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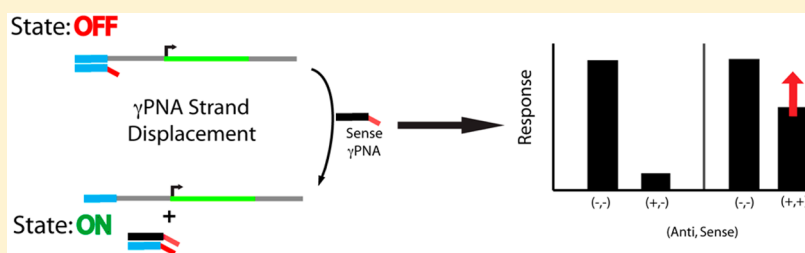
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In Vitro Reversible Translation Control Using γ PNA ProbesTaylor D. Canady,^{†,§} Cheryl A. Telmer,^{‡,§} Stanley N. Oyaghire,^{†,§} Bruce A. Armitage,^{*,†,§} and Marcel P. Bruchez^{*,†,‡,§}[†]Department of Chemistry, [‡]Department of Biological Sciences, and [§]Center for Nucleic Acids Science and Technology, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, United States

S Supporting Information



ABSTRACT: On-demand regulation of gene expression in living cells is a central goal of chemical biology and antisense therapeutic development. While significant advances have allowed regulatory modulation through inserted genetic elements, on-demand control of the expression/translation state of a given native gene by complementary sequence interactions remains a technical challenge. Toward this objective, we demonstrate the reversible suppression of a luciferase gene in cell-free translation using Watson–Crick base pairing between the mRNA and a complementary γ -modified peptide nucleic acid (γ PNA) sequence with a noncomplementary toehold. Exploiting the favorable thermodynamics of γ PNA– γ PNA interactions, the antisense sequence can be removed by hybridization of a second, fully complementary γ PNA, through a strand displacement reaction, allowing translation to proceed. Complementary RNA is also shown to displace the bound antisense γ PNA, opening up possibilities of in vivo regulation by native gene expression.

■ INTRODUCTION

Methods to irreversibly interfere with gene expression (e.g., antisense targeting of mRNA) are well-known chemical biology tools and potential therapeutic strategies. Reversible regulation of gene expression offers the opportunity to perturb gene regulatory networks and signaling cascades. Through the use of a serial method of translation control, investigations related to the temporal and spatial effects of a specific transcript can be approached. Through the careful attention to chemical probe affinity and selectivity, control over protein expression is possible at the transcript level, which allows for the direct investigation at precise nodes in biochemical pathways. Importantly, achieving reversible control through the supply of an external probe can enable numerous interventions and manipulations of native genetic elements in order to assess or control downstream phenotypic effects. To this end, chemical probes can be introduced at precise time points, allowing for on-demand control of translation locally or systemically, depending on the method of probe delivery.

Synthetic translation regulation has been implemented through the utilization of various engineered mRNAs and antisense-probe manipulations. Facile examples of ligand dependent (i.e., riboswitches¹) protein expression, sequence optimization (ribosome binding site strength²), and direct expression knockdown through exogenous and endogenous probe hybridization³ have provided a way to selectively (de)-activate target transcripts. Translation regulation through a

strand displacement mechanism has also been realized. For example, through the careful design of toehold-driven RNA interactions, conditional RNAi mediated knockdown has been demonstrated.⁴ Furthermore, the mRNA transcript itself has been engineered to display conditional RBS sequestration, whereupon translation is driven by a small noncoding RNA displacement reaction.⁵ Using this approach, Green and co-workers recently reported improved transcript designs for higher-fold GFP activation and generalized the design to detect a much larger set of small RNA targets (triggers).⁶ In terms of antisense reversible regulation, Young et al. suppressed gene expression knockdown and then subsequently reactivated it by manipulating a light-sensitive antisense agent that undergoes an intramolecular strand displacement reaction.⁷ This work demonstrated the feasibility of light-defined control over reporter expression where reversibility was established through application of caged antisense probes. Nevertheless, achieving light-activated translation control is limited to a relatively small sensitized area and penetrance depth, thus precluding systemic translation control (i.e., whole animal). To achieve translation control in a systemic manner, as well as limit any genetic manipulation of target transcripts, we designed a translation control system consisting of an antisense agent that inhibits

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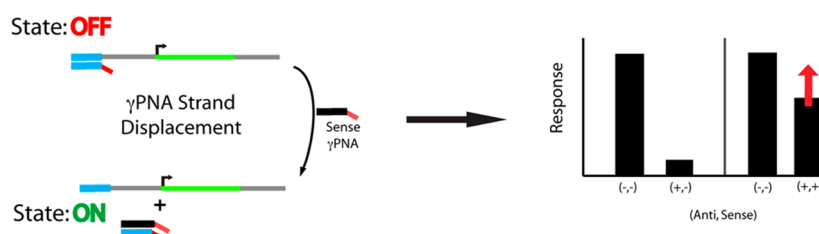


Figure 1. Reversible translational control through use of γ PNA probes. State: OFF is established by the introduction of an antisense γ PNA that binds to a partially cognate mRNA transcript, leaving the adjoining toehold domain free. With the introduction of sense γ PNA, a subsequent γ PNA– γ PNA (toehold nucleation event) binding event initializes strand displacement, rendering the translation to State: ON. The subsequently altered luciferase expression can be plotted to measure the translation recovery (right illustration).

translation but can be subsequently displaced from the mRNA target by a suitable complementary sense agent.

A probe that alters translation must display the requisite affinity and specificity in order to have the desired activity without detrimental off-target effects. Peptide nucleic acids (PNA^{8,9}) provide high affinity for complementary RNA targets, with biological stability owing to the unnatural backbone configuration. The ability of PNA to interfere with gene expression has been well documented,¹⁰ with examples including modulation of transcription,^{11–13} pre-mRNA splicing,^{14–16} mRNA translation,^{17–20} and miRNA function.^{21,22} γ PNA, a second generation analogue of PNA,²³ exhibits enhanced target affinity and increased water solubility compared to traditional PNA.²⁴ These features arise from the fact that γ -modifications that introduce an appropriate chirality to the PNA backbone induce right-handed helicity that closely resembles the structure of the γ PNA when hybridized to complementary DNA/RNA, leading to enhanced target affinity. Meanwhile, the diethylene glycol (miniPEG) substituent used in the current work provides significantly improved water solubility relative to PNA.²⁴ γ PNA has shown promising target selectivity with excellent mismatch discrimination,²⁴ and guanidinium-modified γ PNA has been shown to inhibit translation of mRNA within living cells.²⁵ Moreover, γ PNA– γ PNA duplexes are significantly more stable than γ PNA–RNA heteroduplexes, meaning our proposed approach relies on a thermodynamically favorable process.²⁶ Furthermore, PNA molecules are nuclease, protease, and peptidase resistant, which makes them stable in biological milieu, such as the cell lysates we used in the following in vitro translation experiments.²⁷ Although γ PNA's stability under these conditions has not been characterized, we expect it to be similarly resistant to enzymatic degradation. Herein, we demonstrate reversible translation control mediated through a γ PNA– γ PNA strand displacement process.

RESULTS

Reversible translation control is achieved through two separate and fully complementary γ PNA molecules. The first γ PNA is required for direct mRNA target binding (antisense) which represses translation. The second (sense) γ PNA derepresses translation through a γ PNA– γ PNA displacement interaction, mediated by a free toehold sequence on the first (antisense) γ PNA strand (Figure 1).

Reversible control of translation by base-pairing requires two functioning components: (i) a sequence-selective probe that suppresses translation effectively and (ii) an agent that can relieve this suppression. Previous antisense “gene walk” investigations with PNA established two effective native

mRNA target sites^{18,28,29} (i) the 5′-terminal end of the mRNA and (ii) proximal to or at the Kozak initiation sequence and AUG start codon. Relative to eukaryotic translation, probe hybridization at the terminal end of the 5′-UTR is largely believed to attenuate translation by inhibiting the attachment of the 43S ribosomal preinitiation complex to the transcript and thus blocking initiation of translation.^{18,29} When a probe is targeted to the Kozak/AUG region,³⁰ both ribosomal scanning of the 5′-UTR and subsequent translocation into the coding region are prevented.³¹ The removal of the antisense block from the mRNA transcript via strand displacement from either site would release a fully functional native mRNA and allow translation to proceed.

Translation Suppression by PNA and γ PNA. To begin development of the translational switch, two unmodified 15-mer PNA probes were synthesized that were designed to bind to the above-mentioned sites within a luciferase transcript. Both of the PNAs display IC₅₀ values in the low nM range (Figure S1A), indicative of excellent in vitro targeting, while maintaining negligible activity toward an orthogonal 5′-UTR luciferase transcript that lacks the PNA target sites (Figure S1B). To improve target binding, analogous γ PNA (γ = (R)-diethylene glycol, “miniPEG”) oligomers were prepared. As mentioned above, γ PNA has been demonstrated to exhibit favorable RNA duplex stability, while still maintaining target selectivity. Due to the higher thermodynamic stability of γ PNA–RNA interactions, compared to PNA–RNA,²⁴ we synthesized two γ PNAs (10-mer and 15-mer) targeting the 5′ terminus of the mRNA (referred to as γ PNA₁₀ and γ PNA₁₅). For reference, the luciferase mRNA target sequence and the γ PNA sequences are given in Table 1.

The antisense dose response relationship for both γ PNA₁₅ and γ PNA₁₀ probes against the luciferase reporter are presented in Figure 2A. Both probes gave low IC₅₀ values (<50 nM). Furthermore, a dose of 100 nM γ PNA gave >85% gene knockdown for both cases. Unexpectedly, the two γ PNAs gave similar IC₅₀ values despite the difference in length. Nonetheless,

Table 1. 5′-Terminal mRNA Target Sequence^a

mRNA Target	5′-AGACCCAAGCUUUC-3′
γ PNA ₁₅	NH ₂ -K-TCTGGGTTCGAAAGT-H
γ PNA ₁₀	NH ₂ -K-TCTGGGTTCG-H

^aThe γ PNAs sequences are written from the C-terminus to the N-terminus, “K” represents lysine. The 5′-end of the mRNA transcript is hybridized with the C-terminal end of the γ PNA oligonucleotide.

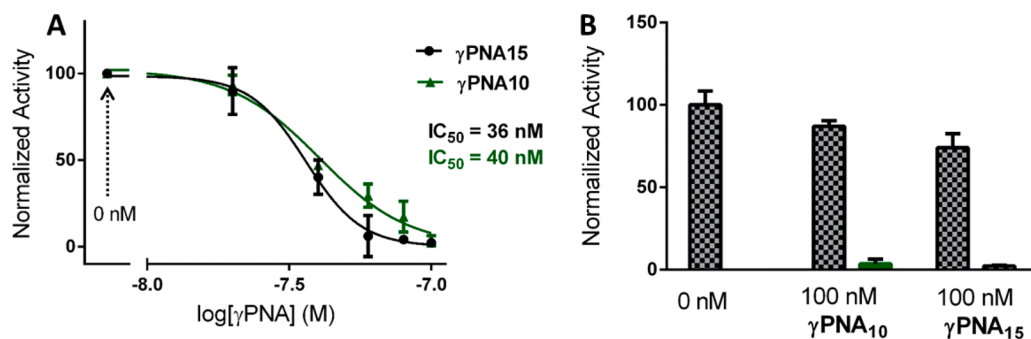


Figure 2. γ PNA antisense gives effective and specific luciferase knockdown. (A) Overlay of antisense dose–response curves for both γ PNA₁₀ and γ PNA₁₅ (both tested at $n = 3$) tested against the luciferase mRNA (10 nM). The γ PNA probe and mRNA were preannealed in a buffered solution (RRL-matching concentration of 79 mM K⁺ and DEPC-treated H₂O) and then placed in a 37 °C for 1 h. (B) γ PNA probes against a luciferase mRNA (10 nM) that has a scrambled γ PNA target site (checked bars). γ PNA₁₀ shows insignificant knockdown ($p > 0.05$) and γ PNA₁₅ shows small (~15%) but potentially significant ($p < 0.05$) luciferase knockdown. The knockdown of the perfect match luciferase is shown for γ PNA₁₅ (100 nM, black bar) and γ PNA₁₀ (100 nM, green bar).

the improved efficacy of the γ PNAs compared to the PNA is consistent with the enhanced γ PNA thermodynamic stability. In addition, by utilizing an mRNA transcript with an orthogonal 5'-UTR, we observed only slight (~10–15%) off-target luciferase knockdown (Figure 2B), again likely due to the higher affinity trade-off. Having observed sufficient antisense knockdown for both lengths of γ PNA, subsequent γ PNA translational control probes were based off the shorter γ PNA₁₀ sequence.

Reversible Translation Suppression with γ PNA.

Because the 10-mer sequence is effective for translational suppression, we designed an extended γ PNA probe containing the same 10-base mRNA binding domain and a contiguous 5-nucleobase toehold that is not complementary to any site on the mRNA (Figure 1). The toehold domain functions as a nucleation site that mediates the displacement of the bound antisense strand by addition of the second sense γ PNA. The sense γ PNA is fully complementary to antisense γ PNA, leading to 5-extra base pairs (for specific sequence design see Table 2).

Table 2. Specific γ PNA Sequence Used in Reversible Experiments^a

mRNA Target	5' -AGACCCAAGC-3'
Antisense γ PNA	H ₂ N-K-TCTGGGTTCG TGATA -H
Sense γ PNA	H-AGACCCAAGC ACTAT -K-NH ₂

^aComplementary toehold domains indicated in red. K = lysine.

The use of a 5-nucleobase toehold was determined by previous reports that show this to be a suitable length³² for strand displacement reactions in DNA-based manipulations. Hence, upon the introduction of the complementary sense γ PNA and through binding of the sense toehold to the complementary antisense toehold, a strand displacement reaction should free the mRNA transcript and toggle translation back ON.

To characterize the γ PNA– γ PNA displacement reaction, we designed a series of surface plasmon resonance (SPR) experiments to verify and quantify the displacement reaction. Unfortunately, SPR is not amenable to direct immobilization of

RNA due to the harsh chip regeneration conditions (i.e., NaOH wash) needed to remove bound γ PNA prior to the next injection of sample. Given this, the SPR chip is functionalized with an immobilized biotinylated-DNA target, where the DNA sequence matches the target mRNA probe binding domain. Antisense γ PNA is then injected (20 nM) into the flow cell to allow the target DNA-antisense γ PNA hybridization reaction to occur (Figure 3). Subsequently, a short buffer wash is used to remove any unbound antisense γ PNA, and then a titrated concentration (0–200 nM) of sense γ PNA is flowed over the chip to allow for the γ PNA– γ PNA displacement reaction. A successful displacement reaction causes an overall loss of mass on the chip and generates a discernible dissociation curve on the SPR sensorgram (Figure 3). The percent displacement should increase as a function of sense concentration.

Figure 4 demonstrates that the displacement experiment functions as illustrated in Figure 3. Loading of the antisense γ PNA results in strong increase in signal, while subsequent addition of sense γ PNA decreases the signal in a dose-dependent manner. Interestingly, only ca. 50% of the antisense γ PNA could be displaced, based on the decrease in the observed response units. However, subsequent control experiments revealed that high (i.e., >100 nM) concentrations of sense γ PNA led to nonspecific binding to the SPR chip, which evidently compensates for the loss in mass due to specific displacement of the antisense γ PNA. (These experiments are described in detail in the Supporting Information.)

To verify that the presence of the antisense toehold was necessary for displacement, we repeated the SPR displacement reaction by considering two separate experimental toehold-control conditions. The first control removed the toehold from the antisense γ PNA to test if displacement driven by the sense γ PNA can still proceed without proper toehold nucleation to the γ PNA-10mer sequence. Here, we found that the removal of the antisense toehold gave no measurable displacement on the SPR (Figure 4C and Figure S4A). The second control experiment utilized a longer immobilized target that completely base-pairs with the antisense γ PNA toehold. When the longer target was applied, there was minimal detection of sense γ PNA driven displacement (Figure 4C and Figure S4B) presumably due to sequestering the antisense toehold.

After observing γ PNA-driven displacement on the SPR, in vitro translation control studies were then conducted. The initial antisense γ PNA probe was preannealed to the target

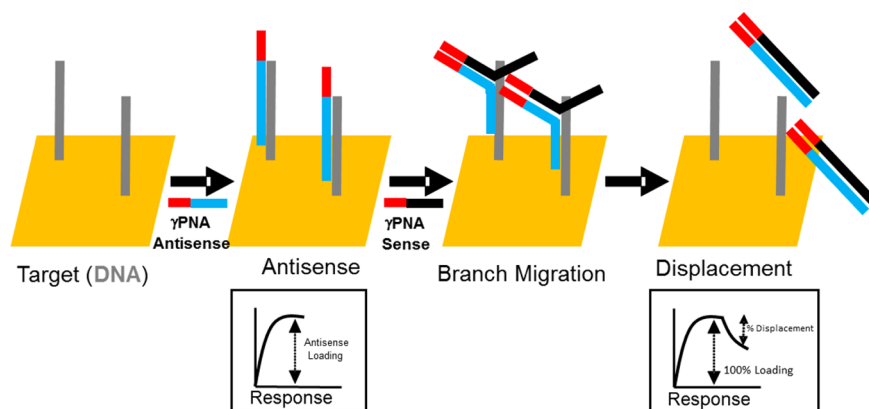


Figure 3. Surface plasmon resonance (SPR) investigation of γ PNA– γ PNA strand displacement. Antisense γ PNA (light blue), with toehold (red), is loaded (association phase) onto the SPR chip via a hybridization reaction with the surface bound DNA (gray) capture strand. A wash step is included to remove any residual antisense γ PNA. The toehold nucleation and strand displacement reaction is initiated through sense γ PNA (black) injection. Branch migration phenomena (unresolvable by SPR) leads to complete strand displacement, resulting in overall mass loss from the chip. The ratio of loading versus loss due to displacement is taken as the percent displacement.

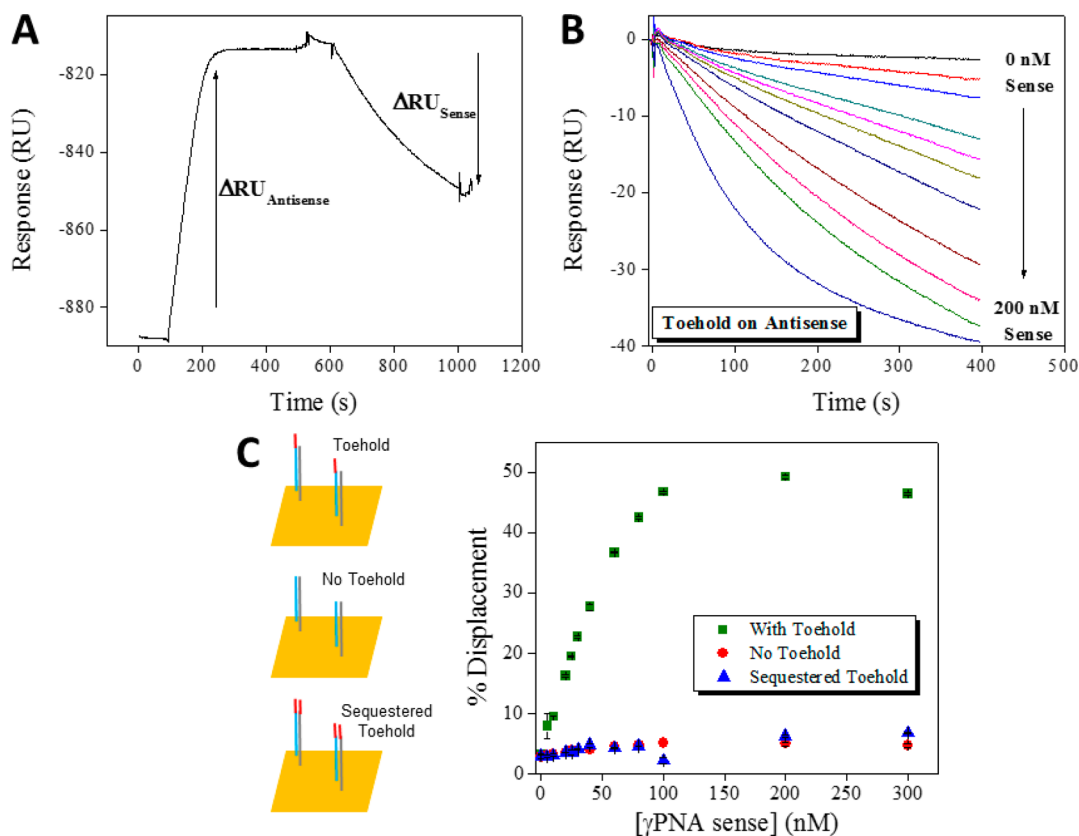


Figure 4. Surface plasmon characterization of γ PNA displacement from complementary DNA. (A) Calculation of percent displacement. The response unit change due to sense γ PNA dissociation (ΔRU_{Sense}) is divided by initially hybridized antisense γ PNA ($\Delta RU_{\text{Antisense}}$). The experiments were conducted so that $\Delta RU_{\text{Antisense}}$ is constant for all runs. (B) Dissociation of antisense γ PNA through increasing sense γ PNA concentrations over time. Every SPR run used a constant antisense concentration of 20 nM, which was injected onto the SPR chip to hybridize the immobilized target DNA. Sense γ PNA was then titrated (0–200 nM), to allow for displacement of the antisense γ PNA from the chip. (C) Various antisense conditions were considered including: toehold, no toehold, and sequestered toehold (left). The total displacement of the achieved by the various conditions is shown (right). (All data in panel C is presented as average of $n = 2 \pm \text{SD}$).

mRNA (1 h at 37 °C in 79 mM K^+ buffer). The sense γ PNA was then introduced and allowed to interact with the antisense γ PNA–mRNA complex over time. The total mixture (mRNA, antisense/sense γ PNA) was subsequently added to the Rabbit Reticulocyte Lysate (RRL) and luciferase bioluminescence was measured using a TECAN plate reader after 90 min of

translation. Following this approach, γ PNA translation recovery was observed across a range of sense γ PNA incubation times at equimolar sense–antisense (100 nM) concentrations in a time-dependent fashion (Figure 5A), with ca. 20% translation recovery observed in 1 h, increasing to ca. 75% recovery in 3 h.

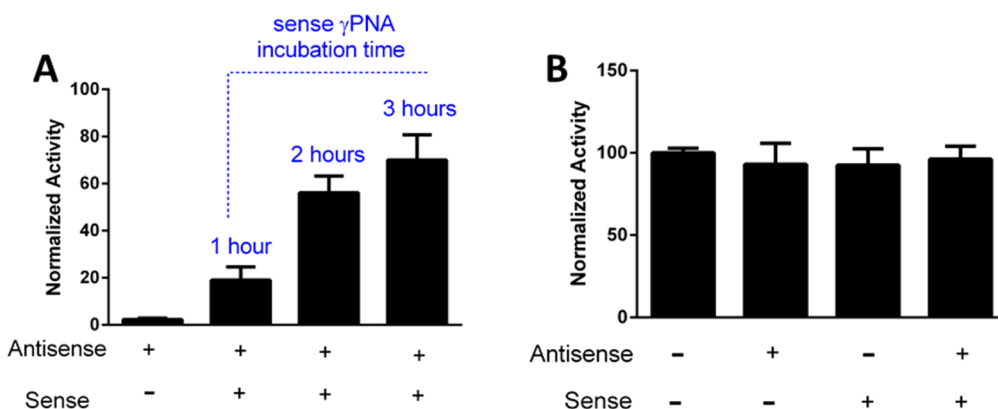


Figure 5. Time dependent translation recovery via γ PNA– γ PNA displacement. (A) The amount of luciferase translation recovery observed was directly related to the sense γ PNA incubation times. At 3-h sense γ PNA translation recovery approached $\sim 75\%$ activity opposed to only $\sim 20\%$ when 1-h incubations were performed. Equimolar (100 nM) antisense γ PNA/sense γ PNA was used with 10 nM of target mRNA for all samples (data is plotted as $n = 3$ average \pm SD). (B) When the antisense γ PNA alone or sense alone or both were incubated (1 h at 37°C) against an orthogonal mRNA transcript (scrambled antisense γ PNA target site), we observed insignificant ($p > 0.05$) luciferase knockdown ($[\gamma\text{PNA}] = 100$ nM, $[\text{mRNA}] = 10$ nM) (data is plotted as $n = 3$ average \pm SD).

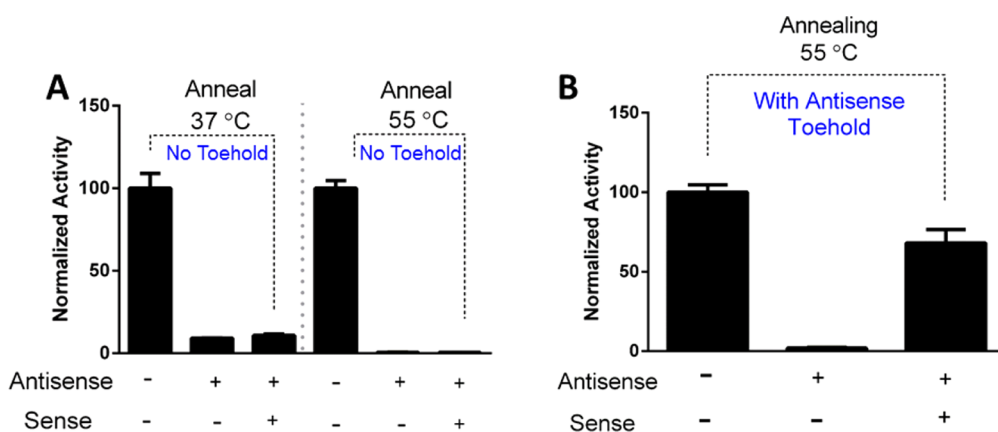


Figure 6. An antisense toehold is required to reverse γ PNA-mediated translation inhibition. (A) The absence of the antisense γ PNA toehold domain eliminates any observed sense γ PNA translation recovery (2-h sense incubation $[\gamma\text{PNA}] = 100$ nM, $n = 3$); however, the toehold loss does not affect the antisense potency, see (+, -) bar. Additionally, the loss of the antisense γ PNA toehold eliminates any observed translation recovery regardless of applying an elevated sense γ PNA incubation temperature (55°C) see (+, +) bar (data is plotted as $n = 3$ average \pm SD). (B) When the antisense γ PNA toehold is available, applying an elevated temperature (55°C) sense γ PNA incubation yields high values of translational recovery ($\sim 70\%$) compared to the knockdown ($p = 0.0002$) (data is plotted as $n = 3$ average \pm SD).

The time dependence almost certainly indicates that our probe-driven reversibility has kinetic limitations. To ensure that the antisense γ PNA does not knockdown an orthogonal luciferase, we incubated the toehold extended antisense γ PNA at the high end of the dose range (100 nM) against a luciferase (10 nM mRNA) transcript with a scrambled target-binding site. We observed no knockdown of the luciferase expressed from the orthogonal transcript when compared to the 0 nM antisense γ PNA control (Figure 5B). These results reflect the high degree of affinity and specificity antisense γ PNA has for the target sequence of the transcript. Lastly, to show that the sense γ PNA/mRNA interaction (no antisense present) does not cause elevated expression, the sense γ PNA was incubated with the mRNA (100 nM sense, 2-h incubation, 10 nM mRNA) and then added to the RRL. Again, the sense γ PNA showed no effect on protein expression levels when compared to the 0 nM γ PNA control (Figure 5B).

The reversibility of an antisense γ PNA that lacks the toehold domain was evaluated to determine if the increased γ PNA– γ PNA duplex stability alone was sufficient to reactivate

translation. Initially, we annealed the “toehold-less” antisense γ PNA to the luciferase mRNA at 37°C . Next, we introduced the sense γ PNA to the annealed mRNA/antisense γ PNA and let the ternary mixture incubate for 2 h before adding the entire mixture to the RRL for translation. While the antisense-driven luciferase knockdown was conserved without the presence of a toehold domain, the sense-mediated reversibility was abolished completely (Figure 6A), indicating the importance of the antisense toehold as a nucleation site required to recover gene translation. Furthermore, similar results were observed even at elevated temperatures (55°C) applied over a 2-h sense incubation time. Applying the same reaction conditions (sense incubation at 55°C) to a toehold-containing antisense γ PNA showed increased recovery of translation (Figure 6B) when compared to recovery values obtained at 37°C sense incubation (70% versus 55%).

An alternative to regulation of translation by exogenous sense γ PNA is to utilize expressible RNA (e.g., miRNA, mRNA, or lncRNA) or exogenously supplied RNA (i.e., siRNA). Therefore, a sense RNA was tested for its ability to displace the

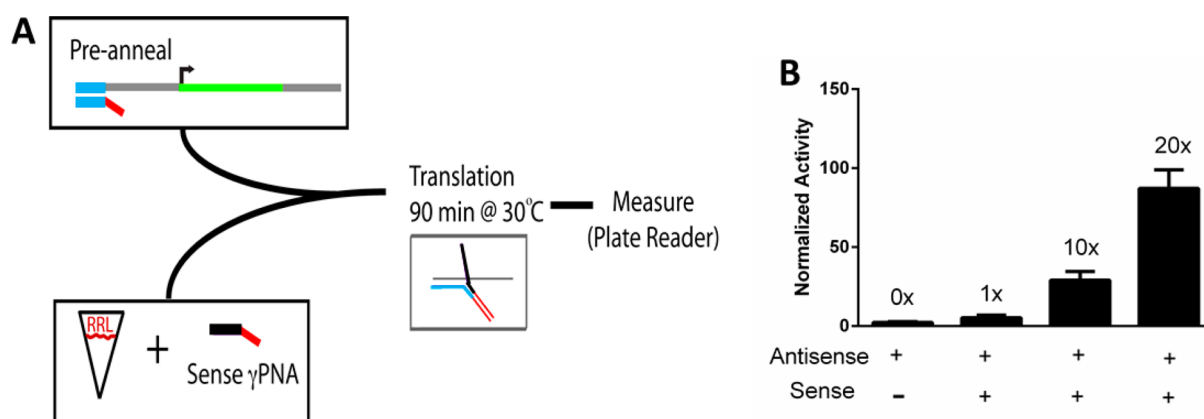


Figure 7. (A) Reversible translation through addition of sense γ PNA (1–20 \times) into rabbit reticulocyte lysate (RRL). The antisense γ PNA (100 nM) is preannealed (1 h at 37 $^{\circ}$ C) to the mRNA prior to the introduction into the RRL. The translation reaction then proceeds for 90 min at 30 $^{\circ}$ C. Translation recovery as a function of sense γ PNA concentration ($N = 3$ for all concentrations). (B) 0 \times represents 100 nM antisense γ PNA (+, -), with no added sense γ PNA. Significant ($p < 0.05$) translational recovery is seen at 10-fold and 20-fold sense concentration compared to antisense (100 nM) alone ($\sim 98\%$ knockdown) (data is plotted as $n = 3$ average \pm SD).

antisense γ PNA (Figure S7A). The sense RNA (200 nM) was incubated with preannealed antisense γ PNA/mRNA sample over the course of 2 h. A 2-fold excess (200 nM) of sense RNA was required to obtain $\sim 30\%$ translation recovery (Figure S7B). This differs from the sense γ PNA experiments where 2-h equimolar sense–antisense (100 nM) incubations gave $\sim 55\%$ recovery. Furthermore, sense RNA concentrations that were 10-fold higher (1 μ M sense) than antisense γ PNA yielded similar translation recovery results to 200 nM sense RNA. Additionally, we tested sense RNA driven-displacement on the SPR (Figures S5 and S6); however, we did not observe antisense γ PNA displacement, which could be due to nonoptimized toehold design (e.g., length, sequence, etc.) or the much shorter time scale of the SPR experiment, where the sense RNA only has 400 s during which to displace the antisense γ PNA versus a 2 h incubation prior to beginning in vitro translation.

To demonstrate translational control under physiological translation conditions, we tested sense γ PNA reversal directly in a functional translation lysate system (Figure 7A). The sense γ PNA was added at various concentrations to the RRL solution. These lysates were then charged with the antisense γ PNA/mRNA complex (100 nM of antisense, 10 nM mRNA), and translation proceeded for 90 min. As expected, when no sense γ PNA is present, $\sim 98\%$ of luciferase activity is blocked by the antisense strand (Figure 7B). Translation of luciferase is restored in a concentration-dependent manner, ultimately achieving $\sim 90\%$ recovery at a 20-fold (20 \times) excess of sense γ PNA (Figure 7B). Considering the antisense reversal in lysates, this suggests that such control may be possible in native physiological environments (e.g., cells), provided the excess sense strand does not exert significant off-target effects.

DISCUSSION

Reversible and selective translation control will provide a tool that is useful in the study of fundamental biological processes as well as in synthetic biology. Here, we demonstrated reversible translational regulation of a luciferase reporter in a cell-free system using γ PNA strand displacement driven by toehold recognition.³³ Translation blocked by an antisense γ PNA targeted to the 5'-terminus of the mRNA could be restored through addition of either a sense γ PNA or a synthetic RNA

designed to be fully complementary to the antisense γ PNA. The latter result opens the door toward using endogenous sense molecules (e.g., miRNA) to reverse translational inhibition by an antisense γ PNA, although further optimization of the toehold is needed to enhance the potency of reversal by RNA. In addition, successful demonstration of PNA as an inhibitor of transcription,³⁴ splicing^{14,15,22} and miRNA function^{21,22} suggests that our γ PNA-based approach can be extended to allow reversible control over other steps in gene expression.

In addition to the luciferase reporter experiments, we developed a new SPR method to monitor strand displacement reactions in real time without the need for fluorescent labels.³⁵ This method should be useful for studying the kinetics of strand displacement for any other natural or synthetic nucleic acid. With growing interest in nucleic acid–based computing, SPR provides an attractive platform on which to study hybridization reactions in a label-free, automated, medium-throughput manner.

We anticipate that the γ PNA displacement method may be useful in more elaborate cell-free experiments that include multiple gene targets, which are to be controlled discretely and independently, or to exert control over multiple open reading frames on a single transcript. Nevertheless, the simultaneous regulation of multiple genes will require careful sequence design of the γ PNA system in order to limit off-target binding and maximize translation activation. Future improvements upon the γ PNA design, for instance by altering toehold sequence and length, or by increasing probe affinities by chemical modification, may lead to enhanced toehold nucleation and subsequent displacement in these types of reactions.

The next logical step in this work is to demonstrate reversible antisense effects in cell culture, which will require effective delivery of γ PNAs into cells. Cell-penetrating peptides,^{36–38} cationic side chain modifications^{25,29,39,40} or small molecule conjugates⁴¹ have all been used to deliver PNAs into cells. In addition, a recent paper reported site-specific genome editing in vivo by γ PNA delivered via a polymeric nanoparticle vehicle.⁴² A similar nanoparticle formulation could be integrated with our technology to achieve intracellular translation recovery. In practice, this would involve delivering the antisense and sense

γ PNAs in separate transfections. Alternatively, a single nanoparticle bolus that delivers both γ PNAs simultaneously could be used if the sense γ PNA is prevented from hybridizing to the antisense γ PNA, e.g., through introduction of a photoreleasable caging group.⁴³

Another concern with moving our reversible translation inhibition to a cellular context is the possibility of off-target binding due to the exceptionally high affinity of γ PNA,²⁴ which could lead to weaker antisense inhibition of the targeted mRNA and/or slower, less potent reversal due to competitive hybridization of one or both γ PNAs to unintended sites. However, designing structure into the γ PNAs, for example, in the form of a hairpin⁴⁴ or competitor strand,⁴⁵ could limit such off-target binding while preserving sufficient affinity to drive the key γ PNA– γ PNA hybridization reaction.

Finally, it could be anticipated that the cellular mRNA surveillance pathways would interfere with our approach by degrading the antisense-targeted transcript before the sense γ PNA could be introduced. However, the activation of nonsense-mediated decay (NMD), nonstop-mediated decay (NSD), and no-go decay (NGD) mechanisms are dependent on the presence of a transcript-docked ribosome.⁴⁶ In the work presented here, we selected an mRNA target site (terminal 5'-UTR) that prevents 5'-UTR eukaryotic ribosomal initiation. Under this γ PNA targeting strategy, we anticipate that little mRNA will be lost due to ribosome-dependent degradation pathways. Similarly, RNaseH-mediated mRNA degradation is unlikely to occur due to the unnatural structure of γ PNA. Thus, it should be possible, within cell culture, to trap a transcript using an antisense γ PNA and then release it for translation at some later time through addition of the corresponding sense γ PNA.

MATERIALS AND METHODS

(γ)PNA Synthesis, Purification, And Characterization. PNA oligomers were synthesized and purified using common protocols. Mass characterization was conducted by MALDI-TOF, and subsequently analyzed for purity by reverse phase HPLC. γ PNA oligomers were obtained from PNA Innovations.

Luciferase Plasmids for T7 RNA Polymerase Generation of RNA and in Vitro Protein Synthesis in a Rabbit Reticulocyte Lysate. The T7 Luciferase Control Plasmid, Promega part number L482A, was modified to remove alternate start codons, add the CMV transcription start sequence, a restriction site upstream of the Kozak-ATG and a restriction site at the start codon to enable luciferases to be inserted. The Promega plasmid was digested with BamHI and SacI and the Gaussia luciferase was ligated into the Promega vector. This also introduced an NcoI site at the start codon. The firefly luciferase was PCR amplified, digested, and ligated into NcoI and SacI digested vector.

NcoFFlucF 5'-TATATACCATGGGAAGACGCCAAAAACATAAAGAAAGG-3'

SacFFlucR 5'-TATATAGAGCTCGCCCCCTCGG-3'

This plasmid was then digested with HindIII and BamHI and annealed oligos were ligated to introduce an NheI site and CMV transcription start sequence.

HindCMVtransF 5'-AGCTTTTCAGATCCGCTAGCGCTACCGGG

BamCMVtransR 5'-GATCCCCGGTAGCGCTAGCGGATCTGAA

The firefly luciferase plasmid has the T7 promoter, CMV transcription start sequence and restriction sites, HindIII, NheI, BamHI and NcoI at the start codon. There is a SacI site 66 bases from the stop codon and a 30 base polyA sequence following the SacI site.

PCR Amplification of the Firefly Luciferase Plasmid. The firefly plasmid was digested (linearized) using an ApaLI restriction site

located downstream of the encoded polyA tail sequence. The linearized product was purified using the Thermo Scientific GeneJET Gel Extraction Kit protocol. The DNA was then PCR amplified using the NEB PCR Protocol for Phusion High-Fidelity DNA Polymerase (cycled 35 times, PCR program 98 °C, 2 min; 98 °C, 10 s; 45 °C, 15 s; 72 °C, 2 min; 72 °C, 1 min; hold at 4 °C).

Primer design: T7 transcription site 5'-TACGACTCACTATAGGG-3'

3'poly A tail site 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'

The products were purified using the Thermo Scientific Gel Extraction Kit protocol and verified using agarose gel electrophoresis (1.8 kB).

Transcription Reaction and Purification. The transcription reaction followed the Thermo Scientific conventional transcription protocol (50 μ L final volume) and consistently gave high RNA product yield (~2.5 μ M, determined via NanoDrop spectrophotometer). The transcription reaction was conducted at 37 °C for 2 h. The transcription products were purified using the Thermo Scientific GeneJET RNA Cleanup and Concentration Micro Kit and concentration was measured using a NanoDrop spectrophotometer.

γ PNA/mRNA Annealing. The γ PNA and RNA were annealed together in the presence of 79 mM potassium chloride (designed to match the K⁺ concentration in the rabbit reticulocyte lysate, RRL Promega) and DEPC-treated water. The RNA concentration for all translation experiments is set at 10 nM in the final translation reaction. The probe concentration varies depending on the desired final concentration of probe. The initial antisense probe is annealed at 37 °C for 1 h. In the case of the reversible translation experiments, an additional preincubation time (1–3 h) is given for sense γ PNA displacement at 37 °C being careful to maintain the incubation salt concentration (79 mM).

Translation Conditions and Luciferase Readout. The translation reaction was conducted using the Promega Luciferase Assay System (E1500) (rabbit reticulocyte lysate). The PNA concentration is determined by considering the 50 μ L final translation reaction volume. The translation reaction is conducted at 37 °C for 2 h. Immediately after, the samples are stored in ice to quench any further protein synthesis. Following the Promega Rabbit Reticulocyte Lysate System technical manual, 5 μ L of lysate solution is mixed into 50 μ L of Promega Luciferase Assay Reagent (E1483) added to a Thermo Scientific Nunc 96 well plate (flat white). The bioluminescence reading was collected on a TECAN Infinite M1000 plate reader.

Surface Plasmon Resonance (SPR). All SPR experiments were performed on a Biacore T100 instrument (GE Healthcare) equipped with a four-channel sensor chip. The commercially available chip is coated with a carboxymethyl dextran matrix that allows further functionalization with streptavidin via a standard NHS-EDC coupling procedure.⁴⁷ Immobilization of streptavidin was continued until 7000 response units (RU) of the protein were captured on each of the four channels (flow cells). The final step of the sensor design involved noncovalent capture of the 5'-biotinylated DNA targets (~150 RU) on the respective flow cells bearing immobilized streptavidin.

Each experiment was preceded by injection of a solution containing 20 nM of the antisense γ PNA oligomer for 400 s (flow rate = 30 μ L/min). A dissociation time of 1 s was incorporated after the injection to allow for diffusion of unbound antisense oligomers from the sensor surface. The subsequent displacement assay was then performed by injecting a solution containing a fixed concentration of either the sense γ PNA or RNA oligomer (flow rate = 30 μ L/min) and monitoring the sensor response over 400 s. Each displacement cycle was ended by introducing a pulse of a regeneration cocktail (1 M NaCl, 10 mM NaOH) for 30 s at a flow rate of 50 μ L/min. The aforementioned cocktail serves to release any residual antisense/sense oligomers and is followed by a buffer injection (150 s, flow rate = 30 μ L/min) to reestablish a baseline prior to the next displacement cycle.

The attenuation in response units following introduction of the sense oligomer was taken as evidence of displacement of the antisense γ PNA from the sensor surface. Therefore, we established a quantitative estimate of the displacement reaction by the ratio of signal attenuation

upon introduction of the sense γ PNA, Δ RU (sense), to enhancement upon introduction of the complementary antisense γ PNA, Δ RU (antisense) eq 1.

$$\% \text{ Displacement} = \frac{\Delta \text{RU (sense)}}{\Delta \text{RU (antisense)}} \times 100 \quad (1)$$

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05351.

In vitro translation results for antisense PNAs and RNA, control experiments for SPR displacement assays (PDF)

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Notes

The authors declare the following competing financial interest(s): One of the authors (Bruce Armitage) has a potential competing financial interest associated with this work. He holds equity in PNA Innovations, Inc., which is commercializing PNA technology invented at Carnegie Mellon University.

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