Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP

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RNA transcripts containing the hammerhead ribozyme have been engineered to self-destruct in the presence of specific nucleoside 3',5'-cyclic monophosphate compounds. These RNA molecular switches were created by a new combinatorial strategy termed 'allosteric selection,' which favors the emergence of ribozymes that rapidly self-cleave only when incubated with their corresponding effector compounds. Representative RNAs exhibit 5,000-fold activation upon cGMP or cAMP addition, display precise molecular recognition characteristics, and operate with catalytic rates that match those exhibited by unaltered ribozymes. These findings demonstrate that a vast number of ligand-responsive ribozymes with dynamic structural characteristics can be generated in a massively parallel fashion. Moreover, optimized allosteric ribozymes could serve as highly selective sensors of chemical agents or as unique genetic control elements for the programmed destruction of cellular RNAs.

In recent years, various combinatorial and rational design strategies have been used to expand the structural and catalytic complexity of RNA beyond that exhibited by natural ribozymes. The catalytic repertoire of natural and artificial ribozymes now ranges from classical abiotic chemical reactions to biologically relevant reactions that are also catalyzed by modern protein enzymes[1–4]. The diversity of structure and of catalytic function exhibited by RNA is consistent with hypotheses that propose the emergence of modern metabolic function from an earlier, yet complex, metabolic state that was guided entirely by RNA enzymes[5–8]. The capacity of nucleic acids to form a great variety of intricately folded structures also supports the view that nucleic acids, like proteins, might have a substantial potential for sophisticated catalytic function that could be harnessed for novel biochemical applications.

Although allosteric regulation of catalytic function by small organic effectors is a critical feature of many protein enzymes, allosteric function has not been observed to date with any of the different classes of natural ribozymes. However, molecular design strategies similar to those used to expand the repertoire of catalytic RNAs have also been used to confer precise allosteric control over the kinetic properties of several natural ribozymes. For example, the catalytic activity of ribozymes can be modulated by effectors such as small organic compounds[9] and oligonucleotides[10,11]. The catalytic activity of each of these allosteric ribozymes responds either positively or negatively to binding of its corresponding effector molecule. Catalytic rate usually is modulated by rapid conformational changes that are brought about by ligand binding to a position located apart from the active site of the enzyme. Considering the tremendous potential of nucleic acids for structure formation[12,13], catalytic function[1–4] and molecular recognition[14,15], it seems reasonable to speculate that an enormous assortment of allosteric ribozymes could be created using various enzyme engineering methods.

Previously, we created a series of self-destructing allosteric ribozymes that are sensitive to various effector molecules such as ATP, flavin mononucleotide (FMN) and theophylline[16–18]. Several of these engineered allosteric ribozymes, derived from the self-cleaving hammerhead RNA, display greater than 100-fold modulation of catalytic rate upon introduction of their corresponding effectors. In some cases, the maximum rate constants (k_{max}) approach the observed k_{max} of the unmodified hammerhead ribozyme. These findings suggest that ribozymes

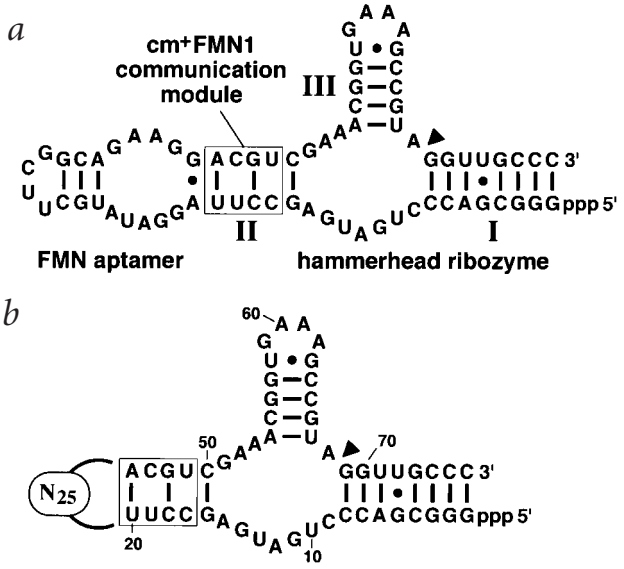

Fig. 1 The tripartite design for allosteric ribozyme construction. ***a***, Sequence and secondary structure for an FMN-sensitive allosteric ribozyme[17]). In this construct, the cm+FMN1 communication module (boxed) separates the ribozyme and aptamer domains. This communication module (cm) is the first sequence class (1) that was previously identified to undergo allosteric activation (+) in the presence of flavin mononucleotide (FMN). Base-paired elements that are required for hammerhead ribozyme activity (I, II and III) are labeled according to Hertel and coworkers[30]. An arrowhead identifies the site of hammerhead-mediated cleavage. ***b***, A tripartite construct carrying a randomized aptamer domain used as the pool to initiate *in vitro* selection. N_{25} represents 25 nucleotides with random base identity.

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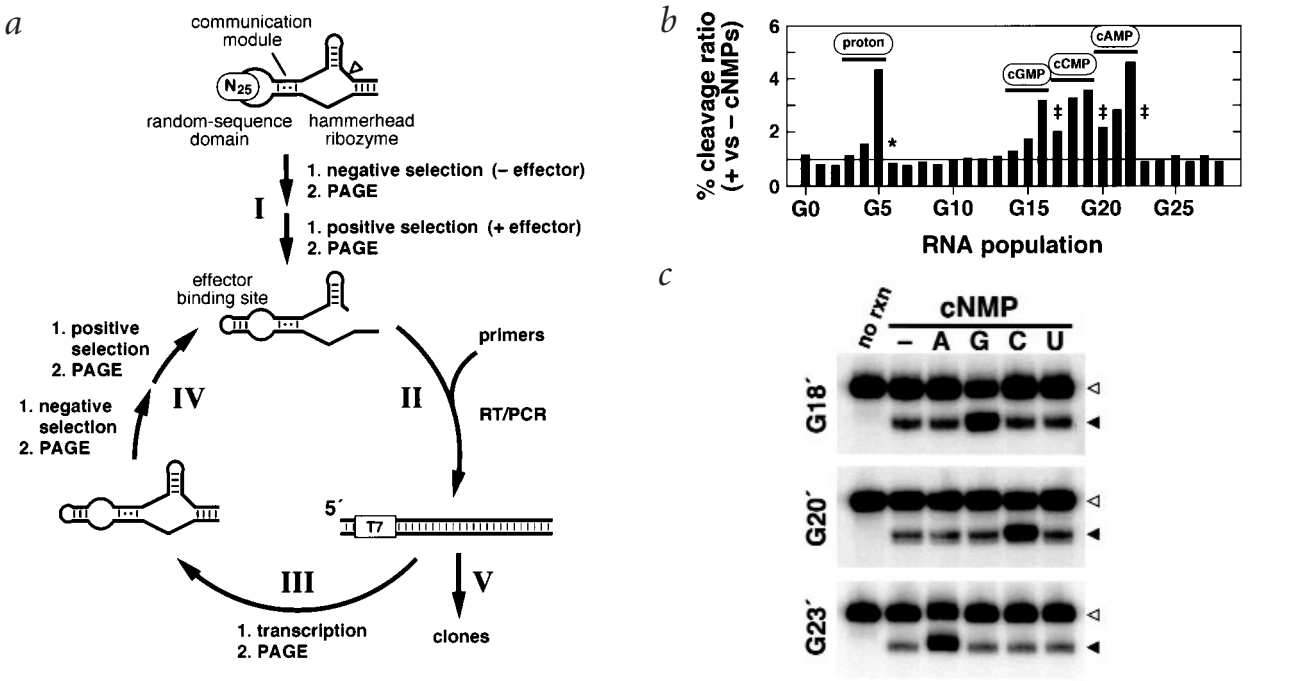

Fig. 2 Allosteric selection scheme and the isolation of RNA molecular switches with new effector specificities. ***a***, Precursor RNAs are (I) subjected to negative selection in the absence of effector. Uncleaved RNAs are isolated by PAGE and subjected to positive selection in the presence of a mixture of the four cNMPs. Cleaved RNAs are (II) amplified by RT-PCR to generate double-stranded DNA templates. The resulting DNAs are (III) transcribed using bacteriophage T7 RNA polymerase (T7 RNAP) to generate a new population of RNA molecules that are (IV) subjected to the next round of negative and positive selections. (V) Double-stranded DNAs from the desired rounds of selection are cloned and sequenced for further analysis. The boxed T7 represents a double-stranded promoter sequence for T7 RNAP. ***b***, Emergence of ligand-specific allosteric ribozymes over the course of *in vitro* selection is reflected by plotting the ratio of cleavage yields (presence *versus* absence of effectors) for each round of selection (G1 through G28). Specificity of the ligand-sensitive populations that emerge throughout the selection are designated by the bars. Asterisk denotes a change in the selection protocol to avoid acidifying the RNA sample before initiating the positive selection reaction. Daggers identify the rounds of selection where the cNMP that functions as an effector in the previous round is added to the negative selection reaction in subsequent rounds. Line indicates a cleavage ratio of 1, which represents the value expected if the cleavage activity of the population as a whole were to exhibit no preference for the effector mixture. ***c***, Selective activation of RNA cleavage by cNMPs. Trace amounts of internally ^{32}P-labeled RNAs representing the populations G18', G20' and G23' were incubated for 15 min in the reaction buffer used for *in vitro* selection (50 mM Tris-HCl, pH 7.5 at 23 °C, and 20 mM $MgCl_2$) in the absence of effector (-) or in the presence of 500 μM of the 3',5'-cyclic mononucleotides A, G, C and U as indicated. Reaction products were separated by denaturing 10% PAGE, and the bands were visualized and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). Open and filled arrowheads identify the precursor and 5' cleavage products, respectively. The 3' cleavage products have greater electrophoretic mobility than the significantly larger precursor RNAs and 5' cleavage fragments, and therefore are not present on the images.

with new allosteric binding specificity and refined kinetic characteristics could be made to function inside cells with a level of catalytic performance that is of biological significance. Acquiring precise kinetic control over the activity of self-cleaving ribozymes in particular would facilitate the construction of a new class of controllable genetic elements. Specifically, incorporation of allosteric ribozymes into cellular RNAs would allow the programmed self-destruction of RNA transcripts by signaling with the appropriate effector compound.

The allosteric modulation of protein enzymes typically results from the binding of an effector molecule to a site located apart from the active site of the enzyme[19]. Likewise, engineered allosteric ribozymes have been designed to carry separate RNA domains that mediate effector-binding and catalytic functions[16,18,20]. A common characteristic of many aptamers is the effect of 'adaptive binding,' whereby conformational reorganization occurs upon binding of the ligand to its RNA receptor[21]. These ligand-responsive RNA aptamers thus serve as an excellent source of effector-binding sites for the creation of novel allosteric ribozymes using modular rational design strategies. In addition to designed molecules, novel allosteric ribozymes have also been isolated using *in vitro* selection methods[17,22].

In nearly all examples reported to date, allosteric ribozymes have been created by joining preexisting ligand-binding domains or 'aptamers' with ribozyme domains to produce the ligand-responsive construct of choice[9]. Since these methods require the use of preexisting ribozyme and ligand-binding structures, the limited number of RNA domains that are currently available restricts the versatility of allosteric ribozyme engineering. We set out to establish a generalized *in vitro* selection protocol that would facilitate the isolation of new allosteric ribozymes with specificity for nearly any effector molecule. Using this 'allosteric selection' strategy, we targeted the four natural 3',5'-cyclic mononucleotides, including the second messengers cGMP and cAMP. This collection of molecules provides a diverse set of targets that are of biological importance and that challenge the structure formation and molecular recognition capabilities of RNA. In this manner, we intend to isolate prototypic molecular switches that, with further refinement, could be used to construct a new class of genetic control elements that respond to natural second messengers.

The allosteric selection strategy

Previously[17], we generated a series of allosteric ribozymes using a

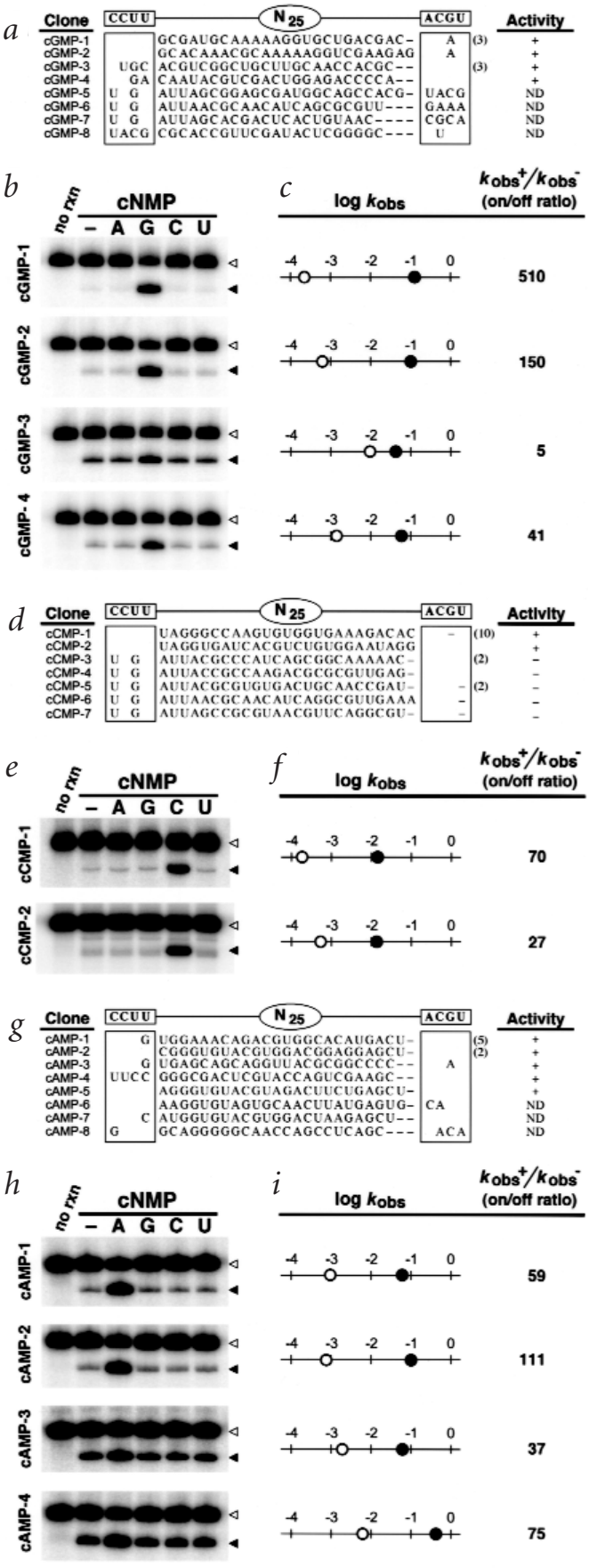

Fig. 3 Allosteric modulation of hammerhead ribozymes by cNMPs. ***a***, Sequences of the original communication module domains (boxed) and the original random sequence domains (N_{25}) for eight distinct clones isolated from the G18' RNA population. Dashes within the N_{25} domain represent nucleotide deletions that have occurred somewhere within this region. Numbers in parentheses report the number of clones with identical sequences. All isolates are identified as (+) having effector-responsive allosteric function, (-) showing no response to the addition of effector, or (ND) not having had the allosteric function determined. Note that in nearly all cases, the communication module domains have acquired a minimum of one mutation. ***b***, Ligand-dependent cleavage of individual allosteric ribozymes isolated from the G18' RNA population. RNA precursors (open arrowheads) produce greater amounts of 5' cleavage product (filled arrowheads) in the presence of 500 μM cGMP than in its absence. The assays were conducted under *in vitro* selection conditions, and as a result, the product yields in the presence of effector *versus* the absence of effector reflect the advantage that each ribozyme maintains during the selection reaction. Reaction products were separated and visualized as described in Fig. 2*c*. ***c***, The initial rate constants for the clones depicted in (*b*) in the presence (k_{obs}+, filled circles) or absence (k_{obs}-, open circles) of 500 μM effector are depicted on a log scale. These rate constants reveal 'on/off' ratios that range between 5- and 510-fold under *in vitro* selection conditions. ***d–f***, Allosteric modulation of G20' hammerhead ribozymes by cCMP. ***g–i***, Allosteric modulation of G23' hammerhead ribozymes by cAMP. Details for the analysis of the cCMP- and cAMP-dependent ribozymes are as described in (*a–c*).

three-domain construct (Fig. 1*a*). These tripartite allosteric ribozymes are each composed of a hammerhead self-cleaving ribozyme and an RNA aptamer that are linked by a small bridge domain. This bridge domain or 'communication module' acts as an information conduit through which the binding status of the aptamer is relayed to the ribozyme through conformational changes that affect catalytic activity. For several of the communication modules identified, we observed that replacing the original aptamer domain with different aptamer domains having various ligand specificities produced new allosteric ribozymes with the corresponding effector dependencies. In other words, certain communication modules including the class I communication module (cm^+FMN1) depicted in Fig. 1*a* appear to serve as generic reporters of the occupation state of different appended aptamers regardless of the particular ligand specificity.

Considering the versatility of the cm^+FMN1 module, we speculated that many as yet undiscovered aptamers could trigger ribozyme function if they were judiciously integrated into the effector-binding site of the tripartite RNA construct. Therefore, we generated a new construct in which the entire effector-binding site is replaced with a 25 nucleotide domain composed of random sequence (Fig. 1*b*). The organization of this RNA construct was expected to facilitate the isolation of allosteric ribozymes with novel effector specificities using a selective amplification process that we have termed 'allosteric selection' (Fig. 2*a*). This process is expected to favor the enrichment of the RNA population for those ribozymes that remain inactive in the absence of effector, but that are activated upon effector addition.

Beginning with a pool of 10^{15} RNA molecules representing nearly all possible sequence variants within the random sequence domain of the construct, we conducted successive negative and positive selection reactions using a mixture of the four 3',5'-cyclic mononucleotides (cNMPs; 500 μM each) as potential effector molecules. Each RNA population was prepared by *in vitro* transcription in the absence of the cNMP mixture, and the full-length precursor RNAs were purified by denaturing 10% polyacrylamide gel electrophoresis (PAGE). The isolated RNA precursors were incubated in the absence

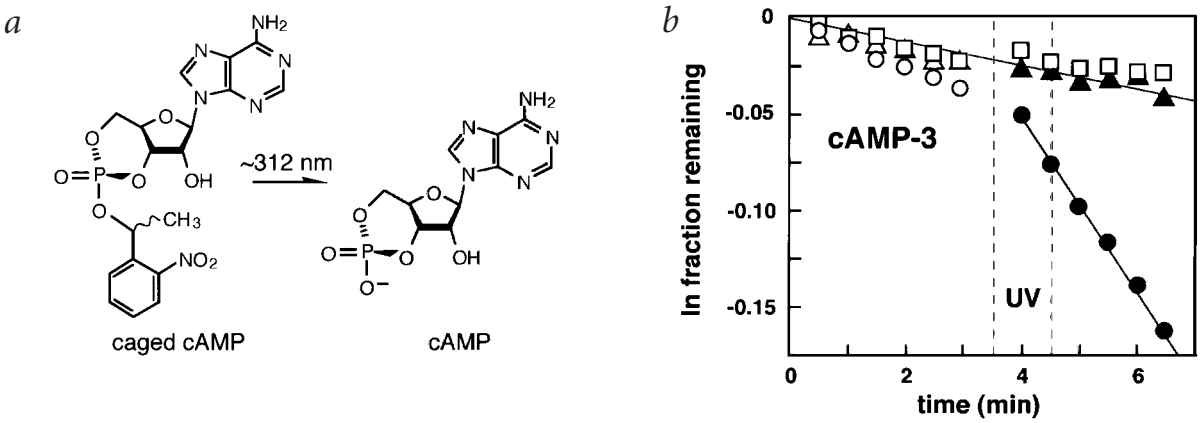

Fig. 4 Molecular recognition of cAMP by cAMP-3 RNA. ***a,*** The caged cAMP analog adenosine 3',5'-cyclic monophosphate, P¹-(2-nitrophenyl)ethyl ester is converted to 3',5'-cAMP by brief irradiation with long-wave UV light. ***b,*** Allosteric activation of cAMP-3 RNA by uncaged cAMP. The plot depicts the natural logarithm of the fraction of precursor RNAs that remain uncleaved at different incubation times in the presence (squares and circles) or absence (triangles) of 2 mM caged cAMP. Shaded and filled symbols represent data collected during or after UV irradiation, respectively. Irradiated mixtures were exposed between t = 3.5 and 4.5 min (dashed lines). The ribozyme is activated only when irradiated (filled symbols) in the presence of cAMP.

of the effector mixture under otherwise permissive reaction conditions (reaction buffer: 50 mM Tris-HCl, pH 7.5 at 23 °C, and 20 mM Mg^{2+}) for an extended period of time. Uncleaved precursors from this negative selection reaction were again isolated by PAGE and subjected to positive selection by brief incubation under the permissive reaction conditions containing the cNMP mixture. The resulting 5' cleavage products were purified by PAGE and amplified by reverse transcription followed by the polymerase chain reaction (RT-PCR). This selective amplification process was repeated to favor the enrichment of allosteric ribozymes that respond to any of the four cNMPs.

Acid-sensitive and effector-independent ribozymes

After only six rounds of selective amplification (G6), the RNA pool exhibited a significant positive response to the addition of the cNMP mixture (Fig. 2*b*). However, upon further examination, we found that the G6 RNA population does not specifically recognize any of the cNMPs but is dominated by ribozymes that are triggered to function by a brief acidic treatment. Over the first six rounds of selection, the pH of the RNA mixture had been unintentionally lowered by adding an acidic mixture of cNMPs immediately before the addition of the reaction buffer (unpublished data). To prevent acidification, the RNA pool used for the positive selection was buffered with 50 mM Tris-HCl (pH 7.5 at 23 °C) preceding the addition of the cNMP mixture and the 20 mM Mg^{2+} used to initiate the reaction.

Two additional classes of selfish RNA molecules also became evident in the early stages of selection. One class of selfish ribozymes promote the RNA cleavage reaction with substantially reduced catalytic rates in both the negative and positive selection steps. The other class distributes into properly folded and misfolded states. In both cases, the ribozymes are not completely self-processed during the negative selection reaction, and therefore are enriched by the selective amplification process without responding to the effectors. These two types of selfish RNAs contributed to the high background level of RNA

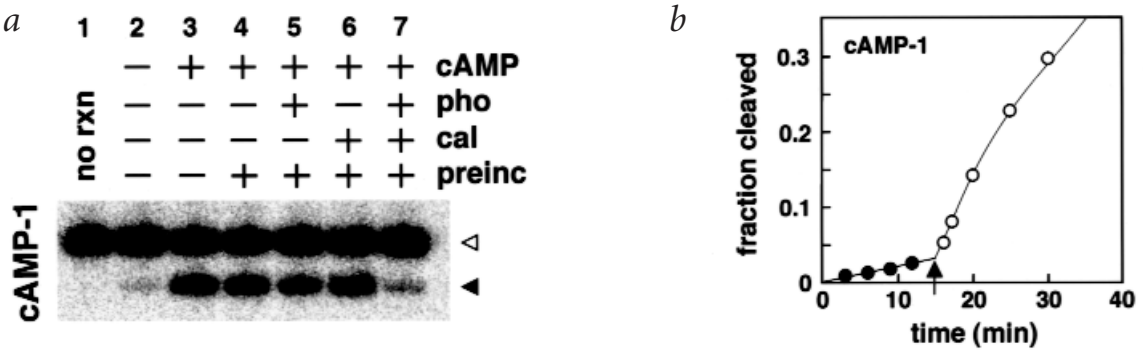

Fig. 5 Molecular recognition of cAMP by cAMP-1 RNA. ***a,*** The effects of *in situ* depletion of cAMP from the reaction buffer before the addition of the cAMP-1 allosteric ribozyme were determined by using 3',5'-cyclic nucleotide phosphodiesterase and calmodulin. Precursor RNAs (o pen arrowhead) undergo activation when incubated in reaction mixtures containing cAMP (+, lanes 3 and 4) or when incubated in reaction mixtures containing cAMP and including either phosphodiesterase (pho) or calmodulin (cal) (lanes 5 and 6, respectively). When combined, the phosphodiesterase and its activator calmodulin promote the hydrolysis of >90% of the cAMP to yield 5'-AMP during a 40 min preincubation (preinc) at 30 °C. The cAMP-1 RNA, which does not accommodate 5'-AMP as an effector (see Fig. 6), is no longer activated under these conditions (lane 7). Reaction products were separated and visualized as described in Fig. 2c. ***b,*** Plot depicting the activation of cAMP-1 by the addition of cAMP to 500 μM (indicated by the arrow) after exhaustive depletion of an original sample of cAMP. This reaction is derivative of that depicted in lane 7 of (*a*), but where an 80 min preincubation with the phosphodiesterase/calmodulin mixture was used to deplete the initial input of cAMP more completely. Filled and open circles identify data points collected before and after addition of the second aliquot of cAMP, respectively.

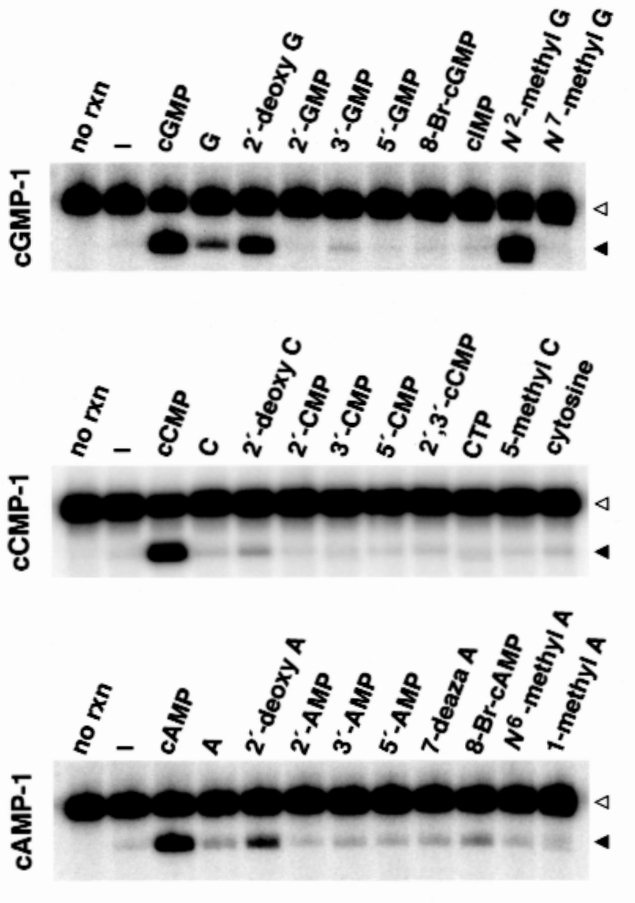

Fig. 6 Selective molecular recognition by cNMP-dependent allosteric ribozymes. Each of the three allosteric ribozymes cGMP-1, cCMP-1 and cAMP-1 were incubated for 2, 2 and 5 min, respectively, under *in vitro* selection conditions in the absence of effector (-), in the presence of 2 mM of its cognate cNMP effector or similarly with a panel of different effector analogs. Internally ^{32}P-labeled precursor RNAs and the resulting 5' cleavage fragments are identified by open and filled arrowheads, respectively. G, C and A represent the nucleosides guanosine, cytidine and adenosine, respectively. cIMP represents inosine 3',5'-cyclic monophosphate. Reaction products were separated and visualized as described in Fig. 2*c*.

catalysis that was observed in the positive selection reaction, and this rendered the efficiency of the allosteric selection process less than optimal.

Fortunately, ribozymes that specifically activate by recognizing an effector molecule attain a significant selective advantage over ribozymes that employ the effector-independent strategies just described. Extension of the incubation time for the negative selection reaction was used to further exclude ribozymes that cleave more slowly. However, ribozymes that persist using a misfolding strategy were more difficult to eliminate. Presumably, certain portions of these molecules partition into active and inactive conformational states after each denaturation event. Therefore, only part of the population cleaves during the negative selection. Upon purification of the uncleaved precursors by denaturing PAGE, the RNAs have another chance to refold and distribute between the two conformational states. This allows a significant portion of the population to cleave during the subsequent positive selection reaction. To select against ribozymes that employ this strategy, we conducted multiple rounds of negative selection and purification. Alternatively, we interspersed negative selection reactions with thermal or chemical denaturation steps to cleave and refold the RNAs repetitively (see Methods).

Isolation of cNMP-dependent hammerhead ribozymes

A measurable response to the cNMP mixture was once again exhibited by the selected RNA populations after a total of 14 rounds (Fig. 2*b*). We observed that the G16 RNA pool is dominated by allosteric ribozymes that are activated specifically upon the addition of cGMP (data not shown). Therefore, we conducted an additional two rounds of selection using only cGMP as the effector. The resulting population, termed G18' RNA, is highly responsive to the addition of cGMP (Fig. 2*c*).

To recover ribozymes that respond to the remaining cNMPs, we added cGMP to the negative selection reaction at G17 and supplied the remaining three effectors in the positive selection reaction. By G19, the RNA pool no longer responds to cGMP, but shows specificity for cCMP (data not shown). Therefore, we conducted an additional round of selection using only cCMP as the effector to produce G20' RNA. This RNA population preferentially cleaves in the presence of cCMP (Fig. 2*c*).

In a repetition of this strategy, we included both cGMP and cCMP in the negative selection beginning with G20, while supplying cAMP and cUMP in the positive selection. This process yielded a population of RNAs at G22 that now responds positively to cAMP. An additional round of selection using only cAMP gave rise to G23' RNA, a population that exhibits allosteric activation exclusively by this effector (Fig. 2*c*). However, after conducting an additional six rounds of selection using only cUMP in the positive selection reaction, specific enhancement in RNA cleavage by this effector was not observed. This finding indicates that cUMP-specific ribozymes were not present in the initial population and that ribozymes with this effector specificity did not by chance emerge as a result of mutations acquired during the selective amplification process.

Kinetic modulation of ribozymes with cGMP, cCMP, cAMP

Clones from the G18', G20' and G23' populations were sequenced in order to further characterize the function of the selected RNAs. Of the 12 clones examined from the G18' population, eight display considerable diversity within the original random sequence domain (Fig. 3*a*). Interestingly, all individuals sustained at least one mutation within the regions that define the communication module, and all but one clone carry deletions within the random sequence domain. This finding indicates that the original pool may not have offered a significant representation of allosteric ribozymes for the cNMP targets despite our efforts to bias the design of the RNA construct in favor of allosteric function.

Clones cGMP-1 through cGMP-4 were tested for catalytic activity and each responds positively to the addition of cGMP with distinctive characteristics (Fig. 3*b*). Comparison of the initial rates of hammerhead cleavage measured in the absence and the presence of effector (without regard for nonlinear kinetics) reveals that cGMP-1 is activated ~510 fold under the conditions used for allosteric selection (Fig. 3*c*). The remaining three clones are activated to a lesser magnitude, however each exhibits selective activation with cGMP and shows no cross-reactivity with the remaining noncognate effector molecules.

Similarly, individual clones from the G20' and G23' populations demonstrate specific activation with cCMP and cAMP effectors, respectively. As observed with the cGMP-specific

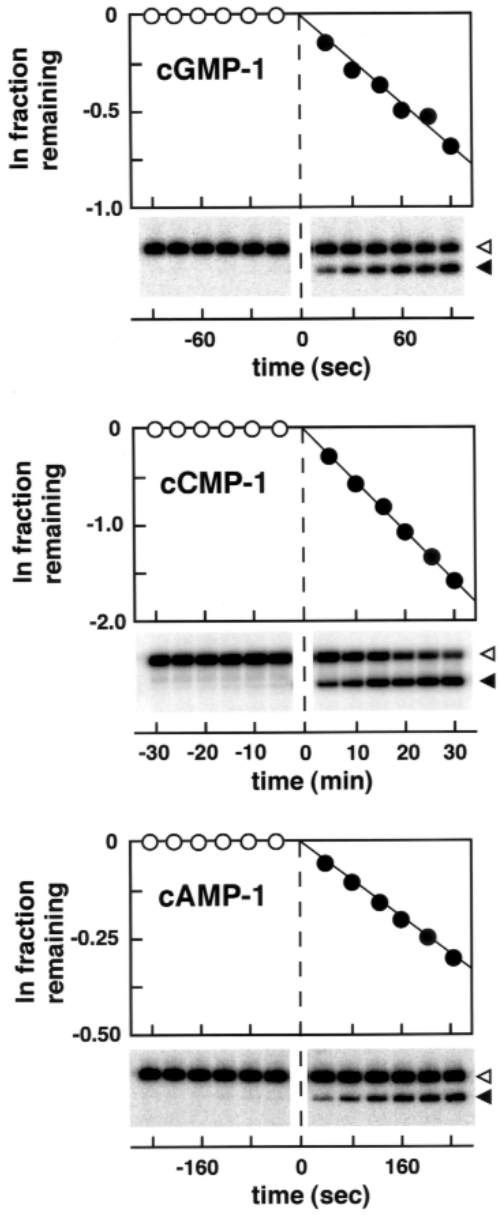

Fig. 7 Rapid effector-mediated activation of allosteric ribozymes. Reactions containing internally ^{32}P-labeled precursor RNAs as indicated were incubated for a brief time in the absence of effector, then 5 mM of their corresponding effector was added (dashed line) and the reaction was continued. The x-axis reflects the time relative to the addition of effector. The precursor (open arrowheads) and resulting 5' cleavage fragments (filled arrowheads) were separated, visualized and quantitated as described in Fig. 2c. The natural logarithm of the fraction of precursor remaining is plotted for each data point generated before (open circles) or after (filled circles) addition of effector, where the change in slope reflects the allosteric response of each ribozyme.

RNAs, the sequences of the isolated G20' RNAs reveal the acquisition of significant mutations or deletions over the course of the selection process, indicating that these changes may have been necessary to give rise to allosteric function (Fig. 3*d*). Although we examined the catalytic performance of all seven clones sequenced from G20', we observed that only cCMP-1 and cCMP-2 were activated by its corresponding effector (Fig. 3*e,f*). The remaining clones manifest weak catalytic activity without regard to the presence of any effector, indicating that these RNAs have persisted to this stage in the selection process without utilizing an allosteric activation strategy.

Eight distinct individuals were also identified among the 13 clones sequenced from the G23' population (Fig. 3*g*). Again, the clones have acquired significant mutations within the original communication module or deletions within the random sequence domains. Each of the five clones examined from the G23' population respond positively to the presence of cAMP (Fig. 3*h*). Moreover, the clones cAMP-1 through cAMP-4 display allosteric reaction kinetics that are similar to those observed with the previous allosteric constructs (Fig. 3*i*). Although no cUMP-dependent ribozymes were isolated from this RNA population, the diversity of sequences and kinetic characteristics of the allosteric ribozymes that were recovered indicate that significant potential exists for the generation of novel effector-modulated RNAs.

Molecular recognition by effector binding sites

Of primary concern is whether the representative cGMP-, cCMP- and cAMP-dependent ribozymes directly recognize the atomic structures of their corresponding effectors, or whether they respond to some other physicochemical signaling agent that might be unintentionally introduced into the reaction mixture. Precedence for alternative effectors for allosteric activation is provided by the observation that the first ribozymes that dominated the RNA population did not respond specifically to any of the four cNMPs, but were sensitive to acidification of the reaction mixture. To determine if the mechanism of ribozyme activation is mediated through direct molecular recognition of cNMPs, we made use of adenosine 3',5'-cyclic monophosphate, P^1-(2-nitrophenyl)ethyl ester, a 'caged' form of cAMP (Fig. 4*a*). The caged cAMP is a triester analog of cAMP similar to those reported by Nerbonne *et al.*[23] and is uncaged by cleavage of the added phosphoester linkage by irradiation with ultraviolet light. This caged effector provides a means to test whether an individual cAMP-dependent clone can be activated upon releasing the effector by irradiation.

The cAMP-dependent clones cAMP-1, cAMP-2 and cAMP-4 (Fig. 3*g–i*) each cleave when presented with the caged effector (data not shown), suggesting that the allosteric binding sites of these RNAs accommodate the chemical alteration present in this analog of cAMP. In contrast, the cAMP-3 clone exhibits the same rate constant whether it is incubated with 500 µM caged cAMP or whether it is incubated in the absence of effector (Fig. 4*b*). Presumably, the allosteric binding site of cAMP-3 excludes the caged cAMP compound from binding and activating the adjoining ribozyme. However, brief irradiation of a mixture containing cAMP-3 RNA and the caged cAMP with long-wave UV light centered on ~312 nm results in a significant activation of ribozyme function. The finding that UV-induced production of cAMP *in situ* triggers ribozyme activation is consistent with a mechanism whereby cAMP is directly recognized as an effector by this particular allosteric ribozyme.

To further investigate whether molecular recognition of cNMP effectors by RNA mediates allosteric ribozyme function, we established an assay wherein cAMP is depleted from the reaction mixture *in situ* (Fig. 5). The *in situ* depletion of cAMP was achieved using cyclic nucleotide phosphodiesterase[24] and its activator calmodulin. These proteins do not deplete the effector when incubated independently, but when combined they efficiently hydrolyze 3',5'-cyclic AMP to yield 5'-AMP. Under our assay conditions less than 10% of the cAMP is destroyed during a 40 min preincubation in the presence of the phosphodiesterase

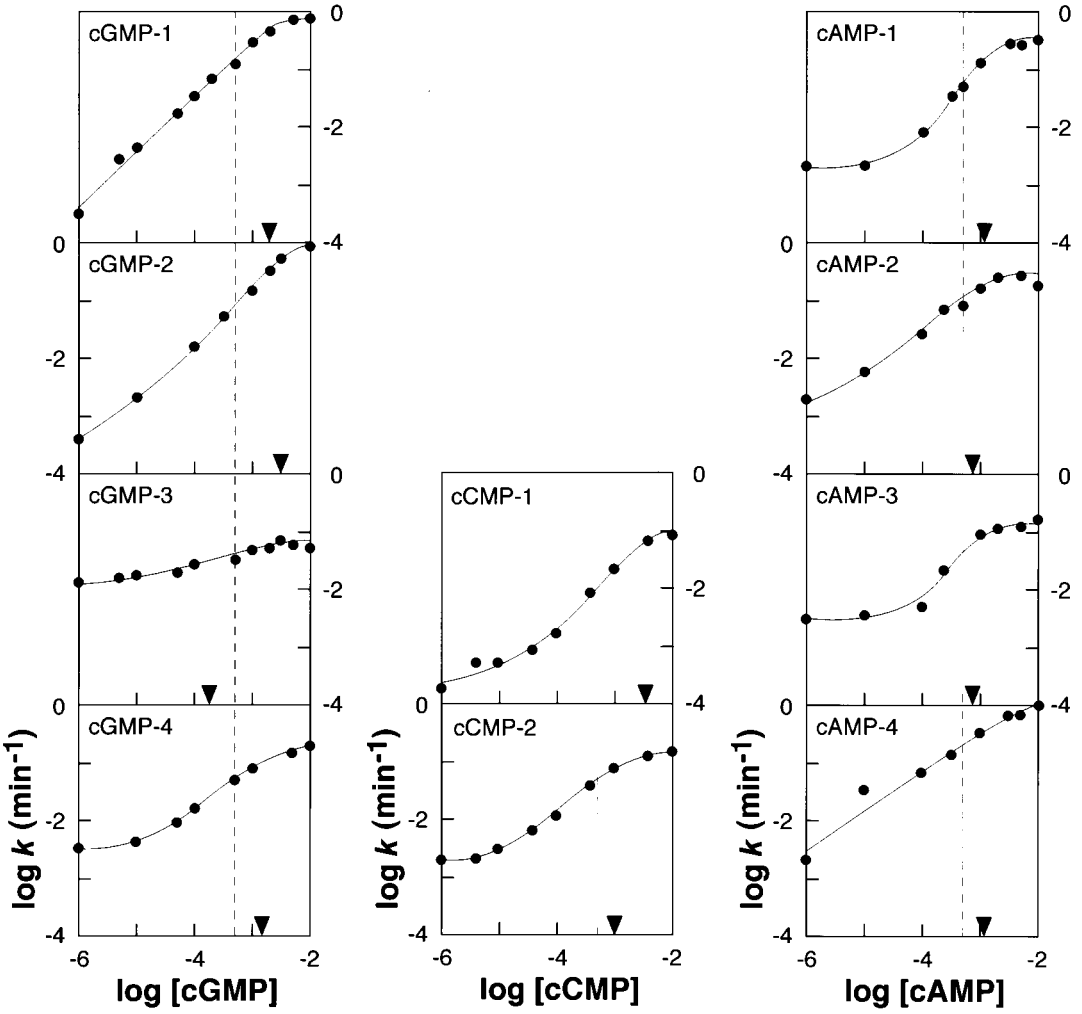

Fig. 8 Effector-binding affinities and the dynamic ranges for various allosteric ribozymes. The logarithm of the rate constant for ribozyme cleavage *versus* the logarithm of the effector concentration is plotted for each of the 10 clones depicted in Fig. 3. The minimum possible values for apparent K_d for each clone are represented by the location of the shaded arrowhead on the x-axis of each plot (assuming that k_{obs} at 10 mM effector reflects k_{max}). The difference in rate constants that is brought about by progressively increasing the concentration of the effector reflects the dynamic range for each clone. For example, log k_{obs} for cAMP-1 increases from -3 in the absence of effector (Fig. 3*i*) to -0.5 upon saturation of effector. Variation in the rate constant brought about by different concentrations of effector corresponds to a dynamic range for cAMP-1 of ~300 fold. Dashed lines reflect the concentration of effector (500 μM) used during *in vitro* selection.

alone; however, more than 90% is destroyed in a similar reaction containing calmodulin, an activator of cyclic nucleotide phosphodiesterase activity (data not shown).

The allosteric ribozyme cAMP-1 does not accommodate 5'-AMP as an effector (Fig. 6). As a result, this ribozyme should not be activated if cAMP is first depleted by the catalytic action of phosphodiesterase–calmodulin complexes. As expected, we found that neither phosphodiesterase nor calmodulin alone inhibited allosteric activation of cAMP-1 RNA (Fig. 5*a*, lanes 5 and 6). In contrast, the allosteric ribozyme was not significantly activated when added to a reaction mixture containing cAMP that had been preincubated with both phosphodiesterase and calmodulin (Fig. 5*a*, lane 7). Moreover, we observed that cAMP-1 ribozymes in a reaction mixture equivalent to that used for lane 7 could be activated upon addition of a second aliquot of cAMP (Fig. 5*b*). This indicates that the loss of ribozyme activation upon preincubation with both protein factors is caused by the depletion of cAMP effector and is not due to any inhibitory effects that are inherent to the protein complex. Both studies described above, which involve either *in situ* production or depletion of cAMP, provide evidence that at least some of the many ribozymes isolated by allosteric selection directly recognize their corresponding cNMP effector molecules.

Molecular discrimination by allosteric binding sites

A preliminary survey of the molecular recognition determinants was conducted using representative clones cGMP-1, cCMP-1 and cAMP-1. In each case, the RNAs exhibit significant discrimination against closely related analogs of their corresponding effector (Fig. 6). For example, cGMP-1 RNA shows significant discrimination against 3'-GMP and 5'-GMP, the hydrolyzed analogs of cGMP. Likewise, the cCMP-1 and cAMP-1 clones also exhibit this same ability to distinguish whether the cyclic phosphodiester structure of their corresponding cNMP effectors has been opened by hydrolysis of the 5'O-P or the 3'O-P bonds.

Although additional experimentation is necessary to define more clearly the determinants of molecular recognition for these

allosteric ribozymes, it appears that in each case the discrimination against opened-ring analogs could be due to steric interactions. The observation that all three clones remain at least partially active when supplied with the corresponding nucleoside and deoxynucleoside analogs of cNMP indicates that the phosphate moiety is not absolutely required for allosteric activation. In contrast, alteration of many of the functional groups on the nucleotide base of each effector adversely affects allosteric ribozyme function (Fig. 6). Therefore, the base moieties of the cNMP effectors appear to be essential for molecular recognition by the different effector binding domains.

Rapid activation of cNMP-dependent ribozymes

A common characteristic of the small-molecule dependent allosteric ribozymes created to date is the rapid activation or deactivation of ribozyme function upon addition of the effector[16,17,20]. The rapid allosteric response is a kinetic feature that is highly desirable for RNA molecular switches that are to find practical application. Therefore, we examined the activation kinetics for the three representative clones cGMP-1, cCMP-1, and cAMP-1. In each case, the ribozymes appear to be activated within seconds after introduction of their corresponding effector molecules (Fig. 7). Rapid activation of ribozyme function is indicative of a dynamic RNA structure that quickly forms active effector binding and ribozyme conformations only upon introduction of the appropriate signaling agent.

Each of the clones described above maintains linear cleavage kinetics through at least one half-life (Fig. 7 and data not shown), indicating that greater than 50% of an individual clone's copies are activated upon addition of the appropriate effector. However, we find that self-cleavage for some individuals reaches a plateau after only a short reaction time, which might be indicative of significant misfolding problems. Upon allosteric activation, most clones examined undergo between 20% and 90% processing before cessation of catalysis.

Binding affinities and dynamic ranges

The effector-binding site of each allosteric ribozyme is expected to bind its ligand with a distinct affinity that can be described by a dissociation constant (K_d) for the RNA–ligand interaction. If occupation of the effector-binding site indeed correlates with the level of activation for a particular allosteric ribozyme, then an apparent K_d for effector binding can be established for this interaction by examining the dependency of catalytic rate on the concentration of effector.

To provide a comprehensive analysis of the binding affinities displayed by the allosteric ribozymes that were isolated in this study, we determined the effector concentration-dependent activities of all 10 allosteric ribozymes described in Fig. 3. Apparent K_d values were determined by establishing the effector concentration that produces a rate constant that is half maximal ($^1/_2\ k_{max}$). In all cases, the apparent K_d falls near the concentration of each effector used during *in vitro* selection (Fig. 8). These constants range from ~200 μM (cGMP-3) to ~4 mM (cCMP-1). By comparison, most ligand-binding RNAs isolated by SELEX methods[14] bind with higher affinities, indicating that improvements in the sensitivity of these allosteric ribozymes to lower concentrations of effector could be achieved.

The plots used to define the apparent K_d for each allosteric ribozyme (Fig. 8) also reveal the range of rate constants that are exhibited for different concentrations of effector. This 'dynamic range' for allosteric responses is highly variable between the different clones, suggesting that the diversity of functional characteristics that can be manifested by allosteric ribozymes is substantial. As expected from our preliminary analysis (Fig. 3*b*), the cGMP-3 ribozyme has a poor rate enhancement or 'allosteric response' to cGMP. As a result, this individual exhibits an overall dynamic range of less than one order of magnitude. In contrast, the clone that displays the best dynamic range is cGMP-1, which maintains a linear increase in the logarithm of its rate constant from 1 μM through 1 mM. Although the increase in the rate constant for cGMP-1 under *in vitro* selection conditions is ~500 fold, the overall rate increase upon saturation of the effector binding site with cGMP is ~5,000 fold. This corresponds to a dynamic range for cGMP-1 of greater than three orders of magnitude.

Engineering novel RNA molecular switches

The allosteric selection strategy (Fig. 2*a*) employed in this study provides an alternative approach for the isolation of novel RNA molecular switches and for the isolation of new ligand binding RNA structures. Herein, we demonstrate the simultaneous isolation of numerous allosteric ribozymes that respond to particular cNMP targets. Similarly, allosteric selection could be used for the isolation of molecular switches on a massively parallel scale by using mixtures of metal ions and metal complexes or by using complex mixtures containing hundreds of organic compounds, proteins or nucleic acids as candidate effector molecules in the positive selection reaction. Indeed, any physicochemical impulse that can influence RNA structure folding might be a signaling agent for allosteric ribozyme function.

Although our initial implementation of the allosteric selection strategy yielded many allosteric ribozymes, two aspects of the current protocol can be modified in order to create a more efficient selective amplification process. First, the emergence of effector-independent ribozymes must be avoided so that only those ribozymes that respond positively to a target effector are selected and subsequently amplified. For example, increasing the incubation time during the negative selection reaction and decreasing the incubation time for the positive selection reaction can disfavor slow-cleaving ribozymes. However, this procedural modification will not disfavor the amplification of ribozymes that distribute between active and inactive conformations. Presumably, ribozymes that use a differential folding strategy can be disfavored by avoiding denaturation and refolding of RNAs after the negative selection is completed. To achieve this effect, we propose the use of a chromatography based selection system that allows separation of cleaved ribozymes from uncleaved precursors without employing denaturing PAGE.

Second, the design of the starting RNA pool must be reorganized to provide a more productive sampling of ligand specific allosteric ribozymes. The original RNA pool used in this study (Fig. 1*b*) was based on the observation that the cm$^+$FMN1 communication module provides a connective bridge between aptamer and ribozyme domains and a general switching mechanism for allosteric function[17]. Moreover, comprehensive representation of the variants derived from the 25 random sequence nucleotides is expected to provide a substantial number of ligand binding structures. However, the sequence data generated for each of the populations of cNMP-dependent ribozymes (Fig. 3) indicates that significant levels of mutation were necessary for allosteric function to emerge from the original RNA pool. Preliminary analysis of the cGMP-1 RNA confirms that the single G to A mutation in the communication module is required for allosteric ribozyme function (data not shown). If this observation holds for other clones as well, then the use of the cm$^+$FMN1 communication module was a burden on allosteric selection.

In addition, the selection failed to produce any ribozymes that are activated by cUMP. Most likely, RNA can adapt to use cUMP as an effector for an allosteric ribozyme; however, our pool design failed to yield such a construct in this *in vitro* selection effort. Therefore, it may be advantageous to use a construct that provides a larger random sequence domain attached directly to the ribozyme domain such that there is no bias in favor of a distinct communication module sequence. This should facilitate the emergence of more diverse examples of effector binding domains that modulate the activity of the adjoining ribozyme by other mechanisms for allosteric function.

Structural and functional versatility of RNAs

In contrast to the limited functions of natural ribozymes, protein enzymes catalyze a tremendous array of chemical transformations with extraordinary precision and enormous rate enhancements. Included among the diverse biochemical functions of protein enzymes are conformational changes that in some instances provide effector-dependent allosteric modulation[19]. Unlike their protein counterparts, natural ribozymes are not known to undergo allosteric modulation of catalytic activity. However, the results of this study and several earlier studies[10,11,16–18,22,25,26] provide evidence that nucleic acids are quite capable of modulating catalytic activity in response to various effector compounds. These findings are consistent with earlier suggestions[5–8] that RNA may have significant untapped potential for complex catalytic function. Presumably, the true catalytic potential of nucleic acids can be harnessed for the construction of synthetic ribozymes that enable unique biochemical applications.

It is important to note that the allosteric ribozymes described in this study have not been subjected to any efforts to optimize their allosteric responses and catalytic function. We elected to feature representative clones that were generated by this initial *in vitro* selection process, regardless of their kinetic characteristics, in order to give a sense of the properties of allosteric ribozymes that first proved successful. The ribozymes described in this report should be considered prototypic because in most cases their effector binding affinities and catalytic rates are most likely inadequate to serve in most applications. Presumably, individual classes of allosteric ribozymes isolated by allosteric selection will be amenable to further optimization using *in vitro* selection strategies similar to those in this study. This would ultimately allow their development as efficient molecular switches for various applications.

Implications for the control of gene expression

Precise control over gene expression is of profound importance to the normal function of all cells. Likewise, the purposeful manipulation of gene expression that is directed with precise temporal or spatial command is of great interest to those who desire to control biological systems at the molecular level. Conceivably, the regulation of gene expression can occur at any stage of the process of information transfer from DNA to RNA and from RNA to the final protein product. In fact, natural systems have evolved an abundance of strategies that are used to adjust the levels of gene accessibility and to modulate the molecular processes that occur after transcription[27]. Many of these mechanisms have become targets for the development of small molecule regulators that can be used to control gene expression[28].

A number of genetic control mechanisms of cells are exerted at the level of RNA. Natural antisense interactions and the modulation of RNA stability, for example, are two mechanisms that are known to impact gene expression. Antisense oligonucleotides and ribozymes are widely used by investigators to purposefully influence the expression of specific genes by exploiting these two mechanisms. These approaches modulate RNA function either by sterically blocking access to the RNA target or by targeting the RNA for destruction. Recently, it was shown that mRNA translation could be blocked by exploiting specific interactions between aptamers and certain dye compounds[29]. Specifically, RNA aptamers that selectively bind Hoechst dyes H33258 and H33342 were integrated into mRNAs such that gene expression was selectively blocked when these ligands were introduced into the cell. Similarly, allosteric ribozymes could be fused to mRNAs so that when the corresponding effector molecule is added to the cell, the ribozyme domain adjusts its catalytic activity. Therefore, allosteric effector molecules could be used to modulate the stability of mRNAs and thus influence the expression of a target gene.

The allosteric selection protocol described herein makes possible the simultaneous selection of new allosteric ribozymes that respond to any of hundreds or even thousands of compounds. This provides a means to test whether self-cleaving ribozymes such as the hammerhead can be made to respond to a wide range of effector stimuli and whether the resulting allosteric constructs can be integrated with mRNAs as new genetic control elements. If this proves feasible, then nearly any natural or bioavailable compound is a candidate for the purposeful control of gene expression in genetically transformed organisms.

Methods

RNA pool preparation. DNA templates for the RNA pool depicted in Fig. 1*b* and the oligonucleotides used for RT-PCR were prepared by automated DNA synthesis (Keck Biotechnology Resource Laboratory, Yale University). All DNAs were purified by denaturing (8 M urea) PAGE before use. The DNA template 5'-GGGCAACCTACGGCTTTCACCGTTTCGACGT(N_{25})AAGGCTCATC-AGGGTCGCC (4.15 nmol) was made double-stranded by extension in the presence of 'primer 2' (5'-TAATACGACTCACTATAGGGCGACCCTGATGAG, 8.3 nmol), which introduces the promoter for T7 RNA polymerase (T7 RNAP). The DNA extension reaction (300 μl) was carried out using SuperScript II reverse transcriptase (RT, Gibco BRL) according to the manufacturer's directions.

The resulting double-stranded DNAs were recovered by precipitation with ethanol and resuspended in a 2 ml transcription mixture containing 50 mM Tris-HCl (pH 7.5 at 23 °C), 15 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 2 mM each of the four NTPs, 200 μCi [α-^{32}P]UTP, and 60,000 U T7 RNAP (His-tag construct supplied by T.E. Shrader). The transcription mixture was incubated at 37 °C for 1 h and the resulting uncleaved precursor RNAs (internally ^{32}P-labeled) were isolated by denaturing 10% PAGE. Note that PAGE purification eliminates ribozymes that have undergone self-cleavage during the *in vitro* transcription reaction. This inherently introduces an additional negative selection step that disfavors the isolation of ribozymes that function without activation by an effector. Moreover, this step disfavors the isolation of allosteric ribozymes that cannot distinguish between the intended cNMP target effectors and the NTPs that are required for *in vitro* transcription.

Allosteric selection. *In vitro* selection for allosteric ribozymes that respond to the cNMPs (Sigma) was carried out using repeated rounds of negative and positive selection. For the first round of negative selection, an initial pool of RNA precursors (9.3 nmol, 5.6×10^{15} molecules) was incubated at 23 °C for 5 h in a reaction mixture (930 μl) containing 50 mM Tris-HCl (pH 7.5) and 20 mM $MgCl_2$ in the absence of the four cNMPs. Precursor RNAs that resist cleavage during this incubation were isolated by denaturing 10% PAGE. Purified precursor RNAs were then subjected to the first round of positive selection at 23 °C for 30 min in the same reaction buffer (930 μl) containing 500 μM each of the four cNMPs. At this stage, cleaved products were purified by denaturing 10% PAGE and the 5' cleavage fragments were recovered from the gel by crush-soak elution and amplified by RT-PCR. Reverse transcription was conducted in a reaction buffer (400 μl total) using SuperScript II RT according to the manufacturer's directions cDNA and using

primer 1 (5'-GGGCAACCTACGG-CTTTCACCGTTTCG). Subsequent PCR amplification of the resulting cDNA using primers 1 and 2 (500 pmol each) was conducted in a reaction mixture (2 ml total) containing 10 mM Tris-HCl (pH 8.3 at 23 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dNTP and 50 U *Taq* polymerase (Promega). The reaction was thermocycled for the desired number of iterations at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s.

Additional rounds of selective amplification were repeated in a similar fashion using 15 min positive selection reactions until effector-sensitive ribozyme function was detected. Subsequent rounds of selection included both negative and positive selection steps that were conducted as described above using smaller RNA pools and with the reaction sizes scaled down accordingly. For the first five rounds of selection, a 10× stock mixture of cNMPs was added to the RNA pool before the addition of the remaining components of the reaction buffer. In subsequent rounds, the cNMP mixture was added after the reaction buffer to preclude the isolation of acid-sensitive ribozymes. In addition, negative selections were altered to select more aggressively against ribozymes that cleave slowly or that distribute between active and inactive conformations upon refolding. To disfavor slow cleaving ribozymes, the negative selection time was increased from 5 h to as much as 48 h, and multiple negative selection steps were occasionally employed before conducting positive selection. To disfavor misfolding ribozymes, periodic thermocycling was employed as described[17], or chemical denaturation with urea or mild alkali were used in an iterative fashion between periods of negative selection to induce multiple cycles of denaturation, renaturation and self-cleavage. Interestingly, ribozymes that use a misfolding strategy for survival also resisted the negative selection strategies that rely on thermal and urea-mediated denaturation (unpublished observations). Therefore, the use of alkaline denaturation proved most effective for negative selection.

Allosteric ribozyme characterization. RNA populations displaying cNMP-dependent self-cleavage were cloned (TOPO TA Cloning Kit, Invitrogen), sequenced (Thermo Sequenase Cycle Sequencing Kit, USB) and further analyzed by establishing the effector-mediated modulation of ribozyme kinetics. Double-stranded DNA templates for individual allosteric ribozyme clones were prepared either by PCR amplification of the plasmid DNA using primers 1 and 2, or by preparation of the appropriate synthetic DNA template. Internally ^{32}P-labeled RNAs were prepared by *in vitro* transcription as described here.

Initial rate constants for RNA self-cleavage were established by incubating trace amounts (~100 nM) of internally ^{32}P-labeled RNA precursors in selection buffer containing different concentrations of cNMP effectors as indicated for each experiment. Reactions were terminated by the addition of 2× PAGE loading buffer containing additional EDTA to sequester the Mg^{2+} cofactor[18]. For each clone, a plot of the fraction of precursor cleaved (<20% processed) versus time gave a straight line, where the slope reflects the initial rate constant for the ribozyme under the particular reaction conditions used. In all cases, duplicate experiments gave rate constants that varied by less than 50%.

The caged cAMP analog, adenosine 3',5'-cyclic monophosphate, P^1-(2-nitrophenyl)ethyl ester (Calbiochem), was resuspended in dimethylsulfoxide (DMSO) to yield a 100× stock solution (200 mM). Dissolved analog was delivered to the ribozyme reaction to yield final concentrations of 2 mM, and the resulting reaction mixture was supplemented with DMSO to give a final concentration of 5% to prevent its precipitation. This concentration of DMSO had no effect on the function of the clone cAMP-3. UV irradiation of the samples contained in a polycarbonate microtiter plate (USA Scientific) was conducted using a UV transilluminator (Spectroline model TVC-312A) that produces light centered at 312 nm. Under these conditions, greater than 80% of the analog is converted to cAMP.

The cAMP depletion reactions were prepared by delivering cAMP (500 μM), 3',5'-cyclic nucleotide phosphodiesterase (activator deficient from bovine brain, Sigma) and calmodulin (3',5'-cyclic nucleotide phosphodiesterase activator, Sigma) as indicated for each reaction. Lyophilized phosphodiesterase and calmodulin samples were separately resuspended in a buffer containing 50 mM 2-(4-morpholino)ethane sulfonic acid (MES) (pH 6.5 at 23 °C), 100 mM NaCl and 60% glycerol. Phosphodiesterase was delivered as indicated to a final concentration of 5×10^{-5} U μl^{-1}, and calmodulin was delivered as indicated to a final concentration of 0.3 U μl^{-1}. Reactions for the cAMP depletion studies contained 50 mM Tris-HCl (pH 7.5 at 23 °C), 20 mM $MgCl_2$, 30 μM $CaCl_2$, and 2.7% glycerol. Trace amount of internally ^{32}P-labeled cAMP-1 RNA was added immediately (no preincubation) or was added after a 40 or 80 min preincubation that was carried out at 30 °C.

Acknowledgments

We thank J. Shin for contributions to Figures 3 and 8, and we thank members of the Breaker laboratory for helpful discussions. We also thank T.E. Shrader at the Albert Einstein College of Medicine for the kind gift of the construct to express His-tagged T7 RNAP. Funding for this work was provided by grants from the National Institutes of Health (NIH), the Defense Advanced Research Projects Agency (DARPA) and the Yale Diabetes and Endocrinology Research Center (DERC). M.K. was supported by Sankyo Co. Ltd. of Japan and G.A.S. was supported by a Seessel postdoctoral fellowship from Yale University. R.R.B. is the recipient of a Hellman family fellowship and a fellowship from the David and Lucile Packard Foundation.

Received 22 June, 1999; accepted 16 August, 1999.

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