Allosteric Aptamers: Targeted Reversibly Attenuated Probes†

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ABSTRACT: Aptamers are unique nucleic acids with regulatory potentials that differ markedly from those of proteins. A significant feature of aptamers not possessed by proteins is their ability to participate in at least two different types of three-dimensional structure: a single-stranded folded structure that makes multiple contacts with the aptamer target and a double-helical structure with a complementary nucleic acid sequence. We have made use of this structural flexibility to develop an aptamer-based biosensor (a targeted reversibly attenuated probe, TRAP) in which hybridization of a cis-complementary regulatory nucleic acid (attenuator) controls the ability of the aptamer to bind to its target molecule. The central portion of the TRAP, between the aptamer and the attenuator, is complementary to a target nucleic acid, such as an mRNA, which is referred to as a regulatory nucleic acid (regNA) because it regulates the activity of the aptamer in the TRAP by hybridization with the central (intervening) sequence. The studies reported here of the ATP−DNA TRAP suggest that, as well as inhibiting the aptamer, the attenuator also acts as a structural guide, much like a chaperone, to promote proper folding of the TRAP such that it can be fully activated by the regDNA. We also show that activation of the aptamer in the TRAP by the complementary nucleic acid at physiological temperatures is sensitive to single-base mismatches. Aptamers that can be regulated by a specific nucleic sequence such as in an mRNA have potential for many in vivo applications including regulating a particular enzyme or signal transduction pathway or imaging gene expression in vivo.

Transcriptional changes are some of the earliest events that signal the presence of disease or change in the differentiated state of a cell. Consequently, there is great interest in developing probes that can be used to identify altered gene expression or that can be triggered by a specific mRNA to initiate a cellular activity. Here we describe a novel nucleic acid probe design that contains an aptamer linked to a complementary attenuator via an intervening sequence. The aptamer is activated by hybridization of the intervening sequence with a complementary nucleic acid sequence that disrupts the aptamer/attenuator stem (Figure 1A). We refer to these probes as TRAPs[1] (targeted reversibly attenuated probes). The design of the TRAP relies on the ability of single-stranded nucleic acids to adopt alternative structures. In its primary structure the TRAP includes the sequence of an aptamer that can interact with its target molecule with high specificity and affinity. In the absence of a complementary nucleic acid sequence the TRAP folds to hinder aptamer activity by virtue of hybridization between a portion of the aptamer and its complementary sequence, the attenuator. The presence of a nucleic acid that is complementary to the intervening antisense sequence results in hybridization and the formation of a rigid double-stranded DNA structure. The resulting structural constraint on the intervening sequence forces apart the shorter terminal stem, releases the aptamer from its attenuation constraint, and allows it to fold into its active configuration.

Ribozymes that are regulated by molecular interactions at an allosteric site have been reported on several occasions. Various extensions to the hammerhead ribozyme have rendered it regulated by oligonucleotides (*1*−*3*), ATP (*4*), theophylline (*5*), FMN (*6*), and cyclic nucleotides (*7*). Similarly, RNA and DNA ligases have been produced that are regulated by ATP and theophylline (*8*, *9*). Aptamers have been used to form riboswitches that regulate translation or transcription in bacteria or yeast (*10*−*12*). They have also been applied as part of the allosteric mechanism for regulating ribozyme activity (*5*, *13*). At least two examples of allosteric aptamers have been described. The one, a hemin-regulated aptamer, binds cytochrome *c* more tightly when hemin binds to a region of the aptamer that does not interact with cytochrome *c* (*14*). The likely mechanism for this allosteric regulation proposed by the authors on the basis of their studies is that the aptamer adopts an alternative structure when hemin binds to a G-quartet that is adjacent in the aptamer sequence to the cytrochrome *c*-binding region. A second allosteric aptamer involves regulation by the aminoglycoside neomycin, which inhibits binding of an aptamer to *Escherichia coli* formamidopyrimidine glycosylase (*15*). As for the hemin-binding aptamer, binding of neomycin alters the activity of the ligand-regulated aptamer (LIRA). In both cases cited, the allosteric regulators of aptamer activity were small molecules, either hemin or neomycin.

Regulation of an aptamer activity by an allosteric mechanism involving a nucleic acid regulator has not yet been reported. Here we describe a general approach to developing an aptamer that is regulated by a specific nucleic acid sequence. In this paper, as an example, we have used the DNA−ATP aptamer (*16*). We show that, in a TRAP

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[1] Abbreviations: regDNA, regulatory DNA; TRAP, targeted reversibly attenuated probe.

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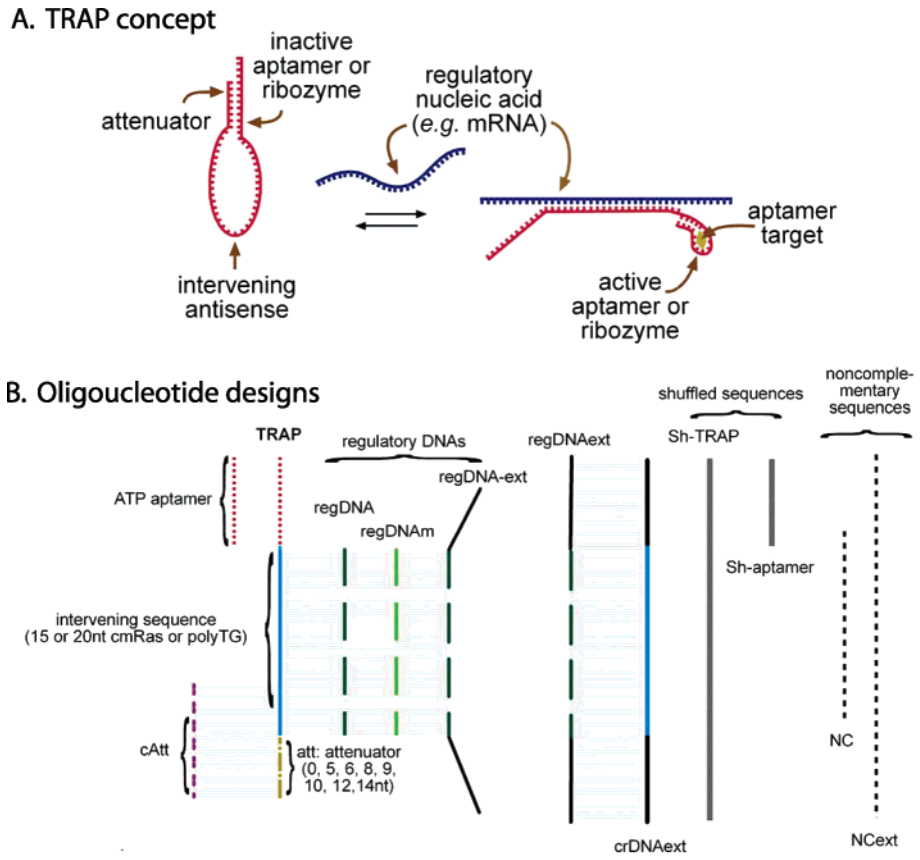

FIGURE 1: Design and function of the TRAP and oligonucleotide designs for this study. (A) Design of the TRAP. The TRAP consists of three segments: aptamer, intervening antisense, and attenuator. Binding of the complementary sense regulatory nucleic acid to the antisense sequence in the TRAP forces the aptamer and attenuator apart, providing the aptamer sequence with an opportunity to fold and interact with its target molecule. (B) Oligonucleotide designs. The oligonucleotides used in this study are shown graphically. Parallel lines indicate complementarities between sequences. Lines of the same color have the same nucleotide sequence. Sequences of the TRAPs are found in the Materials and Methods, and all other sequences are found in the Supporting Information.

configuration, the activity of the ATP aptamer is specifically and quantitatively regulated by its target nucleic acid.

Previous studies of the TRAP concept were done with the hammerhead ribozyme in place of the aptamer in Figure 1A. In these studies also, a simple polyAC intervening sequence was used. Studies of the ribozyme in this configuration showed that we could regulate the activity of the ribozyme by an attenuator (*2*, *3*). But they did not evaluate the use of natural sequences in the intervening sequence. The critical distinction is that natural sequences are likely to fold into a variety of structures that might coexist and could involve interactions of the intervening sequence with the aptamer or ribozyme or with the attenuator to create three-dimensional structures that differ from that proposed in Figure 1A. To understand the structural considerations that guide formation of regulated nucleic acids such as the TRAPs, it is important to evaluate the distribution of folded structures in the population fold. These questions cannot be evaluated with a ribozyme as the functional nucleic acid in the TRAP because, in assays for ribozymes, the substrate is the only radiolabeled component, the ribozyme is in excess (often 10-fold or more) of the substrate, and the assay follows the cleavage of the substrate RNA. Thus, the only ribozyme TRAPs that have folded "properly" and hybridized to the substrate are detected. Ribozyme TRAPs that adopt alternate three-dimensional structures that do not bind the substrate are not detected by the assay even if they constitute 90% of the molecules in the population. With an aptamer as the functional nucleic acid, the assay involves radiolabeling the TRAP and all molecules in the population are detected. Thus, with an aptamer, aspects of uniformity of folding can be addressed with the TRAP design. The results of this study of aptamer TRAPs show that the attenuator inhibits aptamer activity and also suggest that the attenuator serves as a structural guide for forming the correct TRAP structure and to prevent unproductive folding of the oligonucleotide. We also show that the TRAP design allows discrimination at a physiological temperature between nucleic acids that differ by only a single nucleotide in sequence.

EXPERIMENTAL PROCEDURES

Materials and Equipment. ATP−agarose affinity resins and ATP were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP was purchased from ICN (Costa Mesa, CA). Biotinylated ATP (adenosine 5′-triphosphate [γ] biotinyl-3,6,9-trioxaundecanediamine) was from Affinity Labeling Technologies, Inc. (Lexington, KY). Streptavidin−agarose CL-4B was from Sigma. HT-450 Tuffryn membrane filters (0.45 μm pore size) were from Pall (Ann Arbor, MI). Isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC isothermal titration calorimeter (Microcal, Inc., Northhampton, MA). Imagequant software (Amersham-Pharmacia) was used to analyze radioactive bands in gel scans obtained using a Typhoon scanner (Amersham-Pharmacia).

Oligonucleotides. The oligonucleotides used in this study are represented in Figure 1B. The sequences of the three TRAPs most frequently used in the work are given here. All other sequences can be found in the Supporting Information. 8att-polyTG20 TRAP: CCTGGGGGAGTATTGCGGAGGAAGG**TTTTTTGTTTTTGTTTTGTT***CCGCAATA*. 8att-cmRas20 TRAP: CCTGGGGGAGTATTGCGGAGGAAGG**GTACTCCTCAGGGCCGGCGG***CCGCAATA*. 9att-cmRas15 TRAP: CCTGGGGGAGTATTGCGGAGGAAGG**GTACTCCTCAGGGCC***CCGCAATAC*. Oligonucleotides were purchased as PAGE-purified preparations (Integrated DNA Technologies, Coralville, IA).

ATP Binding Assays. Single-stranded ATP−DNA TRAPs or the ATP−DNA aptamer (2−20 pmol, 5′-labeled with ^{32}P) was incubated in the presence or absence of other ssDNAs, as defined in each experiment, at 75 °C for 5 min in binding buffer (20 mM Tris·HCl, 300 mM NaCl, 5 mM $MgCl_2$, pH 7.6) and then cooled to room temperature slowly for 40−60 min. These samples were either loaded onto an ATP affinity column for the column assay or mixed with 40 μM biotinylated ATP and 10 μM streptavidin linked to agarose for the filter assay. For the column assay, each sample was incubated for 10 min on the column and then washed with 20 mL of binding buffer and the retained DNA was eluted with 15−16 mL of 5 mM ATP in binding buffer. For the filter assay, each sample was equilibrated at room temperature for 10 min, then filtered through an HT-450 filter, and washed with 5 mL of binding buffer. For both assays, fractions were analyzed by scintillation counting or measuring Cerenkov radiation. The radioactive cpm bound to ATP was divided by the total cpm in the sample to give the fraction bound. Reported error values are the standard deviations of the averages.

Isothermal Titration Calorimetry. Titrations of 50 μM regDNA-cmRas20 into a cell containing 5 μM 20-mer 0att-cmRas20 TRAP or 8att-cmRas20 TRAP, respectively, were carried out at 37 °C in titration buffer (300 mM NaCl, 5 mM $MgCl_2$, and 20 mM HEPES (pH 7.6 at 23 °C)). For each titration, 5 μL (250 pmol) of regDNA-cmRas20 was injected from a computer-controlled syringe at intervals of 300 or 600 s into the reaction cell that contained an initial volume of 1.43 mL of the appropriate oligonucleotide in the same buffer as the regDNA. Reverse titrations (oligonucleotide tritrated into regDNA) were also run. The syringe was rotated at 310 rpm. Data points were collected every 4 s. Each titration involved a total of 25 injections. Control experiments to determine the heats of dilution were performed using the same injection protocol of regDNA into a cell containing buffer but no oligonucleotide. The heat of dilution for each titrating oligonucleotide in buffer was determined experimentally. These values were subtracted from the corresponding experimentally obtained values for the appropriate oligonucleotide pairs to obtain the heats of interaction of each pair. The subtraction of the heats of dilution did not significantly change the values for heats of interaction for the oligonucleotides. All titrations were carried out at least three times to ensure consistency of the data. Before each titration the oligonucleotide was heated to 85 °C and incubated for 5 min in the titration buffer. The oligonucleotide was then cooled to room temperature slowly for 40 min. The data were analyzed using Origin 7.0 (Microcal, Inc.) with ΔH (enthalpy change, kcal/mol), K_a (association constant, M^{-1}), and N (number of binding sites per molecule in the sample cell) as adjustable parameters. Thermodynamic parameters were calculated from the equation $\Delta G = \Delta H - T\Delta S$, where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature.

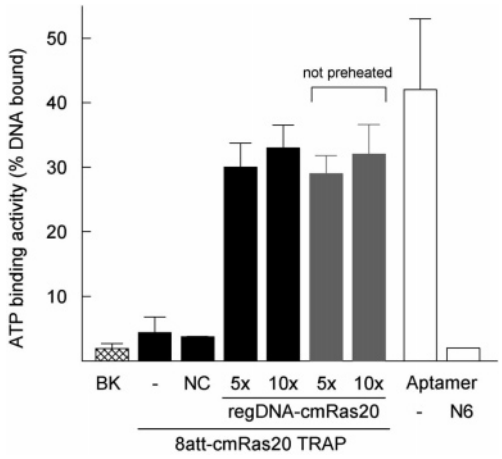

FIGURE 2: Sequence specificity of activation of the TRAP activity by regDNA. The 8att-cmRas20 TRAP was tested for its ability to bind ATP in the presence and absence of complementary regDNA-cmRas20 (5×, 10×) or noncomplementary NC20 (NC) compared with its activity in the absence of any other nucleic acid (−). For some samples the TRAP and regDNA were not heated and cooled together prior to measurement of aptamer activity (not preheated). The background binding activity was determined using a shuffled TRAP sequence alone (BK). The binding activity of aptamer alone was evaluated on the same column and on N6-linked ATP−Sepharose (N6). Averages and standard deviations are shown. All but two values are averages from four or more independent experiments. For NC and N6, the results are the average of two independent experiments. Key: hatched bar, background; black bars, 8att-cmRas20 TRAP that was melted and annealed slowly with the regDNA-cmRas20 or noncomplementary DNA before measurement of aptamer activity; gray bars, 8att-cmRas20 TRAP that was not melted and annealed before measurement of aptamer activity; white bars, ATP aptamer.

Polyacrylamide Gel Electrophoresis. Nucleic acids and their hybrids were separated by electrophoresis through 12% nondenaturing polyacrylamide gels under a constant current of 35 mA at room temperature. The gel buffer and electrode buffers consisted of 89 mM Tris·HCl, 89 mM boric acid, and 2 mM EDTA. The relative amounts of radiolabeled DNA in each band were quantified using Imagequant software after scanning of the gel using a Typhoon scanner. Reported errors are the standard deviations of the averages.

RESULTS

Allosteric Regulation of the ATP Aptamer by a Regulatory Nucleic Acid. To determine if an aptamer activity could be controlled in the TRAP design by an oligonucleotide complementary to the intervening antisense loop, we tested an allosteric DNA TRAP (8att-cmRas20 TRAP) containing an ATP aptamer at the 5′ end, followed by a 20 nucleotide (nt) antisense that is complementary to a portion of a mutant Ras oncogene mRNA and then an 8 nt attenuator sequence at the 3′ end. As predicted, incubation with a 20 nt regulatory DNA oligonucleotide (regDNA-cmRas20) resulted in increased aptamer (ATP binding) activity of the TRAP (Figure 2). Oligonucleotide sequences that were not complementary to the attenuator or to other portions of the TRAP were not

effective in increasing TRAP activity (Figure 2). These results show that the TRAP functions as designed. Regulation of aptamer activity is achieved by the presence of an oligonucleotide with a sequence complementary to the intervening antisense portion of the TRAP. The TRAP aptamer was also activated by regDNAs without prior melting and cooling (Figure 2), suggesting that this form of regulation could occur in vivo.

Proportionality and Reversibility of TRAP Activation. To be useful as a sensor for a complementary nucleic acid such as a cellular mRNA, allosteric regulation by the antisense sequence in the TRAP should be proportional to the concentration of the complementary regulatory nucleic acid and reversible. This was shown for the 8att-cmRas20 TRAP that was activated in a linear fashion by an increasing concentration of complementary regDNA-cmRas20 (Figure 3A). Control experiments showed that the regDNA itself did not display aptamer-like activity and that sequences that were not complementary to the TRAP did not increase aptamer activity (Figure 2 and data not shown). The increase in TRAP activity is stable for at least 24 h (data not shown) but can be readily reversed if an equilibrium is established with a third single-stranded nucleic acid that has a higher association constant for the regDNA than the regDNA has for the antisense segment of the TRAP. In vivo, reversal would be achieved by expressing an RNA that included additional sequence of the targeted regulatory mRNA that surrounds the sequence complementary to the intervening sequence of the TRAP. Here, we have demonstrated this property by using a regDNAext containing a central sequence that is complementary to the TRAP intervening sequence and 15 A's on either end. Reversal of TRAP activation occurred at 23 °C within 20 min of adding the complement of the regDNAext (crDNAext) to the TRAP that had been activated by a 10 min incubation with regDNAext (Figure 3B). The observed proportional increase in TRAP aptamer activity with increasing regDNA:TRAP molar ratio and the ability to reverse this activation show that the TRAP could be used to report the presence of an mRNA sequence or to mediate a signal from a particular mRNA sequence and that the activity of the TRAP can be reversed.

Attenuator Regulation of Aptamer Activity in the TRAP. The TRAP design dictates that allosteric regulation by the regDNA involves a balance of the strength of hybridization of the attenuator/aptamer stem and the antisense/regDNA hybrid. The impact of this balance of stability between two alternative structures was tested by varying the length of the attenuator (Figure 4). As predicted, the ability of the regDNA to activate the aptamer in the TRAP decreased with increasing length of the attenuator (Figure 4, gray bars). In this set of experiments a polyTG20 TRAP was used in place of the cmRas20 TRAP. Whereas an internal structure is predicted for the cmRas intervening sequence by the Mfold software (*17*), no structure is predicted for polyTG. By using the polyTG20 TRAP, we were able to test the effect of varying the length of the attenuator in the absence of complications that could be introduced by the inclusion of structure in the intervening sequence. A practical advantage of the polyTG20 TRAP was also that, under the conditions tested, the basal activity was high enough to observe the incremental decrease in aptamer activity as a result of increasing the attenuator length (black bars).

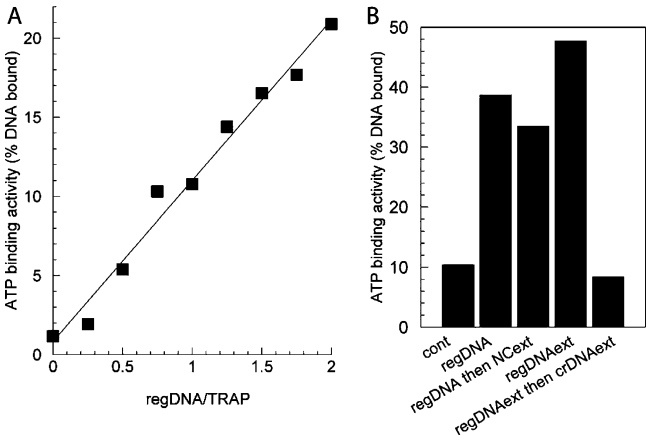

FIGURE 3: Relation between TRAP activity and regDNA concentration and reversibility of TRAP activation. (A) Relation between TRAP activity and regDNA concentration. The molar ratio of the regDNA-cmRas20 to the 8att-cmRas20 TRAP was varied to determine the effect of the regDNA on aptamer activity. The results are compiled from a series of experiments in which the TRAP concentration was a value between 0.075 and 0.15 μM, depending on the experiment. In each experiment a similar linear relationship was observed between ATP binding activity and regDNA concentration. The filter assay employing ATP−biotin and streptavidin−Sepharose was used to assess the ATP binding activity. The R^2 obtained by linear regression for this line was 0.98. The molar ratio of regDNA-cmRas20 to 8att-cmRas20 TRAP (regDNA:TRAP) is plotted (■). (B) Reversal of TRAP activation. The property of reversal (shown in the bars to the right) was tested by first incubating the 8att-cmRas20 TRAP for 10 min at 23 °C with regDNAext (same sequence as regDNA-cmRas20, but with 15 A's on either end), then adding crDNAext (complementary to regDNAext), and waiting another 30 min. All conditions shown in this figure included 0.067 μM 8at-cmRas20 TRAP. Control conditions were the TRAP without regDNA added (cont), with regDNA-cmRas20, or with regDNA-cmRas20 for 10 min followed by NCext (noncomplementary sequence with 15 T's on either end) for 30 min. The purpose of the latter two controls was to test that, under the conditions of the assay shown in Figures 2 and 3A (regDNA and TRAP interactions), the use of an oligonucleotide with polyT extensions had no effect on the interaction between TRAP and regDNA. The molar ratio of TRAP to regDNAext to crDNAext was 1:5:25. These results are from a single experiment in which duplicate independent measurements were averaged to obtain each value. Similar results were obtained in three independent experiments. For both figures, a background value of 4% subtracted from each data point was determined from the average retention of oligonucleotides containing either random sequences or a shuffled 8att-cmRas20 TRAP sequence. Noncomplementary DNA was unable to activate the aptamer in the TRAP. Background values were determined using random and shuffled oligonucleotides.

If, as these data suggest, the TRAP is activated by disrupting hybridization between the aptamer and its complementary attenuator, then a sequence complementary to the attenuator (cAtt) should also activate the aptamer in the TRAP by competing for the attenuator sequence as shown (Figure 4, white bars). The activity of the TRAPs with 12 and 14 nt attenuators increased more with cAtt than did the activity of the TRAPs with shorter attenuators. The likely explanation for this observation is that the base compositions of the cAtt's for these TRAPs were higher in percent GC and therefore the TRAP/cAtt hybrids were more stable than for TRAPs with shorter attenuators. Another factor is that the ratio of cAtt to TRAP for these two TRAPs with the longest attenuators was 10:1 compared with 5:1 for the remaining TRAPs. However, this change in ratio is unlikely to be responsible for the much higher activity of these latter

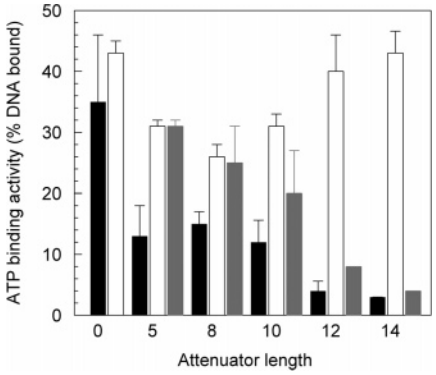

FIGURE 4: Effect of blocking the attenuator sequence on TRAP aptamer activity. The polyTG20 TRAPs with 0, 5, 8, 10, 12, and 14 base long attenuators were incubated in the absence of other oligonucleotides or in the presence of either (1) a 20 nt oligomer complementary to the entire attenuator region and a variable length of the adjacent intervening sequence (cAtt) or (2) the regDNA-polyAC20 complementary to the 20 nt intervening antisense sequence. The ratio of cAtt or regDNA to TRAP was 5:1 for TRAPs with attenuators of 0, 5, 8, and 10 nt and 10:1 for TRAPs with 12 and 14 nt attenuators. Key: black bars, activity in the absence of other oligonucleotides; white bars, TRAP in the presence of cAtt; gray bars, TRAP in the presence of regDNA.

two TRAPs in the presence of cAtt because the ATP binding activity of the 8attpolyTG TRAP was identical at a ratio of cAtt to TRAP equal to 10:1 and 5:1 (data not shown).

Effect of the Intervening Sequence on TRAP Activity. Previous studies of the hammerhead ribozyme TRAP were exclusively done with intervening antisense sequences designed not to interact with the ribozyme or the attenuator (*2*, *3*, *18*). We have also examined the activity of the polyTG20 TRAP with a similar noninteracting intervening sequence for which there are no predicted hydrogen-bonded pairs between bases in the aptamer and in the intervening sequence. However, the use of the TRAP to detect or respond to mRNA sequences will require that the mechanism function with a variety of base sequences in the intervening loop. Some of these intervening loop sequences will provide options for hydrogen bonding between the aptamer and the intervening sequence in addition to the hybridization between the aptamer and attenuator.

To examine the effect of the intervening antisense sequence on the ability of the TRAP to detect the presence of a variety of regDNAs, we compared three TRAPs: (1) the 8att-polyTG20 TRAP with an intervening sequence of a polyT sequence interspersed with three G's for maintaining register with the regDNA (structure 1, Figure 5A), (2), the 8att-cmRas20 TRAP with an intervening sequence containing all four nucleotide bases and with options for base pairing within the sequence (structures 2a−c, Figure 5A), and (3) the 0att-cmRas20 TRAP (structure 3, Figure 5A) with the same sequence as the 8att-cmRas20 TRAP except lacking the 8 nt attenuator. The polyTG intervening sequence is similar to that which was used with the hammerhead ribozyme TRAP and does not correspond to a known RNA sequence (*2*). The cmRas intervening sequence corresponds to a portion of a mutant Ras mRNA and is predicted by the Mfold software (*17*) to have internal structure.

Compared with that of the 8att-polyTG20 TRAP, the activity of the 8att-cmRas20 TRAP was lower in the absence of the complementary regDNA (Figure 5B). However, both

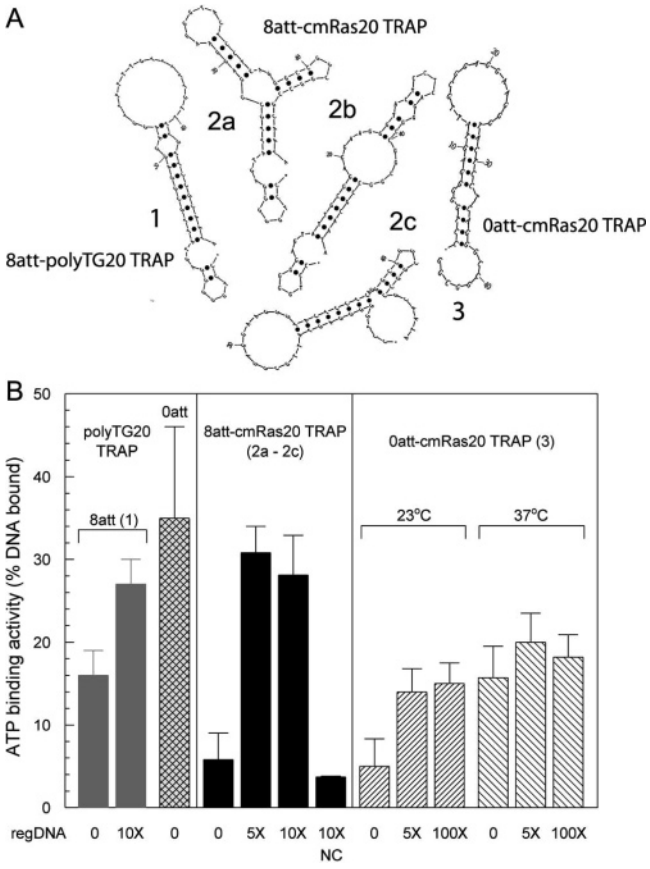

FIGURE 5: Impact of the intervening sequence and attenuator on TRAP function. (A) Two-dimensional structures predicted by the Mfold program (http://www.bioinfo.rpi.edu/applications/Mfold/) for oligonucleotides containing the ATP aptamer and a cmRas antisense sequence with or without an 8 nt attenuator. (1) The polyTG20 TRAP has a single predicted structure with a ΔG of −10.6 kcal/mol. (2a−c) The three predicted structures for the 8att-cmRas20 TRAP with an 8 nt attenuator have ΔG values of −6.2 (a) or −6.1 (b, c) kcal/mol. (3) The single predicted structure for the 0att-cmRas20 TRAP with no attenuator has a ΔG of −4.2 kcal/mol. (B) The activities of four TRAP constructs are compared: 8att-polyTG20 TRAP with an 8 nt attenuator (gray bars), 0att-polyTG20 TRAP (hatched gray bars), 8att-cmRas20 TRAP with an 8 nt attenuator (black bars, middle section), and 0att-cmRas20 TRAP with no attenuator (right and left slashed white bars, right section). The TRAPs were incubated alone (0) or in the presence of a 5-, 10-, or 100-fold molar excess of the complementary regDNA (either regDNA-polyAC20 or regDNA-cmRas20) or a 10-fold excess of noncomplementary NC20 (NC). The ability of the regDNA-cmRas20 to control 0att-cmRas20 TRAP activity was tested at 23 and 37 °C (right section). All other incubations were at 23 °C.

TRAPs were activated to about the same maximum activity by excess complementary regDNA. These results suggest that action of the attenuator to inhibit aptamer activity might be aided by interactions within the intervening sequence that stabilize the closed (aptamer inactive) form of the TRAP but that these latter interactions do not prevent the regDNA from hybridizing with the intervening antisense sequence and fully activating the aptamer.

Role of the Attenuator as a Structural Guide for regDNA Hybridization with the TRAP. In the course of these studies we discovered that, in some instances, the 0att-cmRas20 TRAP being an example, the antisense sequence can interact with the aptamer and strongly suppress its activity in the absence of an attenuator (Figure 5B). However, unlike the

Table 1: Attenuator Effect on the Thermodynamics of the Interaction of regDNA-cmRas20 and 8att-cmRas20 TRAP[a]

oligonucleotide	assumption of the fitting model	N	ΔH (kcal/mol)	ΔS (kcal mol^{-1} K^{-1})	ΔG (kcal/mol)
20-mer	1n	1.0	−110	−313	−12.1
0att-cmRas20 TRAP	2n	0.5	−81	−217	−14.0
		0.7	−67	−178	−12.1
8att-cmRas20 TRAP	1n	0.9	−82	−225	−11.5

[a] Thermodynamic parameters were obtained from fitting ITC data for the interaction of the regDNA-cmRas20 with either the 0att-cmRas20 TRAP or the 8att-cmRas20 TRAP (Materials and Methods). In each case the first oligonucleotide was placed in the cell at 2.5 or 5 μM initial concentration. The second oligonucleotide was titrated into the cell with 25 injections and to a final ratio of about 1.8 over the first oligonucleotide. Each oligonucleotide pair was tested in two protocols, each protocol using a different member of the pair titrated into the cell. The plots were fit mathematically using algorithms that assume a single (1n) or two (2n) alternate binding complexes between the interacting oligonucleotides. The results from both protocols were averaged for the results shown in this table.

TRAP that includes an attenuator, the aptamer linked to the antisense in the absence of an attenuator (0att-cmRas20 TRAP) was not fully activated by regDNA. Even a 100-fold excess of regDNA only increased the 0att-cmRas20 TRAP aptamer activity to 50% of the aptamer binding activity achieved by the 8att-cmRas20 TRAP or the ATP aptamer alone (Figure 5B). This same inability of the 0att-cmRas20 TRAP to be fully activated was observed at 23 and 37 °C.

Analysis by the Mfold program of the two-dimensional structure of the 0att-cmRas20 TRAP predicted that the folded structure of this oligonucleotide ($\Delta G = -4.2$ kcal/mol) was less stable than for the 8att-cmRas20 TRAP, which has the same sequence with the addition of the 8 nt attenuator ($\Delta G = -5.5$ to -5.8 kcal/mol). If correct, this analysis suggested that the thermodynamic basis for the resistance of the 0att-cmRas20 TRAP to activation by the regDNA-cmRas20 could not be accounted for by a much more stable secondary structure of the former. We also tested the possibility that the observed difference in maximal activation of the 0att- and 8att-cmRas20 TRAPs was due to a kinetic difference in the rate of hybridization with the complementary regDNA. However, even after a 24 h incubation of the TRAPs with the regDNA-cmRas20, the same relative activations of the 0att- and 8att-cmRas20 TRAPs were observed.

To examine further the basis for the inability of the 0att-cmRas20 TRAP to be fully activated, we used ITC to compare the thermodynamic parameters of the interaction of the regDNA with three oligonucleotides: (1) a complementary oligonucleotide corresponding to the intervening antisense sequence of the TRAP, (2) the 8att-cmRas20 TRAP, and (3) the 0att-cmRas20 TRAP. The results (Table 1) showed that the calculated stabilities (ΔG values) of the complex between the regDNA-cmRas20 and either the 0att- or 8att-cmRas20 TRAP were similar. However, whereas the thermodynamic parameters for the 8att-cmRas20 TRAP/regDNA-cmRas20 interaction could be fit assuming a single hybrid molecular species, the 0att-cmRas20 TRAP/regDNA-cmRas20 data could only be fit if it was assumed that there were two types of interaction, each representing about 50% of the DNA molecules. Representative data from which the values for this table were obtained are shown in Figure 6. From this analysis it seems that the 0att-cmRas20 TRAP can adopt at least two tertiary conformations that interact differently with the regDNA for hybridization. Such structures were not predicted by the Mfold program and may involve more complex structural elements than are entertained by this algorithm for two-dimensional structural predictions.

Alone, the ITC analysis does not explain why only 50% of the aptamer activity is activated on hybridization with the regDNA. Although there are at least two predicted interactions that might involve two or more folded structures of the 0att-cmRas20 TRAP, neither interaction shows a stability very different from that of the other or from that of the 8att-cmRas20 TRAP. The most likely explanation for these results is that one or more of the hybridized 0att-cmRas20 TRAP structures involve interaction between the regDNA-cmRas20 and the aptamer that inhibits aptamer activity. Thus, the presence of the attenuator in the 8att-cmRas20 TRAP may act as a structural guide to appropriately align the antisense sequence in the "closed" TRAP, making it available for invasion by the regDNA to form the correct structural intermediate for the subsequent separation of the attenuator and aptamer.

To test the hypothesis that the 8att-cmRas20 TRAP forms predominantly one structure and the 0att-cmRas20 TRAP forms more than one structure when hybridized with the regDNA-cmRas20, hybridized and radiolabeled oligonucleotides were separated by nondenaturing acrylamide gel electrophoresis. The results demonstrate that the vast majority of the 8att-cmRas20 TRAP runs as a single band, in the presence and absence of regDNA-cmRas20, suggesting the presence of a predominant structure (Figure 7). By contrast, the 0att-cmRas20 TRAP, with or without regDNA-cmRas20, separates into one major band with the remainder distributed among four to nine bands and sometimes in a smear of unresolved bands near the major band. The results suggest that the 0att-cmRas20 TRAP exists as multiple structural species. These experiments were performed with variations in incubation temperature (10 min incubation at 23 vs 37 °C), times of incubation (10 min and 24 h incubation at 23 °C), and temperatures at which electrophoresis was performed (electrophoresis at 4 and 23 °C with samples that had been incubated at either 23 or 37 °C). In all trials the same observation was made that the 0att-cmRas20 TRAP separated into many bands on the gel whereas the 8att-cmRas20 TRAP remained mainly as a single band. An average of 86 ± 7.8% ($N = 8$) and 89 ± 5.6% ($N = 12$) of the total 8att-cmRas20 TRAP DNA resolved as a single band in the absence or presence of a 1−100-fold excess of regDNA, respectively. By contrast, 46 ± 15% ($N = 10$) and 57 ± 8.5% ($N = 30$) of the 0att-cmRas20 TRAP was found in the most intense band in the absence and presence of a 1−100-fold excess of regDNA, respectively. Thus, these results, along with the ITC data and the measurements of TRAP aptamer activity after hybridization with regDNA, suggest that only about 50% of the 0att-cmRas20 TRAP molecules adopt a structure that is able to interact with the regDNA in a productive way to activate the aptamer.

TRAP's Ability To Distinguish Single-Base Mismatches. Although not practical with oligonucleotides, single-base mismatches can be detected by molecular beacons, nucleic acids with a stem−loop structure similar to that of the TRAP

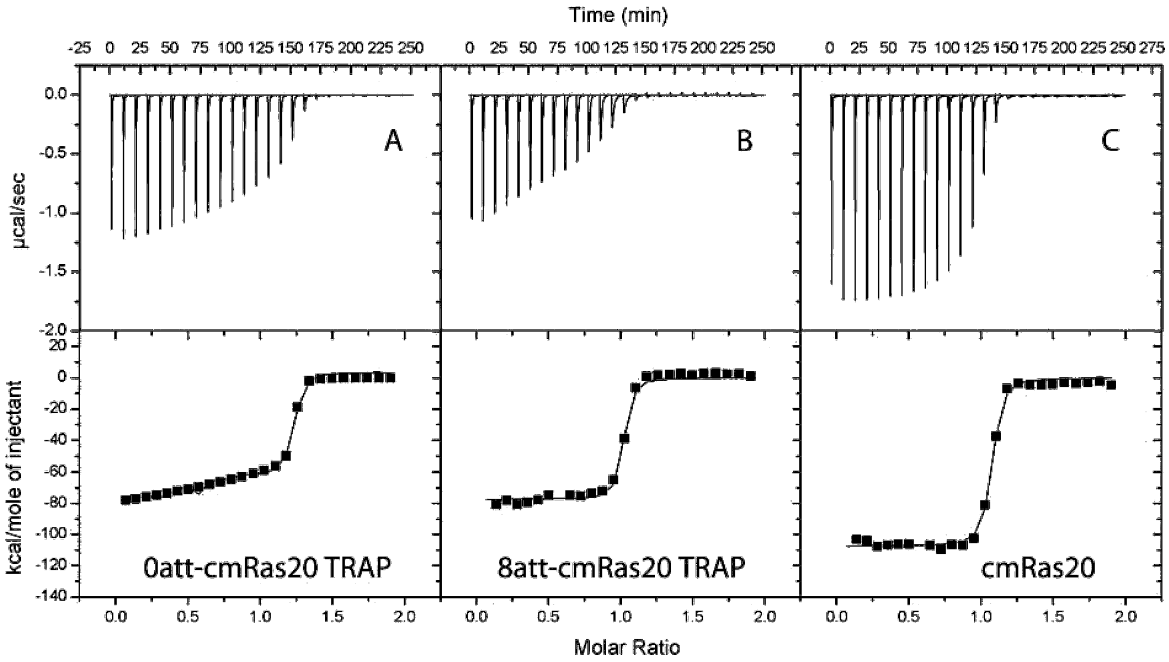

FIGURE 6: ITC data for hybridization of regDNA-cmRas20 with 8att-cmRas20 TRAP or 0att-cmRas20 TRAP. Representative plots and the fitted ITC data are shown in this figure. The 0att-cmRas20 TRAP (A), 8att-cmRas20 TRAP (B), and the 20 nt intervening sequence of the cmRas20 TRAPs (C) were titrated with regDNA-cmRas20, and the heats of reaction were measured by isothermal titration calorimetry. For these examples, the first oligonucleotide was initially at 5 μM in 1.4 mL and the final concentration of regDNA-cmRas20 was 7.6 μM in 1.65 mL with a resulting final molar ratio of 1.8 for regDNA-cmRas20:first oligonucleotide. For all three of these examples, regDNA-cmRas20 was titrated into the cell with 600 s between injections. The upper set of three graphs show the time courses for the three titrations. The lower set of graphs show the heat absorbed as a function of the molar ratio of the interacting species. The compiled data from these and other analyses are shown in Table 1.

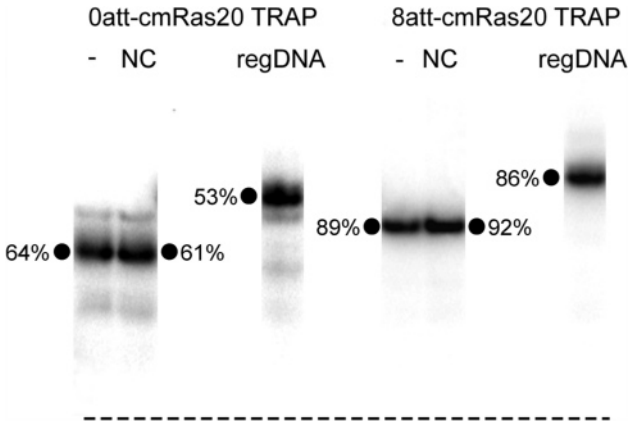

FIGURE 7: Effect of the attenuator on structural forms of cmRas20 TRAP/regDNA hybrids. End-labeled 0att- and 8att-cmRas20 [^{32}P]-TRAPs (0.8 μM) were hybridized with the regDNA-cmRas20 in the absence of ATP by incubating the oligonucleotide pair together for 10 min at 23 °C. The hybridized pairs were then resolved by nondenaturing polyacrylamide gel electrophoresis, and the distribution of [^{32}P]DNA was determined. The 0att-cmRas20 TRAP or the 8att-cmRas20 TRAP was incubated alone (−) or with a 10-fold excess of noncomplementary NC20 (NC) or complementary regDNA-cmRas20. The dashed line shows the position of [^{32}P]-ATP in the gel after electrophoresis. The numbers show the percent of the total [^{32}P]TRAP in the corresponding band in this experiment. Average numbers for all experiments are given in the main text.

(*19*−*21*). However, optimal conditions for distinguishing single-base mismatches by molecular beacons are at temperatures well above 37 °C and also generally include high concentrations of Mg^{2+}. Whereas the molecular beacon is generally regulated by a stem of five to six bases, the TRAP has a longer stem by which it is regulated. We reasoned that the apparent higher stability of the TRAP structure would make its activation more likely to be influenced at physiological temperatures by single-base mismatches between the regDNA and the antisense intervening sequence of the TRAP. Therefore, regDNAm-cmRas15 with one or two base-pair mismatches was tested for its ability to activate a 9att-cmRas15 TRAP at 37 °C and in the presence of only 5 mM $MgCl_2$. The results showed that the 9att-cmRas15 TRAP was capable of distinguishing single-nucleotide mismatches between the regDNAm-cmRas15 and the intervening antisense sequence over at least a 10-fold range (Figure 8).

DISCUSSION

We have described an oligonucleotide design in which a complementary nucleic acid can regulate an aptamer activity by an allosteric mechanism. The aptamer activity in the TRAP is increased by the presence of complementary regDNA. In the absence of regDNA, the aptamer is inhibited by the attenuator (Figure 4). Activation of the TRAP is sequence specific and is proportional to the amount of regDNA present (Figures 2 and 3).

Activation of the TRAP aptamer is determined by the balance in stability of the attenuator/aptamer stem and intervening sequence compared with the stability of the aptamer−target complex and regDNA/intervening sequence hybrid. This equilibrium resembles that established by the molecular beacon with its complementary DNA target. A systematic thermodynamic study demonstrated that the molecular beacon can discriminate between a complete match and a single mismatch in the target DNA sequence over a broader temperature range than can a linear nucleic acid probe (*20*). As the stem length was increased from four to six bases, the window of discrimination moved from a midvalue of about 65 °C to about 50 °C. Very little

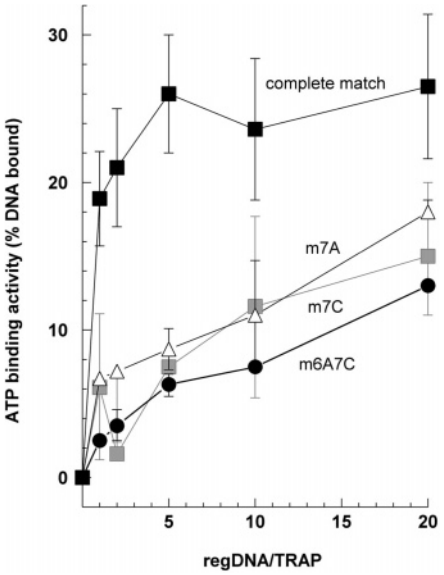

FIGURE 8: Discrimination of single-base mismatches by a 9att-cmRas15 TRAP. A 9att-cmRas15 TRAP with a 15 nt antisense intervening sequence and a 9 nt attenuator was tested for its ability to be activated at 37 °C by four regDNA-cmRas15's: a perfect match (black squares) or a single-base-pair mismatch at position 7 on the 15 nt regDNAm-cmRas15 in which the perfect match (G) was changed to A for a C::A mismatch (gray squares, regDNAm7A-cmRas15) or to C for a C::C mismatch (white triangles, regDNAm7C-cmRas15) or in which the perfect match (TG) at positions 6 and 7 was changed to CA for a CA::AC mismatch (black circles, regDNAm6C7A-cmRas15). The regDNA to TRAP ratio was varied over a 20-fold range. The incubations were performed at 37 °C, and the results are expressed as the percent of 9att-cmRas15 TRAP DNA bound to ATP with a background of 13% (nonspecific binding) subtracted. The background binding was determined using two oligonucleotides with randomized sequences, one of the TRAP and the other of the aptamer. The same average percent bound was found for both randomized sequences. Each data point is an average of the results from between two and eight experiments in which each experimental value was the average of two independent estimates.

discrimination was observed at 37 °C. By contrast, we found good discrimination between single-base mismatches at 37 °C using a 9att-cmRas15 TRAP (Figure 8). For optimizing the discriminatory capability of the TRAP, it was important to find the correct thermodynamic balance between the attenuator/aptamer heteroduplex and the antisense/regDNA hetroduplex. For example, 9 nt in the attenuator and 15 nt in the intervening sequence showed good discrimination single-base-pair mismatches. Whereas the 8att-cmRas20 TRAP showed some discrimination between a complete match and a two-position mismatch, there was no significant difference for the 8att-cmRas20 TRAP between a complete match and a single-base-pair mismatch (data not shown).

Although increasing the length of the stem of the molecular beacons moved the window of sequence discrimination toward physiological temperatures, it also increased the response time such that a molecular beacon with a six-stem base was only a little over 60% opened after 200 s compared with a four-stem beacon, which was completely opened in less than 10 s (*20*). By extension, an eight-stem molecular beacon would be expected to take many minutes to completely open. Our results suggest that the TRAP is not as sluggish in its response to the regDNA as the molecular beacon is to its target DNA. Incubating the TRAP with its target molecule and the regDNA for 5 min results in the same amount of activity as obtained with the aptamer alone. The difference between the TRAP and molecular beacon probably lies in the stability of the aptamer structure when bound to its target molecule. Whereas the beacon has only the ΔG of hybridization between the antisense and target DNA to balance the ΔG of the stem helix, the TRAP equilibrium balances the ΔG of the stem helix against the combined ΔG values of aptamer interaction with its target molecule and regDNA hybridized with the intervening sequence. The additional stability of the released aptamer bound to its target molecule is expected to make the reverse reaction less favorable.

Our results show that maximally activated TRAP has the same activity as the aptamer alone when the TRAP is tested at 37 °C (Supporting Information). Consequently, maximizing the range of change in aptamer activity due to regDNA will be achieved by decreasing the background activity of the TRAP in the absence of regDNA. Here we show that the intervening sequence can play a role in decreasing the TRAP background activity (Figure 5). Identical except for the 20 nt intervening sequence, the 8att-polyTG20 TRAP showed a much higher background activity than the 8att-cmRas20 TRAP. Whereas there is no predicted internal structure of polyTG, the cmRas intervening sequence was predicted to have internal structure. The internal structure introduced by the cmRas intervening sequence may lower the background activity of the TRAP by stabilizing the attenuator/aptamer stem.

Depending on its sequence, the intervening sequence can also interact with and inhibit the aptamer. In the 8att-cmRas20 TRAP example, the absence of an attenuator sequence in the TRAP resulted in an aptamer that was inhibited in the absence of regDNA. But the 0att-cmRas20 TRAP could not be fully activated by hybridization with regDNA even if the regDNA and TRAP were incubated together over a 24 h period. Analysis by ITC suggested that the 0att-cmRas20 TRAP interacts with the regDNA to form more than one structure (Table 1, Figure 6). By contrast, the 8att-cmRas20 TRAP showed evidence of only a single hybridized structure as did the two oligonucleotides with sequences of the regDNA and the intervening antisense of the TRAPs. Further structural analysis by gel electrophoresis of the regDNA hybrids with these two TRAPs supported the hypothesis that the 0att-cmRas20 TRAP forms several structures in both the presence and absence of the regDNA, whereas the 8att-cmRas20 TRAP forms only one major structure in both instances (Figure 7). We believe it is likely that one or more of the hybridized forms of the 0att-cmRas20 TRAP include structural features that prevent the aptamer from properly folding and from recognizing its target ATP. These results suggest that the attenuator plays three important roles in the TRAP. The first role is to inhibit aptamer activity in the absence of the regulatory nucleic acid as demonstrated for the polyTG TRAP in Figure 4. The second role of the attenuator is to act as a structural guide, much like a chaperone, to promote the folding of the remainder of the molecule into a three-dimensional structure that can be effectively invaded by the regulatory nucleic acid in a single and productive mode to fully activate the aptamer. In acting as a structural guide, the attenuator also performs its third role, which is to prevent unproductive folding of the TRAP oligonucleotide. These latter two roles, which are interde-

pendent, are proposed as an explanation of the results of the ATP binding, ITC, and gel electrophoresis analyses of the 8att-cmRas20 TRAP and the 0att-cmRas20 TRAP (Figures 5−7 and Table 1).

The TRAP has many potential applications. One example is in imaging gene expression in vivo. Activation of the aptamer in the TRAP by a particular mRNA would provide a means of imaging cells in vivo that express the complementary mRNA. For imaging gene expression, molecular beacons make particularly good use of fluorescence options for signaling the presence of a specific sequence by coupling a fluorophore and a quenching agent, located at opposite ends of a nucleic acid probe, with a stem−loop structure. Although it can function in vivo, the molecular beacon's usefulness for in vivo applications is limited by the penetration of light through tissue and by the stability of the beacon inside cells (*22*−*24*). Not limited by the need for a fluorescent signal, the TRAP can be designed to bind a target molecule labeled with a radioisotope such as ^{99m}Tc or ^{18}F. The radiolabeled targets would be concentrated by the TRAP in cells in which the mRNA is expressed that is complementary to its intervening sequence. Unlike for the molecular beacon, degradation of the TRAP in vivo will not create a background signal. Also, because of its sensitivity to a single-base mismatch at temperature and salt concentrations close to those of the living cell, we anticipate that the TRAP might also be able to distinguish point mutations in mRNAs of living cells.

Although they both rely on a similar stem−loop structural concept, there are some fundamental differences between the TRAP and the molecular beacon. Both the TRAP and molecular beacon can be taken in by cells as synthetic oligonucleotides. However, unlike the molecular beacon, a TRAP containing an RNA aptamer can be synthesized by a living cell to create a steady-state intracellular concentration of TRAP. TRAPs could also be used to modify cellular processes in response to specific nucleic acid sequences. Aptamers have been selected to regulate enzyme activity, signal transduction cascades, and protein structural transitions (*25*−*29*). The TRAP design described here would provide a means of regulating these aptamers by changes in gene expression. A normal gene expression pattern could be harnessed to regulate the activity of an enzyme or an intermolecular interaction by way of a TRAP containing the appropriate aptamer.

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SUPPORTING INFORMATION AVAILABLE

Lists of oligonucleotides, comparison of the activity of a fully activated TRAP with the unmodified aptamer (Figure S1), and effect of attenuator length on the ATP binding activity of cmRas TRAPs (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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