# Catalysis Based on Nucleic Acid Structures

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Since the discovery that RNA molecules can possess catalytic activities, ribozymes have become a fascinating field both for academic researchers and the pharmaceutical industry. In this review, we emphasize the latest progress made in structure determination of ribozymes as well as the generation of DNA and RNA enzymes with novel catalytic properties by combinatorial approaches.

**Keywords:** Ribozymes, In vitro selection, Nucleic acid libraries, Metallo enzymes, Aptamers.

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## 1 Introduction

For a long time it was thought that among all known biopolymers, proteins are the only ones that are capable of catalyzing chemical transformations. In 1982/1983, however, it was discovered that naturally occurring RNA sequences, the group I introns [1] and the catalytic RNA subunit of RNase P [2], are able to catalyze the hydrolysis and formation of phosphodiester bonds. Since then, a large number of natural ribozymes have been described. Remarkably, it has recently become evident that the peptidyl transferase activity of the ribosomal protein/RNA-complex which is responsible for protein synthesis in living organisms is carried out by the RNA rather than the protein portion [3]. While this discovery has widened the scope of natural RNA catalysis from phosphodiester chemistry to the formation of peptide bonds, another dimension was introduced into nucleic acid catalysis by applying the techniques of in vitro selection of combinatorial nucleic acid libraries to isolate non-natural ribozymes, deoxyribozymes, or chemically modified nucleic acids with novel catalytic properties. These techniques have led to nucleic acids that catalyze a broad range of chemical transformations, ranging from cleavage of carboxylic ester- [4] or amide bonds [5] to C–C-bond-forming reactions such as the Diels-Alder reaction [6] or the catalysis of $\sigma$-bond-rotation involved in isomerization reactions [7]. While these examples of nucleic acid catalysis have an enormous implication for the support of theories about the origin of life on our planet, there are also several impressive examples that show that ribozyme catalysis opens up the possibility for completely novel therapeutic approaches [8–14]. Furthermore, the combination of specific ligand-binding nucleic acid sequences, the so-called aptamers, with catalytic RNAs have allowed to use ribozyme catalysis in a controlled and designed fashion [15–18]. In this review we will summarize the state-of-the-art of nucleic acid catalysis.

## 2 Natural Ribozymes

Since the discovery of the first ribozyme, the self-splicing intron of the ribosomal large subunit RNA of the ciliate *Tetrahymena thermophila* [19], far more than 100 other sequences that belong to the family of group I introns have been identified. To these can be added a considerable number of group II introns [20, 21], which act by a different RNA-cleavage mechanism, a large variety of virus- or viroid-derived ribozymes, namely the hammerhead- [22], the hepatitis delta virus- [23], the hairpin-ribozyme [24, 25], the M1-RNA subunit of RNase P which is responsible for tRNA 5′-end maturation in prokaryotes [26], and the neurospora mitochondrial VS RNA [27]. All these ribozymes have been the subject of a number of excellent reviews [28] giving us the opportunity in this chapter to focus on the more recent developments.

## 2.1
## Structure and Mechanism of Some Natural Ribozymes

In mid-1997 an international conference took place in Santa Cruz, USA, in which, for the first time, the exclusive topic was structural aspects of RNA molecules. A report covering this meeting contains an impressive graphic which shows the RNA structures, RNA/DNA complexes, and RNA/protein complexes contained in the brookhaven database as a function of the year of their publication [29]. Between 1988 and 1993 there were just 20. However, in 1996 alone no less than 41 structures appeared. These new dimensions were headed by the crystal structural elucidation of the first larger RNA molecule since the first crystal structure of tRNA in 1973 [30], the 48 nucleotide long hammerhead ribozyme (HHR) [31–33]. This landmark achievement was followed by a crystal structure analysis of the P4-P6-domain of a group I intron [34–36] and, more recently, a crystal structure of the hepatitis delta virus ribozyme [37].

All these studies established that the folding of RNA molecules is organized in a hierarchical manner. The assembly of complex RNA structures occurs in discrete transitions which build upon the folding of sub-systems that can then self-organize to even bigger and more complex structures [38–40].

### 2.1.1
### *The Hammerhead Ribozyme*

In 1994 and 1995, two crystal structures of hammerhead ribozymes [31, 32] and a structural analysis based on fluorescence resonance energy transfer studies [41] were published. In case of the crystal structure analyses, both ribozyme variants contained certain modifications that had been introduced to avoid self-cleavage [31, 32]. In one case a DNA-analog of the substrate oligonucleotide was used [31], in the other case the all-RNA substrate contained a 2′-*O*-$CH_3$ modification at the attacking 2′-OH group to avoid cleavage in the crystal [32]; for reviews see [8, 42, 43].

While these structures gave important insights into the HHR-structure, the question remained to what extent the folding of a catalytically active HHR would differ from these structures (Fig. 1). A third crystal structure, this time using an unmodified HHR which was crystallized in the absence of any divalent metal ions, turned out to be quite conclusive [33]. After addition of $Mg^{2+}$ the ability of the HHR for self-cleavage in the crystal was restored. In this active conformation the ribozyme folded in the same way as did the 2′-O-$CH_3$-modified HHR. When the $Mg^{2+}$ was added at low pH the ribozyme could be obtained in a metal-bound uncleaved form. This structure was compared with a conformational intermediate which contained a second $Mg^{2+}$-ion bound at the cleavage site and which could be analyzed at an active pH in its uncleaved form after shock-freezing. The most notable differences between this and the inactive form were located at the active site. These structures allowed the identification of five metal binding centers. They were also used to predict binding pockets for metal ions in any RNA molecule by measuring the distribution of electronegativity potentials [44]. Further insight into the cleavage mechanism could be obtained

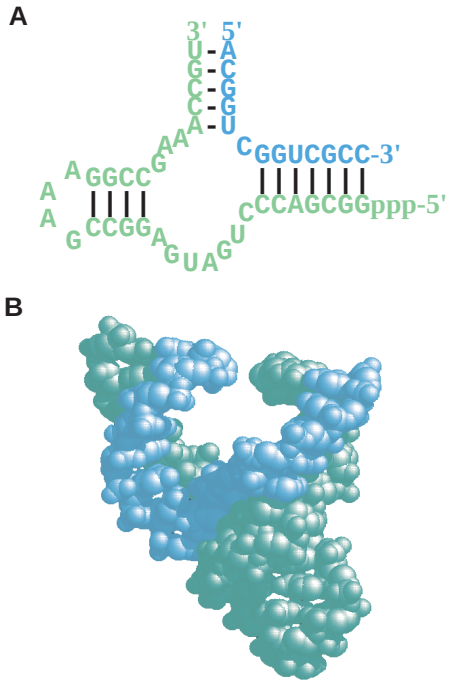



**Fig. 1.** The Hammerhead ribozyme. **A** Sequence and secondary structure. **B** Three dimensional structure of the HHR according to Scott et al. [33]. The substrate oligonucleotide (*blue*) is hybridized to the catalytic part (*cyan*)

by molecular dynamics simulations on the basis of the structure of the active ribozyme [45]. This analysis established that the two $Mg^{2+}$-ions in proximity to the cleavage site are bridged by an $OH^-$-ion which activates the attacking 2′-OH group after the ribose at the phosphodiester group to be cleaved from C3′-*endo* into the C2′-*endo* conformation. Taira and his colleagues also significantly contributed to the understanding of the cleavage mechanism by the HHR. They provided evidence that the departure of the 5′-oxygen is the rate-limiting step in HHR-cleavage [46].

### 2.1.2 *The Group I Introns*

A landmark achievement in RNA structure determination was the solution of the crystal structure of the 160 nucleotide long P4-P6 domain of the *Tetrahymena* group I intron [19, 34, 35, 39]. The P4-P6-domain folds into a compact structure with a sharp turn that is stabilized by tight packing of the helices. This newly discovered structural element was designated as the “ribose-zipper” because of the hydrogen bonds between ribose residues of the helices that participate in the structure. In addition, stabilization of RNA folds in P4-P6 occurs mainly via

tertiary interactions of the eleven base long 'tetraloop receptor' with certain extra-stable GAAA hairpin loops that also contribute to the stability of the motif. In most natural RNAs, interactions of that sort play an important role in the stabilization of RNA tertiary structures [47], e.g. in the group II intron [48–50].

The crystal structure of the P4-P6 domain (Fig. 2) disclosed another novel structural motif [51], the "adenosine platform" [52]. This motif is unusual because it contains two consecutive adenosine residues arranged in the same plain. Adenosine-platforms appear three times in the crystal structure and are part of

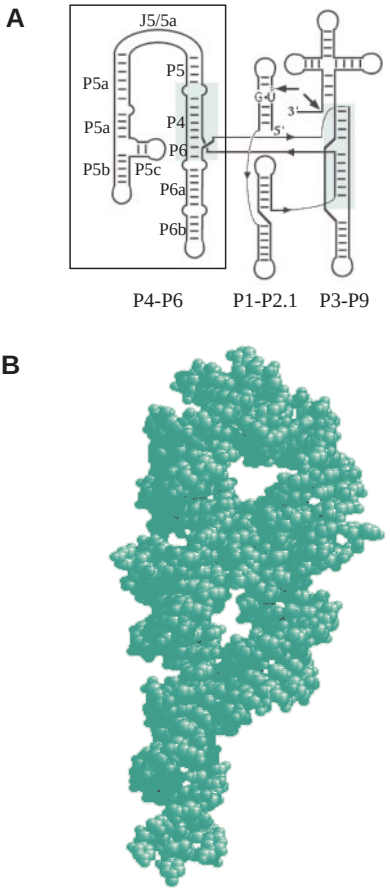



**Fig. 2.** The P4-P6-domain of the group I intron of *Tetrahymena thermophila*. **A** Schematic representation of the secondary structure of the whole self-cleaving intron (modified after Cate et al. [34]). The labels for the paired regions P4 to P6 are indicated. The grey shaded region indicate the phylogenetically conserved catalytic core. The portion of the ribozyme that was crystallized is framed. **B** Three dimensional structure of the P4-P6 domain. Helices of the P5abc extension are packed against helices of the conserved core due to a bend of approximately 150° at one end of the molecule

the recognition motif for the GAAA-tatraloop. The metal-binding within this ribozyme fragment was investigated as well [35, 36]. It appeared that the P4-P6-domain contained a core of five $Mg^{2+}$-ions and it was suggested that this might be the RNA-version of a hydrophobic core which is similar to analogous elements in proteins.

### 2.1.3
### *The Hepatitis Delta Virus Ribozyme*

The hepatitis delta virus (HDV) ribozyme is part of the circular single stranded RNA genome of the hepatitis delta virus which consists of a total of 1700 nucleotides. The HDV ribozyme is required for the processing of multimers of the genomic linear RNA transcripts to unit length by catalyzing a transesterification reaction that results in self cleavage [23].

A 72-nucleotide self-cleaved version of that ribozyme was the third large catalytic RNA from which a crystal structure was determined recently (Fig. 3) [37]. The RNA was crystallized together with protein U1 A, a subunit of the U1 small nuclear ribonucleoprotein particle of the eukaryotic splicing machinery [53], to which it binds and which facilitated crystallization without affecting its activity. The structure determination revealed that the ribozyme folds into a nested double "pseudoknot" structure. Pseudoknots are widespread structural motifs in many functionally different RNA molecules that are defined by the Watson-Crick base-pairing of a single stranded loop region with a complementary sequence outside this loop [40, 54].

The double pseudoknot fold enables the HDV ribozyme to form a deep cleft protecting the active site from the solvent. This had been suggested previously by Rosenstein and Been based on biochemical data [55]. The X-ray structure established that buried deeply in this cleft lies the 5′-hydroxyl leaving group that results from the self-cleavage reaction. This 5′-OH group is surrounded by a number of important functional groups required for activation of the attacking 2′-hydroxyl group and for the transition state stabilization by neutralization of the negative charges that develop during the cleavage process in the leaving group.

### 2.1.4
### *The Hairpin Ribozyme*

Another naturally occurring ribozyme which catalyzes phosphodiester transfer reactions is the hairpin ribozyme. The hairpin ribozyme has been the subject of a number of excellent review articles [24, 25]. Several independent studies performed recently have indicated that the hairpin ribozyme has an interesting feature which distinguishes it from the aforementioned ribozymes mechanistically: While the HHR, the group I intron, the HDV ribozyme and many other ribozymes that we are going to meet in this review are metalloenzymes and require divalent metal ions in their active sites for functional group activation, divalent metals ions only play a passive role (they are mainly required for cor-

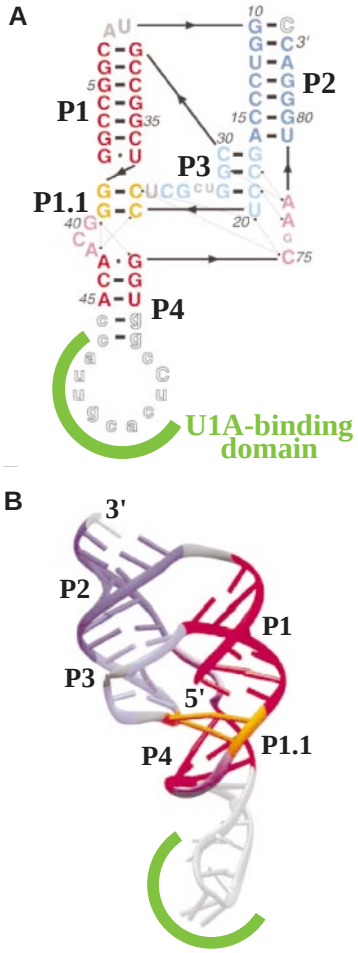



**Fig. 3.** The hepatitis delta virus ribozyme. **A** Secondary structure of the genomic HDV ribozyme RNA used for the determination of the crystal structure [37]. The color code is reflected in the three dimensional structure **B** of this ribozyme. P1 to P4 indicate the base-paired regions. Nucleotides in small letters indicate the U1 A binding site that was engineered into the ribozyme without affecting the overall tertiary structure. The yellow region indicates close contacts between the RNA and the U1 A protein

rect folding of the nucleic acids) in the cleavage reaction catalyzed by the hairpin ribozyme (Fig. 4) [56–58].

This is also true for a number of in vitro selected DNA enzymes which were selected under divalent metal-free buffer conditions [59, 60]. These results contradict the common assumption that all ribozymes are metalloenzymes and provide a number of ribozymes for which it will be very interesting to determine their exact catalytic mechanisms at high resolution.

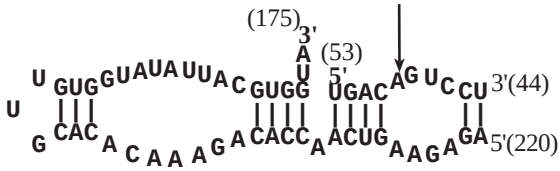



**Fig. 4.** The self-cleaving "hairpin" motif from the satellite RNA of tobacco ringspot virus (sTobRV). The *arrow* indicates the cleavage site. The numbers in brackets indicate the nucleotide positions within the sTobRV satellite RNA

### 2.2
### Ribosomal RNA as a Catalyst

In 1993, Noller and his colleagues published a frequently cited study in which it was suggested that the 23 S ribosomal RNA might be capable of performing the peptidyl transferase activity of the ribosome without any ribosomal proteins [61]. They had removed most of the ribosomal proteins from the RNA portion by extensive phenol extraction and found that the remaining nucleic acid-containing aqueous phase retained peptidyl transferase activity. As it could not be excluded that this activity might have been due to some residual traces of protein that could not be removed, there was no final proof that peptidyl transfer is an RNA-catalyzed process [62]. This was achieved recently by testing six individually synthesized domains of the 23 S ribosomal RNA [63, 64]. These fragments, when complexed together, were shown to be capable of performing the peptidyl transfer reaction. Moreover, the authors were able to demonstrate that the catalytic activity is largely depended on the presence of domain V, which lies in the heart of the peptidyl transferase center of the 23 S rRNA. This reconstitution experiment suggested that fragments of an RNA molecule have the ability to associate into a functional complex and that it is indeed the RNA portion in 23 S rRNA that is responsible for peptide bond formation in the ribosome. As we will discuss below, additional support for this notion comes from a study by Zhang and Cech who recently succeeded in the in vitro selection of a ribozyme that catalyzes a reaction analogous to the 23 S ribosomal peptidyl transferase activity [65, 66].

## 3
## In Vitro Selection of Catalytic Nucleic Acids

The discovery of catalytic RNAs has encouraged hypotheses about the origins of life on earth (see below) and has raised questions whether or not RNA is capable of catalyzing a much broader range of chemical reactions than those suggested by the naturally occurring ribozyme activities. One extremely powerful tool to answer these questions has been provided in the form of the in vitro selection technology. By in vitro selection, a number of novel ribozymes were selected which impressively enlarge the spectrum of chemical transformations catalyzed

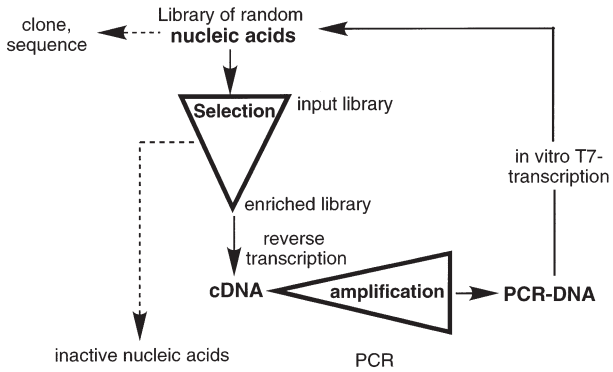



**Fig. 5.** Selection scheme for the in vitro selection of RNA libraries. The RNA library is subjected to a selection criterion suitable for the enrichment of functionally active sequences. The few selected individual sequences are amplified by reverse transcription (RT) and polymerase chain reaction (PCR). The PCR-DNA is then subjected to in vitro transcription with T7 RNA polymerase. The resulting enriched and amplified RNA library can be used as the input for the next selection cycle. This process is repeated until active sequences dominate the library. At this point, individual sequences can be obtained by cloning and their sequence can be determined by sequencing

by RNA or single-stranded DNA molecules. Several excellent review articles summarize them [67–75].

In vitro selection is a combinatorial approach in which functional molecules are selected from large libraries of randomized RNAs or DNAs by selection techniques that are suitable for the enrichment of a particular property such as the binding to a target molecule or a particular catalytic activity (Fig. 5).

The starting pool is generated by standard DNA-oligonucleotide synthesis by which up to $10^{15}$ different DNA molecules can be synthesized at once. The design of pool oligonucleotides involves a completely random base-sequence which is flanked by defined primer binding sites to allow amplification by the polymerase chain reaction (PCR). The synthetic DNA can be transcribed into RNA in vitro because it contains an appropriate promoter, usually the promoter for the RNA polymerase from the phage T7. The selections are performed on the assumption that some molecules in the original pool must have the right receptor structure to bind a substrate or have the correct folding to perform catalysis of a particular chemical reaction. These rare sequences are separated from the vast majority of non-functional molecules and are amplified by the polymerase chain reaction. Since a complete enrichment of functional molecules cannot be achieved in a single step several iterative cycles of selection and amplification are required.

### 3.1
### In Vitro Selection of Catalytic RNA

The most recent progress in this field of catalytic RNA, modified RNA, or ssDNA-selections include modified RNAs that catalyze a Diels-Alder reaction

[6] or amide bond formation [76, 77], RNAs that catalyze the formation of peptide bonds similar to the reaction catalyzed by the ribosome [65, 66], RNA and DNA sequences with various ligase activities [18, 78–83], a ribozyme for the formation of 5′-5′-diphosphate-bonds [84, 85], ribozymes for ester transfer reactions [76, 86], a ribozyme that catalyzes the formation of a glycosidic bond between uracil and phosphoribosyl pyrophosphate [87], and many more.

### 3.1.1 *Indirect Selections*

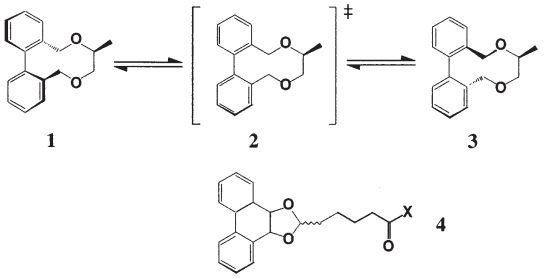
In vitro selection strategies can be sub-divided into two types: direct and indirect selections. These two types of selection experiments directed at the isolation of synthetic catalytic nucleic acids differ mainly by their technical concept, their design and their outcome.

The idea of indirect selections goes back to John Haldane [88], Linus Pauling [89], and Bill Jencks [90]. According to them, every compound that is capable of stabilizing a transition state of a given chemical transformation should also be able to catalyze the reaction itself. Thus, if compounds that bind a transition state analog (TSA) can be isolated they can then be tested for their ability to catalyze the corresponding reaction. This principle has been applied successfully for the isolation of catalytic antibodies which can be obtained by immunizing animals with transition state analogs [91]. After a population of TSA-binding antibodies has been obtained, each antibody-clone is screened for catalytic activity in subsequent experiments.

While indirect selections work quite well for antibodies they have been less successful in the case of catalytic nucleic acids. There are only three examples which prove that it is possible in principle to obtain a ribo- or deoxyribozyme by selecting an aptamer that binds to a TSA: A rotamase ribozyme [7], a ribozyme capable of catalyzing the metallation of a porphyrin derivative [92], and one catalytic DNA of the same function [93]. Another study reported the selection of a population of RNA-aptamers which bind to a TSA for a Diels-Alder reaction but the subsequent screen for catalytic activity was negative for all individual RNAs tested [94]. The attempt to isolate a transesterase ribozyme using the indirect approach also failed [95].

Following the TSA-based strategy, RNA aptamers were selected that specifically complexed the TSA for the isomerization of an asymmetrically substituted biphenyl derivative (Scheme 1) [7]. The selection was performed by affinity chromatography of a randomized pool on the TSA immobilized on agarose. After seven rounds of selection, the RNA pool accelerated the basal reaction 100-fold and was completely inhibited by the planar TSA.

The second example of a catalytic RNA obtained by the indirect selection approach is the isolation of a 35 nucleotide RNA molecule which binds mesoporphyrin IX and catalyzes the insertion of $Cu^{2+}$ into the porphyrin with a value of $k_{cat}/K_M$ of 2100 $M^{-1}$ $s^{-1}$ [92]. Remarkably, the $k_{cat}/K_M$ achieved by the RNA was close to that of the $Fe^{2+}$-metallation of mesoporphyrin catalyzed by the protein enzyme ferrochelatase.




Scheme 1

The only indirect selection that led to a catalytic DNA is a deoxyribozyme that catalyzes the same class of porphyrin metallation as the aforementioned ribozyme. The ssDNA oligonucleotide showed a $k_{cat}$ of 13 $h^{-1}$ for the insertion of $Cu^{2+}$ into mesoporphyrin IX [93, 96–99]. This corresponds to a rate enhancement of 1400 compared to the uncatalyzed reaction which is as good as a catalytic antibody for the same reaction.

Recently, another example of a DNA-aptamer that was selected for binding to a small molecule and that was found to accelerate weakly a chemical transformation was reported [100]. These aptamers selected to bind to the fluorophor sulforhodamine B with high affinity were also capable of promoting the oxidation of a related molecule, dihydrotetramethyl rosamine, albeit with low efficiency.

### 3.1.2
### *Direct Selections*

The more successful strategy for the isolation of RNA- and DNA-based catalysts involves the direct screening of nucleic acids libraries for catalytic activity. This approach is called direct selection [6, 65, 77, 78, 86, 101–107]. In direct selections, nucleic acids that are capable of catalyzing a particular chemical transformation modify themselves with a tag or other characteristic that allows their preferential enrichment over those molecules which are catalytically inactive [108]. The design of ribozyme-selections involving reactions between two small substrates requires that one reactant be covalently attached to every individual member of the starting RNA pool. After the reaction with another substrate which usually carries the selection-tag has occurred, the self-modified RNA is immobilized on a solid support, separated from non-active molecules, and then cleaved off the support.

The principle of direct selections was first introduced by Gerald Joyce and his coworkers. From a library of mutagenized sequences of the *Tetrahymena* group I intron, a series of RNA variants were isolated which had evolved to cleave a non-natural DNA substrate corresponding to their normal RNA substrate oligonucleotides [109]. Since this pioneering work, a large number of in vitro selections with very different goals have been carried out. Thereby, not only variants of natural ribozymes with altered functionalities and substrate specificities were obtained [74, 110], but also ribo- and deoxyribozymes with completely

**Table 1.** Novel ribozymes and deoxyribozymes[a]

| Reaction | References |
|---|---|
| Phosphor transfer reactions | |
| 2′,5′- and 3′,5′-ligation | [78, 81] |
| Oligonucleotide phosphorylation | [102, 105] |
| Cleavage of DNA/RNA chimeric oligonucleotides | [59, 60, 111–114][c] |
| RNA cleavage | [104, 115], [116][c] |
| DNA ligation | [80][c] |
| 5′,5′-RNA ligation | [79] |
| DNA cleavage | [117, 118][c] |
| RNA polymerization | [83, 119] |
| RNA "capping" | [84, 85, 120, 121] |
| Ligation of AMP-activated RNA | [82] |
| Cofactor-dependent RNA cleavage | [113, 122][c] |
| Other reactions | |
| Isomerization (Sigma rotation) | [7] |
| *N*-alkylation | [103] |
| Aminoacylation | [76, 86, 106, 123, 124] |
| *S*-Alkylation | [107] |
| Porphyrin metallation | [92, 93, 98, 99] |
| Amide-bond formation | [76], [77][b] |
| Peptidyl transfer | [65, 66] |
| Diels-Alder reaction | [6][b] |
| Nucleotide/phosphoribosyl transfer | [87] |
| Redox activity | [100] |

[a] catalytic RNAs derived from natural ribozymes are not listed in this table.
[b] the library contained modified bases.
[c] deoxyribozyme.

novel catalytic properties [67–75]. Table 1 summarizes in vitro selected nucleic acid catalysts known to date.

#### *3.1.2.1*
#### *Ribozymes Catalyzing Reactions at Phosphordiester Bonds*

The first ribozymes evolved completely de novo was an RNA ligase [78] and a polynucleotide kinase [102, 105]. In analogy to the natural ribozymes, these RNAs catalyzed phosphodiester transfer reactions as well. For the ligase selection, a library of $>10^{15}$ different RNA sequences was screened for ribozymes which catalyze the formation of a phosphodiester bond between themselves and an external RNA oligonucleotide. The 5′-end of the library was designed as a hairpin loop motif, to be able to fold into close spatial proximity to the 3′-end of the substrate oligonucleotide. The concept was that only those sequences which catalyze the nucleophilic attack of a 2′- or 3′-hydroxy group on the adjacent 5′-triphosphate were able to transfer the substrate oligonucleotide onto themselves. Thus, active catalysts "labeled" themselves with the covalently attached oligonucleotide which enabled their separation from bulk unreacted pool RNA

simply by affinity chromatography on an immobilized sequence complementary to the substrate oligonucleotide. Indeed, after ten rounds of successive selection and amplification, active ligase ribozymes became enriched. The library that now consisted mostly of active sequences catalyzed the ligation of the short RNA oligonucleotide with a rate constant of 0.06 $min^{-1}$ which is about seven million times faster than the template directed background reaction. Cloning and sequencing revealed that several classes of ribozymes had been enriched which either catalyzed the formation of 2′,5′- or 3′,5′-phosphodiester bonds [81, 125]. Interestingly, it turned out that the selected ribozymes had evolved alternative binding sites for their substrate and did not make use of the pre-designed substrate binding site. One of the ligase ribozymes, designated as "the class I ligase", was extensively characterized and its secondary structure was elucidated [125]. Furthermore, a variant of this ribozyme was constructed which was able to perform the template directed extension of an external RNA primer by using mononucleotide triphosphates (Fig. 6) [119].

A modified version of the class I ligase which contained a different substrate hybridization site was also used for the development of a continuous RNA evolution system (Fig. 7) [83]. The hexameric substrate hybridization site of the original class I ligase, 5′-GACUGG-3′ had to be changed to 5′-UAUAGU-3′ in order to make it complementary to an oligonucleotide substrate which corresponded to the sequence of the T7-promoter. This switch in substrate specificity was necessary because the continuous evolution scheme was designed to evolve ribozymes which were capable of ligating a T7-promoter sequence onto their 5′-end with very high efficiency in the presence of reverse transcriptase, the 3′-primer, dNTPs, NTPs, and T7-RNA polymerase. In this way, a competing

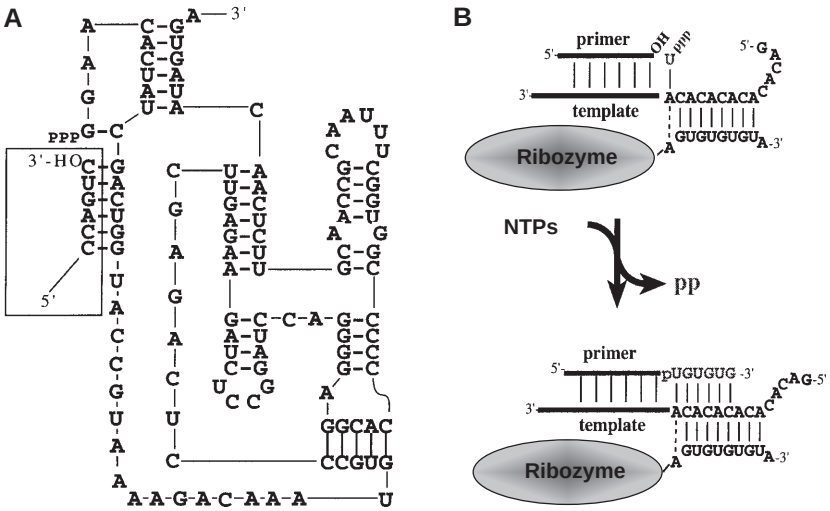
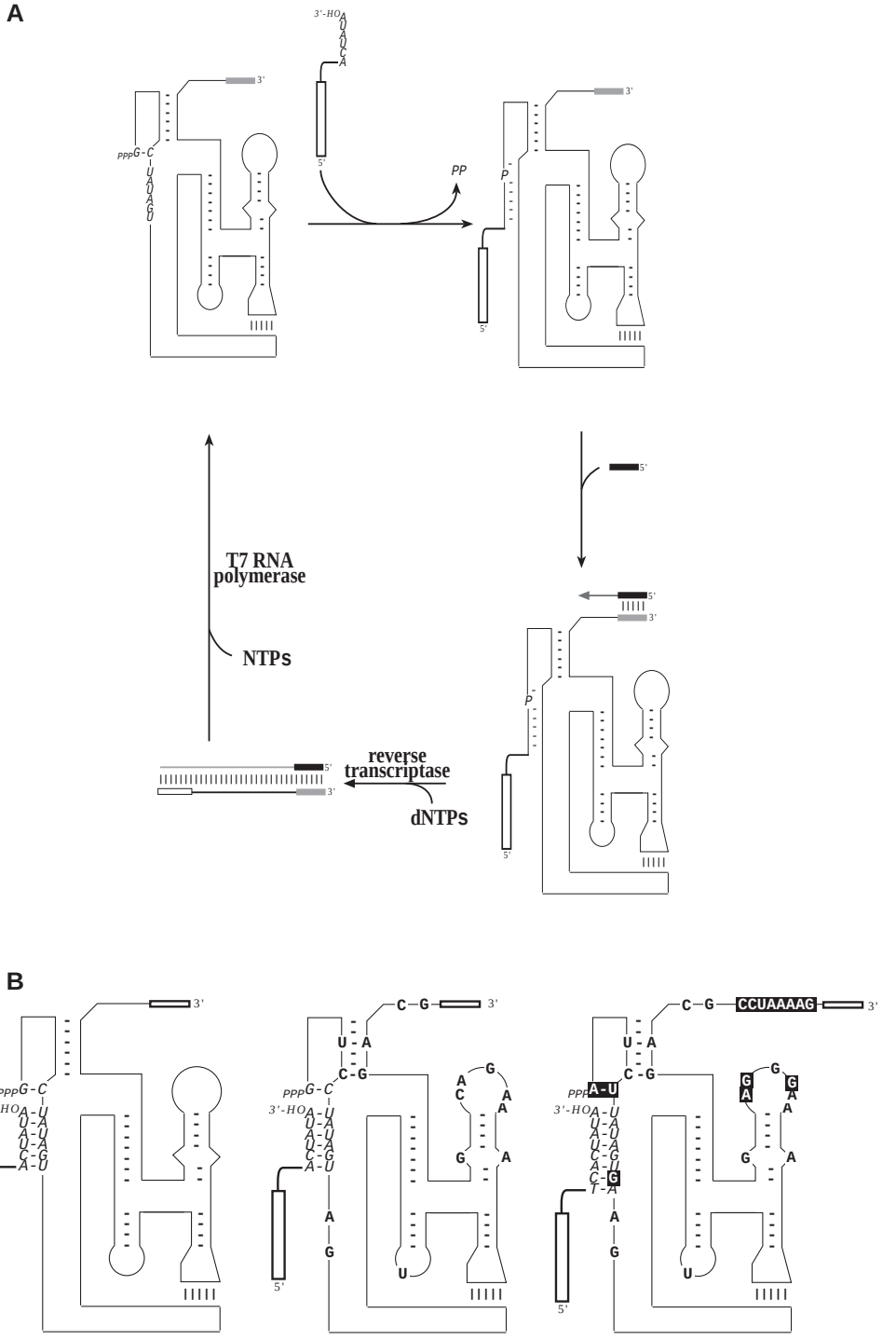



**Fig. 6.** **A** Secondary structure of the class I ligase. **B** Template-directed RNA polymerization of up to six nucleotides catalyzed by the class I ligase (Ribozyme)


A
T7 RNA polymerase
NTPs
reverse transcriptase
dNTPs
PP
B

situation was generated, in which only those ribozymes were replicated by T7-polymerase that had ligated the T7-promoter onto their 5′-end *before* reverse transcriptase would generate too much "sense"-DNA from the ribozyme-template making catalysis impossible. With their system, Wright and Joyce have developed a continuous evolution scheme similar to the Q$\beta$-replicase system used by Spiegelman [126], with the difference that the actual catalytic step is carried out by a ribozyme. The amplification reaction, however, still depends on the "helper proteins" reverse transcriptase and T7-RNA polymerase. The continuous evolution reaction might also be used to develop a ribozyme with RNA polymerase activity, which, perhaps, some day makes the use of a proteinaceous RNA-polymerase obsolete. This process enables the replication, mutation, and selection of many ribozyme generations within a very short period of time and will be of great interest for molecular evolution studies.

In another attempt to provide a starting point for the evolution of self-replicating ribozymes, Chapman and Szostak designed a selection experiment for the generation of RNA molecules that ligate their 3′-end to a hexanucleotide with a 5′-phosphate activated as phosphorimidazolide [79] (see Fig. 8). However, the isolated ribozyme catalyzed the attack of the 5′-terminal $\gamma$-phosphate group on the 5′-phosphorimidazolide of the substrate oligonucleotide forming a 5′-5′ tetraphosphate linkage. Depending on whether a 5′- mono-, di-, or triphosphate was present, the 54 nucleotide long pseudoknot motif was also capable of generating di- or triphosphate linkages.

Hager and Szostak used an RNA library in which each member was "capped" by an adenosine-5′-5′-pyrophosphate group at the 5′-end to isolate ribozymes that catalyze the ligation of an oligoribonucleotide to this activated group. This reaction results in the formation of a 3′-5′-ligation and the release of AMP [82].

A ribozyme activity that led to RNA-modifications that are analogous to the 5′-5′ pyrophosphate "caps" of eukaryotic RNA transcripts was selected by Huang and Yarus [84]. Actually the author's intention was to isolate ribozymes which catalyze the formation of a mixed anhydride between an amino acid carboxylate and a 5′-terminal phosphate of an RNA, an activity that is chemically analogous to the activation of amino acids by ATP catalyzed by aminoacyl tRNA synthetases. However, while the selected ribozymes did

**Fig. 7.** Continuous evolution of the class I ligase ribozyme. **A** Schematic for the continuous evolution system leading to enrichment of a highly active class I ligase. **B** *Left panel*: schematic secondary structure of the class I ligase with variant substrate binding specificity (sequences shown) compared to the original class I ligase. Because this change in substrate specificity resulted in a 1000-fold reduced catalytic activity the new class I ligase construct had to be evolved by stepwise and rapid in vitro evolution to improve the ligation rate. From this, ribozymes emerged which were not only capable of performing fast enough catalysis with the modified substrate hybridization sequence, but also of accepting a DNA-3′-r($N_4$)-substrate. A ribozyme which was mutated at 17 positions (mutations shown) compared to the starting sequence was used to start the continuous evolution reaction (*middle panel*). The *right panel* shows those positions which had changed after continuous evolution

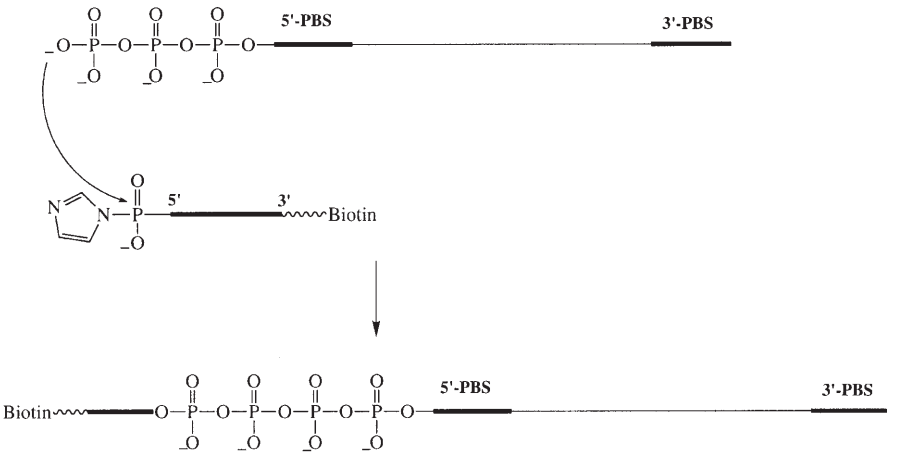



**Fig. 8.** Reaction catalyzed by the RNA to generate a 5′-5′-tetraphosphate linkage

catalyze a reaction that led to the generation of pyrophosphate – the expected by-product of an aminoadenylation reaction – Huang and Yarus showed in a set of control experiments that their activity was independent of the presence of amino acids. Further characterization established that the ribozymes catalyze the formation of 5′-5′-polyphosphate linkages employing substrate molecules such as oligonucleotides or nucleotide mono-, di-, or triphosphates that contain 5′-terminal phosphate groups. Even 5′-phosphate containing biological cofactors such as FMN, $NADP^+$, CoA, PRPP, or thiamine pyrophosphate could be utilized as substrates. These ribozymes also promoted the competing hydrolysis of phosphoanhydrides [85, 120]. In a recent study it was shown that one of the ribozymes, when engineered properly can also use two small-molecule substrates and ligate them together in an intermolecular reaction [121].

#### *3.1.2.2*
#### *Ribozymes Catalyzing Non-Phosphodiester Chemistry*

Many examples of catalytic nucleic acids obtained by in vitro selection demonstrate that reactions catalyzed by ribozymes are not restricted to phosphodiester chemistry. Some of these ribozymes have activities that are highly relevant for theories of the origin of life. Hager et al. have outlined five roles for RNA to be verified experimentally to show that this transition could have occurred during evolution [127]. Four of these RNA functionalities have already been proven: Its ability to specifically complex amino acids [128–132], its ability to catalyze RNA aminoacylation [106, 123, 133], acyl-transfer reactions [76, 86], amide-bond formation [76, 77], and peptidyl transfer [65, 66]. The remaining reaction, amino acid activation has not been demonstrated so far.

The central role aminoacylated RNAs play in translation processes suggests that acyl transfer reactions catalyzed by RNA might have facilitated the devel-

opment and optimization of the translation apparatus during early evolution. The three ribozymes described below expand the scope of RNA catalysis towards this direction.

Lohse and Szostak recently described an in vitro selected ribozyme which catalyzes the transfer of a biotinylated methionine residue from an oligonucleotide substrate to the 5′-OH of a pool RNA molecule [76]. Their design of the acyl donor molecule involved the linkage of the amino acid to the 3′-end of a short 6-mer oligonucleotide capable of hybridizing to the ribozyme. By substituting the 5′-OH group of the ribozyme for an amino group the ester transferase could be engineered to perform a corresponding transfer of the amino acid to the 5′-$NH_2$-modified ribozyme, resulting in the first example of an RNA that catalyzes amide-bond formation.

A second example of a similar activity is a ribozyme which catalyzes the transfer of an amino acid ester from a biotinyl-*N*-phenylalanyl-2′(3′)-adenosine-5′-monophosphate (Bio-Phe-AMP) substrate onto a specific ribose 2′-OH group (Fig. 9) [86] This ester transferase ribozyme is thus an example of an RNA which catalyzes a reaction at a carbon center by utilizing a low molecular weight cofactor. The reaction depends strongly on the presence of divalent metal ions and can be inhibited by AMP, but not with GMP, indicating that a specific binding pocket for the Bio-Phe-AMP substrate exists. The transformation reaches equilibrium due to a significant level of the corresponding reverse reaction, 2′(3′)-aminoacylation of AMP.

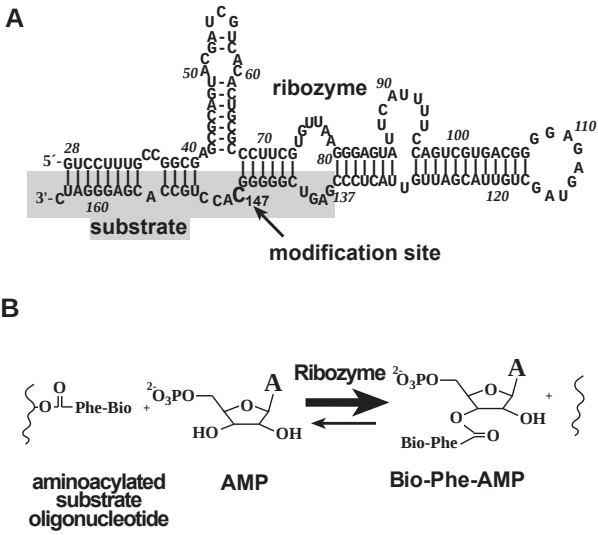



**Fig. 9.** The transacetylase ribozyme. **A** Secondary structure of the clone 11 transacylase ribozyme based on the Zuker RNA folding algorithm Mfold. The oligonucleotide substrate is shaded in *gray*. The 2′-OH group of cytosine 147 (*arrow*) is the site of modification of the oligonucleotide substrate. **B** Reaction catalyzed by the clone 11 transacylase ribozyme. Note that the equilibrium of the reaction lies strongly on the side of the Bio-Phe-AMP substrate

A prerequisite for peptide and protein synthesis in all modern-day life forms is the formation of aminoacyl-tRNAs catalyzed by aminoacyl-tRNA synthetases. These enzymes first activate the $\alpha$-carboxy group of the amino acid by forming an aminoacyl-adenylate containing a highly activated mixed anhydride group which is then used to transfer the amino acid to the 3′(2′)-hydroxy terminus of the cognate tRNA. Illangasekare et al. used an in vitro selection strategy to obtain an RNA that catalyzes the esterification of an activated phenylalanine to its own 3′(2′)-end [106, 123]. An RNA library consisting of ~ $10^{14}$ different molecules was incubated with synthetic phenylalanyl-5′-adenylate. RNA molecules which had catalyzed their own aminoacylation thus became self-modified with a free $\alpha$-$NH_2$-group from the amino acid. This nucleophilic amino group was selectively reacted with the *N*-hydroxysuccinimide of naphthoxyacetic acid. Thus, only those RNAs with the amino acid covalently attached to themselves contained the naphthoxy residue and therefore differed significantly in their hydrophobicity from inactive molecules allowing their separation by reversed phase HPLC (Fig. 10). Eleven cycles of selection resulted in a variety of self-aminoacylating ribozymes of which the most active showed a rate enhancement of $10^5$-fold compared to the background rate.

There are three examples which show that RNAs can be selected that catalyze amide bond- [76, 77] and even peptide bond formation [65, 66]. Among of them is also the first example in which a modified RNA library was used for the selection of a catalytic RNA. In order to enlarge the functional group diversity Wiegand et al. replaced all uridine residues in an RNA library for 5-imidazolyl uridines thus mimicking histidine, a building block in proteins that is capable of performing general acid base catalysis [77]. The isolated amide synthetase exhibited a catalytic rate enhancement between $10^4$- and $10^5$-fold for the amide bond forming reaction. The catalytic domain of the ribozyme contained three 5-

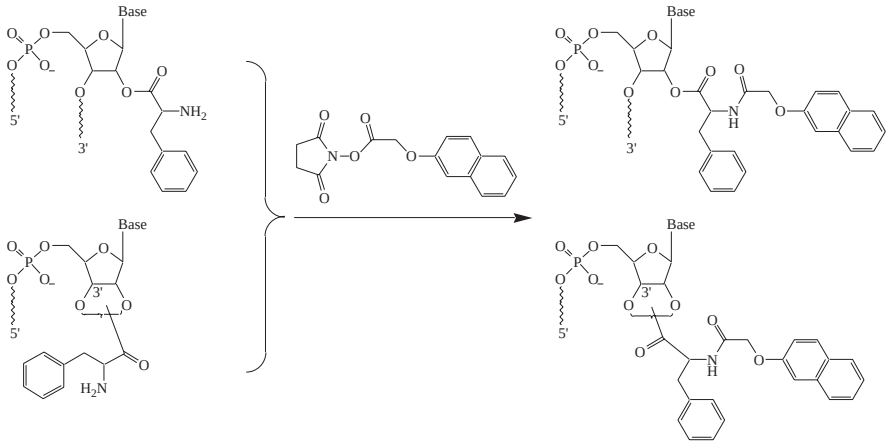



**Fig. 10.** 2′- and 2′,3′-aminoacylation of catalytic RNAs. Catalytic RNAs that can self-aminoacylate can be distinguished by non-modified ones by their increased hydrophobicity by the naphthoxyacetyl label

imidazolyl uridine residues which were absolutely necessary for activity as replacement of these modified residues with uridines abolished catalytic activity. This study also showed that the lack of functional group diversity in nucleic acids can easily be overcome chemically by applying chemically synthesized nucleotide triphosphate (NTP) derivatives that contain additional functionalities [134]. The only requirements with respect to the selection process are that the chemically modified NTPs are accepted as substrates by the nucleic acid replicating enzymes and that the modification does not interfere with the ability of the NTP to pair in the Watson-Crick sense. Researchers have investigated the ability of natural polymerases to accept a number of synthetic dNTPs as substrates [135]. For example, in a recent study Barbas and Sakthivel introduced various functional groups that are normally not found in a nucleic acid, such as carboxylic acids, imidazoles, amines, phenols, and pyridines which were introduced into uridine triphosphates at the 5-position of the base via a rigid spacer arm [136].

Zhang and Cech used a library of $1.3 \times 10^{15}$ different RNAs with a length of 196 nucleotides to isolate novel peptidyl transferase ribozymes [65, 66]. For their direct selection scheme they coupled the $\alpha$-carboxy group of a phenylalanine covalently to the 5′-end of all members of the RNA library. This modified pool was then incubated with a synthetic molecule that mimicked the 3′-end of a tRNA aminoacylated with the formyl methionyl residue. In their minimal version of a formylmethionyl tRNA, the formyl group was substituted by a biotin group and the tRNA was minimized to a single 3′-adenosine residue. After 19 cycles, ribozymes were obtained which catalyzed the formation of a peptide bond between the methionin carboxy group and the amino group of phenylalanine – in analogy to the peptidyl transfer mechanism in the ribosome (Fig. 11). Some of the selected ribozyme variants observed rate constants ($k_{obs}$) of almost 0.1 $min^{-1}$, which translates into a rate enhancement of $10^5$-fold compared to the uncatalyzed reaction. The amino acid/AMP ester is mainly bound to the ribozyme by the adenosine moiety independently of which amino acid is used, allowing amino acids other than methionine to be utilized in this reaction [66].

Eaton and his colleagues have demonstrated that it is also possible to obtain ribozymes that catalyze the formation of carbon-carbon bonds by isolating a "Diels-Alderase"-ribozyme. While the indirect selection approach failed [94] the ribozyme isolated in a direct selection accelerates the Diels-Alder reaction between a diene which is covalently attached to its 5′-end via a long polyethylene glycol linker and a biotinylated maleimide dienophile by a factor of 300 [6]. As with their previous example of amide-bond forming ribozymes, a modified RNA library was used in which all uracil residues were substituted by pyridyl methyl-modified UMPs. Interestingly, the selected Diels-Alderase ribozyme is also dependent on the presence of $Cu^{2+}$, as was the amide synthetase. $Cu^{2+}$ may either be required for forming the proper structure of the catalyst and/or may actively participate in the catalytic step by providing Lewis acid sites which are known to be advantageous for the acceleration of Diels-Alder reactions in water [137].

One of the most interesting ribozymes isolated by in vitro selection techniques is a novel catalytic RNA that promoted the formation of a glycosidic bond

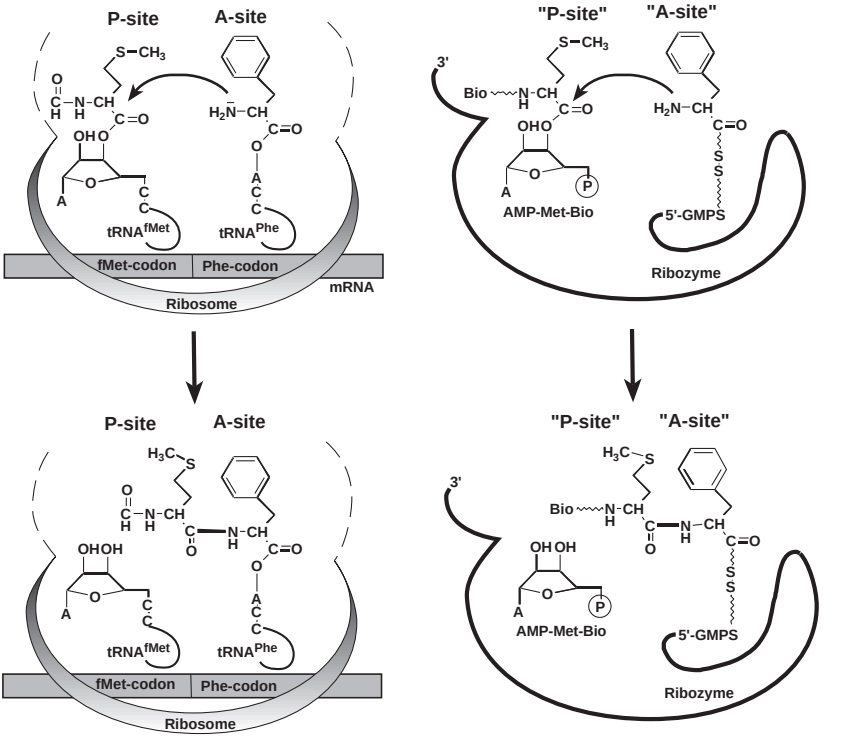
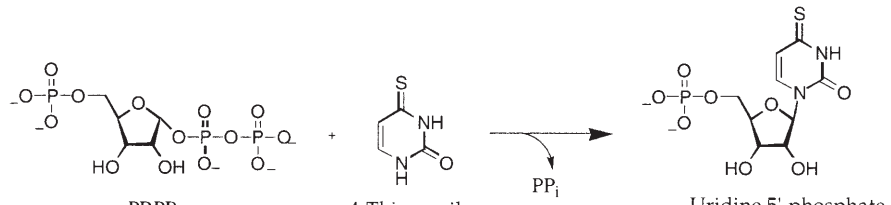



**Fig. 11.** Comparison of the peptidyl transfer reaction in the ribosome and in the selected peptidyltransferase ribozyme. The ribosome contains a binding site for the peptidyl-tRNA (P-site) and for the aminoacyl-tRNA (A-site). In the selected ribozyme the binding site for the AMP-Met-Bio substrate would be analogous to the P-site. The attacking $\alpha$-amino group which is bound in the A-site in the ribosome is covalently attached to the 5′-end in the ribozyme. Catalytically active RNAs preferentially attach the biotin tag onto themselves and can thus be separated from the inactive ones

between 4-thiouracil (4-S-Ura) and 5′-phosphoribosyl-1′-pyrophosphate (PRPP), linked to the 3′-end of an RNA library [87] (Scheme 2).

While the PRPP substrate was covalently attached to the RNA pool, the 4-S-Ura substrate was incubated with the library in solution. Active molecules were selected with a special gel electrophoretic technique on the basis of the




**Scheme 2**

attached thio group [138]. Nucleotide formation catalyzed by the selected catalytic RNAs was at least $10^7$ times faster than the uncatalyzed reaction. The ribozymes showed remarkable specificity for the 4-S-Ura substrate. No reaction occurred with 2-thiouracil, 2,4-thiouracil, 2-thiocytosine, 2-thiopyrimidine, 2-thiopyridine or 5-carboxy-2-thiouracil. This study showed that RNA can perform reactions by binding substrates that are smaller than purine nucleotides. The study by Unrau and Bartel is therefore an important contribution to the RNA-world hypothesis which requires that ribozymes would have needed to promote many metabolic reactions which involve organic molecules of low molecular weight.

### 3.1.3 *Direct Selections From Aptamer-Based Libraries*

Some of the ribozyme selections discussed above require the reactants to be attached covalently to the nucleic acid. In other cases, substrates were incubated with the library in solution and the resulting ribozymes had to evolve defined binding sites for the substrates and to provide the scaffold for its correct positioning. The isolation of new ribozymes might be facilitated by first selecting an aptamer-sequence for binding to a cofactor needed in the reaction and including a highly mutagenized aptamer sequence in addition to a completely randomized portion as a basis for the selection of functional molecules. This method of direct selection may provide a more focused sequence space to increase the number of sequences in the library that are capable of binding to the substrate.

Lorsch and Szostak [102] generated an RNA-library in which the central sequence of a partly randomized ATP-binding RNA aptamer, which had previously been selected by affinity chromatography on ATP agarose [139, 140] was surrounded by three completely randomized regions of a total of 100 bases. This library was used for the selection of an ATP-dependent oligonucleotide kinase by incubation with ATP-$\gamma$-S [102, 105]. RNA molecules onto which the $\gamma$-thiophosphate group of the ATP-$\gamma$-S had been transferred could be separated from the rest of the library on activated thiopropyl agarose, since they specifically formed a disulfide bond between the thiophosphate group and the agarose. These covalently bound RNAs were then eluted by washing with an excess of 2-mercaptoethanol, amplified and reselected. After thirteen cycles of selection, seven classes of ribozymes were characterized which catalyzed various reactions. Five of these RNA classes catalyzed the transfer of the $\gamma$-thiophosphate onto their own 5′-hydroxyl group and are therefore 5′-kinases. The other two classes phosphorylated the 2′-hydroxyl groups of specific internal base positions. Starting from one of these sequences, a ribozyme was developed which can phosphorylate the 5′-end of oligonucleotides in an intermolecular reaction.

An analogous strategy was used by Wilson and Szostak to isolate self-alkylating ribozymes using an iodoacetyl derivative of the cofactor biotin [103]. After isolating biotin-binding RNA aptamers by repeated rounds of affinity chromatography and amplification, a second library was generated which consisted of the mutagenized aptamer sequence flanked on either side by 20 random nucleotides. Molecules from this library which were able to self-alkylate with the

biotin derivative were separated from inactive sequences by binding to streptavidin. By the seventh round of selection, more than 50% of the RNA performed the self-biotinylation reaction. The sequencing of individual clones revealed that a majority of the ribozymes was derived from a single ancestral sequence. To optimize the activity, a third selection was carried out in which the incubation time as well as the concentration of the cofactor were progressively lowered. The resulting ribozyme alkylated itself at the N7 position of a specific guanosine residue within a conserved region with a rate acceleration of $2 \times 10^7$ compared to the uncatalyzed reaction.

The self-biotinylating ribozymes which originated from a biotin-binding aptamer show an astounding structural change compared to their ancestor. A highly conserved nucleotide stretch of the biotin binder which seems to directly mediate the interaction between biotin and the aptamers as well as the catalytically active molecules is retained in the self alkylating ribozymes with only a single point mutation. Yet, the secondary structural context of this consensus sequence is highly unrelated in the two classes of molecules. While the biotin aptamer contains a pseudoknot motif, the ribozyme forms a cloverleaf which resembles the structure of tRNAs.

The fact that, in both selection experiments, new solutions regarding the structure of the functional molecules have been adopted demonstrates that the best sequence for binding is not necessarily the best sequence for performing catalysis. It seems likely that many of the sequence solutions could also have been selected from completely randomized pools. This notion is confirmed by the aforementioned study by Hager and Szostak [82], in which the mutated ATP-aptamer motif was also included in the starting library but where the resulting ribozyme had no relationship to the parent ATP-binding motif.

### 3.2
### In Vitro Selection of Catalytic DNA

A new dimension in the development of nucleic acid based catalysts was introduced by Breaker and Joyce in 1994 when they isolated the first deoxyribozyme [111]. It is not unexpected that DNA is also able to catalyze chemical reactions because it was shown previously that ssDNA aptamers which bind to a variety of ligands can be isolated by in vitro selection [141]. In the meantime, several deoxyribozymes have been described which expand the range of chemical transformations accelerated by nucleic acid catalysts even further and raising question whether even catalytic DNA might have played some role in the prebiotic evolution of life on earth [69–71].

Breaker and Joyce selected a deoxyribozyme that specifically cleaved the phosphodiester bond of a single ribonucleotide embedded within an all DNA oligonucleotide [111]. The library of single stranded DNA contained the single ribonucleotide at a specific position within a primer binding site to avoid the possible effect of RNA on catalysis. Guided by the assumption that a DNA-dependent cleavage at neutral pH would require a metal ion as a cofactor, they included $Pb^{2+}$ in their selection. The starting pool contained about $10^{14}$ double-

stranded DNAs with a randomized region of 50 nucleotides. One of the strands had a biotin group covalently attached to its 5′-end, followed by a 43 nucleotide position of defined sequence which also contained the cleavage site in the form of a single ribose adenosine unit. The biotin moiety was used to immobilize the single-stranded DNA on streptavidine agarose which was then incubated in $Pb^{2+}$-containing buffer. In a small fraction of active sequences, this treatment resulted in the cleavage of the phosphodiester bond at the ribose phosphate "point of fracture". Because these sequences were separated from the biotin anchor group after cleavage, they could be partitioned from the inactive ones by washing the streptavidine matrix, Fig. 12.

After five cycles of selection and amplification, a population of single-stranded DNAs was enriched that catalyzed the $Pb^{2+}$-dependent cleavage at the ribose residue. This intramolecular cleavage activity was transformed into an intermolecular reaction by separating the 38-nucleotide long catalytic domain from the 21-mer substrate which was cleaved specifically and with high turnover rates. Remarkably, the deoxyribozyme can perform well only with the special DNA/RNA chimeric oligonucleotide substrate and cannot cleave a pure RNA substrate of the same sequence.

This goal was recently achieved by Santoro and Joyce who isolated a deoxyribozyme which is able to hybridize to and cleave any RNA sequence under phy-

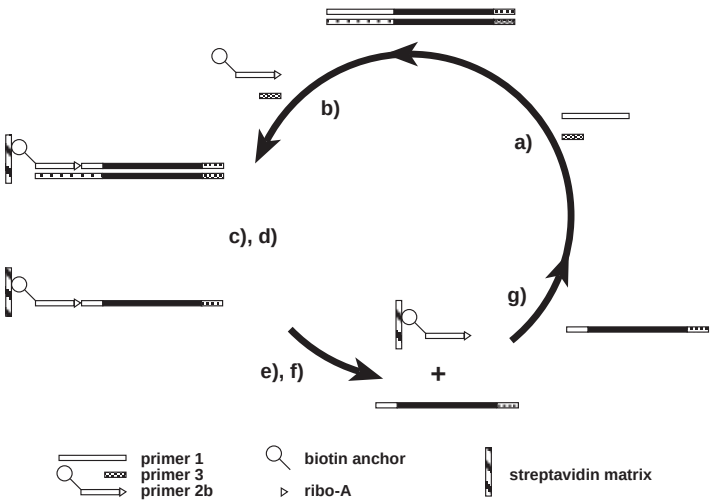



**Fig. 12.** Selection scheme for deoxyribozymes which cleave RNA/DNA chimeric oligonucleotides. In a first PCR *a*), the starting pool was amplified using primer 1 and primer 3. *b*) In a second PCR, the 5′-end of the pool was biotinylated and the ribonucleotide serving as cleavage site was introduced using primer 2b and primer 3. The double-stranded pool was then loaded on a streptavidin column *c*). The antisense strand is removed by raising the pH of the solution *d*) and the remaining pool of single-stranded DNA allowed to fold *e*) by rinsing the column with equilibration buffer. The equilibration was followed by addition of cleavage buffer *f*). Cleavage products were eluted from the column *g*) and amplified by PCR using the same primers as above

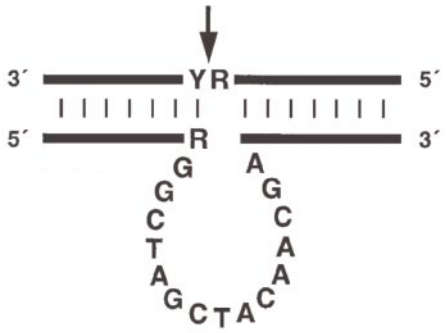



**Fig. 13.** The deoxyribozyme (*bottom strand*) hybridizes to the RNA substrate oligonucleotide (*top strand*). The site of cleavage is indicated by the *arrow* (R = A or G; Y = U or C). The sequences of the helical parts of the enzyme can be chosen as desired, so that almost any RNA sequence can be targeted by the catalytic DNA

siological conditions with remarkable efficiency [116]. The enzyme consists of a 15-mer catalytic domain which is flanked by sequences that can hybridize to the RNA target through Watson-Crick pairing (Fig. 13). Because of its small size and its high cleavage efficiency under multiple turnover conditions, it is a very attractive candidate for therapeutic and biotechnological applications. This catalytic DNA might be particularly useful when incorporated into antisense oligonucleotides to facilitate cleavage of the targeted mRNA.

Meanwhile other experiments have been carried out to isolate DNA molecules with either RNA- or DNA-phosphoesterase activity [69–71, 142, 143]. Since $Mg^{2+}$-dependent rather than $Pb^{2+}$-dependent cleavage is compatible with intracellular conditions and thus, more suitable for possible medical applications, deoxyribozymes were selected that used $Mg^{2+}$ instead of $Pb^{2+}$ for cleavage [112]. One optimized deoxyribozyme that emerged in this selection showed a cleavage rate of 0.01 $min^{-1}$ and was also capable of intermolecular cleavage.

The catalytic potential of DNA was further examined by Carmi et al. with the same in vitro selection strategy to generate catalytic DNAs that facilitate DNA-cleavage by a redox-dependent mechanism [117, 118]. The design of this selection was based on the fact that DNA is more sensitive to cleavage by oxidative mechanisms than by hydrolysis. Therefore, a library of single-stranded DNAs (ssDNA) which was immobilized on streptavidin by a covalently attached biotin-tag was equilibrated in a buffer solution and then incubated with $CuCl_2$ and ascorbate to initiate cleavage of active sequences. The pool isolated after seven rounds of selection consisted of two distinct classes of self-cleaving ssDNA molecules. While one class performed strand scission in the presence of both $Cu^{2+}$ and ascorbate, the other class only required $Cu^{2+}$ as a cofactor. An optimized version of one of the deoxyribozymes shows a rate enhancement of more than $10^6$-fold compared to the uncatalyzed reaction.

To develop deoxyribozymes that make use of a non-metal cofactor rather than divalent metal ions for the cleavage of a ribonucleotide residue we performed an in vitro selection under conditions of low magnesium concentration, or

even without any divalent metal ions, by incubating the immobilized single-stranded DNA library in an excess of the amino acid histidine [114]. Surprisingly, non of the resulting eight classes of deoxyribozymes utilized histidine as a cofactor for cleavage. They either depended on the presence of divalent metal ions or accelerated phosphodiester cleavage even in the absence of divalent metal ions [59]. Remarkably, one of the catalysts, showed higher cleavage activity in the presence of $Ca^{2+}$ than of $Mg^{2+}$, even though calcium was never present during the selection process. We hypothesized that, in this special case, $Ca^{2+}$ might be more suitably positioned at the cleavage site than the magnesium ion. This suggestion is supported by the observation that two $Ca^{2+}$-ions are bound cooperatively by the catalytic DNA.

In vitro selection technology also allowed the isolation of ribozymes that utilize non-metal ion cofactors to facilitate phosphodiester cleavage. It certainly was known long before these studies that ribozymes can do that: The group I intron from *Tetrahymena thermophila* uses guanosine derivatives to initiate the first step of phosphodiester cleavage [19], i. e. the attack of the 2′(3′)-OH-group of a non-covalently bound guanosine cofactor for which the ribozyme contained a specific binding pocket [144]. More recently, still using the successful selection strategy of biotin/streptavidin-immobilization of ssDNA libraries and subsequent elution of cleaved product, deoxyribozymes that cleave phosphoramidate bonds have been reported [122]. These DNA enzymes depend on the presence of trinucleotides as cofactors for cleavage and represent the first examples of DNA enzymes that need a non-metal cofactor for activity.

Another class of cofactor-dependent deoxyribozymes that use the amino acid histidine to promote phosphodiester cleavage was isolated recently by Roth and Breaker [113]. One of the isolated DNA requires L-histidine or a closely related analog to support RNA phosphodiester cleavage with a rate enhancement of nearly a million-fold over the basal substrate cleavage rate (Fig. 14).

Presumably, the L-histidine cofactor is loosely bound to the DNA-enzyme which positions its imidazole group to serve as a general base catalyst. The histidine/DNA complex thus works by a mechanism that is similar to the first

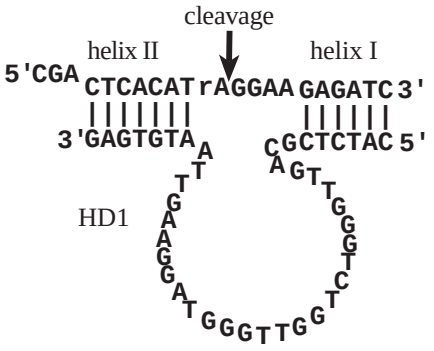



**Fig. 14.** Sequences and secondary structures of one of the selected L-histidine-dependent catalytic DNAs

step of the proposed catalytic mechanism of RNase A. This protein enzyme also has a histidine residue positioned in its active site which serves as a general base catalyst for RNA hydrolysis. The study by Burmeister et al. [122] and particularly the one by Roth and Breaker [113] show that catalytic nucleic acids can recruit the functional groups of complexed small organic cofactors for catalysis thereby dramatically increasing their own limited structural diversity and their catalytic potential [145].

However, even without small-molecule cofactors the catalytic scope of DNA is not restricted to phosphoresterase activity. Cuenoud and Szostak designed a selection scheme to isolate DNA molecules that catalyze the ligation of their free 5′-hydroxyl group to the 3′-phosphate group of a substrate oligonucleotide activated by imidazolide [80]. The resulting deoxyribozyme depends on the presence of a $Zn^{2+}$ or $Cu^{2+}$-metal ion cofactor. The enzyme contains two conserved domains which position the 5′-hydroxyl group and the 3′-phosphorimidazolide of the substrate oligonucleotide in close proximity. Based on the selected sequences, a truncated version of the DNA ligase was designed that is able to ligate two DNA substrates in a multiple turnover reaction with a 3400-fold rate enhancement compared to the template directed ligation.

## 4
## Allosteric Ribozymes and "Aptazymes"

One of the remarkable characteristics of ligand complexation by in vitro selected RNA aptamers is that ligand binding by the RNA molecule is always accompanied by significant structural changes in the binding RNA. Ligands seem to become an integral part of the RNA-aptamer structure once they are bound [146, 147]. This property of aptamers might have inspired the idea of fusing aptamer sequences with known catalytic RNAs to introduce the principle of allosteric regulation into ribozyme catalysis. None of the ribozymes described so far were known to operate as allosteric enzymes in vitro or in vivo.

The hammerhead ribozyme was transformed into an allosteric ribozyme by attaching the ATP- [15] or FMN-binding aptamer sequences [148–150] to its 5′-end [17]. These allosteric HHRs are capable of phosphodiester cleavage only in the absence of the cognate aptamer ligand. In the case of the conjoined aptamer/ribozyme construct the presence of ATP or FMN results in a ligand-induced conformational change. In the case of the ATP-regulated HHR-construct, ligand binding causes a steric clash between aptamer and ribozyme domains [151] which prevents the hammerhead ribozyme from adopting its active structure. The inhibition ratio obtained by this strategy was up to 180-fold. Tang and Breaker also used the principle of allosteric control of ribozyme catalysis to try to select variants of the hammerhead ribozyme that would be more active in phosphodiester cleavage than the wild-type HHR by including the inhibitory ligand ATP during the in vitro transcription [16]. In this selection, however, only the natural HHR sequence was isolated suggesting that this sequence represents a motif which has been optimized by nature for the purpose of phosphodiester cleavage.

Robertson and Ellington recently described the in vitro selection of a novel ligase ribozyme that requires allosteric activation of an oligonucleotide effector molecule for activity [18]. The allosteric ribozyme ligase is activated up to 10000-fold by the oligonucleotide effectors. "Rational" engineering of the ribozyme by incorporation of the aptameric ATP binding sequence [139] allowed its transformation into an enzyme that requires ATP in addition to the oligonucleotide effector for activity. This approach of effector-controlled ribozyme activity, when linked to RT-PCR technology, has the potential to be applied wherever accurate diagnostic quantification of oligonucleotide- or small-molecule-analytes is desired. Such allosteric ribozymes or 'aptazymes' might possess a significant application potential in medicine, diagnostics or biotechnology.

## 5
## Ribozymes and the Origin of Life

One of the most fascinating problems many scientists, philosophers, and theologists deal with is that of the origin of life on Earth and maybe on other planets in the universe. According to the now widely accepted "RNA-world hypothesis", the way from the earliest evolving prebiotic entities to the first cells might have been accompanied by an era in which chemical transformations of primitive metabolisms were catalyzed explicitly by RNA molecules [68, 127, 152]. Most likely, life on earth did not come into existence in a single event, but emerged as the result of permanently ongoing improvement of molecules that are thought to have had the property to catalyze a wide variety of chemistries including their own synthesis. Therefore, besides their ability to self-replicate the ability to evolve in cumulative selection processes is a necessary condition for the generation of living entities from those first replicators and catalysts. The reason why RNA molecules are believed to have played a dominant role in the origin of life is that they can store information and mutate while at the same time being catalytically active. As shown by many examples of novel in vitro selected ribozymes, RNAs also might have had the potential to carry out a primitive metabolism by folding into complex three-dimensional structures that allow selective binding of other molecules and the catalysis of chemical reactions. The features of those molecules involved in the first metabolic processes were altered by mechanisms of Darwinian evolution, mutation and selection within geological timescales, as biological evolution is a slow process. In vitro selection enables the researcher to investigate the scope of RNA catalysis, or nucleic acid catalysis in general, many of which we have met in this review. As we have seen, among the artificial ribozymes that have been selected there are a number which catalyze some "key reactions of life" that have been suggested to be possible evolutionary precursors of ribosomal RNA, or RNA-based metabolism. These results provide an experimental basis for the plausibility of the RNA-world hypothesis.

## 6 References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