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Thrombin-Mediated Transcriptional Regulation Using DNA Aptamers in DNA-Based Cell-Free Protein Synthesis

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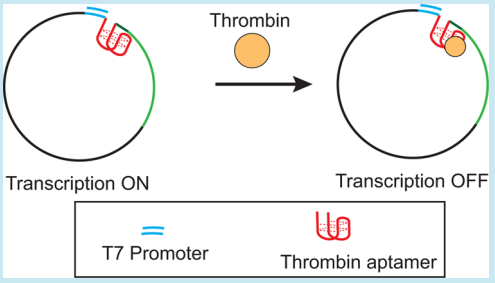
Supporting Information

ABSTRACT: Realizing the potential of cell-free systems will require development of ligand-sensitive gene promoters that control gene expression in response to a ligand of interest. Here, we describe an approach to designing ligand-sensitive transcriptional control in cell-free systems that is based on the combination of a DNA aptamer that binds thrombin and the T7 bacteriophage promoter. Placement of the aptamer near the T7 promoter, and using a primarily single-stranded template, results in up to a 6-fold change in gene expression in a ligand concentration-dependent manner. We further demonstrate that the sensitivity to thrombin concentration and the fold change in expression can be tuned by altering the position of the aptamer. The results described here pave the way for the use of DNA aptamers to achieve modular regulation of transcription in response to a wide variety of ligands in cell-free systems.



KEYWORDS: *T7 promoters, cell-free systems, DNA aptamer, thrombin, transcriptional regulation*

Synthetic gene circuits comprised of novel genetic regulatory mechanisms have emerged as powerful tools for understanding and harnessing biological function.[1] Engineered arrangements of genetic components have resulted in systems capable of predetermined functions such as bistability,[2] logic control[3−5] and oscillation of gene expression.[6,7] Synthetic gene circuits also offer the opportunity to redesign biological systems for the production of biofuels and other chemicals as well as for constructing devices for sensing and responding to biomedical conditions.[8,9] In practice, the majority of synthetic gene circuits have been implemented in cell-based systems. While these demonstrations benefit from natural mechanisms to sustain a living cell, such as protein synthesis and degradation, creating predictable engineered systems can be complicated by interference from endogenous host machinery and selection pressures that act against unneeded, resource-consuming systems.[10,11] Additionally, conflicts occur when sensing or generating materials that can compromise cell viability and survival. Therefore, alternative strategies to harness and understand biological complexity are a needed complement to existing cell-based approaches.[12]

Cell-free systems, which include both plamsid-based cell-free protein synthesis systems and *in vitro* transcription systems, provide a versatile platform for understanding and applying the design elements that underlie cellular efficiency.[13−15] Cell-free approaches employ select cellular components, produced naturally or synthetically, to carry out defined biological processes. Issues related to plasmid compatibility, protein toxicity or maintenance of a living cell can be mostly ignored, allowing focus on defining essential system components[16] and implementing predictable dynamic behavior.[17] The flexibility, simplified context, and precise specification of system components are distinct advantages of the cell-free approach. A number of cell-free, *in vitro* gene circuits have been demonstrated. For example, simplified nucleic acid templates, in which transcription was regulated by DNA hybridization, were co-opted to build bistable switches and oscillators that reasonably agree with quantitative predictions.[18,19] Additionally, expression cascades,[20] negative feedback[21] and logic gates[22] have been realized using circuits involving protein intermediates in plasmid-based cell-free protein synthesis systems.

Well-characterized molecular tools for signal sensing and tuning gene expression are essential for the design and construction of synthetic gene circuits in cells and cell-free systems.[21,22] In particular, ligand responsive gene regulation strategies are key. While a myriad of gene regulatory mechanisms are used in natural cells, ligand-dependent transcriptional control strategies that function in cell-free extracts remain fairly limited. This is partly due to the prevalent use of bacteriophage RNA polymerases. For example, T7 RNA polymerase is commonly used to drive transcription due to the enzyme's stability and high processivity.[23,24] While these characteristics are desirable for achieving high yield protein

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synthesis, synthetic biology applications can be compromised by the lack of sufficient ligand-sensitive T7 promoters.[20] Attempts to engineer ligand regulatable T7 promoters have used either protein-based transcription factors or modified nucleic acid bases to confer ligand sensitivity.[25−27] For example, control of T7 RNA polymerase can be accomplished by placing a cis acting promoter element, which binds to a repressor, downstream to the transcription start site.[28] Other approaches have considered incorporation of photoresponsive elements[29] or triplex DNA formation.[27,30]

The use of nucleic acid aptamers can potentially allow regulation of gene expression in response to a wide variety of small molecules and proteins. Aptamers are single-stranded DNA or RNA molecules that can be engineered to bind to specific target molecules with high affinity and specificity.[31] Accordingly, RNA aptamers have found extensive application and often couple the binding event and the ensuing conformational change for regulation of transcription or translation.[32−38] Nucleic acid aptamers offer several practical advantages. Aptamers can potentially be selected against any ligand of interest from a combinatorial library using an iterative affinity selection procedure.[31,39] In addition, known hybridization rules facilitate predictive and rational design of nucleic acid domains. The ease and predictability of engineering nucleic acid domains make DNA and RNA molecules particularly useful substrates for engineering flexible platforms for achieving tunable sensing and actuation.[32,40,41]

Here we describe a new approach to using aptamers to control gene expression at the transcriptional level using viral promoters. The approach involves the insertion of a DNA aptamer sequence proximal to the T7 promoter such that binding prevents transcription (Figure 1). The required single-stranded regions are easily accommodated by bacteriophage polymerases[42,43] and employable in a cell-free context. Thrombin binding DNA aptamer (TBA) was selected for demonstrating analyte specific transcriptional control. TBA is well-characterized and is known to bind to human α-thrombin with high affinity (K_d of 10−100 nM) and specificity.[44] Presence of additional flanking sequences and surface immobilization are not detrimental to thrombin binding, which facilitates the insertion of the aptamer sequence into the DNA template.[45,46] These features have enabled the incorporation of thrombin aptamers in several designs for biosensors and DNA circuits.[46−49] We show that thrombin can be used to effectively repress expression from thrombin aptamer containing templates in a cell-free context. In addition, the sensitivity of the promoter to thrombin concentration and relative expression level can be tuned.

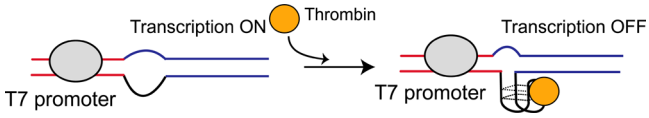

Figure 1. Design for aptamer-mediated transcriptional regulation. A ssDNA aptamer that binds thrombin is placed downstream to the T7 promoter. The transcriptional template contains a noncomplementary, ssDNA region that harbors the aptamer sequence. In the absence of thrombin, T7 RNA polymerase transcribes the template. Upon thrombin binding to the DNA aptamer, transcription from the T7-aptamer promoter is repressed.

To evaluate transcriptional regulation using DNA aptamers, double-stranded, largely double-stranded and largely single-stranded DNA templates were created. The largely double-stranded template contains an unpaired "bubble" DNA region after the double-stranded promoter where DNA aptamers on the template and the nontemplate strands are located (Supporting Information, Figure S2). These structures were created from hybridization of single-stranded DNA templates. Several strategies for generating linear ssDNA templates such as affinity purification of biotin-labeled ssDNA generated from PCR[50] and rolling circle amplification[51] were evaluated. However, these approaches resulted in only small amounts of ssDNA, which were insufficient for optimizing protein synthesis reactions (data not shown). Consequently, the ssDNA templates were generated from phagemids, which offer the advantage of producing high yield ssDNA that is long enough to code for a protein.[52] Restriction digests of the resulting bubble template confirmed proper hybridization (Supporting Information, Figure S2). In addition, to the bubble templates, largely single-stranded templates, which contain only a 19-base, double-stranded T7 promoter, were generated by annealing an oligonucleotide to the ssDNA generated from the phagemid. For all templates, the aptamer sequence was placed downstream from the transcription start site along with a 4 bp stem loop structure to facilitate aptamer formation and increase its stability.[44] Finally, DNA encoding green fluorescent protein (GFP) was located downstream from the aptamer. Fluorescence anisotropy experiments with oligonucleotide analogues of the ssDNA and bubble DNA constructs confirmed thrombin binding to the aptamer (Supporting Information, Figure S1).

The ability of thrombin to repress transcription from double-stranded, bubble and ssDNA templates was monitored by cell-free protein synthesis reactions (Figure 2). Not surprisingly, constitutive expression from purely double-stranded plasmid templates was 10 times greater than from the bubble template and about 4 times greater than from the ssDNA template. Upon incubation of the entirely double-stranded template with 1.8 μM thrombin, only a modest change in gene expression is observed. This indicates that thrombin binding to the aptamer region is low, presumably due to the stable double-stranded structure obscuring aptamer recognition. We also note that addition of thrombin to double-stranded plasmid DNA templates without the aptamer did not result in repression from T7 promoters. This indicates that thrombin does not interfere with transcription and translation in the *E. coli* cell-free extract. The lower baseline expression from bubble template indicates that this structure serves as poor template for gene expression. In contrast, the largely ssDNA aptamer templates are suitable structures for gene expression and a 6-fold decrease in gene expression is seen when in the presence of thrombin. Further, rapid repression of expression can be observed without prior incubation with thrombin (Supporting Information, Figure S3).

Previous reports have shown that protein binding at a position proximal to the transcription start site is essential for achieving effective repression from T7 promoters.[21,27,28] This suggests that aptamer placement that facilitates ligand binding close to the transcriptional start site is important for specific control of gene expression. When the aptamer is present along with its complementary sequence, resulting in a fully base-paired structure, addition of thrombin results in little to no change in expression. This result is presumably due to the stability of the double-stranded structure that prevents significant formation of the aptamer. Placing the aptamers

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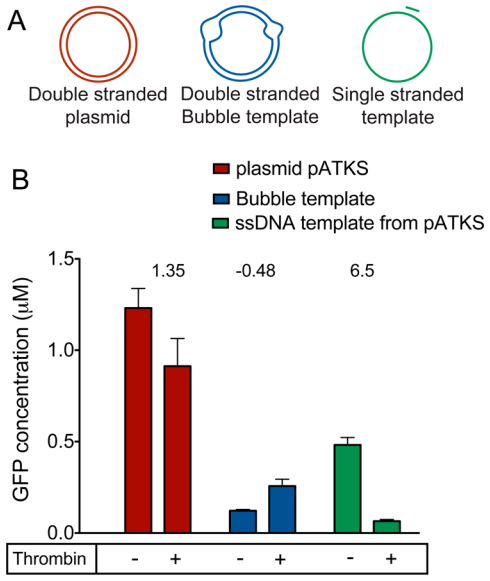

Figure 2. Effect of promoter design on aptamer-mediated repression. (A) Schematic of templates used to test the different designs. The red structure represents the double-stranded plasmid with the thrombin aptamer in a duplex form downstream from the promoter. The blue template is a "bubble template" that contains an aptamer structure both on the template (derived from pATKS) and nontemplate strand (generated from pANTKS) in an unpaired context downstream from the promoter. The second bubble in the diagram corresponds to the unpaired region that results from the phagemid origins of replication. The green schematic corresponds to the mostly ssDNA template derived from hybridizing an oligonucleotide to pATKS. (B) GFP concentrations measured after a 6 h reaction from these templates, in the absence or presence of 1.8 μM thrombin. The numbers above the bars indicate fold change in expression upon addition of thrombin to the DNA template.

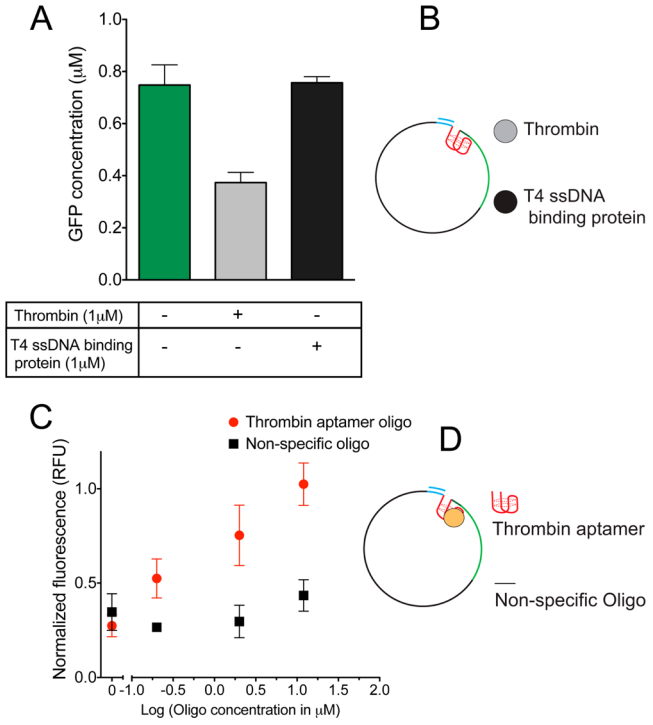

Figure 3. Testing specificity of aptamer-mediated transcriptional regulation. (A) Response of ssDNA template to 1 μM thrombin and 1 μM T4 ssDNA binding protein. (B) Schematic of the aptamer template along with thrombin and T4ssDNA binding protein. (C) Results of addition of thrombin aptamer oligo and the nonspecific oligo in trans to 10 nM of the ssDNA aptamer template complexed with 2 μM thrombin. Fluorescence values have been normalized to expression from ssDNA templates in the absence of thrombin and after 6 h of expression. Error bars correspond to standard deviations of triplicate measurements. (D) Schematic of the aptamer template bound to thrombin and the nonspecific oligonucleotide and an oligonucleotide containing the thrombin aptamer.

within unpaired, bubble regions offers a strategy to increase accessibility of the aptamer. However, as shown in Figure 2, this template was a poor substrate for transcription. T7 RNA polymerase is known to bypass gaps and discontinuities in the template strand with the aid of the nontemplate DNA strand.[43,53] Further, DNA topology is known to adversely affect transcription *in vitro*.[54,55] Several studies have shown that noncanonical DNA structures such as DNA quadruplexes present an obstacle to transcription from T7 promoters, with the effect being more pronounced when the structures are located proximal to the transcriptional start site.[56−58] Collectively, structural blockages on both strands at positions near to the promoter likely lead to poor gene expression and performance of this promoter design. The final template design consisted primarily of single-stranded DNA, corresponding to the template strand, and hybridization to a short, synthetic oligonucleotide to create a double-stranded T7 promoter. This design proved to be the most responsive to thrombin-mediated repression of gene expression.

To test the specificity of thrombin-dependent repression, the nonspecific, single strand DNA binding T4 gene 32 protein[59] was tested for transcription repression on the single-stranded T7 aptamer promoter. The addition of ssDNA binding protein did not have a significant effect on transcription demonstrating that specific protein binding to the aptamer is required for effective transcriptional repression (Figure 3A). The change in gene expression upon the addition of thrombin as a function of DNA concentration was also investigated. The highest fold change in expression from ssDNA templates (pATKS) was observed at a concentration of 20 ng/μL (Supporting Information, Figure S5).

The specificity of the transcriptional repression was further evaluated using exogenously added thrombin aptamer oligonucleotides to competitively inhibit gene repression when in the presence of thrombin (Figure 3B). Thrombin aptamer concentrations in excess of thrombin protein relieved thrombin-mediated gene repression, whereas the addition of a nonspecific DNA oligonucleotide did not affect repression. Therefore, the addition of exogenous DNA aptamer allows for "induction" of expression from these promoters in the presence of thrombin. This observation provides additional support that repression is mediated selectively by thrombin and that the DNA aptamer can bind to thrombin in a complex cell extract system. This result also highlights a mechanism for reversing thrombin-mediated gene repression from aptamer templates. The presence of additional aptamer sequences can allow the turning off of gene repression or for tuning the ligand concentration required for expression. Such tools may prove useful for effectively designing gene circuit behavior.

The effect of aptamer position on transcriptional efficiency was also explored by placing the thrombin aptamer at positions +2, +9 and +28, relative to the transcriptional start site (Table 1). Results from protein expression assays show that placement of the DNA aptamer further away from the transcriptional start

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Table 1. Effect of Position of the Aptamer on Thrombin-Mediated Response

Plasmid template	Aptamer postion	Promoter Design	Relative expression *	Fold change **
pATKS	+2	TAATACGACTCACTATAGG ATTATGCTGAGTGATATCCG CTACTGCCACCGCCGGCGAGAT CTT	0.45	6.78
pETAKS	+9	TAATACGACTCACTATAGG ATTATGCTGAGTGATATCCTCTAGAG CTACTGCCACCGCCGG CGAGATCTTTATT	0.7	2.78
pETA26KS	+28	TAATACGACTCACTATAGG ATTATGCTGAGTGATATCCCGCTTAACCTCGAGGTGGCTCTAGA G CTACTGCCACCGCCGGCGAGATCTT	1.22	2.01

*Relative expression indicates expression levels normalized to expression from the ssDNA template generated from pKSGFP plasmid. **Fold change in expression indicates the change in expression upon the addition of thrombin.

site leads to an increase in basal gene expression levels. However, the magnitude of thrombin induced gene repression decreases. Dose response curves with the aptamer at +2 and +9 positions show a half maximal repressor concentration of 218.9 ± 0.04 and 567.90 ± 1.2 nM, respectively (Figure 4). Dose

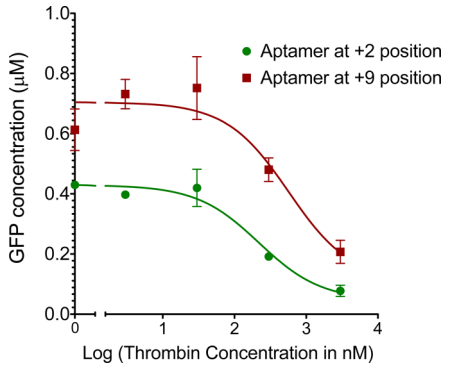

Figure 4. Effect of position of the aptamer on thrombin-mediated response. Dose response curves for the +2 and +9 aptamer ssDNA constructs where the *X*-axis corresponds to the log of the thrombin concentration. Only nM concentrations of thrombin are needed before a logarithmic transformation is observed.

responses of the aptamer placed at +28 position were not tested given the small fold change in the expression even to 2 μM thrombin concentration. To test if the addition of a second thrombin aptamer results in improved gene repression, a dimeric DNA aptamer template was constructed and tested. However, basal expression from the template was low (Supporting Information, Figure S4).

Engineered placement of the promoter offers another means for tuning gene expression in response to a ligand. Repression efficiency is expected to decrease as the aptamer is moved away from the transcription start site.[28] Accordingly, placement of thrombin binding aptamer 2 bases away from the transcriptional start site results in up to a 6- to 7-fold thrombin concentration-dependent change in gene expression in the ssDNA template design. While moving the aptamer 28 bases away from the transcription start site increases basal transcriptional levels, only up to a 2-fold change in gene expression upon thrombin addition is observed. T7 promoters are highly conserved in the region between −17 and +6. Aptamer placement at the +2 position disrupts the viral promoter sequence, while placement at the +9 and +28 positions do not. Further, the thrombin binding aptamer is known to form a DNA quadruplex structure.[60,61] Taken together with the known effects of secondary structures that can block transcription *in vitro*,[56,62,63] these results indicate that disruption of the native promoter sequence and the formation of secondary DNA structures close to the transcriptional start site combine to lower basal expression levels when the aptamer is placed in the +2 position. The finding that placement of operators more proximal to the transcriptional start site achieves effective repression from T7 promoters at the expense of lower basal expression mirrors results obtained with T7lacO and T7tetO promoters.[21,28]

Cell-free systems are promising platforms for implementing engineered networks from defined components.[18,19] Such systems are not constrained to native forms of biomolecules and can employ conditions that are incompatible with living cells. Moreover, the open nature of cell-free systems allows users to optimize numerous parameters including the cell-free extract components and their preparation, the reaction substrates and the format of the reaction such that cost-effective, industrial scale protein production can be considered.[64−66] Effective application of network designs in these systems will require the availability of a library of environmentally responsive promoters.[67] In particular, control of gene expression at the transcriptional level will be critical for many applications. Control at the transcriptional stage offers several

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advantages over control at the translational or post-translational levels.[10] Being the first step in gene expression, multiple downstream targets can be regulated simultaneously. In addition, signal amplification can be achieved since binding of a single transcriptional factor regulates the expression of several hundred resulting RNA and protein molecules.[68] Since nucleic acid aptamers can be selected against any molecule of interest that otherwise does not have a double-stranded DNA binding domain, the use of DNA aptamers for transcriptional repression in cell-free systems paves the way for creation of feedback circuits with novel sensory capabilities.

Here, an effective strategy for implementing aptamer control elements has been demonstrated and relies on a simple combination of a single-stranded template with an oligonucleotide to define the promoter element. Defining the placement of the aptamer sequence relative to the promoter enables engineering of gene expression levels and tuning sensitivity to ligand concentration. The approach to template synthesis offers the possibility of providing sufficient DNA substrates for larger scale cell-free protein synthesis reactions. Further, tuning the strength of the T7 bacteriophage promoter in combination with changing the aptamer position can allow users to tune fold changes in response to ligand addition.[69] The T7 bacteriophage promoter tolerates the single-stranded template but requires placement of the aptamer near the promoter sequence. This placement can compromise promoter strength. Potentially, DNA-based aptamer control elements can be extended to other bacteriophage or bacterial promoter sequences and allow designs that lead to even greater dynamic range in gene expression control. For this study, we tested the use of thrombin binding aptamer to evaluate our aptamer-mediated control of gene expression. This strategy can potentially be extended to other aptamers and their associated ligands provided they function in cell extracts.[70] Selection and use of DNA aptamers that work with known promoter elements will result in new approaches to regulating gene expression in response to a wide range of molecules.

MATERIALS AND METHODS

Plasmid Construction. All plasmid constructions were carried out using standard techniques.[71] GFP was cloned into pBluescript KS II (+) and pBluescript KS II (−) vector backbones, and the aptamer sequences were then inserted downstream to T7 promoters using inverse PCR. The aptamer constructs are listed in Supporting Information, Table S1, and sequence information is provided in Supporting Information, Section S6. Plasmids will be made available upon request.

Single-Stranded DNA Template Preparation. ssDNA templates were assembled by annealing template strands generated from pBluescript KS (−) II variants with T7 promoter oligo in annealing buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, and 1 mM $MgCl_2$). Thrombin aptamer sequence was cloned into the phagemid vectors pBluescript KS II (+) and pBluescript KS II (−) at different locations downstream to the transcription start site. These two backbones differ from each other only with respect to the orientation of the F1 origin. ssDNA molecules were then generated using a standard procedure.[71] For the preparation of double-stranded templates with bubble regions, ssDNA generated from pANTKS was annealed to an oligonucleotide that is complementary to a HindIII site on the pBluescript KS II (+) DNA backbone prior to digestion with HindIII restriction endonuclease. The resulting template was purified and annealed to the single-stranded DNA template derived from pBluescript KS II (−) by slow cooling from 95 °C to room temperature in a thermocycler in annealing buffer. The resulting construct contains a mismatched bubble region corresponding to the F1 origin region. The efficiency of annealing and dsDNA generation was verified by digesting the DNA template using restriction endonucleases. For preparation of the largely single-stranded templates, the oligonucleotide 5′ TAATACGACTCACTATAGG 3′ was hybridized to the single-stranded DNA template generated from plasmids pATKS, pETAKS and pETA26KS in the presence of annealing buffer.

Cell-Free Protein Synthesis Experiments (CFPS). The Promega S30 T7 High-Yield Expression System kit (Promega TM306) was used for CFPS experiments. The S30 premix and the cell extract were mixed in proportions recommended by the manufacturer, and template was used at 20 ng/μL concentration per reaction. DNA concentrations used were based on previous optimizations.[21] Reactions were set up in Corning CLS3820 plates following manufacturer's instructions except that the final reaction volume was scaled to 15 μL. Samples were incubated at 30 °C with shaking, and fluorescence measurements (485/20 nm excitation, 528/20 nm emission) were made every 7 min in a Biotek Synergy 2 plate reader. Error bars associated with the fluorescence measurements represent standard deviation of three replicates. Values indicated in the graphs represent GFP concentrations obtained after 6 h of cell-free protein synthesis reactions.

Thrombin-dependent gene repression was tested by incubating the DNA templates with thrombin (diluted from stock solution into 10 mM Tris-HCl pH 7.5 and 50 mM KCl) along with 0.01% Tween-20 for 1 h at room temperature, followed by the addition of the cell extract. Thrombin protein stocks were supplied in 50% glycerol solution. Thrombin concentrations used in this study ensured that the final glycerol concentration in cell-free protein synthesis reactions were less than 0.5%.[72] Human α-thrombin was purchased from Haematologic Technologies, Essex Junction, VT. The thrombin aptamer sequence used to relieve repression was 5′ GGTTGGTGTGGTTGG 3′ and the nonspecific oligonucleotide sequence was 5′ GGCGTAACTGATCGATCGT 3′. To test the effect of different thrombin aptamer and nonspecific oligonucleotide concentrations on thrombin-mediated repression, different oligonucleotide amounts were heat denatured and slowly cooled to room temperature in the presence of 10 mM Tris-HCl pH 7.5, 5 mM KCl and 1 mM $MgCl_2$ before they were added to cell-free protein synthesis reaction.

ASSOCIATED CONTENT

Supporting Information

This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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