RNA Quadruplex-Based Modulation of Gene Expression

Markus Wieland[1] and Jörg S. Hartig[1,*]

[1]Department of Chemistry, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany

*Correspondence: joerg.hartig@uni-konstanz.de

SUMMARY

RNA-based modules such as riboswitches represent straightforward and simplified approaches for the regulation of gene expression, as no additional proteins are needed. G-rich sequences are known to adopt stable four-stranded structures, and such quadruplexes have been suspected to play important roles in key functions such as the control of gene expression. Here we demonstrate that RNA quadruplexes readily form in vivo. We have constructed mRNA-based G-rich elements that mask the ribosome binding site by folding into four-stranded structures. The suppression of gene expression correlates with the stability of inserted G quadruplexes. Moreover, quadruplexes with moderate stability respond to changes in temperature, thus behaving as artificial RNA thermometers. In conclusion, we introduce tuneable mRNA-based devices that enable modulation of gene expression by a predictable but thus far unknown mechanism.

INTRODUCTION

In addition to complex, protein-dependent regulation of gene expression, much more immediate "RNA only"-type mechanisms such as riboswitches, RNA thermometers, and trans-acting noncoding RNAs have recently been discovered in bacteria [1–3]. A variety of such mechanisms function via modulation of translation initiation by sequestering the ribosome binding site (RBS) in stable duplex folds [3–5]. The RBS contains the Shine-Dalgarno (SD) consensus sequence that needs to be single stranded for the recruitment of the small subunit of the ribosome in order to efficiently initiate translation [6]. It is composed of a purine-rich sequence located five to ten positions upstream of the start codon. Although highly degenerated, a consensus sequence of AGGAG or GGAGG has been described [7, 8]. Masking of the RBS by secondary-structure formation is a common mechanism to regulate gene expression. For example, several recently discovered riboswitches shut off gene expression upon binding of a regulatory active metabolite to the 5′ untranslated region of an mRNA (5′UTR) [9]. The binding event within the so-called aptamer platform triggers a conformational change, resulting in masking of the RBS within the expression platform by rearranging hairpin structures [3, 10]. Other examples include temperature-dependent control of gene expression based on sequestering the RBS by RNA thermometers. Here, translational initiation is blocked by a hairpin structure masking the SD at lower temperatures. Increased temperature results in freeing the RBS and induction of gene expression [11, 12]. Further RNA-based mechanisms involve a variety of trans-acting RNAs that regulate gene expression by modulating accessibility of the ribosome binding site [13, 14]. Here we present a novel mechanism for masking the Shine-Dalgarno sequence by forming four-stranded, G-rich structures. The results add strong evidence to the debate as to whether such quadruplexes are able to fold and affect functions in vivo.

Despite the most common element of secondary structure found in nucleic acids, the helical antiparallel duplex arrangement, other interesting folds such as triple- and quadruple-stranded motifs have been observed [15–17]. Among these structures, the quadruplex helix is suspected to play important roles in highly regulated processes such as the modulation of gene expression. There have been several reports of the participation of DNA quadruplexes in gene regulation, such as, in mammals, sequences that bear the potential to form quadruplexes which have been discovered in promoters of proto-oncogenes [18–20]. A computational survey found putative G quadruplex sequences to be enriched in proto-oncogenes, whereas they are less frequently observed in tumor suppressor genes [21]. In addition, small molecules that bind to and thereby stabilize DNA quadruplexes have been shown to suppress gene expression of certain proto-oncogenes, hence presenting a therapeutic potential [18, 22]. Probably the best-characterized G-rich sequences that are able to fold into quadruplex structures are the capping structures at the ends of eukaryotic chromosomes, the telomeres [23–25]. Nevertheless, whether quadruplexes readily fold inside cells and whether functions are generally connected to these unusual sequences are still a matter of debate. Strong evidence for the existence of G-rich quadruplex structures in vivo was observed by the staining of telomeres with quadruplex-specific antibodies [26], selective binding of proteins and small molecules to G-rich mRNAs and telomeric DNA [27–29], as well as formation of quadruplex DNA structures during transcription [30].

In contrast to four-stranded DNA, RNA quadruplexes are less characterized, although G-rich RNA is able to fold into equivalent structures with stabilities comparable to their DNA counterparts [31]. Recently, Balasubramanian and coworkers have described an interesting RNA quadruplex that modulates translation of a proto-oncogene in a mammalian cancer cell line [32]. The authors reported a 4-fold reduction in gene expression using an in vitro translation assay. Furthermore, a potential regulative role of four-stranded G-rich sequences has been suggested recently in alternative splicing of human pre-mRNA [33]. Here we present rationally designed quadruplexes inserted into the ribosome binding site of a bacterial reporter gene. This setup has the advantage that observed effects on gene expression can be compared to data obtained with other structural elements.

RESULTS

Quadruplexes Efficiently Modulate Gene Expression

We have designed a series of sequences that contain G-rich elements surrounding the Shine-Dalgarno sequence of a reporter gene coding for enhanced green fluorescent protein (eGFP). The inserted G-rich stretches were placed immediately up- and downstream of the SD without altering the consensus sequence (Figure 1A). In order to determine whether a stable quadruplex is able to fold in vivo resulting in interference with translational initiation, we have inserted a sequence that should fold into a highly stable four-stranded structure. High stability was realized by choosing a sequence that should be able to fold into a quadruplex composed of three stacks of G tetrads combined with short loops between G stretches flanking the SD sequence containing only one uridine, termed G_3U (Figure 1B). Short loops result in remarkable stability of quadruplexes, as has been demonstrated for DNA quadruplexes previously and is shown for our RNA sequences later [34]. Interestingly, upon insertion of the stable quadruplex sequence (G_3U), eGFP expression was reduced by 96% with respect to the wild-type RBS (WT) (see Figure 1C).

In our design, less stable quadruplexes should repress gene expression less efficiently, as the SD should be more accessible. The stability of quadruplexes depends on the number of G stacks, the loop size, and loop composition [31, 34]. It is therefore possible to fine-tune the stability of a quadruplex fold. Starting from the stable quadruplex G_3U, two possibilities for destabilization were tested. First, increasing the loop length between G stretches flanking the SD from one to two uridines results in a variant termed G_3U_2. In accordance with the proposed stability, eGFP expression increased 3-fold compared to G_3U, but still displaying only 10% of WT eGFP expression. A second possibility consists of the reduction in the number of G tetrad stacks from three to two, resulting in the variant G_2U starting from G_3U (Figure 1). The variant G_2U shows more pronounced expression of eGFP with respect to G_3U and G_3U_2, displaying 30% expression compared to the WT sequence. Next, we designed even more desta-
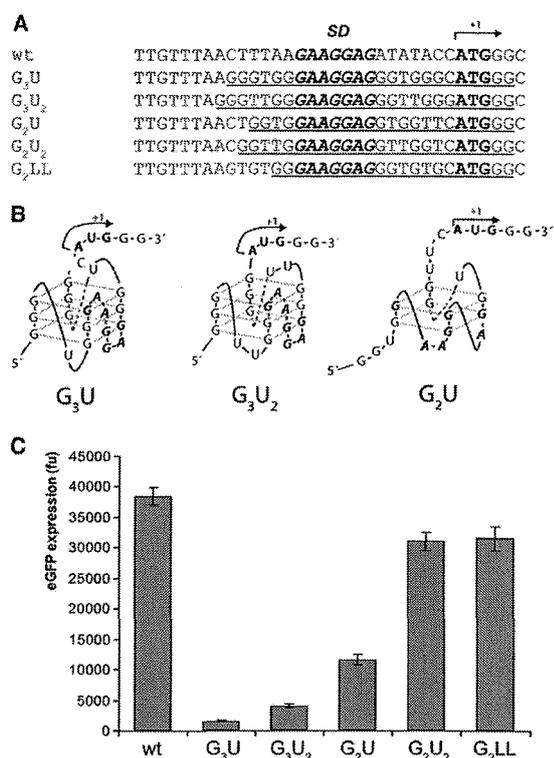

Figure 1. Influence of Quadruplex-Forming Sequences Inserted into the 5′ Untranslated Region of an eGFP Reporter Construct

(A) Sequences that were cloned into the 5′UTR of eGFP. The Shine-Dalgarno sequence (SD) is shown in italics and was not altered. The start codon is shown in bold.

(B) Possible quadruplex folds of selected clones masking the SD sequence (shown in italics). The RNA quadruplexes fold into parallel-oriented quadruplexes as determined by CD spectroscopy.

(C) Influence of quadruplexes on reporter gene expression. Stable quadruplexes strongly suppress gene expression, whereas less stable folds permit efficient translation. Error bars reflect the standard deviation of three independent experiments.

bilized quadruplex sequences by combining the two-stack design with a loop size of two (variant G_2U_2), as well as reducing the number of G stretches that might participate in a quadruplex to four, enforcing a putative quadruplex with a potentially destabilizing long loop (termed G_2LL). In both cases, efficient eGFP expression was observed displaying about 80% of WT activity (Figure 1C).

In order to validate that the reduced gene expression upon insertion of the quadruplexes results from masking the SD sequence and is not due to preliminary termination of transcription, we have carried out in vitro transcription experiments using linearized plasmid templates. For these studies we have used T7 RNA polymerase, which is the same that drives transcription of eGFP in vivo in pET16 constructs. As can be seen in Figure S1 (see the Supplemental Data available with this article online), no difference

is observed for the transcription of the linearized plasmid templates WT and G_3U containing the strongest quadruplex. Both sequences can be transcribed to full length with no preliminary termination visible, except for expected short products resulting from inefficient transcription initiation.

Investigation of Quadruplex Topologies and Stabilities

In order to characterize the inserted G-rich RNA sequences with respect to their structure and stability, we carried out circular dichroism (CD) studies using synthetic RNAs corresponding to the sequences inserted into the mRNAs (underlined in Figure 1A). Monovalent cations, especially potassium, increase the stability of quadruplex structures [35, 36]. Intracellular potassium levels are known to be high in bacteria. In *Escherichia coli*, concentrations of 200–250 mM are usually found, with deviations ranging from 100 mM to a maximum of 600 mM if grown in high osmolarity media [37]. For the structure and stability investigation of the engineered RNA quadruplexes in vitro, we decided to measure their CD spectra in buffered solutions containing 100 mM KCl, a concentration that is already known to highly stabilize quadruplex structures [36, 38]. Figure 2A shows the CD spectra of the inserted G-rich sequences. All sequences fold into parallel-oriented quadruplex sequences, indicated by a minimum at 240 nm and a maximum at 263 nm, which are the expected values for a parallel fold [39]. Quadruplexes composed of three G tetrads (G_3U and G_3U_2) display stronger CD signatures compared to sequences that should fold into four-stranded structures with two G stacks (G_2U and G_2U_2). The sequence G_2LL shows intermediate CD intensities. The fact that all quadruplexes fold into parallel-orientated strands is not surprising, as RNA quadruplexes generally disfavor the antiparallel fold. In order to form an antiparallel topology, guanosines have to adopt in part the syn conformation, which is highly unfavorable for RNA due to the ribose C_3' endo conformation [40].

To access the stability of the investigated RNA quadruplexes, we followed the thermal denaturation of the parallel, four-stranded structures at 263 nm by CD spectroscopy (Figure 2B). As expected, RNA quadruplexes composed of three G tetrads display much higher melting points compared to the two-stack counterparts, that is, >90°C for G_3U and 78.7°C for G_3U_2 compared to 56.5°C for G_2U, 53.7°C for G_2U_2, and 66.2°C for G_2LL. When cooling the samples, no hysteresis was observed, pointing to fast folding kinetics (data not shown). The impressive stability of G_3U is in agreement with the results obtained for DNA quadruplexes composed of three stacked G tetrads containing short loops reported by Balasubramanian, Neidle, and coworkers [34]. Nevertheless, RNA quadruplexes containing only two G tetrads (G_2U, G_2U_2, and G_2LL) still display pronounced stability. Surprisingly, the sequence G_2LL shows higher stability than G_2U and G_2U_2, a result not expected from the expression data. Apart from this exception, the stability of
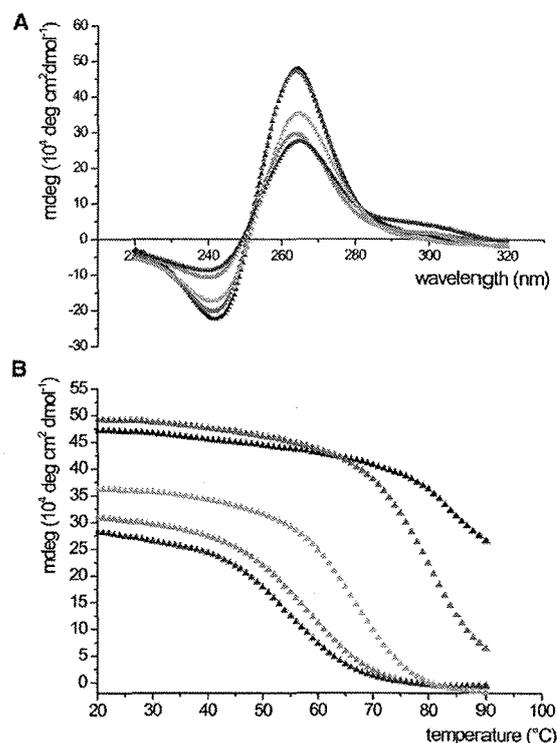

Figure 2. Circular Dichroism Experiments for the Elucidation of the Folding Topology and Stability of RNA Quadruplex Sequences

(A) CD spectra of RNAs (5 µM concentration in 50 mM Tris [pH 7.5], 100 mM KCl; the sequences used for the CD experiments are underlined in Figure 1A and given in Experimental Procedures). G_3U (black), G_3U_2 (red), G_2U (green), G_2U_2 (blue), and G_2LL (cyan) indicate that the engineered RNA sequences containing the Shine-Dalgarno sequence fold into parallel-oriented quadruplex structures indicated by a maximum at 263 nm and a minimum at 240 nm.

(B) Decrease in the maximum CD signal at 263 nm with increasing temperature indicates the thermal stability of the quadruplex structures. The inserted quadruplex sequences display melting points of >90°C for G_3U, 78.7°C for G_3U_2, 56.5°C for G_2U, 53.7°C for G_2U_2, and 66.2°C for G_2LL (color index is similar to Figure 2A).

the quadruplex fold of the isolated RNA strands matches well with the observed levels of gene expression in vivo.

Temperature-Dependent Switching

Duplex structures that mask the ribosome binding site are found in RNA-based modulators of gene expression such as *trans*-acting RNAs, riboswitches, and RNA thermometers [1–3]. In the last genetic devices, a decrease in temperature results in the increased stability of a secondary structure that interferes with ribosomal initiation. This behavior made us wonder whether a decrease in temperature would also stabilize our four-stranded structures in vivo. The results of eGFP expression at different temperatures are shown in Figure 3. Whereas no influence on gene expression was observed for the WT clone, quadruplex-containing variants show pronounced temperature
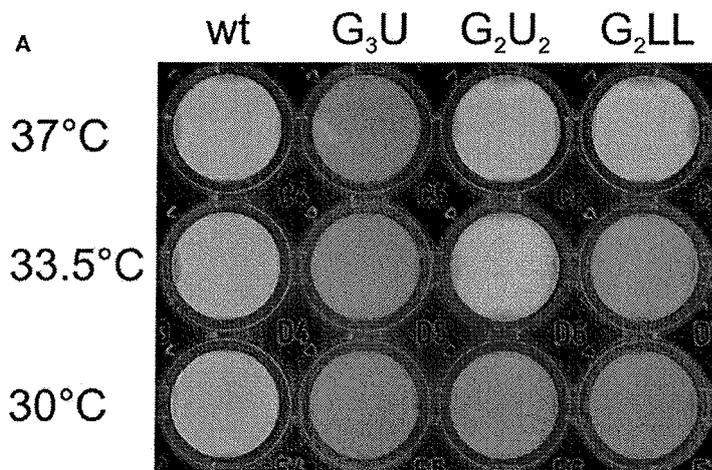
A

wt G_3U G_2U_2 G_2LL

37°C

33.5°C

30°C

B

eGFP expression (fu)

(y-axis: 0, 5000, 10000, 15000, 20000, 25000, 30000, 35000, 40000, 45000)

(x-axis: wt, G_3U, G_3U_2, G_2U, G_2U_2, G_2LL)

Figure 3. G Quadruplexes as Artificial Thermoswitches: Temperature Dependence of Gene Expression

(A) Photograph of *E. coli* liquid cultures. Bacteria were grown overnight at the indicated temperatures. Whereas the WT clone displays highly efficient and temperature-independent expression, clones G_2U_2 and G_2LL show clearly visible temperature dependence.

(B) Absolute fluorescence of eGFP measured in liquid culture. Black columns, 37°C; dark gray, 33.5°C; light gray, 30°C. Error bars reflect the standard deviation of three independent experiments.
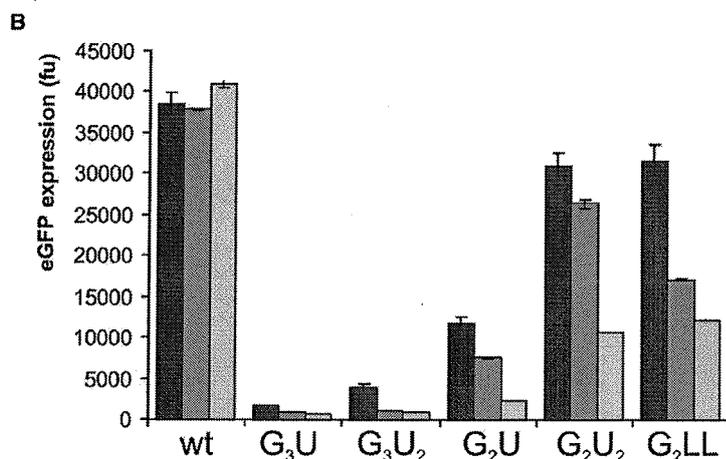
dependence. The variants G_2U_2 and G_2LL show the highest absolute reduction in eGFP expression (50% with respect to the WT expression), while highest relative reduction is observed for the more stable variants G_3U_2 and G_2U (80% reduction at 30°C compared to 37°C). Interestingly, the variant G_2LL responds earlier to changes in temperature (see Figure 3A, 33.5°C) compared to the variant G_2U_2, while both show comparable expression levels at 37°C and 30°C. This finding could be connected to the higher melting temperature as determined by CD spectroscopy, resulting in an earlier onset of quadruplex folding with decreasing temperature.

DISCUSSION

Secondary structures in the form of hairpins masking the ribosome binding site are employed in a variety of mechanisms to modulate gene expression. Only recently, simplified but highly versatile RNA-based mechanisms such as riboswitches and RNA thermometers have been discovered. Here we have shown that four-stranded secondary structures readily fold in vivo and modulate gene

expression if positioned within the RBS. These quadruplex elements provide a novel way to interfere with translational initiation in bacteria. The effects are predictable and tuneable, as interference with gene expression corresponds to the thermal stability of the inserted G quadruplex elements. To the best of our knowledge, with the variant G_3U we have described the most pronounced effects exhibited by a quadruplex structure in vivo, represented by a 98.6% reduction in gene expression (comparing clones WT and G_3U grown at 30°C). This finding could be explained by our strategy to deliberately position the four-stranded structures into elements that have to be non-structured in order to maintain functionality. Interestingly, putative quadruplex-forming sequences are significantly less abundant surrounding the RBS region compared to intergenic and protein-coding sequences in bacterial genomes, as shown by computational approaches. In fact, the likelihood of finding a putative quadruplex-forming sequence surrounding the Shine-Dalgarno sequence and the start codon in *E. coli* exhibits a sharp drop [41]. Despite the pronounced effects of the variant G_3U, the less stable variants have also provided novel insights; for example,

quadruplexes composed of only two stacks of G tetrads can have pronounced influence if placed at critical positions, as shown by the dramatic decrease in expression by the variant G_2U. This is an important finding, as the number of sequences that could potentially form quadruplexes composed of only two G tetrads found in genomes is considerably higher compared to the corresponding numbers of putative structures consisting of three stacks.

The in vitro CD experiments show that under physiological conditions, very stable RNA quadruplexes can exist. Further evidence pointing at stabilization of four-stranded structures compared to duplex structures comes from studies that have investigated competing structures under conditions of molecular crowding. Within such setups mimicking intracellular conditions, quadruplex folds seem to be favored over duplex folds [42]. The CD data point at RNA quadruplexes that fold into parallel-oriented, propeller-type topologies by traversing the grooves in double-chain reversal loops [43, 44]. Nevertheless, the supposed structures in Figure 1B are only models of potential folds. A more detailed structural investigation is necessary to identify the correct structure. The situation is more complicated, as more than one fold is possible due to the presence of more than four G stretches, especially for the less stable sequences consisting of only two G tetrads. To clarify the design, we have tried to reduce the number of G stretches, for example by deleting the last G stretch positioned within the first two codons. Unfortunately, deletion of the G stretch by silent mutations as well as deletion of the complete His tag-encoded N terminus of eGFP had significant effects on eGFP expression even in the WT clone (data not shown). In Figure 1B, we have shown the fold that involves the shortest loops, suggesting the most stable alternative fold. Nevertheless, alternate interactions such as participation of nucleobases other than guanine as well as influences of loop nucleotides can contribute to quadruplex stability and complicates prediction of quadruplex folding [31, 45]. This might also be an explanation for the unpredicted behavior of the sequence G_2LL. In addition, more unstable structures composed of three stacked tetrads might be possible where one corner of guanines within the quadruplex structure is not made up of consecutive guanines [31]. Another possible explanation for the observed discrepancy of in vivo activity and in vitro stability within the variant G_2LL could be the potential dimerization of the oligonucleotide in the CD experiments. Such multimeric structures could contribute to the observed stability. In vivo, this effect would likely be insignificant, as much lower effective concentrations are present.

The observed temperature dependence of such quadruplex elements points at the possibility that G-rich structures could function in regulatory processes. Compared to typical hairpin-based RNA thermometers, the measured temperature dependence is less pronounced [12]. On the other hand, one has to take into account that the sequences are likely not optimized to function as temperature switches. Such an optimization could be carried

out by further sequence design or by combinatorial approaches. Nevertheless, the obtained switches G_2U_2 and G_2LL are very sharp, with drastic reduction in gene expression within a window of only 3.5°C. This is not expected if one takes into account that all quadruplexes remain folded in vitro even at temperatures much higher than 37°C. On the other hand, the CD experiments exclude possible interactions with intracellular components that might weaken the quadruplex folds. The two variants differ in their onset, with G_2LL displaying the highest change from 37°C to 33.5°C and G_2U_2 from 33.5°C to 30°C. Such artificial switches might prove useful with respect to the latest efforts at defining and constructing standardized and predictable biological tools and elements for the construction of biological devices with novel properties in the emerging field of synthetic biology [46–48]. Quadruplex structures seem to be well suited for such purposes, as secondary structures of highest stability composed of only very few nucleotides can be designed. In order to implement a very stable quadruplex, only four stretches of three consecutive Gs are necessary. In addition, small loops contribute more to stability than larger ones [34]. The DNA sequence 5'-GGGTGGGTGGG TGGG-3' as well as the most stable quadruplex used in the present study (G_3U) fold into very stable structures that cannot be melted in aqueous solutions containing potassium [34]. To achieve such stable secondary structures made from classical Watson-Crick-paired hairpins, much longer sequences are required. Such "nucleotide-economic" modules of structural stability might have useful applications in diverse fields ranging from the above-mentioned synthetic biology to nucleic acid-based therapeutics.

SIGNIFICANCE

The intracellular existence of G quadruplex structures is still controversial, with no direct proof to establish its reality. Nevertheless, much convincing evidence points at the existence and diverse functions of quadruplexes in regulatory processes inside cells. Most studies are concerned with the description of naturally occurring sequences and show moderate effects of quadruplex formation. Here we have demonstrated further evidence of the existence of folded quadruplexes by a strategy orthogonal to preceding studies. Introducing artificial rationally designed sequences into a defined environment enabled us to study effects of quadruplex formation in a bacterial model organism. Our results show for the first time, to our knowledge, that quadruplex sequences can have pronounced influences on gene expression in vivo if placed at a crucial position, such as surrounding the ribosome binding site. The results are significant as well in light of recently discovered RNA-based mechanisms for regulating gene expression in bacteria such as riboswitches, noncoding RNAs, and RNA thermometers. The finding that the introduced quadruplexes behave as simplified sensors of temperature

opens up the possibility that quadruplexes might be involved in similar or more sophisticated mechanisms of regulation.

EXPERIMENTAL PROCEDURES

Construction of Plasmids

The G quadruplexes were introduced into the pET16b_eGFP plasmid (kindly provided by Prof. Dr. M. Scheffner) by performing PCR using the Phusion DNA polymerase (Finzyme) and sequence-specific primers with the designed G quadruplex sequences attached to the primer 5′ end (underlined). Prior to PCR, each primer was 5′-phosphorylated using T4 PNK (Fermentas).

The following primers were used: 5′-TTCCCACCCTTAAACAAAAT TATTTCTAGAGGG-3′, 5′-GGAGGGTGGGCATGGGCCATCATCATC ATC-3′ (pET16b_eGFP_G₃U); 5′-TTCCCAACCCTAAACAAAATTATTT CTAGAGGG-3′, 5′-GGAGGGTTGGGATGGGCCATCATCATCATC-3′ (pET16b_eGFP_G₃U₂); 5′-TTCCACCAGTTAAACAAAATTATTTCTAGAG GG-3′, 5′-GGAGGTGGTTCATGGGCCATCATCATCATC-3′ (pET16b_ eGFP_G₂U); 5′-TTCCAACCGTTAAACAAAATTATTTCTAGAGGG-3′, 5′-GGAGGTTGGTCATGGGCCATCATCATCATC-3′ (pET16b_eGFP_ G₂U₂); 5′-TTCCCACACTTAAACAAAATTATTTCTAGAGGG-3′, 5′-GGAGGGTGTGCATGGGCCATCATCATCATC-3′ (pET16b_eGFP_ G₂LL).

Following PCR, the template plasmid pET16b_eGFP was digested using the restriction enzyme DpnI. PCR products containing the G quadruplex sequences were blunt-end ligated (Quick Ligase; New England Biolabs) and transformed into E. coli BL21(DE3) gold culture (Stratagene). Single colonies were picked and grown in LB medium supplemented with 100 μg ml⁻¹ carbenecilline (Roth). To confirm successful cloning, the plasmids were isolated (Miniprep kit; QIAGEN) and sequenced (for complete sequences, see the Supplemental Data).

eGFP Expression Studies

Bacteria were grown in LB medium supplemented with 100 μg ml⁻¹ carbenecilline at 37°C, 33.5°C, and 30°C until outgrown. One hundred microliters of each culture was transferred into 96-well microplates and the fluorescence of the expressed eGFP (excitation wavelength = 488 nm, emission wavelength = 535 nm) was determined using a TECAN M200 plate reader. For background subtraction, an E. coli BL21(DE3) gold culture not expressing eGFP was treated equally.

CD Spectroscopy

CD measurements were made of RNA oligonucleotides synthesized using standard phosphoramidite solid-phase chemistry with the following sequences:

G₃U: 5′-GGGUGGGAAGGAGGGUGGGCAUGGG-3′,
G₃U₂: 5′-GGGUUGGGAAGGAGGGUUGGGAUGGG-3′,
G₂U: 5′-GGUGGAAGGAGGUGGUUCAUGGG-3′,
G₂U₂: 5′-GGUUGGAAGGAGGUUGGUCATGGG-3′,
G₂LL: 5′-GUGUGGGAAGGAGGGUGUGCAUGGG-3′.

RNA samples were prepared at 5 μM concentration in diethylpyrocarbonate-treated water buffered with 50 mM Tris-HCl (pH 7.5), 100 mM KCl. Oligonucleotides were annealed by heating to 95°C for 5 min followed by slow cooling to 20°C over 16 hr. CD spectra were recorded on a Jasco 715 spectrometer in cuvettes with a 1 cm path length, resolution of 0.5 nm, band width of 1.0 nm, and speed of 20 nm/min at 25°C. Each spectrum was accumulated five times and averaged. For thermal denaturation studies, the sample was heated from 20°C to 90°C with a heating rate of 1°C min⁻¹. The CD signal at 263 nm was recorded every 1°C and the melting temperature was obtained by determining the temperature at the half-maximum decrease of the signal.

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