Nucleic Acid Enzymes (Ribozymes and Deoxyribozymes): *In Vitro* Selection and Application

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Natural catalysts composed of RNA were discovered in the early 1980s. After this advance, it was logical to consider that artificial nucleic acid enzymes made from either RNA or DNA could be developed by recapitulating the basic evolutionary principles in the laboratory. In subsequent years, many artificial ribozymes and deoxyribozymes have indeed been identified by *in vitro* selection, and the scope of nucleic acid catalysis now encompasses a much wider range of chemical reactions than is found in nature. The study of artificial ribozymes and deoxyribozymes can provide fundamental insight into the functioning of natural catalytic nucleic acids. In addition, artificial nucleic acid enzymes are increasingly used in practical applications ranging from analytical chemistry to biology.

Advanced Article

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The discovery of natural RNA catalysts has prompted chemical biologists to pursue artificial nucleic acids that have catalytic activities. Such artificial nucleic acid enzymes may comprise either RNA (ribozymes) or DNA (deoxyribozymes). The term "ribozyme" was first used in 1982, when Kruger et al. reported natural catalytic activity by RNA (1). This term is now used universally for catalytic RNAs, whether artificial or natural (see also the WECB review on Catalytic Modes in Natural Ribozymes). The term "deoxyribozyme" was first used in 1994, when Breaker and Joyce reported the first artificial DNA catalyst (2); no natural deoxyribozymes have been identified. With current knowledge, we cannot design nucleic acid enzymes that have completely new catalytic activities from first principles or by rational modification of known enzymes. Instead, we must use combinatorial search techniques to identify functional RNA and DNA sequences by sifting through a large number of random sequences using an appropriate search strategy. This process of "*in vitro* selection" has proven useful for identifying nucleic acid enzymes with a wide range of catalytic activities. In certain cases, we can apply ribozymes and deoxyribozymes for practical purposes such as sensing the presence of an analyte and providing a visible response. This review describes how artificial nucleic acid enzymes are identified by *in vitro* selection and used in practical applications.

Scope of Ribozyme Catalytic Activities

The first artificial ribozyme was reported in 1990 (3). Since that time, *in vitro* selection has been used to discover many artificial ribozymes with a wide range of catalytic activities. The known natural ribozymes catalyze phosphodiester cleavage or ligation, with the exception of the ribosome—made of both RNA and protein—that catalyzes peptide bond formation. Many artificial ribozymes also catalyze phosphodiester exchange reactions (RNA/DNA cleavage or ligation), although a growing number of ribozymes catalyze other reactions. Artificial ribozymes for phosphodiester cleavage or ligation have been emphasized in part because nucleic acid catalysts can readily bind via Watson–Crick base pairs to oligonucleotide substrates. By segregating the binding and catalysis functions to distinct regions of the nucleic acid enzyme (**Fig. 1**), the difficulty of achieving catalysis is reduced. The substrate binding energy is designed directly into the system, and the enzyme needs only to catalyze the reaction.

The scope of artificial ribozyme activities identified to date is provided in **Table 1** (4–52); representative examples of ribozymes and the reactions that they catalyze are shown in **Fig. 2**. Such a compilation indicates that many different types of reactions are amenable to nucleic acid catalysis, which includes such prototypical "organic" reactions as the aldol reaction and the Diels–Alder cycloaddition, which form carbon–carbon

Table 1 Tabulation of artificial ribozymes by type of reaction catalyzed and type of bond formed or broken

Reaction catalyzed	Bond	# Rand nt	Rate enh	M^{2+} req	Selection method	Ref
2′,3′-cyclic phosphate hydrolysis	O–P	0	50	Pb^{2+}	PAGE shift	(4)
RNA cleavage	O–P	0	80	Pb^{2+}	PAGE shift	(5)
RNA cleavage	O–P	100	200	Mg^{2+}	PAGE shift	(6)
RNA cleavage	O–P	30	nd	None	PAGE shift	(7)
RNA ligation	O–P	220	7×10^6	Mg^{2+}	seq tag acquisition	(8)
RNA ligation	O–P	220	8×10^8	Mg^{2+}	seq tag acquisition	(9)
RNA ligation	O–P	210	5×10^5	Mg^{2+}	seq tag acquisition	(10)
RNA ligation	O–P	116	250	Mg^{2+}	biotin tag acq (beads)	(11)
RNA ligation (branch formation)	O–P	80	nd	Mg^{2+}	biotin tag acq (beads)	(12)
RNA phosphorylation	O–P	100	1×10^5	Mg^{2+}	S tag acquisition (beads)	(13)
RNA phosphorylation	O–P	0	6×10^6	Mg^{2+}	S tag acq + PAGE shift	(14)
RNA capping	O–P	90	$\sim 10^3$–10^4	Mg^{2+}	biotin/seq tag (beads)	(15)
RNA capping	O–P	50	nd	Ca^{2+}	PAGE shift + rxn w/bead	(16)
RNA capping	O–P	0	nd	Ca^{2+}	rational modification	(17)
amino acid adenylation	O–P	80	nd	Ca^{2+}	S tag acquisition (beads)	(18)
cofactor synthesis	O–P	30, 60	nd	Mn^{2+}	biotin tag; PAGE shift	(19)
RNA polymerization	O–P	0	nd	Mg^{2+}	rational modification	(20)
template-directed pol.	O–P	76 0	nd	Mg^{2+}	S taq acq + PAGE shift	(21)
RNA-protein conjugation	N–P	152	nd	Mg^{2+}	biotin tag acq (beads)	(22)
Diels–Alder reaction *(nonstd)*	C–C	100	800	Cu^{2+}	biotin tag + PAGE shift	(23)
Diels–Alder reaction *(nonstd)*	C–C	0	1×10^4	$Cu^{2+} + Ni^{2+}$	biotin tag + PAGE shift	(24)
Diels–Alder reaction	C–C	120	1×10^4	Mg^{2+}	biotin tag acq (beads)	(25, 26)
aldol reaction	C–C	142	4×10^3	Zn^{2+}	biotin tag acq (beads; hν)	(27)
alcohol oxidation	C–H	70	1×10^7	$Mg^{2+} + Zn^{2+}$	biotin tag acq (beads)	(28)
aldehyde reduction	C–H	0	3×10^6	$Mg^{2+} + Zn^{2+}$	rational modification	(29)
pyrimidine nt synthesis	C–N	228	1×10^8	Mg^{2+}	S + PAGE; biotin (beads)	(30, 31)
purine nt synthesis	C–N	95	nd	Mg^{2+}	S tag acq + PAGE shift	(32)
N^7G alkylation	C–N	0	3×10^6	Mg^{2+}	biotin tag acq (beads)	(33)
amide synthesis *(nonstd)*	C–N	100	1×10^5	Cu^{2+}	biotin + PAGE (or beads)	(34)
urea synthesis *(nonstd)*	C–N	100	1×10^6	nd	reag tag + PAGE (or beads)	(35)
peptide bond formation	C–N	142	1×10^6	Mg^{2+}	biotin tag acq (beads)	(36)
peptidyl-RNA synthesis	C–N	0	100	Ca^{2+}	rational modification	(37)
acyl transfer	C–O	90	1×10^{10}	Mg^{2+} 0	biotin tag acq (beads)	(38, 39)
acyl transfer	C–O	120	nd	Mg^{2+}	biotin tag acq (beads)	(40)
aminoacylation	C–O	50	2×10^5	$Mg^{2+} + Ca^{2+}$	reagent tag acq + HPLC	(41)
aminoacylation	C–O	70 0	nd	Mg^{2+}	biotin tag acq (beads)	(42)
aminoacylation	C–O	70	2×10^5	Mg^{2+}	biotin tag acq (beads)	(43)
aminoacylation	C–O	0	6×10^7	Ca^{2+}	rational modification	(44)
carbonate hydrolysis	C–O	70	100	None	SELEX for TS analog	(45)
phosphorothioate alkylation	C–S	30	2×10^3	Mg^{2+}	S tag + PAGE (or beads)	(46)
Michael reaction	C–S	142	3×10^5	Mg^{2+}	biotin tag acq (beads; hν)	(47)
porphyrin metalation	Cu–N	50	500	$Mg^{2+} + Cu^{2+}$	SELEX for TS analog	(48)
Pd nanoparticle formation *(nonstd)*	Pd–Pd	40	nd	None	nanoparticle binding	(49, 50)
biphenyl isomerization	None	28	88	Mg^{2+}	SELEX for TS analog	(51)

NOTES: This tabulation is not intended to be exhaustive, in that other examples of ribozymes with the indicated activities may have been reported. nd = not determined in published report. When the reaction type is marked with "(*nonstd*)," the ribozyme required nonstandard nucleotides for activity. The entry "# Rand nt" is the total number of originally random nucleotides in the sequence pool; these may have been in two or more portions interspersed with constant regions. The entry "Rate enh" is the highest rate enhancement reported, often calculated as the ratio of rate constants for the observed versus background reactions (Fig. 4). In some cases, the authors reported the rate enhancement as the ratio of k_{cat}/K_m for the ribozyme and the analogous value for the uncatalyzed reaction. The listed rate enhancement may be a lower limit on the true value, for at least one of two reasons: (1) Only an upper limit was possible on the uncatalyzed rate. (2) The uncatalyzed rate represents a spectrum of reactions, only one of which corresponds to the particular reaction catalyzed by the ribozyme. Under "Selection method," the entry "(beads)" is included if noncovalent binding to beads (or other solid support) of the tagged nucleic acid sequences was an integral part of the key selection step. In some other cases, the substrate was presented on beads to avoid aggregation, but the key selection step did not involve a solid support. The entry "(hν)" is included if a photochemical release step was used.
[0]The experiment started with a known aptamer or ribozyme sequence, which in some cases was partially randomized. In some cases, a new random-sequence domain of indicated length was also included.
[0]The experiment used rational redesign of a known ribozyme sequence.
[0]Further work showed that the ribozyme requires only outer-sphere contacts with the Mg^{2+} ion, because exchange-inert $Co(NH_3)_6^{3+}$ supports full activity (52).

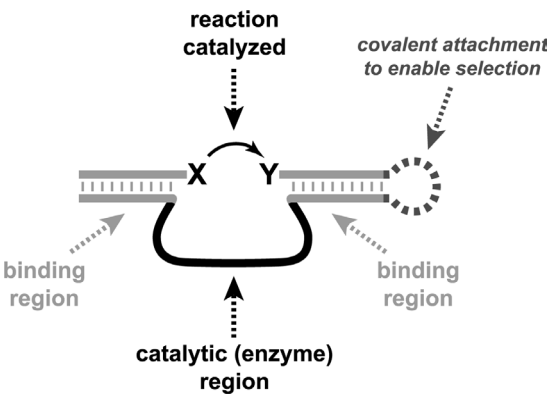


Figure 1 Schematic view of nucleic acid enzyme catalysis, showing separate binding and catalytic regions of the ribozyme or deoxyribozyme (lower strand). At the outset of selection, the binding regions are fixed in sequence, whereas the catalytic region comprises a random sequence. In this example, the two substrates (upper strands) are oligonucleotides, which interact with the binding regions by Watson–Crick base pairs; X and Y are the two functional groups that react with one another (solid arrow). Substrates may be small molecules rather than oligonucleotides, and some nucleic acid enzymes break rather than form bonds in the substrate. During most selection procedures, one substrate is attached covalently to the enzyme strand (e.g., via dashed loop at right), which enables selection to occur by linking genotype (sequence) to phenotype (catalysis). However, for practical application such attachment may not be required, particularly when the substrates are oligonucleotides.

bonds. Although it is impossible to prove that any particular reaction *cannot* be catalyzed by RNA or DNA, and only a small number of reaction types have been investigated to date, the available data do not suggest any inherent limitations on the scope of nucleic acid catalysis. More experiments are needed to probe the limits of catalysis by RNA and DNA.

General Considerations for *In Vitro* Selection Procedures

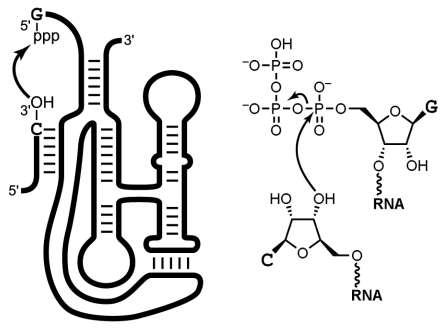
The identification of new artificial ribozymes is impossible without carefully designed *in vitro* selection methodologies. Although the details and even the fundamental elements of the selection procedure can differ for each ribozyme, some general considerations are common. An early approach to ribozyme selection was to identify RNA sequences that bind to a transition-state analog, as has been done for catalytic antibodies. Although such approaches do work in certain cases (45, 48, 51), in other cases the approach was unsuccessful (53), and it was suggested that selecting directly for catalysis is superior (8). Indeed, most ribozyme selections now aim directly for selecting catalytic RNA sequences using the desired substrates rather than transition-state analogs.

Sequence space and length of the random region

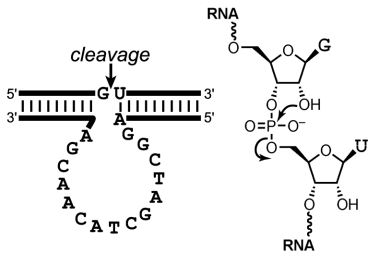
A ribozyme selection experiment almost always begins with solid-phase synthesis of a long DNA oligonucleotide that has two types of sequence elements: constant and random (**Fig. 1**). The constant regions either serve as primer binding sites for a polymerase chain reaction (PCR) step or—after conversion to RNA—provide binding sites for nucleic acid substrates. The random region constitutes the nucleotides that, as RNA, will compose the catalytic portions of any functional ribozymes that emerge from the selection process. For some but certainly not all ribozymes, RNA nucleotides from the constant regions can also contribute to catalysis.

One critical consideration for any selection effort is the nucleotide length of the random region. This length directly determines the possible number of nucleotide sequences—i.e., the size of the "random pool"—in a mathematically straightforward way. Because N is the common designation for a random nucleotide (versus A, G, U, and C for the four standard RNA nucleotides), the random region is usually denoted as, for example, N_{70} for a 70-nucleotide region. For a statistically random N_{70} region, there are $4^{70} \approx 10^{42}$ possible nucleotide sequences. Successful ribozyme selections have used random regions that range in length up to N_{228} (**Table 1**), for which sequence space has the unimaginably large value of $4^{228} \approx 10^{137}$. The number of random-pool molecules actually used to initiate selection is limited by technical considerations such as a manageable PCR volume and is on the order of 10^{13}–10^{16}, which corresponds to 0.01–10 nmol. Therefore, for all but the shortest random regions ($<N_{25}$), sequence space is vastly undersampled. For a relatively small 40-nucleotide random region and starting with 10^{14} molecules, only 10^{-10} of the 10^{24}-molecule sequence space is represented; for a typical N_{70} pool, only 10^{-28} of the 10^{42}-molecule sequence space is covered. Despite such sparse sampling, many selection experiments are quite successful, which implies that catalytically active RNA sequences are relatively common in sequence space, at least for the investigated catalytic activities. Such a conclusion has been reached on the basis of experimental data numerous times (8, 23).

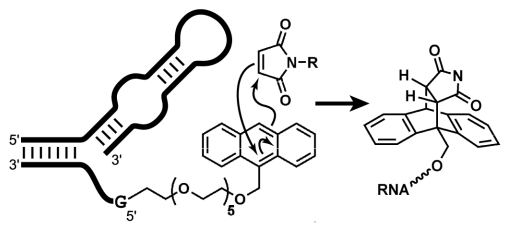
It can be difficult to choose the proper random-region length. This choice requires a compromise between coverage of sequence space (always favoring small random regions) and achieving the structural complexity necessary to support catalysis (probably but not necessarily favoring large random regions; note that excess sequence elements can inhibit catalysis). In selections for identifying aptamers (ligand-binding RNAs), the optimal random-pool length was found to reflect a balance between these factors (54). In ribozyme selections, the structural complexity of the catalytic motif was found to influence strongly the optimal pool size, with complex motifs particularly benefiting from long random pools (55). Of course, before undertaking a new selection effort, one does not necessarily know the structural complexity of the ribozymes that will be identified. Therefore, in practice, the random-pool length is usually chosen based on experience and educated guesswork. To handle the uncertainty, parallel selection experiments that use more than one random-pool length may be performed. Several random pools of various lengths may also be allowed to compete directly with one another in the same tube (19, 56).



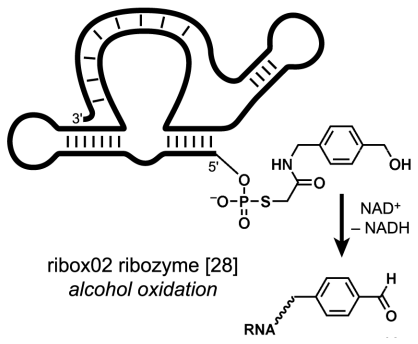
class I ligase ribozyme [8,9]
RNA ligation



10–23 deoxyribozyme [97]
RNA cleavage



Diels-Alder ribozyme [25,86]
Diels-Alder reaction



ribox02 ribozyme [28]
alcohol oxidation

Figure 2 Possible secondary structures and catalyzed reactions of four representative nucleic acid enzymes from **Tables 1** and **2**.

Tolerance of ribozymes to variation in sequence

Many artificial ribozymes do not seem to require specific nucleotides at every position within their initially random regions. This is similar to how many proteins tolerate amino acid changes at numerous sites but not at certain key residues. Due to the hierarchical nature of RNA folding, in which a complex tertiary structure forms on a foundation of Watson–Crick secondary structure elements such as stem-loop structures (57), a ribozyme can probably tolerate nucleotide changes at certain positions as long as Watson–Crick interactions are maintained (see Reference 58 for similar findings with aptamers). At other positions, the identities of the nucleotides may not matter at all. These considerations increase substantially the possibility that a particular ribozyme will emerge from a selection experiment, because many variants of a ribozyme that differ at noncritical nucleotides are essentially equivalent in terms of catalytic activity.

Overview of Experimental Selection Strategies

In general, a successful *in vitro* selection experiment must physically link the information in the catalytic nucleic acid sequence with the desired reaction chemistry, such that the successful sequences can be isolated. Therefore, an experimental strategy must be devised by which the majority of catalytically incompetent random-pool sequences are discarded, whereas the small minority of functional random-pool sequences are retained. Many strategies have been used, and many variations are possible even within the framework of a single general strategy. One possible selection approach is represented by the strategy used to identify the first RNA ligase ribozymes (**Fig. 3**) (8). As a means of illustrating a selection process, the key aspects of the procedure are discussed below, using the RNA ligase ribozyme selection as the specific example.

Preparation of the random-pool DNA

The random-pool DNA is prepared by solid-phase synthesis (SPS), with the random (N) nucleotides provided simply by mixing the four standard DNA nucleotide phosphoramidites together in one bottle. During SPS, random coupling to each growing oligonucleotide chain ensures that the collection of synthetic oligonucleotides has an effectively random region that encompasses all N positions. In practice, the phosphoramidites are mixed in non-1:1:1:1 mole ratio, because each phosphoramidite reacts during SPS with a different rate constant. After correcting for these known unequal coupling efficiencies, the random pool will have an approximately equal proportion of each standard DNA nucleotide at each position. Once the random-pool DNA has been synthesized, the random-pool RNA is made using this DNA as a template for *in vitro* transcription using T7 RNA polymerase (59).

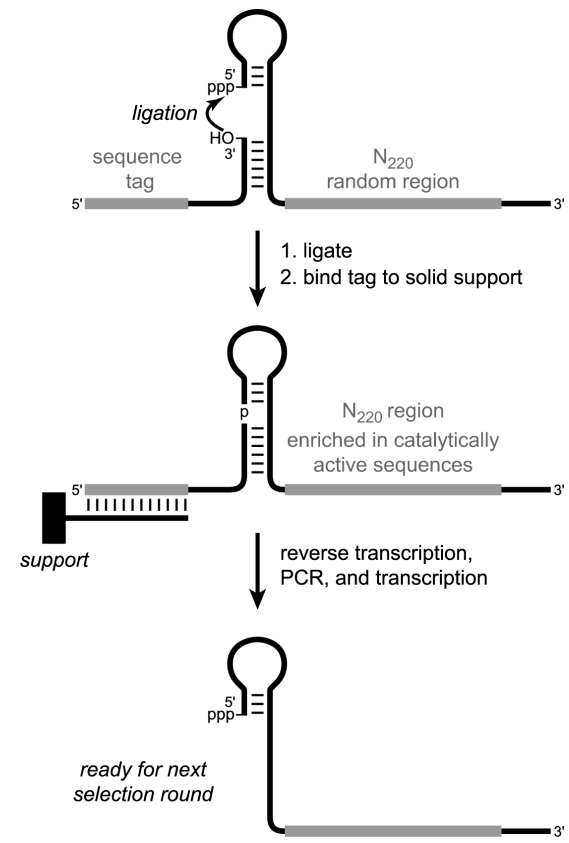


Figure 3 The key selection step of the strategy for identifying the first RNA ligase ribozymes (8). Catalytically active ribozymes join a substrate oligonucleotide to themselves. In this example, acquisition of this substrate ''sequence tag'' and subsequent binding to an oligonucleotide immobilized on solid support is the physical basis of selection, although many other approaches are possible (Tables 1 and 2). After the key selection step, reverse transcription, PCR, and transcription are used to prepare the pool RNA (now enriched in catalytically active sequences) for the next selection round.

Performing a selection round

The key facet of most *in vitro* selection procedures is to arrange the random and constant regions such that if a particular ribozyme candidate is capable of the desired catalysis, the oligonucleotide strand becomes chemically modified in such a way that leads to facile separation from the many other sequences that are catalytically inactive. For the original RNA ligase ribozymes (8), this was accomplished by using a substrate oligonucleotide with a 5′ sequence tag (**Fig. 3**). Because the second substrate oligonucleotide was covalently linked with the random region, the information in the random region became joined with the 5′ tag if (and only if) the random region encompassed a catalytically active ribozyme. The 5′ sequence tag then served as a primer binding site for PCR amplification of the catalytically active sequences after reverse transcription. Along with sequence tag acquisition as the physical basis of selection, other efforts have used the gain or loss of a biotin moiety, the gain of an amino acid moiety, the gain of a thiol group, and band shift on polyacrylamide gel electrophoresis (PAGE); see **Table 1** for annotations regarding the selection methodologies. In many cases, more than one method was used, either in parallel (i.e., more than one method within the same round) or in series (one method in some rounds; another method in other rounds).

Also important are the incubation conditions during the selection step in which the desired chemical reaction is performed. There are many experimental variables, including but not limited to pH, temperature, buffer identity and concentration, organic cosolvent (if any), and metal ion identities and concentrations, in addition to the duration of incubation. For the ribozymes that ligate RNA (8), the selections were performed primarily at pH 7.4, 600 mM KCl, and 60 mM $MgCl_2$ at 25 °C for 16 h, at least for the initial selection rounds. Due to the large number of independent variables, selection experiments often take advantage of parallel processing: Multiple selection experiments that each use a specific set of incubation conditions are performed. By comparing the outcomes of several selections performed in parallel where the only difference is in the incubation conditions, one may gain information on which aspects of these conditions are essential to achieving the desired catalytic activity.

Once the catalytically active nucleic acid sequences have been separated by an appropriate physical method, the selection round must be completed by synthesizing the DNA pool for input into the next selection round, now enriched in those sequences that (as ribozymes) are competent for catalysis. For the RNA ligase ribozymes, this was achieved by reverse transcription and PCR amplification followed by transcription (**Fig. 3**). Similar steps are common to most ribozyme selection procedures.

The overall selection cycle is iterated multiple times until the activity of the pool is sufficiently high that identifying individual sequences within the pool is warranted. The necessary number of rounds can vary greatly but is typically between 5 and 15 (only rarely greater than 20). The need for multiple selection rounds may initially seem mysterious—how come catalytically active sequences do not emerge after just one selection round? The reason is that most of the astronomically large number of possible random-pool sequences are not truly catalytically active, yet they may accidentally survive a particular selection round merely by chance. Only by requiring *reproducible* catalysis over multiple selection rounds can active ribozyme sequences dominate the selection pool.

A hazard of any selection experiment is that certain nucleic acid sequences may be able to survive even though they do not actually catalyze the desired chemical transformation. There is no general solution to this problem, other than to take great care in designing the selection procedure to avoid giving such opportunistic sequences a means of survival. Occasionally "negative selection" pressure can be applied, in which sequences that catalyze an undesired reaction are intentionally discarded before the remaining sequences are offered the opportunity to catalyze the desired reaction.

Testing the catalytic activities of individual ribozymes

Once the activity of the pool has reached an acceptable level after multiple rounds, individual ribozymes are cloned (but not yet

sequenced) and tested for catalytic activity. Like many facets of selection, the decision on when to clone has no firm rules. Many selections have had successful outcomes even when low catalytic activities of the uncloned pools (e.g., just a few percent) were achieved. It is important to note that "activity of the pool" is distinct from "yield of a particular ribozyme," because the uncloned pool encompasses many ribozymes that may have vastly different catalytic rates and yields. An uncloned pool may comprise ribozymes that each has modest catalytic activity. Alternatively, an uncloned pool may have ribozymes with widely varying activities—a limiting case would be some ribozymes that are highly active and others that are nearly inactive, averaging to a modest level of activity for the whole pool. Only by cloning and testing the activities of individual ribozymes can these possibilities be distinguished. Following the testing of activities, successful (e.g., highly active) ribozymes are sequenced and their activities verified after independent synthesis. Comparison of several ribozymes often allows identification of a "consensus" sequence.

Ribozyme optimization

After individual catalytically active ribozyme sequences have been identified, a "minimal" ribozyme is often devised by systematically deleting portions of the sequence. Usually this is guided by computer folding algorithms such as mfold (60) that can predict RNA secondary structure with good reliability, particularly for small RNAs. Empirically, it is often found that certain regions of an initially identified ribozyme (such as a large single-stranded loop) can be shortened or even eliminated without damaging catalysis. This is advantageous because smaller ribozymes are easier to synthesize and less likely to suffer nonspecific degradation, and they are also easier to study mechanistically because fewer nucleotides must have their chemical roles explored. However, as shown with the natural hammerhead ribozyme (61), one must not be overzealous in removing nucleotides that may be catalytically critical. A newer approach to determining a minimal ribozyme motif is to use nonhomologous random recombination in tandem with selection (62). This method is particularly advantageous because it is not biased by preconceived notions of the structural motifs that appear within a ribozyme.

Because all sequence space cannot possibly be covered for random regions of approximately N_{25} or larger, initial ribozyme sequences are often not optimal catalysts—in most cases, one or more nucleotide changes within the enzyme region would make them even better. Therefore, systematic ways to examine such variants would be helpful, and two methods are commonly used. First, "mutagenic PCR" (also called "error-prone PCR") can introduce additional variation (63). Whereas DNA polymerases such as Taq polymerase have nonzero error rates, these error rates are not generally large enough to explore a substantial amount of sequence space other than what is already present within the initial pool. However, suitable PCR conditions (typically changing the concentrations of divalent metal ions Mg^{2+} and Mn^{2+}) can intentionally elevate the polymerase error rate considerably, thereby introducing substantial variation during the DNA amplification step. An important consideration is the types of mutations that are made; some conditions favor certain nucleotide changes, whereas other conditions provide more randomness. Instead of altering the polymerase error rate, nonstandard nucleotide triphosphate analogs may be used to promote random mutations during PCR (64).

Second, a selection effort can be restarted from the beginning using a partially randomized sequence pool. In this "reselection" approach, a new pool is prepared by SPS on the basis of a known ribozyme sequence and with an enzyme region that is partially randomized, meaning that a markedly unequal mixture of the four nucleotide phosphoramidites is used for each N position. A typical reselection experiment might use a partially randomized pool in which all enzyme region nucleotides have the appropriate nucleotide of the parent ribozyme with 70% probability, or one of the other three nucleotides with 10% probability each. As for consideration of initial random-pool length, the extent to which each nucleotide position is randomized is based on both experience and guesswork. As a guide, one can compute the distribution of nucleotide changes relative to the "parent" sequence for any set values of randomization probabilities (65, 66), thereby knowing how many nucleotides (on average) will be changed relative to the parent sequence.

A final, practical consideration for many ribozymes is to convert them from intramolecular (*cis*-acting) to intermolecular (*trans*-acting) catalysts, which allow the possibility of multiple turnover. For ribozymes that catalyze reactions of nucleic acid substrates, this is often achieved by omitting a covalent phosphodiester linkage between the ribozyme and one of its oligonucleotide substrates during selection (**Fig. 1**). For ribozymes that catalyze other reactions, omitting a covalent tether is sometimes, but not always, successful and must be attempted on a case-by-case basis.

Continuous evolution as an alternative method

One principal drawback of many conventional *in vitro* selection procedures is their tediousness, particularly when individual selection rounds are time-consuming. This also limits the number of selection rounds that may be performed. To obviate such issues, investigators have performed "continuous evolution" experiments. In continuous evolution, samples of replicating molecules are diluted serially (e.g., $\sim 10^3$-fold dilution, often with >100 serial transfers) and at constant temperature (isothermal amplification), rather than selected in discrete rounds and with temperature cycling that is characteristic of PCR. The history of continuous evolution goes back to Spiegelman's work in the 1960s on Qβ replicase, in which self-replication by minimal nucleic acid sequences was sought (67). More recently, Wright and Joyce broadened the approach of continuous evolution to encompass evolution of catalytic function (68). In one experiment, a continous evolution approach was used to evolve an RNA ligase ribozyme that is "resistant" to the activity of an RNA-cleaving deoxyribozyme (69). The application of continuous evolution to ribozymes has advantages in terms of both speed and amplification power, but there are disadvantages in terms of susceptibility to contamination and limitations on the types of reactions that may be catalyzed (70).

Multiple turnover using *in vitro* compartmentalization (IVC)

An inherent limitation of most selection approaches is the absolute requirement for a covalent linkage between the catalytic nucleic acid and its substrate during selection. Although in many cases the emergent ribozymes can be converted into intermolecular (*trans*-acting) forms, during the selection process each candidate ribozyme is restricted to two possible chemical yields, 100% or 0%; i.e., each molecule has either performed or not performed the desired chemical reaction. Consequently, selection for multiple turnover is not possible. (In some cases, artificial ribozymes are capable of multiple turnover anyways, but this must be considered as fortuitous.) To alleviate this difficulty, candidate ribozymes have been encapsulated along with unattached substrates within individual droplets in a water-in-oil emulsion. This process is termed *in vitro* compartmentalization (IVC) (71, 72). If the system is arranged such that each droplet contains just one type of ribozyme candidate, then the encapsulation serves the same conceptual purpose as a covalent bond in terms of linking information ("genotype"; the ribozyme sequence) to catalytic ability ("phenotype"). An IVC strategy has been applied to develop several ribozymes with multiple turnover ability (73, 74).

Fundamental Insights Into Nucleic Acid Catalysis from Artificial Ribozymes

A primary motivation for studying artificial nucleic acid enzymes is to gain insight into natural nucleic acid catalysts. These insights include implications for prebiotic chemistry and the RNA World hypothesis, as well as a fundamental mechanistic understanding of nucleic acid enzymes.

Implications for prebiotic chemistry and the RNA world

The RNA World hypothesis posits that before the advent of proteins, there was a period of prebiotic evolutionary history in which RNA both carried information and performed catalysis (75) (see also the WECB review on the Origins of Life: Emergence of the RNA World). Although it is probably impossible to reconstruct a complete RNA World in the modern laboratory, exploring the capabilities of artificial ribozymes can provide information and constraints. For example, the identification of a ribozyme for processive template-directed RNA polymerization (21) provides evidence that relatively small RNA molecules (although they do have hundreds of nucleotides) are capable of catalysis that would have been important in a prebiotic RNA-based era. Many studies have focused on similar considerations (e.g., see References 76 and 77).

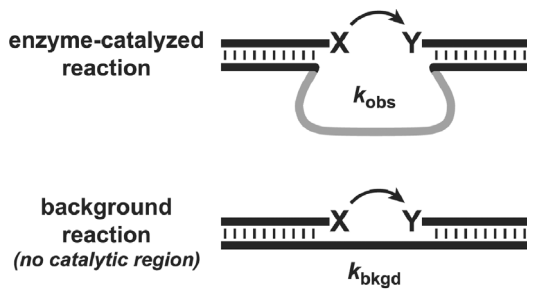


Figure 4 One approach to assessing the rate enhancement of a nucleic acid enzyme by comparing the rate constant of the enzyme with the rate constant for a "splint" oligonucleotide that lacks the catalytic region.

Mechanisms of artificial ribozymes

Little is known about the mechanisms of most artificial ribozymes. The catalytic rate and other basic features of each new ribozyme are generally determined as part of its initial characterization. Detailed mechanistic analyses are infrequent, although on occasion the tools of physical organic chemistry [e.g., kinetic isotope effects (78)] have been applied. Artificial ribozymes are usually, but not always, selected in the presence of divalent metal ions such as Mg^{2+} or many others (**Table 1**). Not surprisingly, artificial ribozymes typically require a modest-to-high concentration of such metal ions for their function. The catalytic tasks of these metal ions are often unknown, and a major challenge is to separate the roles of metal ion participation in structure versus catalysis.

For each ribozyme, it is usually possible to identify an appropriate "background" reaction that has a relatively low rate. For an RNA ligase ribozyme, a relevant background reaction is the analogous ligation reaction when the functional groups are held together by a complementary splint that has no enzyme region whatsoever (**Fig. 4**). The rate enhancements determined for various ribozymes in comparison with suitable background reactions can be low ($\sim 10^2$), but they can also be as high as 10^{10} (**Table 1**). Although ribozyme rate enhancements are often modest relative to those of protein enzymes, the appropriate benchmarks are the natural ribozymes, and on this basis, artificial ribozymes compete well with their natural counterparts.

The substantial rate enhancements observed for artificial ribozymes imply that they do more than passively hold together their substrates. Therefore, ribozyme catalysis is more than an "effective molarity" phenomenon. Consistent with this, almost all ribozymes make just one product even when multiple products are possible. For example, although the initially reported RNA ligase ribozymes could potentially have made either 3′–5′ or 2′–5′ linear RNA linkages, each particular ribozyme was observed to make just one linkage (8). Depending on geometrical constraints, effective molarity alone could lead to a mixture of products, which is not observed. In most cases, it is not yet clear whether ribozymes generally work by preferential lowering of transition-state energies or by precise positioning of the reacting

moieties, i.e., orientation effects. Given that such issues are not yet settled for most of the natural ribozymes [such as the ribosome (79, 81)], mechanistic studies of all kinds of ribozymes will continue to be an active research field.

Structural biology of artificial ribozymes

Most (but not all) natural ribozymes have X-ray crystal structures available, and nuclear magnetic resonance (NMR) spectroscopy has also been extensively applied. Perhaps surprisingly, only two artificial ribozymes have been studied using these methods. First, the Pb^{2+}-dependent RNA-cleaving "leadzyme" has been examined by both X-ray crystallography (82, 83) and NMR spectroscopy (84, 85). Second, a ribozyme for the Diels–Alder reaction has been studied by X-ray crystallography (86), revealing a preformed hydrophobic substrate-binding pocket that is capable of enantioselective catalysis. The application of structural biology methods to understand artificial ribozyme function is a field ripe for increased activity.

Evolving new RNA catalytic activities starting from known ribozymes

One interesting issue that is relevant both for a fundamental appreciation of RNA catalysis and for practical applications is to understand the circumstances in which a known ribozyme activity may be evolved to provide a different type of catalysis. In one report, evolving a self-aminoacylating ribozyme into a self-phosphorylating ribozyme required a substantial number of mutations, such that the new ribozyme could adopt a distinct structure (14). This implies that escape from the parent ribozyme's fold is required for evolution of new activity. Another study found that one designed RNA sequence can adopt either of two different folds, each of which catalyzes a different reaction (87). In nature, bifunctional sequences presumably represented transitional structures for the evolution of one catalytic activity into another. However, most bifunctional sequences would probably not exist today, because they are likely suboptimal for both activities.

Ribozymes Made from Components Other than the Standard Four Nucleotides

The simplest way to select ribozymes is with only the four standard RNA nucleotides A, G, U, and C. However, RNA polymerases can incorporate many chemically modified nucleotides. Recognizing that the four standard nucleotides offer only a limited variety of functional groups, some researchers have pursued ribozymes that include nonstandard nucleotides [see **Table 1**, activities marked with "(*nonstd*)"]. These nonstandard nucleotides are intended to enhance the catalytic properties of the ribozymes. In contrast, for fundamental studies that probe the limits of ribozyme catalysis, ribozymes have been sought that incorporate only a subset of the four standard nucleotides.

Ribozymes with nucleotides other than A, G, U, and C

The first *in vitro* selection effort with nonstandard nucleotides identified RNA that catalyzes the Diels–Alder reaction (23, 24). In this work, a pyridine-modified uridine derivative replaced all uridines throughout the RNA. The pyridine moieties were presumed to assist catalysis by some combination of hydrogen bonding, hydrophobic and dipolar interactions, and metal coordination. Most of the new ribozymes were strictly Cu^{2+}-dependent, suggesting a role for Lewis acid catalysis. Other artificial ribozymes with nonstandard nucleotides (each of which has an unmodified Watson–Crick face that permits use with T7 RNA polymerase and reverse transcriptase) include an RNA ligase ribozyme with N^6-amine-modified adenosines (11); amide synthase ribozymes that incorporate 5-imadazolyl-U (34); and a urea synthase ribozyme that also incorporates 5-imadazolyl-U (35). One of the most intriguing artificial ribozyme activities is the ability to induce palladium nanoparticle crystallization using pyridine-modified uridine nucleotides (49). The various ribozymes create different crystal shapes such as hexagonal plates versus cubes, and they require the pyridine-bearing RNA nucleotides for their activities (50). Presumably the pyridines interact directly with metals during crystal formation. Such ribozymes present an intrinsic mechanistic challenge due to the hetereogeneous nature of their reaction.

Incorporating nonstandard RNA nucleotides into a selection effort can enhance the chemical (or biochemical) stability of the emergent ribozymes. For example, artificial variants of the natural hammerhead ribozyme are resistant to ribonucleases due to 2′-amino and 2′-fluoro modifications (88). Increased stability to natural RNA-cleaving enzymes is useful for certain practical applications, particularly *in vivo* (see below).

Ribozymes with fewer than four kinds of nucleotide: minimal informational systems

In the opposite of adding nonstandard functional groups to RNA's chemical repertoire, ribozymes having three or fewer kinds of nucleotide have been investigated. In one study, RNA ligase ribozymes were identified that exclude cytidine (C) from the enzyme region (89). When one such ribozyme was reselected with inclusion of C, the catalytic rate improved about 20-fold (90), providing a quantitative measure of the importance of variety in ribozyme components. Going to the extreme, an RNA ligase ribozyme was identified with only two different nucleotides: a binary informational system (91) comprising only uridine (U) and 2,6-diaminopurine (D) nucleotides (**Fig. 5**). This ribozyme was inefficient, with only about 8% ligation yield in 80 h at pH 9.0 and 23 °C ($k_{obs} \approx 0.05\,h^{-1}$). Nevertheless, its activity demonstrates that a dramatically minimized and biologically related informational system can encode catalysis. Such types of ribozymes could have been relevant in an RNA World, at a point in time when only one base pair had evolved.

Practical synthesis of ribozymes

For practical synthesis of a ribozyme, two options are generally available. The first method is SPS, which is readily available to nonexperts via several commercial sources and via some academic service facilities. When nonstandard RNA nucleotides are desired, SPS is generally the best approach. The second method is *in vitro* transcription from a DNA template (59), which in most cases is limited to the four standard RNA nucleotides. A new method (albeit one that requires organic synthesis of the components of a nonstandard base pair) allows enzymatic transcription of RNA with unnatural nucleotides (92).

In Vitro Selection of Deoxyribozymes

Many considerations that are important for ribozymes also apply to deoxyribozymes, which differ solely by the lack of a 2′-hydroxyl group at each nucleotide. Natural DNA is almost always found in double-stranded form, which is structurally uniform and does not support catalysis. In contrast, artificial deoxyribozymes are largely single-stranded, and their conformational flexibility allows catalytic activity. Because RNA already seems to have a paucity of functional groups relative to proteins, early speculation was that the lack of a 2′-hydroxyl would render DNA catalytically inferior to RNA (93), if not altogether incompetent. Nevertheless, experiments have shown that DNA is quantitatively as functional as RNA in terms of catalysis. This is particularly evident when comparisons are made between RNA and DNA enzymes that catalyze the same type of reaction. For example, deoxyribozymes that cleave RNA are at least as competent as analogous ribozymes, if not better (94). This similarity between RNA and DNA may relate to common classes of mechanisms. Evidence increasingly suggests that ribozymes often rely on acid–base catalysis involving the nucleobases (95), which are present in both RNA and DNA. Combining this observation on catalysis with the finding that both RNA and DNA aptamers are highly competent for binding to ligands (96), it is unsurprising that the lack of 2′-hydroxyl groups on DNA is still compatible with its catalytic activity.

Scope of deoxyribozyme catalytic activities

To date, many fewer types of reactions have been explored with deoxyribozymes than ribozymes. This reflects both the later discovery of DNA catalysis and possibly the greater fundamental appeal of RNA catalysis as more relevant to the RNA World hypothesis. Nevertheless, when they have been examined, deoxyribozymes have proven quite competent catalytically (**Table 2**) (97–122). This extends not only to the types of reactions that have been investigated but also to important characteristics such as rates. For example, the RNA-cleaving 10–23 deoxyribozyme can achieve a k_{cat} of $10\,min^{-1}$ (rivaling natural ribozymes) and a k_{cat}/K_m of $10^9\,M^{-1}\,min^{-1}$, which is higher than that for the protein enzyme ribonuclease A (97).

Other considerations for deoxyribozymes

The same experimental selection approaches that are used for ribozymes are generally applicable to deoxyribozymes. For deoxyribozymes, there is no need to transcribe a sequence pool from DNA to RNA and then reverse-transcribe the successful RNA back into DNA for amplification during each selection round. Instead, the DNA is both the information and the catalyst throughout the selection process. Mechanisms of deoxyribozymes have been probed even less often than for ribozymes; in general, much work remains to understand how DNA catalyzes reactions. Intriguingly, one RNA-cleaving deoxyribozyme uses ascorbate as a cofactor (116) and another uses histidine (123), indicating that nucleic acid enzymes can use cofactors to expand their limited functional group repertoire. Structural biology efforts have also been very modest for deoxyribozymes. In the sole published experiment, the 10–23 deoxyribozyme crystallized in a catalytically inactive 2:2 stoichiometry complex with its RNA substrate (124). Although this was an interesting four-way structure with implications for understanding Holliday junctions, it highlights the need for future efforts to understand the structure of catalytic DNA. Structural data on DNA aptamers show that they can form ligand-recognition motifs that resemble their RNA counterparts, even without 2′-hydroxyl groups (125).

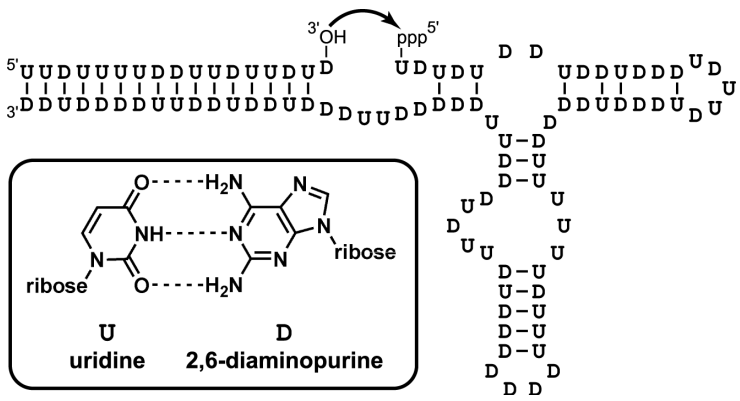


Figure 5 One possible secondary structure of an informationally minimal RNA ligase ribozyme that comprises just two types of nucleotide, uridine (U) and 2,6-diaminopurine (D) (91). The catalyzed reaction is formation of a 3′–5′ linkage by reaction of a 3′-hydroxyl group with a 5′-triphosphate. The inset depicts the structures of U and D along with their Watson–Crick base-pairing interaction.

As for ribozymes, several deoxyribozymes that incorporate nonstandard DNA nucleotides have been identified. For example, an RNA-cleaving deoxyribozyme with three imidazole functional groups has been obtained (100); other examples include RNA-cleaving deoxyribozymes that have both imidazole and amine nonstandard functionalities (101, 102). DNA can be completely modified by incorporation of solely nonstandard nucleotides during PCR (126). This suggests that future efforts should be able to expand considerably the use of chemically modified DNA for *in vitro* selection. A different purpose of nonstandard nucleotides is for regulation of catalytic activity. For example, appending an azobenzene moiety onto an RNA-cleaving deoxyribozyme allows the catalytic activity to be switched photochemically (127, 128).

An interesting conceptual question is whether a ribozyme and deoxyribozyme can be active with the same nucleotide sequence (with, of course, the U nucleobases of RNA replaced with T in DNA). In one study in which a deoxyribozyme with hemin-dependent peroxidase activity was identified, exchanging all DNA for RNA reduced but did not destroy the catalysis (129). More recently, a selection approach was used to convert a known ribozyme sequence into a deoxyribozyme (130). Curiously, this deoxyribozyme was nonfunctional when made as the corresponding RNA, whereas the initial ribozyme was inactive as DNA. The notion of nucleotide sequences that are catalytically active when made as either RNA or DNA is intriguing for its implications regarding "crossover" between two types of informational macromolecule.

For practical synthesis of deoxyribozymes (i.e., single-stranded DNA), SPS is the typical approach. If nonstandard nucleotides must be incorporated, SPS is generally the sole viable approach. In contrast, for DNA containing only the four standard nucleotides, conventional PCR may be used. If so, a main challenge is to separate the desired single-stranded DNA product from its complement. This may be achieved by using one primer with a nonamplifiable 5′-tail, such that the two product strands are of unequal length and therefore separable by PAGE (131). Asymmetric PCR that includes only one primer may instead be applied (132), although because DNA synthesis is linear and not exponential in this process, only a limited amount of single-stranded DNA can be generated. As another option, methods have been described for taking advantage of biotin–streptavidin technology to isolate single-stranded DNA from conventional PCR reactions (133, 134).

Terminology for Nucleic Acid Enzymes

An alternative definition of "nucleic acid enzyme" is a protein enzyme that modifies nucleic acids; e.g., T4 polynucleotide kinase (T4 PNK) for phosphorylation of RNA and DNA. Unfortunately, the term "nucleic acid enzyme" can therefore refer either to ribozymes and deoxyribozymes or to protein enzymes

Table 2 Tabulation of artificial deoxyribozymes

Reaction catalyzed	Bond	# Rand nt	Rate enh	M^{2+} req	Selection method	Ref
RNA cleavage	O–P	50	$\sim 10^5$	Pb^{2+}	biotin tag loss (beads)	(2)
RNA cleavage	O–P	40	$\sim 10^5$	Mg^{2+}	biotin tag loss (beads)	(98)
RNA cleavage	O–P	50	nd	Mg^{2+}	biotin tag loss (beads)	(97)
RNA cleavage	O–P	40	1×10^8	None	biotin tag loss (beads)	(99)
RNA cleavage (*nonstd*)	O–P	50	nd	Zn^{2+}	biotin tag loss (beads)	(100)
RNA cleavage (*nonstd*)	O–P	20	nd	None	biotin tag loss (beads)	(101)
RNA cleavage (*nonstd*)	O–P	50	$\sim 10^5$	None	biotin tag loss (beads)	(102)
RNA ligation (2′–5′)	O–P	40	300	Mg^{2+}	PAGE shift	(103)
RNA ligation (3′–5′ and other)	O–P	40	2×10^4	Zn^{2+}	PAGE shift	(104)
RNA ligation (3′–5′)	O–P	40	$\sim 10^4$	Mg^{2+}	PAGE shift	(105)
RNA ligation (3′–5′)	O–P	40	$\sim 10^5$	Zn^{2+}	PAGE shift	(105)
RNA ligation (branch formation)	O–P	40	5×10^6	Mn^{2+}	PAGE shift	(106, 107)
RNA ligation (branch formation)	O–P	40	$\sim 10^5$	Mg^{2+}	PAGE shift	(108, 109)
RNA ligation (lariat formation)	O–P	40	$\sim 10^5$	Mn^{2+}	PAGE shift	(110, 111)
DNA phosphorylation	O–P	70	$\sim 10^9$	Mn^{2+}	PAGE shift	(112)
DNA adenylation (capping)	O–P	70	2×10^{10}	$Mg^{2+} + Cu^{2+}$	PAGE shift	(113)
DNA ligation	O–P	116	3×10^3	Cu^{2+} or Zn^{2+}	biotin tag loss (beads)	(114)
DNA ligation	O–P	150	$\sim 10^5$	Mn^{2+}	PAGE shift	(115)
oxidative DNA cleavage	C–O	50	$\sim 10^6$	Cu^{2+}	biotin tag loss (beads)	(116, 117)
DNA deglycosylation	C–N	85	9×10^5	Ca^{2+}	PAGE shift	(118)
thymine dimer photoreversion	C–C	40	3×10^4	None	PAGE shift	(119)
phosphoramidate cleavage	N–P	72	$\sim 10^3$	Mg^{2+}	biotin tag loss (beads)	(120)
porphyrin metalation	Cu–N	228	1×10^3	Cu^{2+} or Zn^{2+}	binding to TS analog	(121, 122)

NOTES: See Table 1 legend for description of column headings.

that modify RNA and DNA. In practice, both definitions of nucleic acid enzyme are used by different groups of authors, and the intended meaning must be gleaned from context.

Occasionally, the term "RNA enzyme" is used as a synonym for ribozyme. In contrast, the contraction "RNAzyme" is rarely used, perhaps because "ribozyme" is approximately the same length and is just as simple to write. Similarly, "DNA enzyme" is often used as a synonym for deoxyribozyme. The contraction "DNAzyme" is used by some authors to replace the polysyllabic "deoxyribozyme." However, there is often confusion about the capitalization (i.e., DNAzyme, DNazyme, or Dnazyme), with only the first of these variants generally considered proper.

RNA and DNA molecules with catalytic activity are related to aptamers. Ribozymes and deoxyribozymes bind to their substrates and catalyze chemical reactions, whereas RNA and DNA aptamers simply bind to their ligands (135, 136). Aptamers are most commonly identified by a procedure originally termed "systematic evolution of ligands by exponential enrichment" or SELEX (135). SELEX is a form of *in vitro* selection, but not all *in vitro* selection is SELEX. In particular, SELEX is not generally used to identify nucleic acid enzymes—note the word *ligands* (not *catalysts*) in the full form of the acronym. Therefore, except when SELEX is performed on a transition-state analog, RNA and DNA catalysts are identified by "*in vitro* selection."

One relatively subtle but important distinction for identifying catalytic nucleic acids is the difference between screening and *in vitro* selection. In a screen, all candidates (such as small-molecule compounds from a combinatorial library) are surveyed individually for a desired property. This requires an efficient screening procedure, which for small-molecule combinatorial chemistry often involves multi-well plate experiments or miniaturized systems that allow parallel examination of thousands of spatially segregated samples. (See also the WECB reviews on Chemical Libraries: Screening for Biologically Active Small Molecules and on High Throughput Screening (HTS) Techniques: Applications in Chemical Biology.) However, statistical considerations for nucleic acid enzymes—e.g., 10^{15} sequences examined in a single experiment—obviate any realistic possibility of screening individual sequences one at a time. Instead, selection approaches must be used. The distinguishing feature of a nucleic acid enzyme selection is that a successful candidate sequence must survive through a stringent experimental step based on its catalytic ability. Even though most random-pool sequences have no catalytic activity, the sequences that are functional emerge due to their immense selective advantage, magnified over multiple selection rounds.

Finally, for *in vitro* experiments, the difference between "selection" and "evolution" is that evolution requires the introduction of variation *after* the start of the experiment. In many selection procedures, all variation is present within the random region of the initial pool at the outset of the experiment. Because such procedures merely serve to discard inactive sequences and retain active ones without introducing new variation, they are *in vitro* selection and not *in vitro* evolution. In contrast, when variation is introduced intentionally by mutagenic PCR or by restarting the experiment with a partially randomized pool, *in vitro* evolution is being performed.

Practical Applications of Nucleic Acid Enzymes

As the field of artificial nucleic acid enzymes continues to develop, attention has increasingly turned from conceptually oriented experiments to those with more immediate practical utility. Ribozymes and deoxyribozymes have been used for analytical, biochemical, biological, and chemical applications. In addition to several specific applications that are described below, ribozymes and deoxyribozymes have been used for many additional purposes in biochemistry, nanotechnology, and even molecular computation (94).

In vitro engineering of signaling ribozymes and deoxyribozymes as analytical sensors

Analyte detection is one of the primary research motivations in many disciplines. Numerous efforts have focused on nucleic acid enzymes as the basis for sensors. One of the most productive approaches combines *in vitro* selection with rational design to create allosteric nucleic acid enzymes. In such a case, catalytic activity is regulated by binding of a small-molecule ligand remote to the catalytic site in a modular fashion (**Fig. 6a**). Many artificial allosteric ribozymes have been identified, such as several hammerhead ribozyme variants (**Fig. 6b**) (137–141). Additional work has shown that nuanced behavior such as responsiveness to more than one ligand is possible (142), as is catalysis that is regulated by oligonucleotides (143–145). Laboratory exploration of allosteric ribozymes presaged the discovery of riboswitches, which are naturally occurring RNA regulators of gene expression that generally function by allosteric mechanisms (146). Allosteric deoxyribozymes may be even more practical than their ribozyme counterparts, due to the increased chemical and biochemical stability associated with DNA. A small number of deoxyribozymes have been reported that use various strategies to achieve allostery (147–150).

Many systems have been engineered specifically to optimize their practical signaling properties, often on the basis of regulated RNA cleavage activity. Lu et al. have developed deoxyribozyme sensors for metal ions and small organic molecules whose sensing ability is based on fluorescence or colorimetric signals (**Fig. 6c**) (151–154). Li et al. placed a fluorophore and a quencher close together on the substrate, thereby identifying new RNA-cleaving deoxyribozymes that synchronize fluorescence signaling with catalysis (**Fig. 6d**) (147) and collectively function at a wide range of pH values (155). These deoxyribozymes are advantageous because they were developed specifically to cleave the fluorophore-containing substrate, and therefore catalysis is optimal with the substrate that is directly relevant to sensing applications. Two research groups (156, 157) have placed ribozymes into arrays that offer the potential for investigating complex biochemical phenomena such as gene expression patterns.

Cleavage of RNA *in vitro* and *in vivo* by deoxyribozymes

RNA-cleaving deoxyribozymes have been particularly useful as *in vitro* laboratory reagents for RNA cleavage (94). Because such deoxyribozymes bind to their RNA substrates via extensive Watson–Crick base pairs (e.g., 10–23 deoxyribozyme in **Fig. 2**), selectivity for the substrate sequence is an inherent part of each interaction. With the development of a nearly complete collection of related RNA-cleaving deoxyribozymes (158), it is now possible to cleave almost any desired RNA target sequence with an appropriate DNA enzyme. In some experiments, the secondary structure within the RNA target can interfere with binding of the deoxyribozyme. Measures to improve the RNA–DNA interactions by chemical modification of the DNA can enhance the cleavage yield (159), as can inclusion of "disruptor" oligonucleotides that interfere with the target's secondary structure.

DNA is not susceptible to the cellular ribonucleases that quickly destroy RNA. Due to this stability and to other advantages in cost, toxicity, and potency, deoxyribozymes are particularly useful for *in vivo* mRNA cleavage (see summary in Reference 94), although chemically modified ribozymes may also be employed (160). Such approaches using nucleic acid enzymes are a valuable counterpart to other mRNA-targeting strategies, most notably the application of small interfering RNA (siRNA) or antisense oligonucleotides.

RNA ligation by deoxyribozymes: synthesis of linear and branched RNA

Several studies from the Silverman research group have identified deoxyribozymes that ligate two RNA substrates. Several deoxyribozymes join an RNA 3′-hydroxyl group with an RNA 5′-triphosphate, forming a native 3′–5′ linear RNA linkage (105). Synthesis of RNA incorporating internal chemical modifications often requires ligation of two or more fragments. Therefore, RNA ligase deoxyribozymes should be an important alternative to methods such as "splint ligation" using T4 DNA ligase (161), which often does not work well in particular systems. Other deoxyribozymes create 2′,5′-branched RNA or lariat RNA by mediating the reaction of an internal RNA 2′-hydroxyl group with a 5′-triphosphate (108, 111). In addition to the very high site-selectivity exhibited by these deoxyribozymes (which

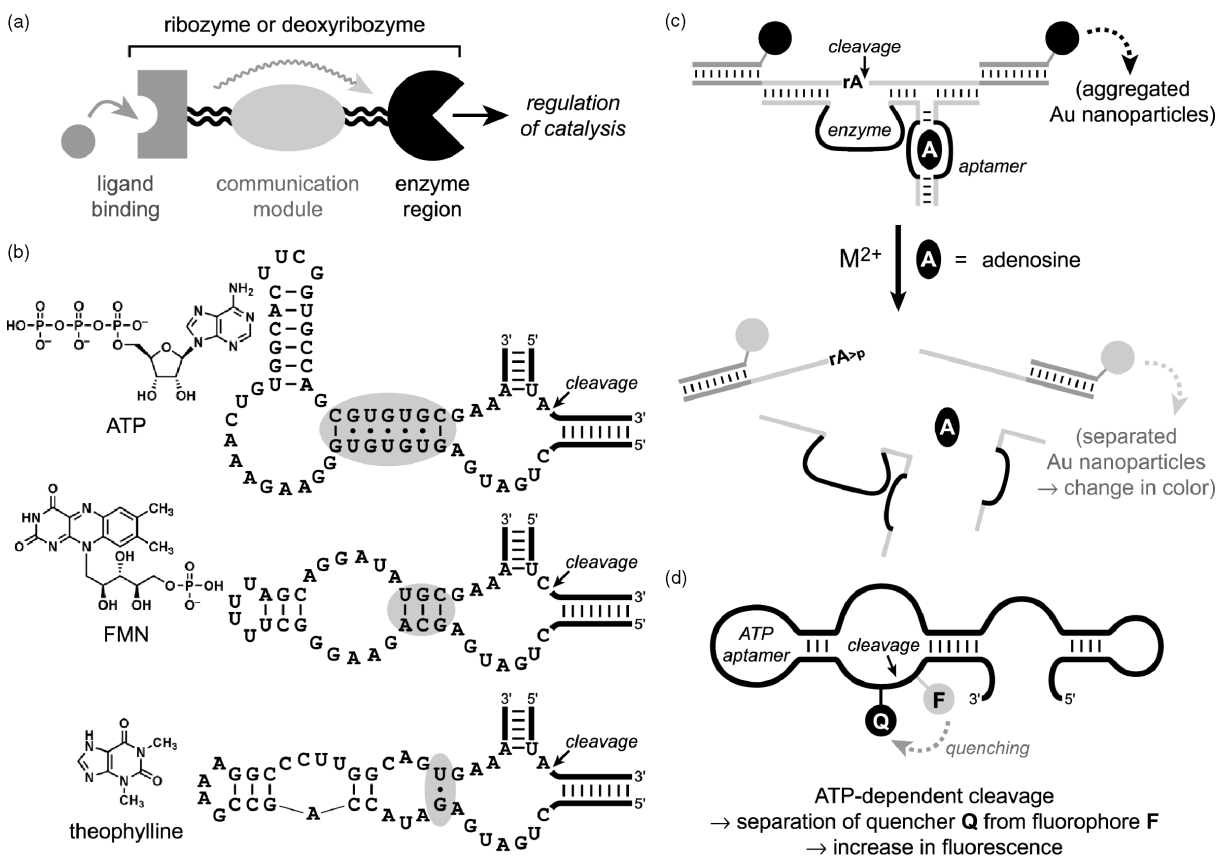


Figure 6 Artificial ribozymes and deoxyribozymes as sensors. (a) Schematic depiction of an allosterically regulated nucleic acid enzyme. (b) Examples of allosteric hammerhead ribozymes that cleave RNA in response to binding of the depicted ligands (137, 139); other examples are known (140, 141). The communication modules are highlighted in gray. (c) Example of a signaling ribozyme that is sensitive to adenosine binding with a colorimetric response due to gold nanoparticle dissocation (153). (d) Example of a signaling deoxyribozyme that synchronizes ligand (ATP) binding and catalysis with a fluorescence response (147).

is interesting in a fundamental sense), the branched RNA products can be applied to enable biochemical experiments that would otherwise be impossible (162).

Catalysis of bimolecular small-molecule reactions by ribozymes and deoxyribozymes

A particularly desirable yet challenging goal is to identify nucleic acid enzymes that mediate bimolecular reactions between two small molecules, i.e., when the substrates are not themselves nucleic acids. For this purpose, no substrate should be covalently attached to RNA or DNA. Very few nucleic acid enzymes have achieved this objective. Of course, when nucleic acid enzymes have been identified by selection for binding to a transition-state analog (**Tables 1** and **2**), the resulting catalysis requires no covalent attachment to the substrate. However, in most selections for small-molecule catalysis, one substrate is tethered covalently to the RNA or DNA. This tether can be very short (17, 20, 28, 30), or a long flexible tether such as PEG can be used to mimic a substrate free in solution (25, 34, 35, 73). In either case, most of the resulting nucleic acid enzymes are nonfunctional when the substrate lacks the tether. Indeed, for only two types of *in vitro*-selected nucleic acid enzymes has catalysis of small-molecule chemistry been achieved with no tethering whatsoever (in *trans*). First, an RNA capping ribozyme is active when the reacting nucleotide is not attached to the remainder of the ribozyme (17). Second, several Diels–Alder ribozymes (25, 26, 73) are active when the PEG tether that links the anthracene substrate to an oligonucleotide is absent. These limited examples are insufficient to draw general conclusions about what is necessary to achieve bimolecular catalysis of small-molecule reactions by nucleic acid enzymes.

Perspective on Artificial Ribozymes and Deoxyribozymes

As noted by Joyce, in the arena of nucleic acid enzymes, "you get what you select for ... and sometimes a whole lot more" (163). (Battle-hardened experimentalists might add, "... but if things don't work, a lot less.") Currently, the study of artificial ribozymes and deoxyribozymes is just under 20 years old and about to leave behind its teenage years. The original motivation to study nucleic acid enzymes was for the fundamental purpose of exploring the scope of nucleic acid catalysis. Artificial ribozymes and deoxyribozymes have already offered substantial insight into such catalysis, and we may expect additional success from ongoing research in this area. As more types of reactions well beyond nucleic acid cleavage and ligation are increasingly the goal of artificial nucleic acid enzymes, including efforts toward catalysis of small-molecule reactions, a practical and important question is to decide what are the most appropriate reactions to be targeted by RNA and DNA catalysis? Which reactions truly *need* nucleic acid catalysts, and how can nucleic acid enzymes be developed as practical chemical reagents? In contrast, what reactions are best performed by other catalysts such as protein enzymes or more traditional organic and organometallic catalysts? The responsibility falls to those of us working actively on selection experiments to develop and demonstrate the synthetic utility of ribozymes and deoxyribozymes for the broader chemical biology community. In parallel with these considerations, elegant experiments have already shown that practical applications such as analytical sensing can be accomplished in spectacular fashion using nucleic acid enzymes. Such techniques will surely be refined and extended in the near future.

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