



REVIEW

Thirty-five years of research into ribozymes and nucleic acid catalysis: where do we stand today? [version 1; referees: 2 approved]

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Abstract

Since the discovery of the first catalytic RNA in 1981, the field of ribozyme research has developed from the discovery of catalytic RNA motifs in nature and the elucidation of their structures and catalytic mechanisms, into a field of engineering and design towards application in diagnostics, molecular biology and medicine. Owing to the development of powerful protocols for selection of nucleic acid catalysts with a desired functionality from random libraries, the spectrum of nucleic acid supported reactions has greatly enlarged, and importantly, ribozymes have been accompanied by DNAzymes. Current areas of research are the engineering of allosteric ribozymes for artificial regulation of gene expression, the design of ribozymes and DNAzymes for medicinal and environmental diagnostics, and the demonstration of RNA world relevant ribozyme activities. In addition, new catalytic motifs or novel genomic locations of known motifs continue to be discovered in all branches of life by the help of high-throughput bioinformatic approaches. Understanding the biological role of the catalytic RNA motifs widely distributed in diverse genetic contexts belongs to the big challenges of future RNA research.



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Introduction

Nowadays, the term ‘ribozyme’ to designate an RNA catalyst is used with the same implicitness as the term ‘enzyme’ has always been used for proteinaceous biocatalysts. The fact that RNA can cleave and ligate itself, that cleavage of the 5′-trailer of tRNA in tRNA processing is mediated by the RNA subunit of RNase P, that introns may undergo self-splicing, and that the spliceosome and, even more impressively, the ribosome are actually ribozymes meanwhile has found entry into the textbooks. The exciting field of research into RNA catalysis started more than 30 years ago and over the first two decades was dominated by the discovery and identification of several classes of ribozymes occurring in nature and the elucidation of their catalytic structures and mechanisms. Apart from the ribosome that catalyses the formation of the peptide bond, all ribozymes discovered so far in nature support cleavage or ligation of a phosphodiester bond or both. However, the powerful method of SELEX (systematic evolution of ligands by exponential enrichment), originally developed for the selection of high-affinity RNA binders (aptamers) from a random library^{1,2}, was adapted to the selection of ribozymes (and, moreover, DNazymes) to catalyse a broad range of reactions, thus greatly enhancing the spectrum of nucleic acid catalysis^{3,4}. Over the years, an enormous amount of data were obtained on high-resolution structures and the mechanisms of ribozymes^{5,6}. All of this contributed to an understanding of ribozyme catalysis to an extent that has allowed engineering of ribozymes and DNazymes with pre-determined functionality. Thus, the past decade has seen impressive developments based on the usage of known catalytic motifs in ribozyme-based switches for therapeutic and environmental diagnostics⁷ and more recently for control of gene expression⁸. In parallel, the ability of RNA to catalyse a wide variety of chemical reactions has revitalised the RNA world hypothesis, a postulated period in the origin of life in which RNA was the main player, for one as carrier of genetic information and for the other as catalyst⁹. Early life may have started with self-replicating RNA, and a great deal of effort has been invested in developing ribozymes capable of self-replication¹⁰ or, even more challenging, of catalysing RNA polymerisation^{11,12}. The interest in RNA-world-relevant ribozyme activities continues, and one may well expect that there will be more to come.

In addition to ribozyme-based applications and RNA world scenarios as central topics of current research in the field, the frequency of ribozymes in nature and their function are of ongoing interest. With the help of high-throughput bioinformatic approaches, new ribozymes or novel genomic locations of known catalytic RNA motifs in highly diverse genetic contexts have been discovered in all branches of life^{13–16}, and current research addresses the question of their biological role.

The field of ribozyme research has changed the focus from discovery and mechanistic/structural characterisation of ribozymes towards functional engineering into application. Nevertheless, the excitement of the first days of ribozyme discovery has carried over throughout the years; the search for new ribozymes or just ribozyme locations continues in all kingdoms of life—in particular, in the human genome. Moreover, as mentioned above, the search for RNA-world-relevant ribozyme activities continues with unchanged curiosity. A number of excellent review articles have summarised the achievements in nucleic acid catalysis (for

recent examples, see 5,17–19). Here, we will concentrate on recent discoveries and developments in the field to draw a concise picture of ribozyme research and RNA and DNA catalysis 35 years after its beginning.

Ribozyme-based switches

Over the past decade, it has become increasingly clear that the conformational flexibility of RNA is an important determinant of cellular function. In this regard, riboswitches located in the 5′-untranslated region (5′-UTR) of specific mRNAs have gained much attention²⁰. Composed of an aptamer and an expression platform, riboswitches regulate, in a ligand-dependent manner, gene expression at the level of transcription or, alternatively, translation. Binding of a specific ligand to the aptamer induces a conformational change in the expression platform, turning gene expression ON or OFF. Interestingly, this principle of allosteric regulation was used in the test tube before it was discovered in nature²¹. By combination of ribozymes with aptamers, ribozyme activity was rendered ligand dependent and, consequently, adjustable. RNA or DNA aptamers for binding to a desired molecule can be produced by SELEX and linked to the ribozyme via a communication module, a sequence that translates the binding event occurring in the aptamer unit into an activity-associated conformational change within the ribozyme part. Thus, ribozyme activity can be used as a readout for a binding event, which in the case of a multiple turnover reaction would even lead to signal amplification. Owing to their modular composition of DNazyme or ribozyme and aptamer, such constructs were termed aptazymes²². Beyond the significance of aptazymes for medicinal diagnostics and therapy, RNA- and especially DNazyme-based biosensors have gained importance as tools in environmental monitoring, in particular to detect environmental pollutants, such as toxic heavy metals, air- and water-borne microbes, and other toxins²³.

Allosteric regulation of ribozyme activity has been used in a variety of contexts in the life sciences. Here, significant effort has been made in artificially modulating gene expression by a chemical signal. A ribozyme-based device positioned in the 5′- or 3′-UTR of a transcript and acting as a regulatory unit is partitioned between two functional conformations: one representing a ribozyme active state, the other an inactive state^{24,25}. Ligand binding would support one of the two states, dependent on the specific design. As a consequence of ligand binding, translation is switched ON or OFF (Figure 1). The advantage is that the effector molecule (ligand) binds directly to the regulatory module, without the involvement of proteins, such as transcription factors, which usually mediate genetic control. After some pioneering work in the early 2000s, important progress has been made in engineering ligand-dependent ribozyme modules that switch expression of suitable reporter genes, often using the hammerhead ribozyme to control stability of the target transcript²⁶. Furthermore, the genomic hepatitis delta virus ribozyme was engineered to control gene expression in mammalian cells and, when placed in tandem configuration, to construct a NOR logic gate device, demonstrating the modular composition of ribozyme-based RNA devices²⁷. Moreover, the recently discovered twister ribozyme, a highly flexible and active endonucleolytic ribozyme, has been used for the development of genetic switches²⁸. In all of these approaches, stability of a target transcript is modulated through conditional control of the

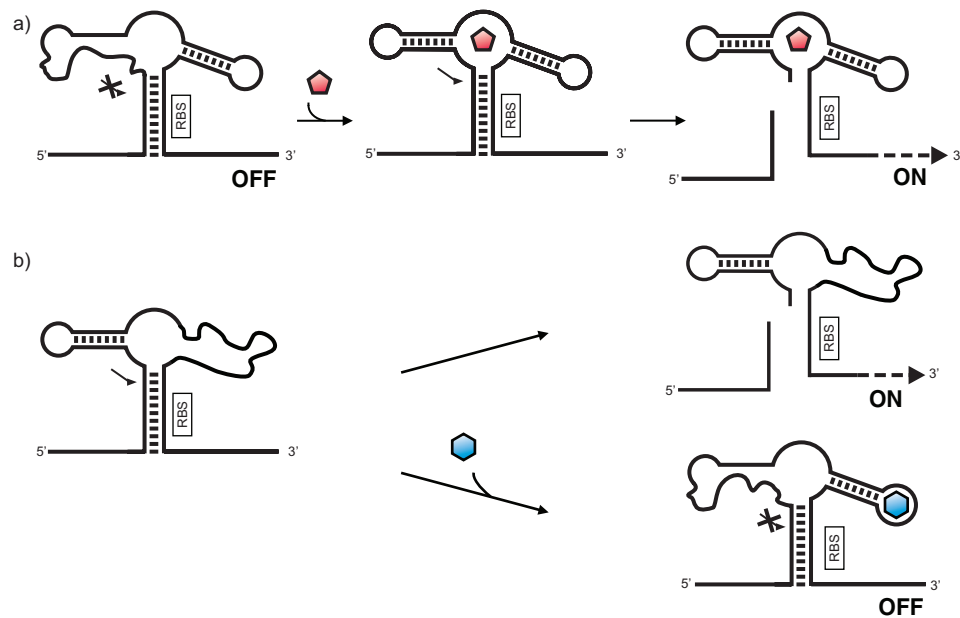


Figure 1. Ribozyme-based ON (a) and OFF (b) switches. The ribozyme-based device is positioned in the 5'-untranslated region (5'-UTR) of the transcript of interest. (a) In the absence of a specific ligand, the ribozyme is inactive and the ribosome-binding site (RBS) is sequestered in a double-stranded region; translation is switched OFF. Upon ligand binding, the ribozyme is activated and cleavage can take place. As a result, the RBS is set free and translation can proceed. (b) In the absence of a specific ligand, the ribozyme undergoes self-cleavage, thereby freeing the RBS and allowing translation to proceed. Binding of the ligand inhibits ribozyme activity, and translation is switched OFF.

cleavage activity of a ribozyme conjugated with a naturally occurring or *in vitro* selected aptamer domain and placed at a suitable position of the transcript. Ribozyme-based genetic control has been performed in different organisms^{27,29,30} and in response to diverse ligands^{26,30–32}. In addition to chemical signals (ligand-responsive switches), physical signals (light or temperature) can be used to control ribozyme activity in such devices³³. Beyond regulation of bacterial or mammalian genes, the potential of ribozyme-based genetic switches for regulation of DNA and RNA viruses has been demonstrated³⁴. In particular, genome replication, infectious particle production and cytotoxicity of adenoviruses, and (in the case of a measles virus) progeny infectivity and virus spread were reduced by aptazyme-mediated control of gene expression, paving the way for future applications in medicine and virology.

Important challenges in the engineering of ribozyme-based switches by modular composition are the link between ribozyme (actuator) and ligand-responsive aptamer (sensor) and the relatively slow kinetics of secondary structure changes induced by ligand binding, thus limiting the regulatory potential of ribozyme-based switches^{24,35}. Therefore, it is all the more important that powerful protocols for *in vivo* selection and screening and for high-throughput cellular RNA device engineering have been developed^{24–26,28,36}. In general ribozyme-based switches allow for the regulation of gene expression by up to 30-fold^{26,27}. However, it can be anticipated that, based on novel protocols for RNA device engineering and on the ever-growing understanding of the underlying structure-function relationships, novel designs will outperform those currently available.

DNAzymes

As mentioned above, protocols for *in vitro* selection of nucleic acid catalysts from random libraries have paved the way for the development of artificial RNAzymes and DNAzymes. One of the most proficient DNAzymes, the so-called 10–23 motif, was selected back in 1997³⁷ and was fully characterised in 1998³⁸ and since then has been used as a scaffold in a large number of re-selections and rational designs. In addition, novel DNAzymes were selected from fully randomised libraries. The chemical repertoire of DNAzymes is surprisingly broad, ranging from cleavage of phosphodiester, ester, and amide bonds over supporting C-C bond-forming reactions up to the repair of thymine dimers, peptide modifications, and others (excellently reviewed in 19). The recently achieved DNA-catalysed amide hydrolysis³⁹ is a good example of the challenges in DNAzyme development. Previous selection experiments had led to DNA-catalysed DNA phosphodiester cleavage instead of the desired amide hydrolysis⁴⁰, and, under conditions that deliberately avoided phosphodiester hydrolysis, no DNAzyme with activity for hydrolysis of an aliphatic amide bond was found. Instead, selection resulted in DNA catalysts that supported hydrolysis of carbonic acid esters or of aromatic amide bonds⁴¹. Only the inclusion, in the selection experiment, of nucleotide derivatives with attached protein-like functional groups allowed the identification of DNAzymes capable of aliphatic amide hydrolysis (Figure 2)³⁹.

There has also been some effort in elucidating the structure of DNA catalysts. A recent breakthrough is the crystal structure of an RNA-ligating deoxyribozyme at 2.8 Å resolution⁴². The structure gives new insight into the principles underlying DNA catalysis

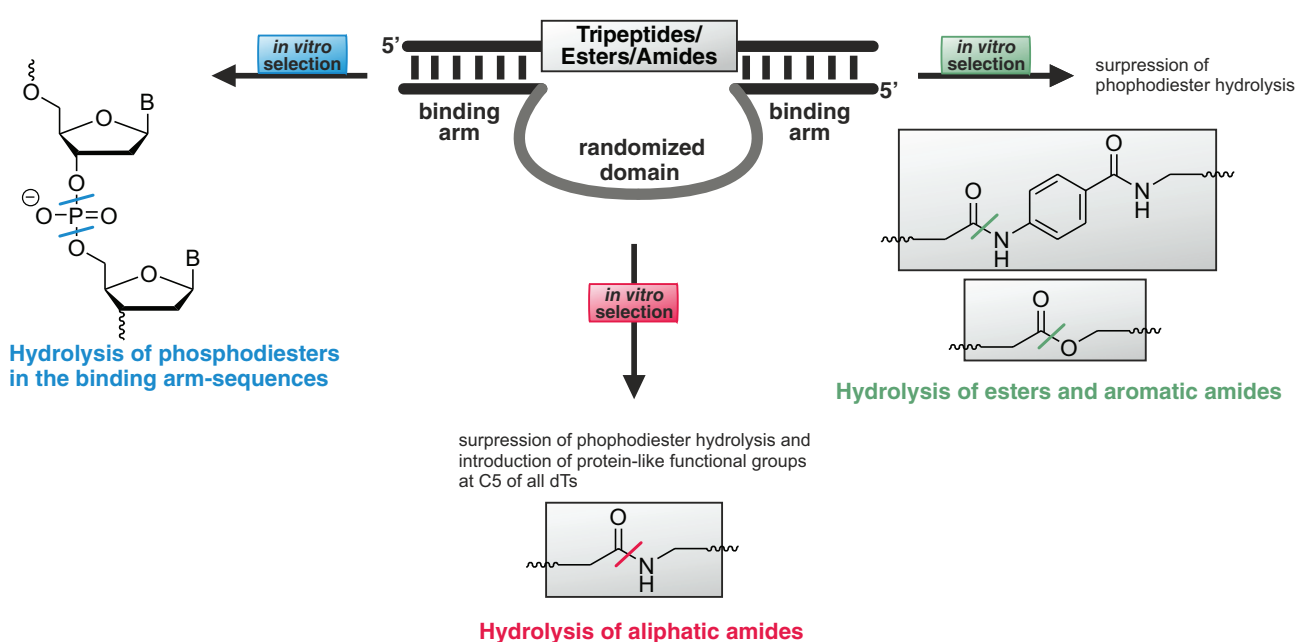


Figure 2. Selection of a DNAzyme from a random library under varying conditions. For more detail, see the 'DNAzymes' section of the main text.

and allows conclusions to be drawn on the similarities and differences between RNAzymes and DNAzymes. Notably, the structure revealed that DNA can explore a wide range of conformations owing to a less restrictive sugar puckering as compared with RNA, and this feature compensates for the lack of the 2'-OH group that is present and structurally important in RNA (Figure 3).

In general, DNAzymes continue to be developed as functional modules in biosensors and computing circuits^{23,43-47} as well as for therapeutic use⁴⁸. For example, recent progress was made in the development of variants of the 10-23 DNAzyme against hepatitis C virus⁴⁹ and for the treatment of basal cell carcinoma⁵⁰ as well as in DNAzyme-mediated modification of allergen-induced asthmatic responses⁵¹.

XNAzymes

Very recently, an exciting new class of nucleic acid catalysts has emerged. Artificial endonuclease and ligase enzymes composed of synthetic genetic polymers, xeno nucleic acids (XNAzymes), were selected from random libraries in a method termed 'cross-chemistry selective enrichment by exponential amplification' (X-SELEX)^{52,53}. As an essential prerequisite of the experiments, a modified DNA polymerase was engineered to tolerate the XNA building blocks (triphosphates) for polymerisation⁵⁴. Four different XNAs (Figure 3) were used in the selection: arabino nucleic acids (ANAs), 2'-fluoroarabino nucleic acids (FANAs), hexitol nucleic acids (HNAs), and cyclohexene nucleic acids (CeNAs), and for all of them catalytically active species were found after 10 to 20 rounds of selection⁵². Moreover, a FANA metalloenzyme with activity for ligation of FANA was identified, thus establishing catalysis in an entirely synthetic system⁵³. These results have strong implications

for the emergence of life on earth, underscoring the possibility that genetic polymers with backbones other than ribose may have pre-dated the emergence of RNA and the RNA world.

Ribozymes in RNA world scenarios

The discovery of ribozymes has led to a renaissance of the RNA world theory, and ever since much effort has been put into the identification of ribozymes with useful activities in a time period when life was based on RNA functioning as both genome and genome-encoded catalyst⁹. Thus, a number of *in vitro* selections aimed at the identification of RNA catalysts supporting reactions that might have been used by RNA world organisms were carried out. The synthesis of RNA certainly would have been a core activity, and ribozymes for reaction steps involved in RNA synthesis have been generated⁵⁵. Recent success has been made in ribozyme-mediated triphosphorylation of RNA-5'-hydroxyl groups using cyclic trimetaphosphate as the energy source^{56,57}, in ribozyme-mediated self-replication^{10,58}, and in polymerisation of activated nucleotides^{11,12,59}. In addition, other recently demonstrated activities, such as ribozyme-mediated RNA processing^{60,61}, recombination^{62,63}, nucleotide addition⁶⁴, and self-alkylation⁶⁵ (some of them illustrated in Figure 4), speak to the capacity of RNA to support a wide variety of reactions with relevance in RNA world scenarios.

New catalytic motifs

Until recently, 10 classes of ribozymes existing among contemporary organisms were known, the hammerhead and hairpin ribozyme probably being the most prominent examples. The years after the discovery of these ribozymes were filled with investigations into their structures and catalytic mechanisms, and it

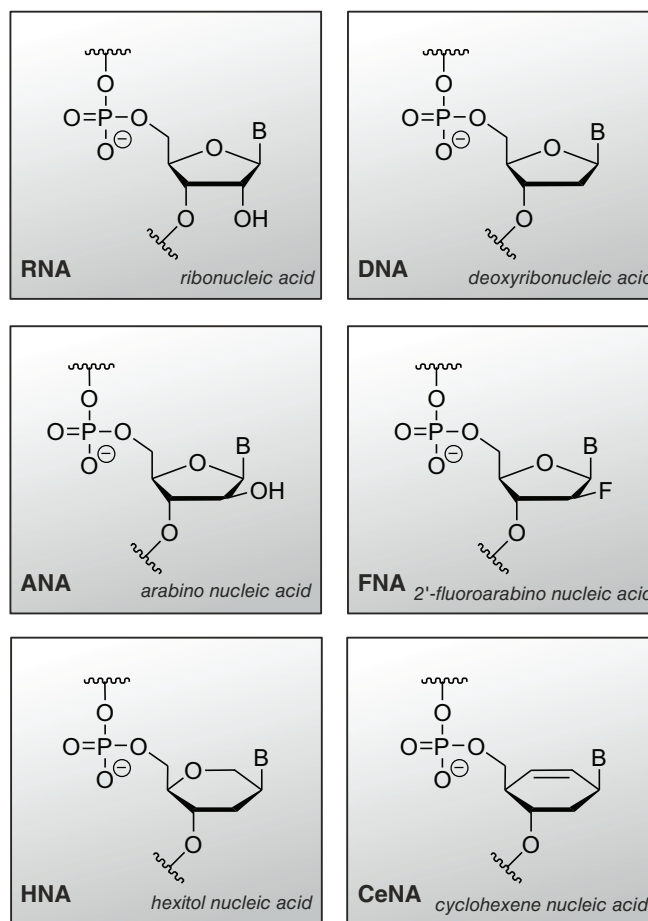


Figure 3. Structure of xeno nucleic acids in comparison with DNA and RNA. ANA, arabino nucleic acid; CeNA, cyclohexene nucleic acid; FANA, 2'-fluoroarabino nucleic acid; HNA, hexitol nucleic acid.

took a rather long time until the question for additional naturally occurring ribozymes was addressed. The first of the recently discovered self-cleaving RNAs constituting the eleventh class of ribozymes is a small catalytic RNA motif, present in many species of bacteria and eukaryotes¹⁵. In keeping with the tradition of giving ribozymes names related to their secondary structure, the new motif was called *twister* because of its small yet complex consensus structure composed of three stems conjoined by internal and terminal loops and a two-pseudoknot tertiary fold (Figure 5)⁶⁶⁻⁶⁸. With an *in vivo* cleavage rate of 1000 per minute, the *twister* ribozyme is one of the fastest self-cleaving ribozymes, and based on biochemical experiments in conjunction with molecular dynamics simulation, a mechanism involving general acid-base catalysis by a conserved active site adenine residue has been proposed⁶⁹. This is in general agreement with the mechanisms of other self-cleaving ribozymes like the hairpin or the hepatitis delta virus ribozyme, which also

require an adenine residue in the active site⁵. However, apparently there is a striking difference: whereas in the hairpin and hepatitis delta virus ribozyme, N1 of adenine is involved in catalysis, N3 of adenine was suggested as a strong candidate to act as general base in *twister* ribozyme-mediated self-cleavage⁶⁹. This is particularly interesting because, if indeed N3 takes this role, it would expand the mechanistic repertoire of the small endonucleolytic ribozymes.

High-throughput bioinformatics assisted the identification of additional self-cleaving candidates named *twister sister*, *pistol*, and *hatchet* ribozyme⁷⁰, which upon *in vitro* characterisation were shown to indeed be ribozymes⁷⁰⁻⁷². All of these new ribozymes support a transesterification reaction yielding a 5'-hydroxyl group and a 2',3'-cyclic phosphate at the cleavage site. A recent review of the chemistry and biology of self-cleaving ribozymes referring also to the four new ribozyme classes can be found in 5.

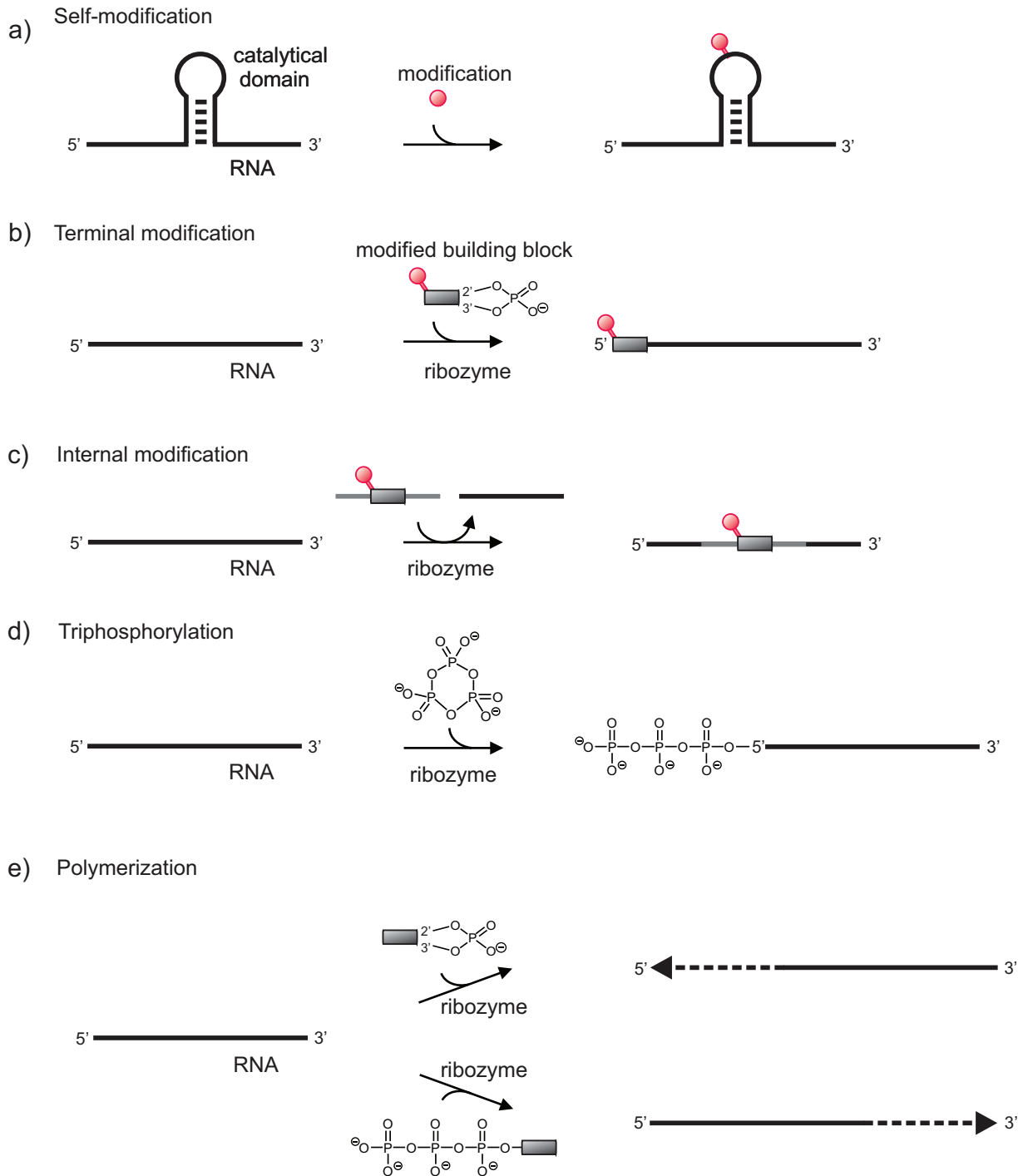


Figure 4. Schematic presentation of ribozyme activities that might have played a role in the RNA world. a) self-modification, e.g. alkylation; **b)** 5'-terminal modification by ribozyme-supported addition of an activated building block; **c)** internal modification by ribozyme-supported fragment exchange; **d)** ribozyme-supported 5'-triphosphorylation with trimetaphosphate; **e)** ribozyme-supported RNA polymerization with nucleoside-2',3'-cyclic phosphates (in 3'→5'-direction) or nucleoside-5'-triphosphates (in 5'→3'-direction) as activated building blocks.

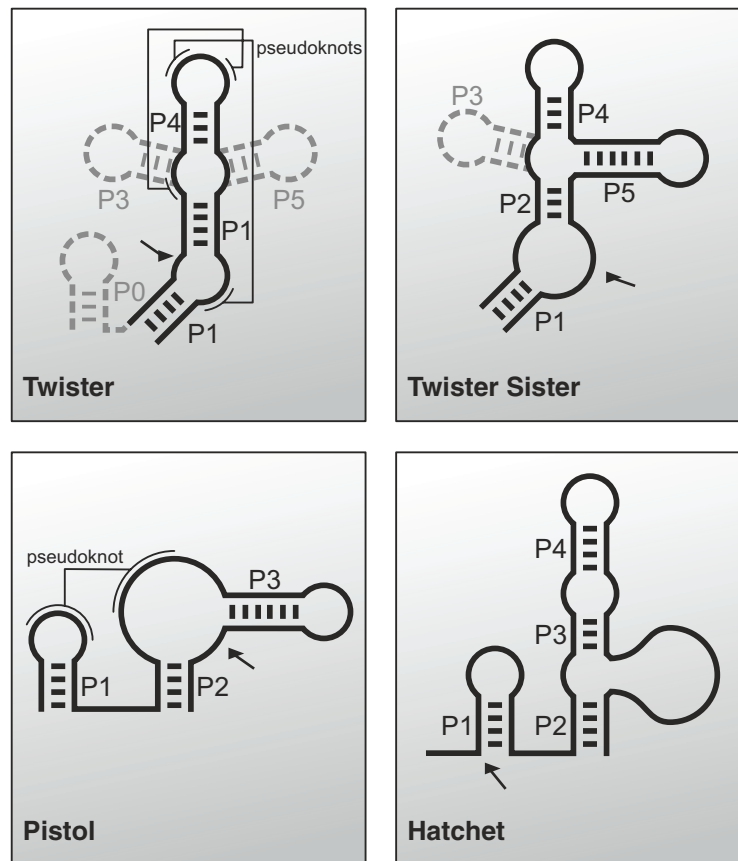


Figure 5. Secondary structures of recently discovered ribozymes. The arrows denote the cleavage sites.

Future prospects

Over the years, ribozyme research and nucleic acid catalysis have remained a very exciting field with unchanged potential for new discoveries. A strong focus of current research is the uncovering and understanding of the role that ribozymes play in biological systems. The first results on the influence of self-cleaving RNA structures on genetic control are just emerging. For example, the hammerhead, the hepatitis delta virus-like, and the twister ribozyme are widespread in nature and appear in rather diverse genetic contexts^{14,15,73}. Ribozymes have been identified in intronic regions and mobile genetic elements, suggesting a role in pre-RNA and transcript processing^{13,74,75}. Understanding this additional level of genetic control and regulation is one of the major challenges of current and future research in this area. The ongoing development of high-throughput bioinformatic approaches will further facilitate the identification of conserved structures and the evaluation of their genetic distribution. In addition to novel genetic locations of known ribozymes, new catalytic RNA motifs may be expected to be discovered, as shown recently for the twister, twister sister, pistol, and hatchet ribozymes^{15,70}. In the area of ribozyme engineering by rational design and *in vitro/in vivo* evolution, exciting results regarding new approaches for the artificial control of gene expression by allosteric ribozymes placed in

non-translated regions of transcripts may be anticipated. Also, the search for ribozymes with RNA world relevant activities can be expected to continue with unbroken excitement. In this regard, the catalytic repertoire of XNAzymes^{52,53} will certainly be further explored.

Thirty-five years after the discovery of the first catalytic RNA, ribozyme research has not lost the intriguing and highly motivating flair of the first days. There are still many questions to be addressed and much is waiting to be discovered.

Abbreviations

FANA, 2'-fluoroarabino nucleic acid; SELEX, Systematic evolution of ligands by exponential enrichment; UTR, untranslated region; XNA, xeno nucleic acid.

Competing interests

The authors declare that they have no competing interests.

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