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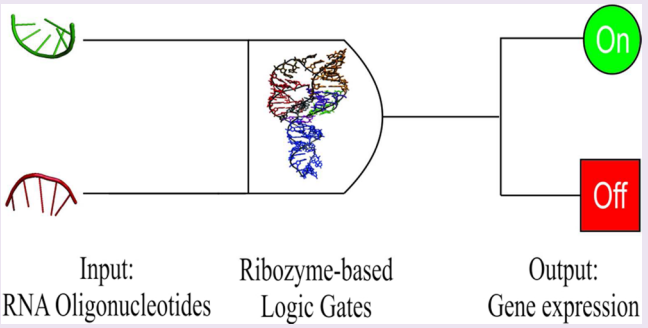
Programming a Highly Structured Ribozyme into Complex Allostery Using RNA Oligonucleotides

Samuel G. Rouleau, Rachel Jodoin, Martin Bisaillon, and Jean-Pierre Perreault*

RNA group/Groupe ARN, Département de biochimie, Faculté de médecine et des sciences de la santé, Pavillon de Recherche Appliquée sur le Cancer, Université de Sherbrooke, 3201 Jean-Mignault, Sherbrooke, Québec J1E 4K8, Canada

Supporting Information

ABSTRACT: RNA possesses great potential for expanding the toolbox currently available to synthetic biologists. Here, the modulation of the Hepatitis Delta Virus ribozyme's activity with a series of rationally designed aptamers and effector RNA oligonucleotides is described. The ribozyme was initially fused with an 18-nucleotide hairpin structure that abolished its self-cleaving activity. The binding of a 14-mer oligonucleotide to the hairpin rescued the self-cleavage in a concentration-dependent manner. This modified ribozyme was inserted into the 5′ UTR of a reporter gene, and the resulting construct was used to demonstrate that it is possible to modulate the ribozyme activity *in cellulo* with the oligonucleotide. Subsequently, ribozymes possessing specialized aptamers respecting other logic gates were also successfully designed and found to be functional *in vitro*. To our knowledge, this is the first example of HDV ribozyme regulation by oligonucleotides, as well as the first allosteric regulation of HDV ribozyme in mammalian cells.



Synthetic biology is a rising field full of promise. RNA can combine many functions within a single molecule. Among other features, it is highly malleable, thus making it an attractive molecule with which to drive a programmable function. Natural and engineered RNA modules perform a variety of biological functions, including hybridizing to targets, binding ligands, undergoing programmed conformational changes, and catalyzing reactions (reviewed in ref 1). Several ribozymes have been transformed through the addition of a communication module coupled to an aptamer that specifically recognizes an effector such as a small metabolite. The resulting allosteric ribozyme's catalytic activity is thus regulated by the presence of an effector. *Cis*-acting versions can be inserted into the untranslated regions (UTR) of mRNA and act as gene expression regulators. The presence of the metabolite turns "*on*" the self-cleavage activity of the mRNA, which in turn leads to the loss of the expression of the corresponding protein. This approach has been demonstrated to work well with the *hammerhead* ribozyme.[2] Hepatitis Delta Virus (HDV) ribozyme is derived from a virus that replicates in hepatocytes and is therefore naturally well adapted to the human cell environment.[3] This makes it particularly attractive for the development of therapeutic tools. HDV ribozyme folds into a double-pseudoknot secondary structure (see Figure 1A; reviewed in refs 3 and 4). This structure is composed of two stems (stem I, which permits binding of the substrate to the ribozyme in the *trans*-acting version, and stem II, which forms a pseudoknot in the *cis*-acting version), two stem-loops (III and IV), and three single-stranded junctions (I/II, I/IV, and IV/II). The junction I/IV and the loop III regions are both single-stranded in the initial stages of folding but are subsequently involved in the formation of the pseudoknot I.I. Several studies of the HDV ribozyme revealed that this catalytic RNA adopts a highly ordered structure (see Figure 1A; refs 5−7), in agreement with previous reports suggesting the presence of some unusual properties. For example, the *cis*-acting version retains self-cleavage activity in solutions containing up to 5 M urea[8] and at temperatures as high as 80 °C.[9] These unique properties limit our ability to transform the HDV ribozyme into an allosteric catalytic RNA. Replacement of the stem-loop IV of a genomic HDV ribozyme version by an aptamer for theophylline coupled to a communication module has been reported to function *in vitro*.[10] However, the same strategy was not successful with an antigenomic HDV ribozyme. Moreover, the plug and play strategy, as well as *in vitro* selection, did not lead to the development of a Tat-dependent HDV version.[11]

One of the observed limits of *in vitro* selection was the inability to isolate inactive ribozymes, most likely because the catalytic center included many tertiary interactions that prevented others, such as those that might occur with a randomized domain, from occurring. Similarly, the HDV ribozyme's tight folding prevents the more subtle conformational changes that take place within the aptamer upon effector binding. To date, only the addition of a sequence at the end of stem II (see Figure 1A), which possesses the ability to form a

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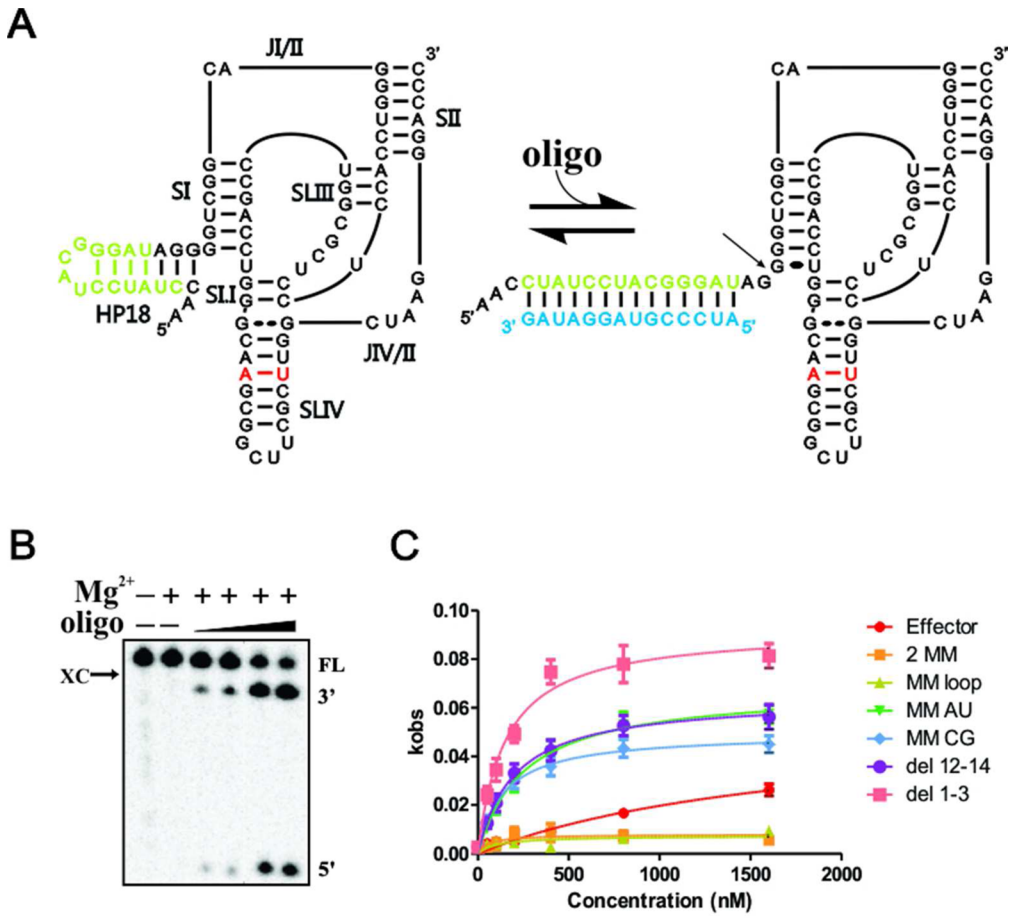

Figure 1. (A) Predicted secondary structure of the HP18 ribozyme both with and without the effector oligonucleotide E (blue nucleotides). The green nucleotides represent E's binding site. The red nucleotides represent those that were mutated (as compared to the original antigenomic ribozyme) to avoid creating alternative start codons. The arrow indicates the cleavage site. (B) *In vitro* cleavage assays with increasing concentrations of E. The arrow indicates the position of the xylene cyanol (XC) tracking dye. FL indicates the band corresponding to the full length RNA, and 3′ and 5′ represent the cleavage products. (C) Kinetic analysis of the different effector oligonucleotides.

highly stable G-quadruplex structure solely in the presence of potassium ions, has led to an efficient allosteric HDV antigenomic ribozyme, namely, the G-quartzyme.[12] However, since the intracellular concentration of potassium cannot easily be modulated, no further progress can be achieved with the G-quartzyme *in vivo*. Importantly, it appears that the modulation of a highly stable ribozyme seems to require an RNA motif that is also very stable. The goal of this study was to develop allosteric HDV self-cleaving ribozymes based on an antigenomic version whose folding pathway has been well described.[3,7]

RESULTS AND DISCUSSION

In order to circumvent the structural hurdles presented by the use of the antigenomic HDV ribozyme, its catalytic activity was modulated using RNA oligonucleotides. This offers several advantages, including the facts that RNA/RNA interactions should be strong enough to compete with the formation of the tight structure, that this system can be used *in vivo*, and that the Watson–Crick base pairing makes it easy to rationally design the aptamers to be bound by the oligonucleotides. A similar strategy was successfully used to obtain allosteric *hammerhead* ribozyme.[13] Moreover, previous work showed that the presence of a double-stranded structure located just upstream of the cleavage site inhibits the ribozyme's activity.[14] This led to the fusion of the self-cleaving HDV sequence to an 18 nucleotide hairpin loop (HP18) that included the first guanine downstream of the cleavage site (which normally forms a Wobble base pair with U35; see Figure 1A on the left). The idea was that modifying the ribozyme in a region that is not part of the highly structured catalytic core should facilitate the development of an inactive ribozyme. The HP18 ribozyme was synthesized *in vitro* by runoff transcription in the presence of radiolabeled nucleotides. This ribozyme was completely deprived of any catalytic activity (Figure 1B lane 2). On the other hand, the presence of the effector, a 14-mer RNA oligonucleotide complementary to a large portion of the HP18 domain, rescued the self-cleavage activity in a concentration dependent manner (see Figure 1A,B, lanes 3–6). Thus, the inactive ribozyme became active solely in the presence of the effector oligonucleotide.

Subsequently, oligonucleotides either of different sizes or harboring mutations that lead to mismatches when they are base-paired with HP18 were tested to characterize the effector/ribozyme interaction. The effectors (E) were named according to whether mismatches (mm) or deletions (del) were present and with respect to their position (e.g., Emm7 indicates an effector with a mismatch at position 7, whereas Edel1–3 indicates an effector from which the first three nucleotides were deleted). The sequences of the oligonucleotides and both the

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Table 1. Kinetic and Thermodynamic Parameters of the Effector Oligonucleotides for the HP18 Ribozyme

					ΔG (kcal mol^{-1})		
oligonucleotide	sequence	K_M' (nM)	k_2 (min^{-1})	k_2/K_M' (min^{-1} nM^{-1})	effector intramolecular	effector homodimer	effector/ribozyme
E	AUCCCGUAGGAUAG	1809	0.05814	3.21×10^{-5}	−2.400	−9.30	−34.30
Emm7	AUCCCCUAGGAUAG				−2.40	−10.80	−28.60
Emm7−12	AUCCCCUAGGAAAG				−1.700	−10.80	−24.50
Emm12	AUCCCGUAGGAAAG	253.2	0.06783	2.67×10^{-4}	−1.70	−7.90	−30.20
Edel12−14	AUCCCGUAGGA___	197.3	0.06414	3.25×10^{-4}	−0.900	−6.30	−29.30
Emm9	AUCCCGUACGAUAG	139.4	0.04956	3.55×10^{-4}	0.80	−9.20	−27.80
Edel1−3	___CCGUAGGAUAG	149.9	0.09238	6.16×10^{-4}	1.300	−4.90	−28.60
E-2	CCGUAGGAUAGGU	65.36	0.08858	1.36×10^{-3}	−0.8	−2.4	−37.1

cleavage activities and the kinetics parameters of the ribozyme are reported in Table 1 and are illustrated in Figure 1C. Self-cleavage experiments were performed using various concentrations of effector to determine both the cleavage rate at saturation (k_2) and a *pseudo* Michealis constant for a given effector (K_M'). The effectors containing either one or two mismatches (Emm7 and Emm7_12) with the HP18 loop did not rescue the self-cleavage activity. In contrast, mutations causing mismatches at other positions, or the deletion of 3 nucleotides at either the 5′ or 3′ extremity, permitted self-cleavage at a level greater than that observed with the original effector. The Gibbs free energies (ΔG) of the various base-pairing possibilities were calculated using secondary structure prediction softwares. Specifically, the intramolecular base-pairing of the effector was evaluated with the mFold software,[15] while the ability of the effector to base-pair either with itself (to form an homodimer) or with the ribozyme was estimated using the Pairfold program[16] (see Materials and Methods for the detailed procedures and Table 1 for the predicted ΔG values). Overall, the less stable were the intramolecular and homodimer structures of the effector, the higher was the self-cleaving activity. Indeed, an effector that is less prone to base pair with itself is more available to bind the ribozyme. The binding energy between the effector and the self-cleaving species appears to be less important. Specifically the differences between the various effectors are only caused by 1 or 2 mismatches, or by the deletion of 3 nucleotides out of 14 base pairs, which modifications do not significantly change the binding energy. It is important to note that the ability of a given effector to activate the self-cleavage cannot be predicted considering only one of these three parameters. It is preferable to optimize the three at the same time in order to develop the best possible effector. To validate these observations, a new oligonucleotide, E-2, possessing a binding site on the ribozyme that is shifted two nucleotides upstream of the cleavage site, was designed (Table 1). According to the predictions, this new effector should be less prone to interact with itself and has a stronger binding to the ribozyme. As expected, this effector yielded a significant improvement in the level of self-cleavage activity. Its k_2/K_M' ratio is 42-fold greater the original oligonucleotide's (Table 1, compare E-2 and E). This experiment led to two important conclusions. First, an easy and efficient way of obtaining an inactive HDV ribozyme is to introduce a hairpin adjacent to the cleavage site. Second, it is possible to modulate the HDV ribozyme's activity simply by using oligonucleotides that act as allosteric effectors.

The ribozyme's ability to regulate gene expression in mammalian cells was examined. Ribozymes were inserted in the 5′UTR of a firefly luciferase reporter gene, and the resulting constructs were co-transfected into HEK 293 cells with 2′-O-methyl RNA oligonucleotides (see Methods for detailed procedures). Wild type HDV ribozyme was found to be active regardless of which oligonucleotide was co-transfected (Figure 2A). The luciferase activity was approximately 2-fold lower with the active self-cleaving sequence than with a C76G mutant, a sequence that folded into the same structure as the wild type ribozyme but whose catalytic cytosine has been replaced by a guanosine, thereby creating a completely inactive ribozyme. Thus, the observed decrease in luciferase activity resulted only from the ribozyme cleavage. The HP18 self-cleaving sequence was inactive when co-transfected with a control oligonucleotide of irrelevant sequence, partially active with the original effector (E), and fully active with the E-2 optimized oligonucleotide (Figure 2A). This is in agreement with the *in vitro* data. To our knowledge, this is the first report of an allosteric activation of HDV self-cleavage *in cellulo*.

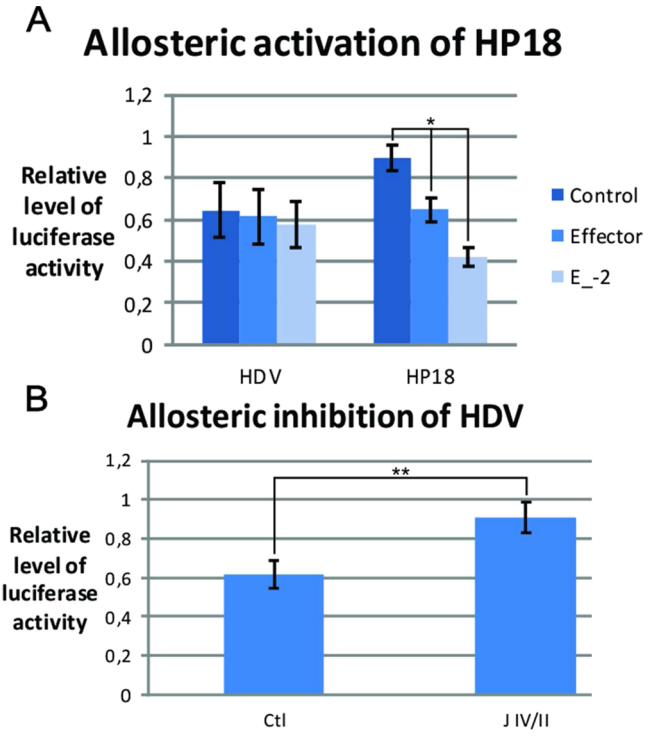

Figure 2. Regulation of luciferase activity within HEK 293 cells. The relative luciferase levels are normalized by comparing wild type constructions with C76G inactive mutants. (A) Activation of the HP18 ribozyme's cleavage activity by effector nucleotides. *$P < 0.05$ (B) Inhibition of HDV ribozyme's cleavage activity by the JIV/II effector. **$P < 0.01$.

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Next, an effector that could inhibit the ribozyme was designed. The strategy was to use an oligonucleotide complementary to the J IV/II region, which must be single-stranded for the catalytic center to be active (Supplementary Figure 1A, upper part). This new effector (JIV/II) inhibits the wild type HDV ribozyme's activity in a concentration-dependent manner *in vitro* (Supplementary Figure 1B, upper part). The JIV/II region could also inhibit the ribozyme's self-cleavage in HEK 293 cells and thus the increased luciferase expression (Figure 2B). These two robust molecular switches (*on* and *off*) possess great potential for use as synthetic biological tools. For example, the insertion of the ribozymes into the 5′ UTRs of multiple genes would permit a coordinated expression of those genes. The reduction in the level of gene expression was not as strong as that reported with some *hammerhead* ribozymes.[1,2] This might be caused by the possible capacity of HDV-like ribozymes to act as non-canonical translation initiators.[17] Nevertheless, the system presented here offers an interesting alternative, namely, a reduction without the complete loss of gene expression. This type of a system could be used to regulate the expression level of an essential gene whose complete abolition of expression is detrimental to the cells.

Importantly, the results presented here demonstrate that it is possible to modulate the activity of the highly stable HDV ribozyme based on rational design using oligonucleotide. Both the HP18:E-2 and the wild type HDV:JIV/II meet the criteria of the YES and NOT logic gates, respectively. In order to further explore this concept various constructs meeting the criteria of other logic gates were designed, and their self-cleavage was evaluated. Stem-loop IV, which has only a structural role and is located outside of the catalytic center,[18] was mutated so as to be complementary to stem-loop III (Supplementary Figure 2A). The base-pairing of the sequences between both stem-loops III and IV produced an inactive species, while the addition of a 19-nt oligonucleotide effector that binds to stem-loop IV rescued the self-cleavage activity (Supplementary Figure 2B). This construct corresponds to another YES function. Both YES modules (*i.e.*, the HP18 and stem loop IV domains) were then combined into the same ribozyme to obtain an AND gate (Supplementary Figure 3A). Either in the absence of effector or in the presence of only one oligonucleotide (regardless of which one), no significant self-cleavage activity was observed. Conversely, in the presence of both effectors, self-cleavage was observed (Supplementary Figure 3B). Similarly, an OR gate was designed by inserting both YES ribozymes into one RNA species. Self-cleavage was observed in the presence of either of the two oligonucleotides alone (Supplementary Figure 4). Analogously, a second NOT gate was obtained using an effector that binds to stem-loop III (Supplementary Figure 1A, lower part). Both NOT gates were then combined to get a NOR gate (*i.e.*, a self-cleaving RNA species that becomes inactive in the presence of only one oligonucleotide when it might be bound by two oligonucleotides). All of these new ribozymes showed robust levels of the desired regulation *in vitro* (Supplementary Figures 1−4B). Further optimization of these ribozymes should lead to better regulation of gene expression *in cellulo*. More importantly, these experiments unambiguously demonstrated that it is possible to program the highly structured HDV ribozyme into complex allostery using RNA oligonucleotides.

METHODS

HDV Ribozymes and DNA Constructs. HDV ribozymes were constructed using a PCR-based strategy that included two complementary and overlapping oligonucleotides as described previously.[19] The primers permit the incorporation of either the T7 RNA promoter for *in vitro* transcription (see below) or restriction enzyme sites for cloning. A histone stem loop (GGCCCTTAT-CAGGGCC) was also inserted upstream of the ribozyme to isolate the ribozyme from the upstream sequence, as it has been shown to not interfere with the ribozyme self-cleavage activity.[20] The 5′ to 3′ elongation of the DNA sequence that produced a double-stranded DNA template was performed using *Pwo* DNA polymerase (Roche Diagnostics). The PCR products were ethanol precipitated, dissolved in water, and then either used for *in vitro* transcription (see below) or digested for cloning purposes.

Cloning. The pGL3 (Promega) plasmid was used as the base vector. The HDV ribozymes were inserted in the 5′ UTR of the firefly luciferase reporter gene. The pGL3 plasmid was digested with the *Hin*dIII and *Nco*I restriction enzymes (NEB), and a cloning cassette consisting of two annealed primers with overhangs compatible with the digested plasmid was added. The cassette full sequence is AAGCTAAATCAATAACCAATAAATCCAACTTATAAGCT-TAAAAAAAAAAGAATTCAATAAATCTAATACATAAAGG-CATTCCGGTACTGTTGGTAAAGCCACCATGG. Unstructured flanking sequences are underlined. The cassette also permits the addition of an *Eco*RI restriction site for easy directional cloning (*Hin*dIII/*Eco*RI). In the case of the OR ribozymes, the two ribozymes were ligated together *via* a *PacI* restriction site, purified on agarose gel, and then cloned in the cassette with the *Hin*dIII/*Eco*RI sites. For each construction, an inactive version where the catalytic cytosine is replaced with a guanine was also constructed.

RNA Synthesis. The ribozymes were synthesized by runoff transcription as described previously.[19] Briefly, transcriptions were performed in the presence of purified T7 RNA polymerase (10 μg), RNase Out (24 U, Invitrogen), pyrophosphatase (0.01 U, Roche Diagnostics), and PCR product (2−5 mM) in a buffer containing 80 mM HEPES-KOH, pH 7.5, 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 5 mM of each NTP, and 1 μL of [α-^{32}P]UTP (3,000 Ci mmol^{-1}; New England Nuclear) in a final volume of 100 μL at 37 °C for 2 h. Upon completion, the reaction mixtures were treated with DNase RQ1 (Promega) at 37 °C for 15 min, and the RNA was then purified by phenol/chloroform extraction and ethanol precipitation. The resulting pellets were dissolved in water and loading buffer (95% formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol and/or 0.025% bromophenol blue). These samples were fractionated through 8% denaturing polyacrylamide gels (PAGE, 19:1 ratio of acrylamide to bisacrylamide) in buffer containing 45 mM Tris-borate, pH 7.5, 8 M urea, and 2 mM EDTA. The reaction products were visualized by ultraviolet (UV) shadowing. The bands corresponding to the correct sizes were cut out, and the transcripts were eluted overnight at 4 °C in elution buffer (500 mM ammonium acetate, 10 mM EDTA, 0.1% SDS). The transcripts were ethanol precipitated, dried, and dissolved in water. The RNA was quantified by absorbance at 260 nm.

***In Vitro* Cleavage Assays.** *Hairpin 18 Ribozyme.* The *in vitro* cleavage assays were performed with a final concentration of 50 nM of ribozyme and increasing concentrations of effector oligonucleotide (0−1600 nM). The reactions were performed in a total volume of 40 μL. For each experimental condition, 20 μL of 100 nM ribozymes were snap-cooled (2 min at 65 °C and 2 min on ice) and then preincubated at 37 °C for 5 min. The cleavage reactions were initiated by the addition of 20 μL of the desired concentration of effector RNA oligonucleotide and 2X reaction buffer (200 mM KCl, 100 mM Tris-HCl, 10 mM $MgCl_2$). At different time intervals, aliquots were transferred in a tube containing 10 μL of loading buffer to stop the reaction. All aliquots were then fractionated on an 8% denaturing PAGE. The results were visualized using a Phosphor Screen and quantified using the ImageQuant software (Molecular Dynamics). The cleavage percentage was calculated (cleaved products counts over cleaved plus uncleaved products counts) for each time point, and the

k_{obs}, K_M', and k_2 were calculated using GraphPad Prism5. Briefly, the rate of cleavage (k_{obs}) was obtained by fitting the data to the equation $A_t = A_{max}(1 - e^{-kt})$, where A_t is the percentage of cleavage a time t, A_{max} is the maximum percent cleavage, and k is the rate constant (k_{obs}). These k_{obs}'s, specific for each concentration, were then plotted in a *pseudo* Michaelis–Menten equation $Y = k_2X/(K_M' + X)$ where Y is the k_{obs} and X is the concentration of effector oligonucleotide.

Other Ribozymes. The same procedure was used, but the concentration of effector oligonucleotides was set at 2 μM for the assays with multiple effectors or ranged from 1 to 5 μM for the assays with increasing concentrations.

Free Energy Calculation and Structure Prediction. The intramolecular structure energy was obtained with the mFold software[15] using the effector oligonucleotide sequences. The intermolecular of the oligonucleotides dimer free energy and the oligonucleotide/ribozyme binding free energy were obtained with the Pairfold software, part of the rnasoft software[16] using the effector oligonucleotide sequences and a partial ribozyme sequence, namely, ggccuauccuacgggauaggggucggcaccgaccu, which contains the HP18 and the P1 stem.

Cell Culture and Dual Luciferase Assay. HEK 293 cells (human embryonic kidney) were cultured in T-75 flasks (Sarstedt) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate (all purchased from Wisent) at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. Cells (1.2×10^5) were seeded in 24-well plates and 24 h later were co-transfected with the specific pGL3 plasmid construction (firefly luciferase, Fluc) (50 ng/well and the pRL-TK vector (Renilla luciferase, Rluc) (Promega) (25 ng/well), and 2′-O-methyl RNA oligonucleotides (750 or 1500 ng/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were lysed, and the RLuc and FLuc activities were measured using the Dual-luciferase Reporter Assay kit (Promega) according to the manufacturer's protocol using a GloMax 20/20 Luminometer (Promega). For each lysate, the value of the Fluc was divided by the value of the Rluc. The ratios obtained for the C76/G mutant version were compared to those obtained with the wild type version of each construction. For each construction, both the mean value and the standard deviation were calculated from at least two independent experiments, which were each done in triplicate. *P* values were determined using GraphPad Prism5.

■ ASSOCIATED CONTENT

Supporting Information

List of primers used and supplementary figures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: Jean-Pierre.Perreault@USherbrooke.ca.

Notes

The authors declare no competing financial interest.

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