI activity is largely determined by the selected 5′-UTR of the mRNA molecules. To better compare the CITI activity of mRNA versus DNA templates, we converted our data into a scatter plot of protein yield (Fig. 5B). Notably, the 6 SC mutants showed a strict correlation between DNA and mRNA template ($R^2 = 0.971$) (Fig. 5B, inset). This was in contrast to the MC mutants and the **Ω** sequence, in which combined transcription and translation activity was higher than translation only. Apparently, the single copy (SC) 5′-UTRs that were directly evolved using yeast ribosome display have little to no promoter activity. As a final direct demonstration that CITIs do not contain promoter activity, we then used DNA templates of the CITI mutants without the T7 promoter sequence in the combined Tx/Tl CFPS reaction. When lacking the T7 promoter, protein synthesis was found to be negligible (Supplementary Table S5).

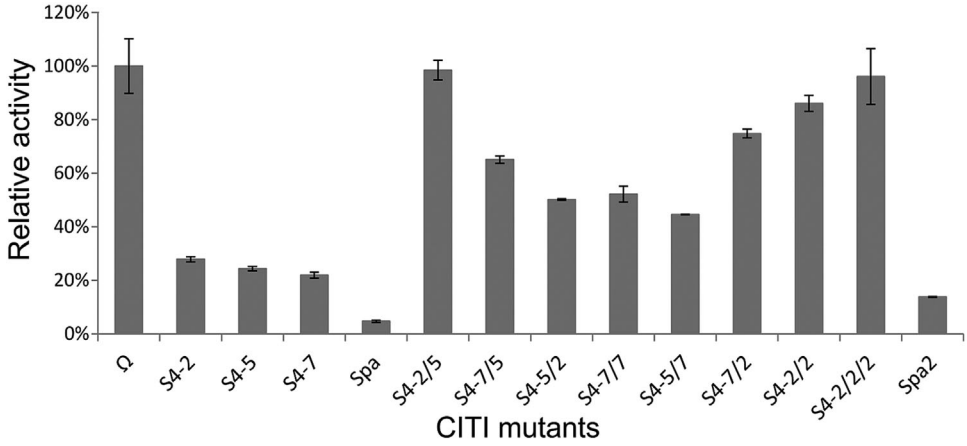

Figure 4. Enhanced CITI activity in yeast CFPS by creating multi-copy CITI mutants enhance. The Ω sequence and three single-copy mutants, S4-2, S4-5, and S4-7, were used as controls. S4-2/5: S4-2 + S4-5; S4-7/5: S4-7 + S4-5; S4-5/2: S4-5 + S4-2; S4-7/7: S4-7 + S4-7; S4-5/7: S4-5 + S4-7; S4-7/2: S4-7 + S4-2; S4-2/2: S4-2 + S4-2; Ω: wildtype Ω sequence; S4-2: single-copy S4-2; S4-5: single-copy S4-5; Spa: only spacer sequence; Spa2: 2-copy spacer sequence. The standard deviation was estimated from three independent reactions.

Protein Expression Tuning via Selected 5′-UTR Sequences

Following development of a novel yeast ribosome display system, we sought to test if we could use selected CITI sequences to permit control of gene expression over a range of values. First, we characterized the expression-tuning ability of CITI mutants in vitro using our Tx/Tl CFPS system. As expected, 12 selected CITI mutants could regulate in vitro protein synthesis over an 80-fold range for luciferase and 10-fold range for GFP (Fig. 6A). Second, we wanted to examine the ability of the same 5′-UTR sequences to tune protein expression in vivo. To test the 12 sequences in living yeast cells, we cloned them into a GFP reporter vector and transformed these constructs into yeast strain ATCC-MYA3666 (Fig. 6B). We observed that fluorescence per OD varied over ∼20-fold without lagging cell growth among all samples (Fig. 6B and Supplementary Fig. S6). Interestingly, for the evolved single-copy (SC) CITI mutants, the expression-tuning pattern showed high similarity between the in vitro and in vivo data ($R^2 = 0.731$) (Fig. 6C, inset). The correlation was somewhat surprising because the in vivo mRNA may be capped at the 5′ end, unlike in our in vitro system, which is not capped. It is interesting that the in vivo activity of many of the CITI mutants exceeds that of the Ω sequence; yet in vitro none of the variants exceed the relative activity of Ω (Fig. 6A). We suspect that the differences in CITI activity in vivo and in vitro are likely due to a different mechanism in vivo rather than cap independent translation.

Discussion

We report on the development of a ribosome display technology that is based on a recently established yeast CFPS system. Our method allows for significant enrichment of target sequences

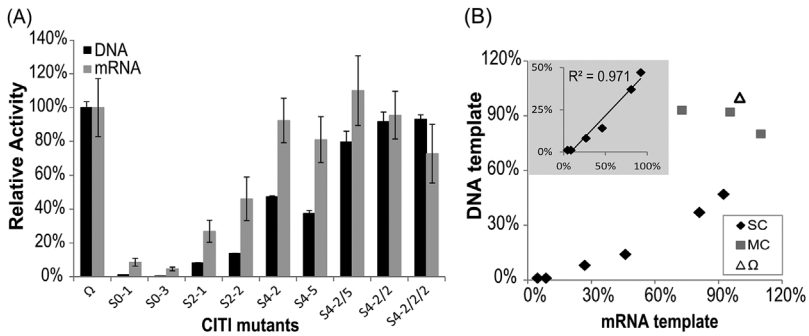

Figure 5. CITI activity validation using mRNA templates demonstrates the lack of cryptic promoter activity. **A**: mRNA templates were prepared for various mutants: S0-1, S0-3 (0-round pool); S2-1, S2-2 (two-round selection); S4-2, S4-5 (four-round selection); S4-2/5 (combined S4-2 and S4-5); S4-2/2 (2-copy S4-2); S4-2/2/2 (3-copy S4-2). The CFPS activities of mRNA templates (gray, translation only) and DNA templates (black, Tx/Tl) were normalized to those of Ω, respectively. The standard deviation was estimated from three independent reactions. **B**: The correlation of CITI activity between mRNA template and DNA template was presented in scatter plot (the gray-shaded smaller window for only single-copy (SC) mutants). SC, single-copy CITI; MC, multi-copy CITI; Ω, Ω sequence.

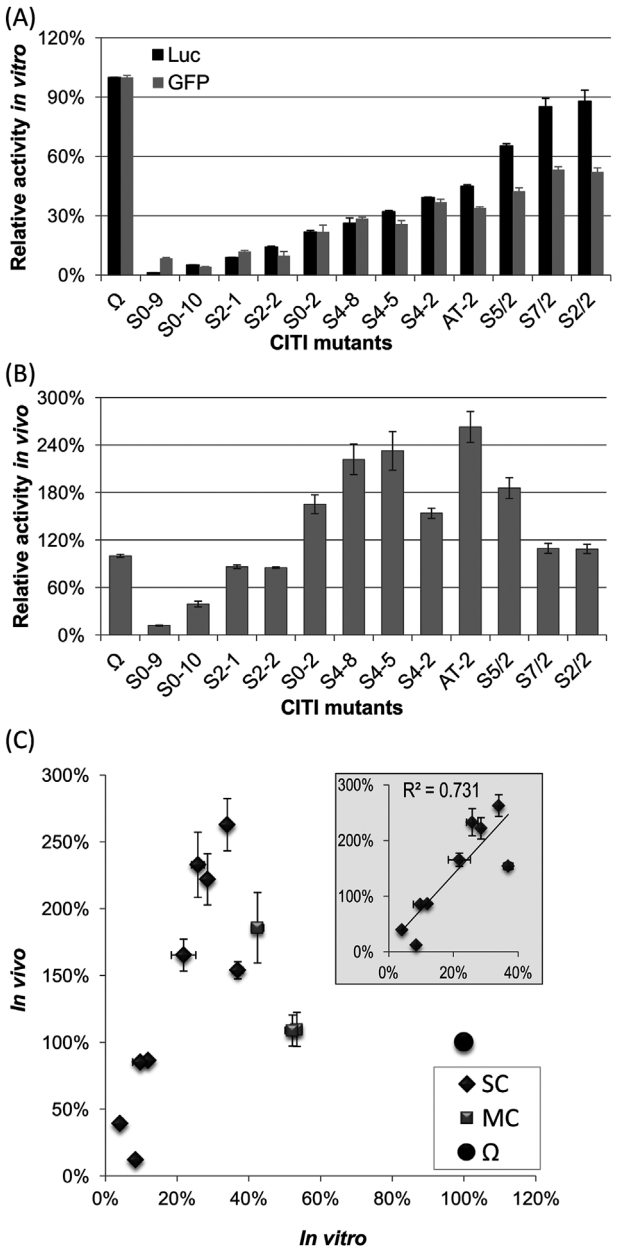

Figure 6. Characterization of the expression-tuning ability of CITI mutants. **A**: The CITI activity of nine single-copy (SC) and three multi-copy (MC) mutants were tested in combined Tx/Tl CFPS using luciferase (Luc, black bar) and GFP (GFP, gray) as reporters. The protein yields of luciferase and GFP driven by the Ω sequence are counted as 100%, respectively. **B**: Selected CITI sequences (with 24-nt spacer) were placed into a yeast plasmid with the TEF promoter and green fluorescence protein. Single colonies were cultivated to mid-exponential phase for in vivo fluorescence assay. The fluorescent readout of each sample was normalized by culture OD_{600}. The protein yields of GFP driven by Ω sequence is counted as 100%. **C**: The consistent expression-tuning pattern of GFP in vitro and in vivo is illustrated by a scatter plot (the bigger window for all samples, and gray-shaded inset window for only single-copy (SC) mutants). Standard deviation accounts for the day-to-day variation in three independent experiments.

without the need for cloning and transformation. Specifically, the single-round enrichment of functional sequences using yeast ribosome display is at least 100-fold, which is on the same order of magnitude of the well-developed *E. coli* based system and slightly less than the only other eukaryotic ribosome display system to be developed from rabbit reticulocyte lysate (RRL) (Boder and Wittrup, 1997; Hanes and Pluckthun, 1997; He and Taussig, 2007; Mattheakis et al., 1994). However, the yeast ribosome display technology that we developed still provides several advantages over current approaches for selection and characterization of eukaryotic DNA regulatory parts and proteins (Boder and Wittrup, 1997; Zhou et al., 2010): (i) the library diversity in our work is above 10^7 as compared to recent in vivo reports that achieved library diversity of $\sim 10^4$–10^6 (Dvir et al., 2013; Zhou et al., 2003); (ii) a single-round of selection can be completed within 4–6 h as compared to 2 days for in vivo systems; and (iii) we use linear DNA templates to encode library members, which remains a challenge in *E. coli* based systems (Gan and Jewett, 2014), and opens the way to rapid process implementation from PCR templates and automation.

As a model demonstration, we applied our ribosome display system to the selection of 5′-UTR sequences that can function over a broad dynamic range of protein expression. One of the major challenges for evolving CITI and/or IRES sequences is that evolved sequences may have cryptic promoter activity within 5′-UTR (Wellensiek et al., 2013; Zhou et al., 2003). In our approach, however, no selected mutants had obvious promoter activity in vitro (Supplementary Table S5). We attribute this feature to the merits of our unique template design for ribosome display. Namely, in eukaryotes, promoters are generally located approximately 30–40 nucleotides upstream of the transcription start site. In our design, the N-terminal FLAG tag immediately follows the mutated CITI sequence. Therefore, if a CITI sequence present in the library happened to be cryptic promoter, the sequence encoding the N-terminal tag could be overlooked by the native polymerase. If the nucleotides encoding the FLAG tag were not present in the mRNA, the tag is not translated, which would result in failure to select such sequences from the library.

The research reported here highlights the potential of yeast ribosome display to identify 5′-UTR regulatory elements for both in vitro and in vivo protein synthesis in high-throughput. Looking forward, we anticipate that our technology may be applied to protein evolution, the workhorse application for other ribosome display systems. It may be, for example, that yeast ribosome display could provide a more amenable evolution environment for eukaryotic protein folding and interactions as compared to bacterial systems. With the increasing ability and cheaper costs of in vitro DNA synthesis and assembly (Gibson et al., 2008), we predict that yeast ribosome display can provide a versatile toolkit for optimizing codon usage, maximizing protein folding efficiency, engineering proteins, and a wide variety of other applications.

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