COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Short DNA Containing χ Sites Enhances DNA Stability and Gene Expression in *E. coli* Cell-Free Transcription–Translation Systems

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ABSTRACT: *Escherichia coli* cell-free transcription–translation (TXTL) systems offer versatile platforms for advanced biomanufacturing and for prototyping synthetic biological parts and devices. Production and testing could be accelerated with the use of linear DNA, which can be rapidly and cheaply synthesized. However, linear DNA is efficiently degraded in TXTL preparations from *E. coli*. Here, we show that double-stranded DNA encoding χ sites—eight base-pair sequences preferentially bound by the RecBCD recombination machinery—stabilizes linear DNA and greatly enhances the TXTL-based expression and activity of a fluorescent reporter gene, simple regulatory cascades, and T7 bacteriophage particles. The χ-site DNA and the DNA-binding λ protein Gam yielded similar enhancements, and DNA with as few as four χ sites was sufficient to ensure robust gene expression in TXTL. Given the affordability and scalability of producing the short χ-site DNA, this generalized strategy is expected to advance the broad use of TXTL systems across its many applications.

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KEYWORDS: *Escherichia coli*; gene circuits; prototyping; RecBCD; synthetic biology; TXTL

Introduction

Over the past decade, cell-free transcription-translation (TXTL) systems have been engineered to address a broad range of novel applications in synthetic biology (Perez et al., 2016). The cell-free TXTL technology has become versatile enough to be employed for biomanufacturing and medicine (Ng et al., 2012; Pardee et al., 2016), metabolic engineering (Dudley et al., 2014), and quantitative disciplines such as biophysics (Tayar et al., 2015). Cell-free TXTL systems have also facilitated the prototyping of regulatory elements and small gene circuits by accelerating the design-build-test cycle (Sun et al., 2014; Takahashi et al., 2015). These TXTL systems have almost entirely been developed from *E. coli*.

While TXTL systems have principally relied on circular plasmid DNA for templating gene expression, chemically synthesized or PCR-amplified linear DNA offers a simpler and more affordable option. However, a major impediment to employing linear DNA is its poor stability in TXTL extracts. In *E. coli* TXTL extracts, the RecBCD complex, or exonuclease V, is the major nuclease responsible for the degradation of linear DNA (Sitaraman et al., 2004). The RecD subunit rapidly degrades DNA templates by the 3′ end at a rate of more than 500 bp/s (Spies et al., 2007). One solution has been preparing TXTL extracts from an *E. coli* strain with *endA* and *recC-ptrA-recB-recD* deleted (Michel-Reydellet et al., 2005). However, this *E. coli* strain has not been optimized for cell-free expression and is limited to transcription from the T7 promoter. A separate solution has been the addition of the Gam protein from λ phage that binds and inhibits the RecBCD complex (Sitaraman et al., 2004). Although effective, this approach requires the recombinant expression and purification of affinity-tagged Gam protein. We therefore were interested in exploring alternative approaches to stabilize linear DNA in *E. coli*-based TXTL reactions.

The RecBCD complex is the major pathway for double-strand break repair in *E. coli* (Smith, 2012). The activity of RecBCD is regulated by the χ sequence (5′-GCTGGTGG-3′), repeated about one thousand times throughout the *E. coli* genome. RecBCD is known to stall on DNA χ sites as part of homologous recombination (Spies et al., 2007). We hypothesized that the addition of short linear DNA containing χ sites to a TXTL reaction could stabilize linear DNA used for cell-free expression without interfering with protein synthesis (Fig. 1A). To test this hypothesis, we first assessed how dsDNA with or without χ sites impacts the stability of linear

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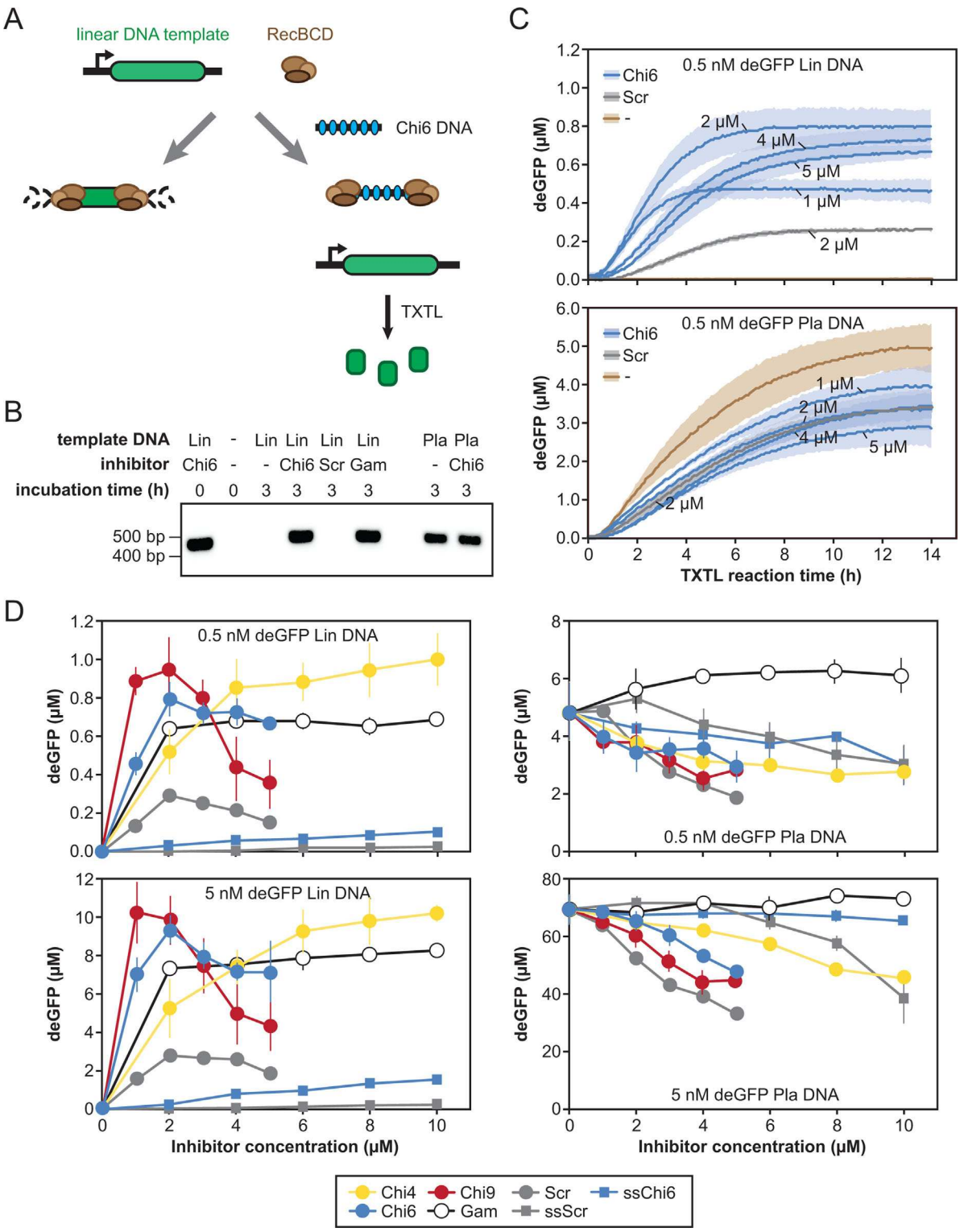

Figure 1. Enhancing the stability and activity of linear DNA templates in TXTL systems with χ-site DNA. **A:** DNA with multiple χ sites is preferentially bound by RecBCD, thereby protecting the linear DNA template and allowing protein production. **B:** Semi-quantitative PCR of the P70a-deGFP DNA template incubated in an *E. coli* TXTL reaction for 0 or 3 h at 29°C. A linear (Lin), circular (Pla), or no (−) DNA template was incubated by itself (−), with 2 μM of dsDNA containing six χ sites (Chi6), 2 μM of dsDNA with scrambled χ sites (Scr), or 5 μM of Gam protein. Semi-quantitative PCR is expected to yield a product of 442 bps. **C:** Fluorescence kinetics of *E. coli* TXTL reactions incubated with the linear or plasmid P70a-deGFP DNA template. The DNA template (0.5 nM) was incubated by itself, with 0–5 μM Chi6 DNA, or with 2 μM Scr DNA. The thick line is the average and the light band is the S.E.M from at least three independent TXTL reactions. **D:** Varying the number of χ sites (4 in Chi4, 6 in Chi6, 9 in Chi9) and concentrations for two linear and plasmid template concentrations of P70a-deGFP. GamS protein, Scr DNA, Ran DNA, and single-stranded Chi6 (ssChi6) and scrambled (ssScr) DNA were also tested. The average and S.E.M. of at least three independent TXTL reactions are indicated.

DNA in the *E. coli* TXTL system. The dsDNA was generated either by annealing two 85-nt oligonucleotides encoding six χ sites (Chi6) with each site separated by a 5-nt linker or by annealing two 85-nt oligonucleotides in which the six χ-site sequences and their spacers have been scrambled (Scr) (Table S1; see Materials and Methods for the Python code used to generate the Scr sequence). We tested the ability of Chi6 or Scr DNA (2 μM) to stabilize a linear DNA template (10 nM) encoding the deGFP reporter gene under the P70a constitutive promoter (P70a-deGFP) (Garamella et al., 2016). The Chi6 DNA or Scr DNA was incubated in a TXTL reaction for up to 3 h at 29°C followed by measuring the template abundance by semi-quantitative PCR (Fig. 1B). Incubating the deGFP DNA template in the absence of Chi6 dsDNA or the presence of Scr DNA yielded no detectable PCR product. In contrast, incubating the P70a-deGFP template with the Chi6 DNA yielded a PCR product with similar levels to the unincubated DNA template or a DNA template incubated with 5 μM of the Gam protein. Finally, incubating a circular deGFP DNA template in the presence or absence of the Chi6 DNA yielded similar levels of the PCR product, in line with the stability of circular DNA in *E. coli* TXTL systems. Therefore, the addition of short, linear DNA containing χ sites can stabilize linear DNA templates in *E. coli* TXTL reactions.

To measure the effect of Chi6 DNA on TXTL-based protein synthesis, we monitored the fluorescence of TXTL reactions incubated with the linear and plasmid P70a-deGFP template DNA on a fluorescence plate reader. The presence of Chi6 DNA resulted in a net production of deGFP from the linear template, while the absence of the dsDNA resulted in no detectable fluorescence (Fig. 1C). Increasing concentrations of Chi6 DNA in reactions with the plasmid template caused a slight decrease in the production rate of deGFP protein (Fig. 1C). This negative effect of Chi6 DNA on gene expression likely explains why protein synthesis from linear P70a-deGFP template peaked and then decreased at higher concentrations of Chi6 DNA. The presence of Scr DNA did yield measurable deGFP production, although we attribute this to the delayed degradation of the DNA template, allowing the transient expression of stable deGFP mRNA and protein. We also tested a random, 85-nt dsDNA sequence (Ran). The kinetics of deGFP expression (P70a-deGFP, 0.5 nM) in the presence of 2 μM Ran DNA was just above background (Fig. S1). Ran DNA has a 50% GC content, whereas Scr has a 66% GC content, suggesting that GC content affects the rate of RecBCD template degradation. We further found that pre-incubating the Chi6 DNA in the TXTL reaction prior to the addition of the DNA template did not enhance deGFP production, suggesting that the Chi6 DNA can rapidly block the degradation of the linear DNA (Fig. S2).

To determine the number of χ sites that allows the greatest protein synthesis recovery in TXTL, we compared the activity of the deGFP DNA template incubated with DNA containing four (Chi4, 60 bp), six (Chi6, 85 bp), or nine (Chi9, 125 bp) χ sites (Fig. 1D). All DNA inhibitors yielded similarly high fluorescence levels, wherein lower concentrations were required for DNA with more χ sites. As expected, each χ-site DNA was more efficient than Scr DNA. All three dsDNAs with χ sites yielded marginally better deGFP production than the Gam protein. Because the RecBCD complex can be inhibited by ssDNA containing χ sites (Kulkarni and Julin, 2004), we also tested a single-stranded version of the Chi6 DNA (ssChi6) or a scrambled version (ssScr). However, neither efficiently inhibited linear DNA degradation. Repeating these experiments with the DNA plasmid template revealed that the inhibitory DNA but not GamS interfered with deGFP production (Fig. 1D), paralleling what we observed following the addition of the Chi6 DNA (Fig. 1C). We thus conclude that dsDNA containing as few as four χ sites can greatly enhance protein synthesis from linear DNA templates, although the Chi6 DNA at 2 μM appears to represent a good compromise with respect to the cost of oligonucleotide synthesis and its optimal working concentration.

To further validate the utility of the Chi6 DNA, we assessed the activity of two transcriptional cascades encoded on linear or plasmid DNA templates (Fig. 2A). Each cascade comprised two DNA templates: one template driving expression of the σ28 sigma factor or T7 RNA polymerase from the P70a promoter, and the other template driving expression of deGFP from the σ28 promoter (P28a) or T7 promoter (T7p14). Both cascades were previously demonstrated in TXTL using plasmid DNA templates (Garamella et al., 2016; Shin and Noireaux, 2012). For both cascades, the addition of the Chi6 DNA resulted in large deGFP production from linear DNA. For plasmid DNA, the addition of Chi6 DNA added a delay in the synthesis of deGFP from the σ28 cascade, but reached the same endpoint concentration. This delay could be due to the slower expression of σ28, which is required to express from P28a-deGFP. The Chi6 DNA only had a slight impact on the synthesis of deGFP from the T7 cascade, and there was less of a delay than that observed for the σ28 cascade. We attribute this delay to the negative impact of the Chi6 DNA and the relative expression strength of σ28 and T7 RNA polymerase.

As a final validation, we assessed the impact of the Chi6 DNA on a substantially larger linear DNA template: the linear, 40-kbp genome of the T7 phage. The genome encodes ∼60 proteins and was previously shown to produce infectious phage particles in the *E. coli* TXTL system incubated with the Gam protein (Garamella et al., 2016; Shin et al., 2012). TXTL reactions were conducted with the T7 genome and varying concentrations of the Chi6 DNA. The number of infectious T7 phages synthesized was then determined by a plaque assay with *E. coli* B. We observed an $\sim 10^6$-fold improvement in phage titers with the addition of 0.5 μM Chi6 (Fig. 2B). There was a slight decrease of phage synthesis at greater concentrations of the Chi6 DNA, suggesting that an intermediate concentration of the Chi6 DNA may be optimal for some applications. These results demonstrate that dsDNA containing χ sites can greatly enhance protein production from large, linear DNA templates in the *E. coli* TXTL system.

Cell-free TXTL is becoming a standard technology for broad applications in bioengineering, especially for the rapid prototyping of synthetic and natural genetic parts and circuit motifs. Using linear DNA that is chemically synthesized or generated by PCR can reduce a design-build-test cycle to less than a day. In this work, we demonstrated that a short dsDNA sequence containing multiple χ sites could stabilize linear DNA templates and greatly enhance the biosynthesis of different protein products, although the protein yield is still smaller from a linear DNA template than a plasmid template. Generating the χ-site DNA is affordable, its usage is straightforward, and it can be incorporated in TXTL reactions as a standard component when linear DNA is used. Because χ sites have been

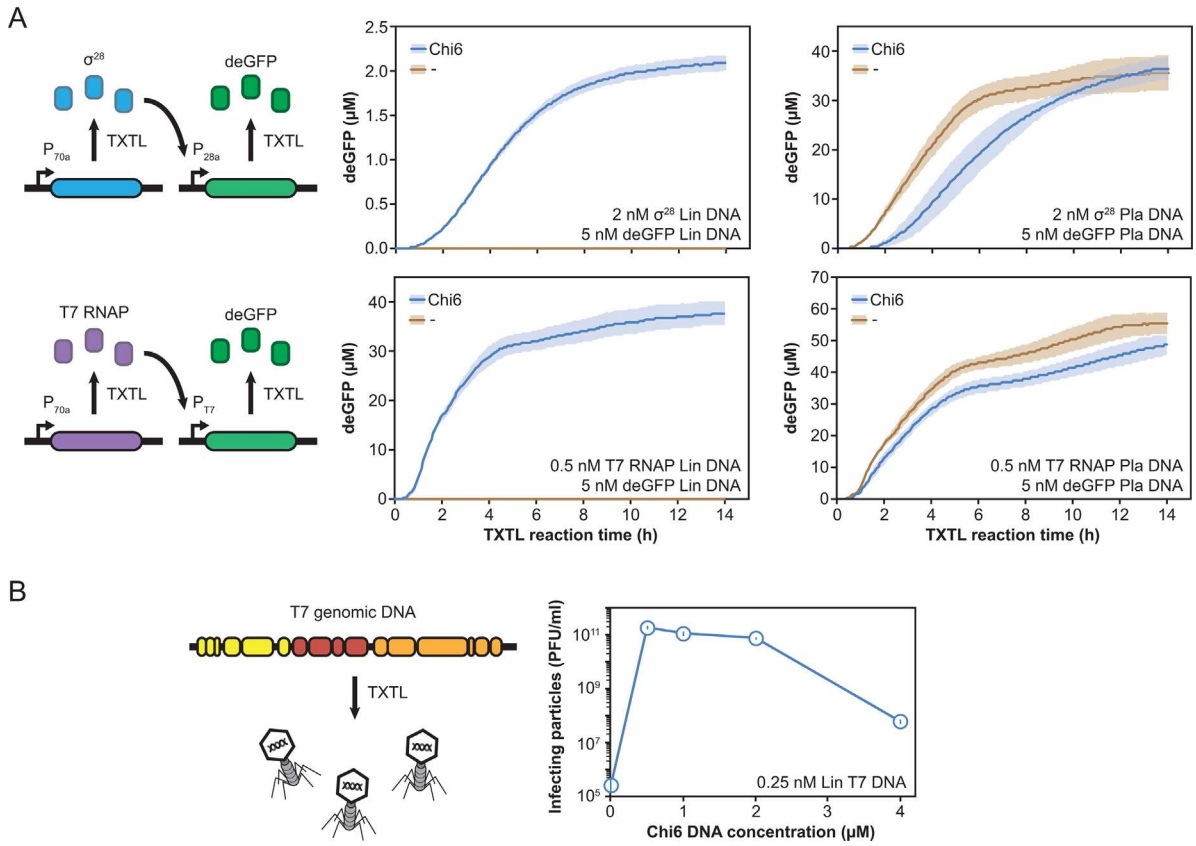

Figure 2. Enhancing regulatory cascades and phage T7 synthesis from linear DNA templates with χ-site DNA. **A:** Left, schematic of the two transcriptional activation cascades. Each cascade is encoded on two linear or plasmid DNA templates. Fluorescence time-course of the cascades in *E. coli* TXTL reactions with or without 2 μM Chi6 for linear (middle) and plasmid (right) DNA templates. The thick line is the average and the light band is the S.E.M from at least three independent TXTL reactions. **B:** Left, schematic of the linear dsDNA genome and synthesis of the T7 phage in TXTL. Right, plaque-forming units (PFUs) as a function of Chi6 concentration. The genome of phage T7 (0.25 nM) was incubated for 12 h in cell-free reactions before assessing phage titers. The average and S.E.M. of at least three independent TXTL reactions are indicated.

associated with *Bacillus subtilis*, *Lactococcus lactis*, and other bacteria and have been shown to protect dsDNA from exonucleolytic degradation (Chedin and Kowalczykowski, 2002), we anticipate that this approach could be implemented as part of the development of many other bacterial TXTL platforms (Kelwick et al., 2016).

Materials and Methods

DNA Construction

The plasmids P70a-deGFP, P70a-S28 and P28a-deGFP, P70a-T7rnap, and T7p14-deGFP were described previously (Garamella et al., 2016). The T7 phage genome was purchased from Boca Scientific. The χ-site DNA was made from oligonucleotides purchased at IDT. The sequence of the scrambled (Scr) χ-site DNA was prepared by drawing random letters from the χ-site DNA without replacement using the following Python code:

```
import random
chi6= NNNNN
shuff= .join(random.sample(chi6, len(chi6)))
```

Where the variable "chi6" is set to be the sequence of the chi6 DNA used in the manuscript (Fig. S1), and the variable "shuff" is assigned a shuffled version of the sequence. Note that this process is random, so the sequence of the shuffled chi DNA produced by this code may differ from the one used in the manuscript. The sequence of the random (Ran) DNA was generated by randomly selecting a nt at each of the positions. Oligonucleotides were annealed using standard procedures.

TXTL Preparation and Reactions

The all *E. coli* cell-free TXTL system was prepared from BL21 Rosetta 2 from Novagen as described previously (Shin and Noireaux, 2010; Sun et al., 2013). TXTL reactions are composed of 33 vol% crude extract and 67 vol% energy and amino acid mixtures, cofactors, and ions. A typical TXTL reaction is composed of 50 mM HEPES pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 2% PEG8000, 10–15 mM maltose or 20–40 mM maltodextrin, 1.5–3 mM of each of the 20 amino acids, 40–120 mM K-glutamate, and 2–7 mM Mg-glutamate. The concentrations of circular or linearized plasmids, linear PCR products, and

linear T7 phage genome were between 0 and 10 nM in the TXTL reactions. Cell-free reactions were carried out in a volume of 5–20 μL at 29–30°C. The λ Gam protein (6His tag in N-terminal) was purified as described previously and used at an optimal concentration of 3–5 μM in TXTL reactions (Sun et al., 2014).

DNA Stability Assays

DNA stability in TXTL was measured using semi-quantitative PCR. Linear DNA was prepared by PCR-amplifying a 1.2 kbp product from the plasmid P70a-deGFP with the Taq polymerase and the primers CSMpr1200 and CSMpr1021 and with the following cycling conditions: 55°C annealing temperature, 30 s extension time, and 25 cycles of amplification. The PCR products were resolved and visualized on a 1% agarose gel. Reactions were incubated for 3 h at 29°C.

Measurements of Gene Expression in TXTL

Quantitative measurements of gene expression were carried out using the reporter protein deGFP (25.4 kDa, 1 mg/mL = 39.37 μM), a modified version of the reporter eGFP that is more translatable in cell-free systems (Shin and Noireaux, 2012). Fluorescence was measured on an H1m plate reader (Biotek Instruments, Ex 485 nm, Em 525 nm, 384-well plates Nunc 142761, or 96-well V-bottom plates Corning Costar 3357). End-point measurements were carried out after 12 h of incubation. Kinetics of gene expression for the cascades were conducted for 14 h, with a time lapse of 3 min between data points. To quantify deGFP on plate readers, a linear calibration of intensity versus deGFP concentration over four orders-of-magnitude was made using pure recombinant eGFP (from either Cell Biolabs Inc., San Diego, CA or Biovision, Milpitas, CA) (Garamella et al., 2016).

Measurements of T7 Bacteriophage Synthesis

The number of infectious T7 phage particles was measured by the standard plaque assay using the *E. coli* strain B as host as previously reported (Shin et al., 2012). Briefly, the cells were grown in Luria–Bertani (LB) broth at 37°C. A 5-mL LB culture of *E. coli* host B was cultured overnight then diluted to 1:50 in 50 mL of LB broth and cultured at 37°C and 250 rpm to mid-log phase. The 50-mL culture was centrifuged for 10 min at 5,000*g* and resuspended in 5 mL of LB broth. TXTL reactions were diluted in LB broth, and 25 μL of the last dilution was added to a 2.6-mL solution of 0.6% liquid LB-agar solution at 45°C and 25 μL of culture. The mixture was then dispensed on a 1.5% LB-agar plate. Plates were incubated at 37°C for 7 h, and plaques were counted on each plate.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

SUPPLEMENTARY INFORMATION FOR:

Short DNA containing χ sites enhances DNA stability and gene expression in *E. coli* cell-free transcription-translation systems

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Contents:

Plasmids:

P70a-deGFP

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P70a-T7rnap

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P28a-deGFP

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T7p14-deGFP

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Oligonucleotides:

Name	Sequence
chi4.fwd	TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCA
chi4.rev	TGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGAAGTGA
chi6.fwd	TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTG GTGGCCACTGCTGGTGGCCA
chi6.rev	TGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCA GTGGCCACCAGCAGTGAAGTGA
chi9.fwd	TCACTTCACTGCTGGTGGCCTCTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTG GTGGCCACTGCTGGTGGCCTCTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCA
chi9.rev	TGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGAGGCCACCAGCAGTGGCCACCAGCA GTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGAGGCCACCAGCAGTGAAGTGA
Scr.fwd	GTTGGATCCCGCCTGGATGGTGATCCGGGACCTCTCGCGCGTTGGCCCGTGGCGGCATATCGTTGC TTCACCTCTCCCGTGGAGGG
Scr.rev	CCCTCCACGGGAGAGGTGAAGCAACGATATGCCGCCACGGGCCAACGCGCGAGAGGTCCCGGAT CACCATCCAGGCGGGATCCAAC
Ran.fwd	GTGAAGGTGTCTCGCGCCACCTCTAAGTAAGTGGGCCGTCGAGACATTATCCCTGATTTTTTCACT ACTATTAGTACTCACGGCGC
Ran.rev	GCGCCGTGAGTACTAATAGTAGTGAAAAAATCAGGGATAATGTCTCGACGGCCCACTTACTTAGA GGTGGCGCGAGACACCTTCAC

Primers:

Name	Description	Sequence
RM01s	Amplifies 1178 bp P70a-deGFP, 1083 bp P70a-S28, 3243 bp P70a-T7rnap, 1103 P28a-deGFP	CACCATCAGCCAGAAAACCG
RM02s	Amplifies 1813 bp T7p14-deGFP	CCTGCCACCATACCCAC
RM03as	Amplifies 1083 bp P70a-S28, 3243 bp P70a-T7rnap	TGTAGCACCTGAAGTCAGC
RM04as	Amplifies 1178 bp P70a-deGFP, 1813 bp T7p14-deGFP	CATGTTCTTTCCTGCGTTATCC
RM05as	Amplifies 1103 bp P28a-deGFP	CAGTGAGCGAGGAAGCGG
CSMpr1120	Amplifies a 1170-bp fragment from the P70a-deGFP plasmid containing the deGFP transcriptional unit	CCGCAGAGTGGATGTTTGACA T
CSMpr1121	Amplifies a 1170-bp fragment from the P70a-deGFP plasmid containing the deGFP transcriptional unit	TTACCGCCTTTGAGTGAGCTG A
CSMpr1200	Amplifies 442-bp fragment of P70a-deGFP	CATCAGCCAGAAAACCGAAT
CSMpr1201	Amplifies 442-bp fragment of P70a-deGFP	AAGTCGTGCTGCTTCATGTG

Strains:

Name	Genotype	Source	Use in Study
E. coli B	wt		T7 phage plaque assay
BL21-Rosetta2	F- *ompT hsdS*B(rB- mB-) *gal dcm* pRARE2 (CamR)	Novagen 71402	TXTL lysate preparation

Table S1. Tables listing plasmids, oligonucleotides, primers, and strains used in this work.

A

Chi6 5′ TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCA 3′

Scr 5′ GTTGGATCCCGCCTGGATGGTGATCCGGGACCTCTCGCGCGTTGGCCCGTGGCGGCATATCGTTGCTTCACCTCTCCCGTGGAGGG 3′

Ran 5′ GTGAAGGTGTCTCGCGCCACCTCTAAGTAAGTGGGCCGTCGAGACATTATCCCTGATTTTTTCACTACTATTAGTACTCACGGCGC 3′

B

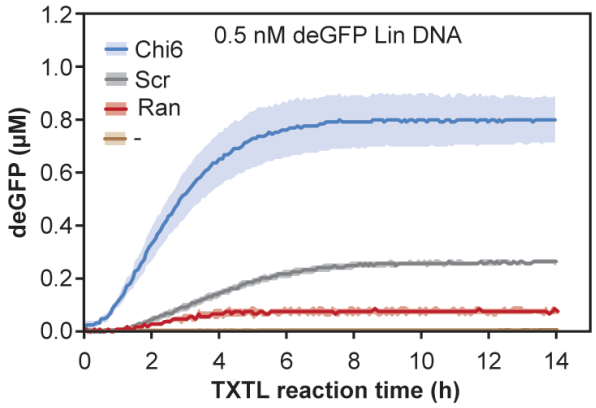

Figure S1. Testing the effect of a random 85 nt dsDNA oligonucleotide as a means of enhancing linear DNA templates in TXTL. **A:** DNA sequences for Chi6, Scr, and Ran (random). χ sites in Chi6 are highlighted in blue. **B:** Fluorescence kinetics of *E. coli* TXTL reactions incubated with 0.5 nM linear P70a-deGFP DNA template and 2 μM either Chi6, Scr, or random dsDNA (the data for Chi6 and Scr DNA is the same as that in Figure 1C). The thick line is the average and the light band is the S.E.M from at least three independent TXTL reactions.

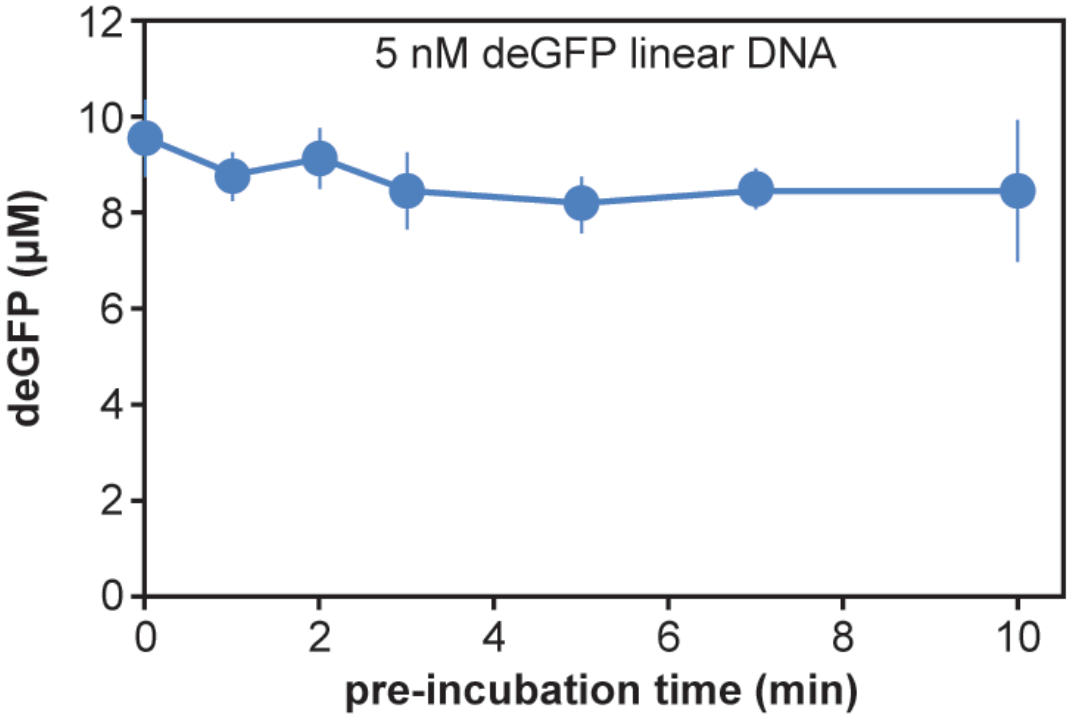

Figure S2. Pre-incubating with Chi6 in TXTL reaction before addition of the linear P70a-deGFP template DNA. The horizontal axis indicates the time between the addition of 2 μM Chi6 DNA and 5 nM DNA template.