Oligonucleotide libraries – *variatio delectat*

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In vitro selection of combinatorial nucleic acid libraries leads to specific target-binding molecules – RNA, single stranded DNA, modified RNA or modified DNA, commonly designated as aptamers – and to novel catalytic nucleic acids. The current state of aptamer and ribozyme technology is such that it establishes itself as a means of obtaining useful tools for molecular biology, diagnostics, molecular medicine and bio-organic chemistry.

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Current Opinion in Chemical Biology 1998, **2**:320–327

http://biomednet.com/elecref/1367593100200320

Abbreviations

HHR	hammerhead ribozyme
NES	nuclear export signal
SECIS	selenocysteine inserting sequence
ssDNA	single stranded DNA
XAP	export aptamer

Introduction

Last year's issue of *Current Opinion in Chemical Biology* on combinatorial chemistry contained five reviews devoted to oligonucleotide libraries [1–5]. Since then an impressive number of new examples that demonstrate the broad applicability of *in vitro* and *in vivo* selected nucleic acids have appeared. The technique of *in vitro* selection was used to address questions of biological interest and previously newly selected aptamers (nucleic acids that can bind target molecules) have been refined and applied for purposes of biotechnological relevance or for applications in molecular medicine in its broadest sense. One study analyzed the importance of the length of molecules in oligonucleotide library design [6] and another described a new *in vitro* selection protocol that allows the selection of nucleic acid aptamers without the need for iterative amplification steps [7]. The remarkable progress in the determination of aptamer solution structure, achieved mainly in 1996 and in the first half of 1997, was reviewed extensively in several articles [5,8••,9•,10•]. Two new solution structures have been published since then: for the theophylline RNA aptamer [11] and the AMP–DNA aptamer [12]. Progress in the design and application of selection schemes for various novel ribozymes has probably been the most impressive. Here we give an update of some novel and promising developments in the field of combinatorial nucleic acid libraries, such as the selection of novel ribozymes and biologically active compounds.

Aptamers for small molecules

Several new RNA motifs that specifically complex molecules of low molecular weight have been isolated [13–16,17•,18••]. Three of these were targeted to antibiotics — streptomycin [16], viomycin [15] and chloramphenicol (Cam) [14]. Welch *et al.* [17•] selected for RNAs that bind a puromycin derivative of 5′-CCdA-3′-phosphate (CCdAp-Puro), a transition state analog for peptide bond formation. They obtained aptamers that were similar to conserved nucleotides in the peptidyl transferase loop domain of 23S rRNA.

Haller and Sarnow [18••] isolated an aptamer that binds 1000-fold better to N7 methyl guanosine residues than to nonmethylated guanosines. As N7 methyl guanosine residues closely resemble the 5′-terminal cap structure of eukaryotic mRNAs, this cap-binding aptamer turned out to specifically inhibit the translation of capped, but not uncapped, mRNAs in cell-free lysates from either HeLa cells or yeast. These aptamers might, therefore, be useful in studies of cap-dependent processes such as pre-mRNA splicing and nucleocytoplasmic mRNA transport.

Aptamers and the dissection of cellular processes

RNA libraries for the identification of binding elements in RNA–protein interaction

A number of *in vitro* selections have been published that were aimed towards understanding the mechanisms of RNA–protein recognition by comparing selected sequences with natural RNA targets for a given protein in order to identify sequence elements critical for binding or for certain biological functions [19••,20–22].

An impressive example of the use of combinatorial RNA libraries to identify natural RNA binding sites for a given target protein was provided by Buckanovich and Darnell [19••]. They selected RNA aptamers that bound to the neuron-specific RNA-binding protein Nova-1, an autoantigen found in individuals with a neurologic disorder associated with breast cancer and dysfunction of brainstem or spinal motor systems. Although it was known that Nova-1 contained three RNA-binding domains, a particular RNA motif recognized by Nova-1 was unidentified. To identify such motifs, an *in vitro* selection for Nova-1 binders was performed using a library of 10^{15} different 52-mer RNAs. Isolated aptamers contained a conserved 15-mer consensus motif $(UCAU[N]_{0-2})_3$ that was found to be absolutely necessary for Nova-1 binding. Remarkably, a GenBank search for this consensus sequence identified natural Nova-1 binding sites in two neuronal pre-mRNAs. One sequence lies within an intron of the glycine receptor α2 (GlyR α2) pre-mRNA. The other sequence corresponds to the pre-mRNA that encodes Nova-1 itself. Both natural

pre-mRNAs specifically interact with authentic Nova-1 protein. These studies established that Nova-1 functions as a sequence-specific nuclear RNA-binding protein *in vivo*. This was one of the first SELEX experiments that identified previously unknown naturally occurring nucleic acid binding sites of an RNA binding protein. The authors suggest that disruption of Nova-1 binding to GlyR α2 pre-mRNA by the autoantibody is involved in the mechanism of this neurologic disease.

In vivo selection and *in vivo* expression of aptamers

Three remarkable selections aimed at determining the mechanisms responsible for the transport of RNAs to specific subcellular compartments have been published [23•,24,25••]. Grimm *et al.* [23•] used an *in vivo* selection approach to identify RNA sequence signals and mechanisms that contribute to the localization of RNAs within nuclei of *Xenopus laevis* oocytes. They prepared a library composed of a truncated version of the U1 snRNA (that is, one of the components of small nuclear ribonucleoprotein particles), which carried a 20 nucleotide randomized insert, as well as the m^7G-cap export signal, but no nuclear import signal. Because of this special design, most of the library members localized in the cytoplasm after injection of the pool into the nuclei. The few molecules that remained localized in the nucleus because their 20 nucleotide insert represented a sequence that served as a nuclear localization signal were isolated, amplified and used for the next selection round. After 12 cycles of injection and amplification a class of sequences was obtained that interacted with the predominantly nuclear phosphoprotein La, which binds to the 3′-U termini of newly synthesized RNA polymerase III transcripts. Furthermore, the selected sequences appear to promote retention of the RNA in the nucleus by masking the 5′-cap export signal. Another population of RNAs contained the consensus sequence 5′-AAUUUUUGG-3′, which bound to Sm proteins. Sm proteins promote import of the spliceosomal RNAs U1, U2, U4, and U5 into the nucleus, implying that this population of RNAs can be re-imported into the nucleus.

To investigate the relationship between nuclear retention and export of RNA, a similar selection was performed, this time in the presence of an inhibitor of RNA export, the matrix protein of vesicular stomatitus virus [24]. The sequences isolated in this selection acted like nuclear export elements in promoting efficient export of RNAs which are otherwise not exported.

Hamm *et al.* [25••] took a different combinatorial approach to obtain RNA sequences that inhibit RNA export. In previous work [26], Hamm had shown that RNA aptamers selected for binding an anti-ligand antibody can mimic the epitope bound by that antibody. An analogous approach was used by selecting RNA aptamers against an antibody recognizing the nuclear export signal (NES) of the HIV-1 Rev protein [25••]. The RNA mimics were shown to be functionally equivalent to the Rev-NES–peptide conjugates [27] in the nuclear cytoplasmic transport in *Xenopus* oocytes. Like the NES–peptide, the RNA mimics — designated 'export aptamers' (XAPs) — blocked Rev-dependent export of a reporter RNA and inhibited the cap-dependent U1 snRNA export. The XAP itself was actively transported and it was suggested that this might be due to the fact that the XAP structurally mimics the NES–epitope that recognizes the receptor for the NES.

An example of using *in vitro* selected aptamers *in vivo* in order to dissect binding from the overall biological function of an RNA-binding protein was a study by Klug *et al.*, who isolated aptamers that bind the special elongation factor SelB of *Escherichia coli* [28•,29]. SelB promotes incorporation of the rare amino acid selenocysteine into formate dehydrogenase H (*fdhF*) at a UGA codon by simultaneous binding to selenocysteyl-tRNA ($tRNA^{sec}$), GTP and a downstream mRNA hairpin structure (the SECIS [selenocysteine inserting sequence] element) [30]. Klug *et al.* [28•] investigated if binding SelB to the SECIS element could be dissected from its overall biological function, the promotion of selenocysteine incorporation into proteins. The isolated SelB-binding RNA aptamers closely resembled the wild type SECIS element with respect to its SelB-binding characteristics. Despite their similarity to the SECIS mRNA hairpin and their tight binding to the special elongation factor SelB, the aptamers did not permit the incorporation of selenocysteine at the UGA selenocysteine codon. On the basis of these results it was assumed that the biological role of the SECIS element includes promotion of a conformational change in the SelB protein, which is necessary for selenocysteine incorporation into proteins.

Another aptamer selected *in vitro* and tested for function *in vivo* is an RNA that binds to the yeast RNA polymerase II (Pol II) [31]. When expressed in a yeast strain with an artificially reduced level of Pol II, the aptamer was shown to inhibit yeast growth specifically.

Aptamers for proteins and their application in biotechnology, medicine and diagnostics

The specificity of molecular recognition combined with the simplicity by which protein-binding aptamers can be obtained, engineered and chemically modified make these molecules very attractive as drugs or tools in biotechnology and diagnostics. Although no aptamer has yet proven successful in daily clinical practice, various recent examples impressively illustrate their potential in affecting cellular processes. Possible obstacles such as lack of stability can easily be overcome, for example by using 2′-fluoro- or 2′-amino-modified oligonucleotides, which have the advantage of being compatible with nucleic acid replicating enzymes such as T7 RNA polymerase and reverse transcriptase [2], thereby allowing the direct selection of modified pools for a given target.

Lee and Sullenger [32•] selected 2′-amino-modified, nuclease-resistant aptamers that recognize autoantibodies

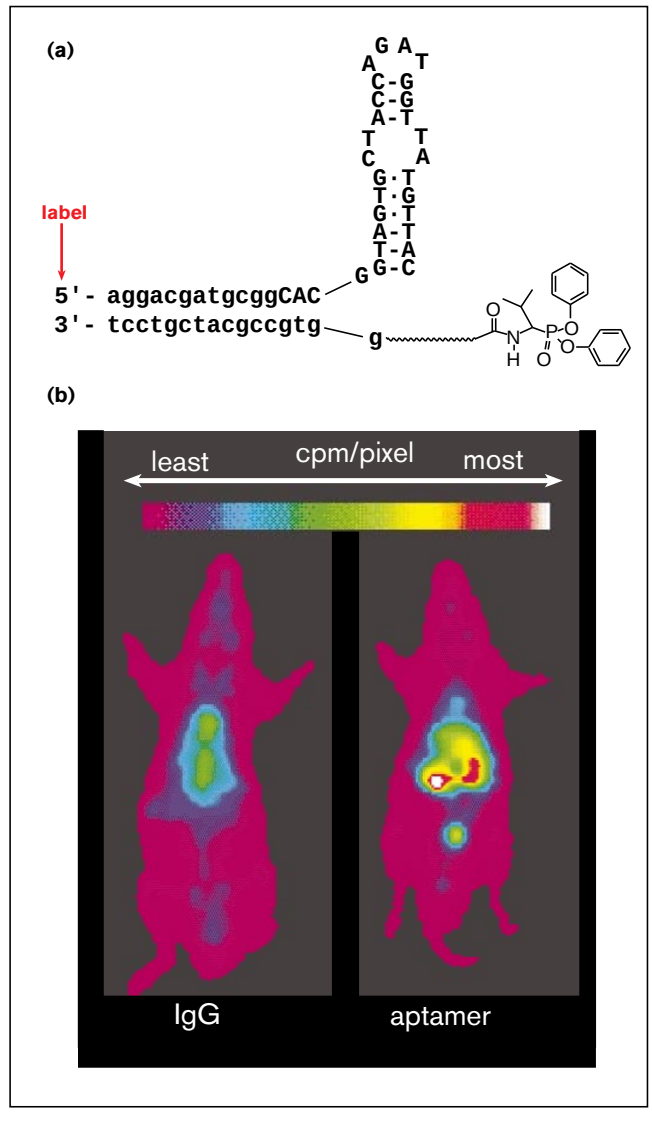
present in the serum of patients suffering from the muscular disease *Myasthenia gravis*. These aptamers inhibit the binding of acetylcholine receptors on human cells to these autoantibodies and may play a future role in blocking the undesired antibody-mediated immune response associated with this disease. Similarly, aptamers which inhibit interferon-γ (IFN-γ) activity were obtained from 2′-fluoro-, 2′-amino- and 2′-fluoro/amino-modified RNA pools [33]. As IFN-γ participates in cell differentiation and is thought to be involved in various diseases, these aptamers might be of interest for therapeutic and diagnostic purposes.

A more indirect approach to degradation-resistant RNA aptamers is the use of mirror-image L-RNA oligonucleotides as peptide receptors or small-molecule receptors. The principle of this technique has previously been described and reviewed [34–36]. Whereas these earlier studies had targeted molecules of low molecular weight, L-arginine and D-AMP, a recent study showed that it is also applicable to peptides. Williams *et al.* [37•] selected natural DNA ligands for an analog of the peptide hormone vasopressin, synthesized from D-amino acids. The natural L-vasopressin was specifically recognized by the L-DNA enantiomer of the originally selected D-DNA aptamer [37•].

A most striking example of aptamer application is in the use of a modified single stranded DNA (ssDNA) aptamer inhibitor of the human serine protease neutrophil elastase for the *in vivo* diagnostic imaging of inflammation. The aptamer was originally isolated by a technique designated as 'blended SELEX' in which the ssDNA library was modified with a valine phosphonate derivative that acts as a weakly reactive elastase inhibitor. Some ssDNAs promoted the covalent attachment of the reactive valine-ester to the elastase, preventing the enzyme from degrading connective tissue [38,39]. The same aptamer was also used in a parallel study of the reduction of lung inflammation in a rat lung alveolitis model [40•]. Finally, a technetium-99m (^{99m}Tc)-labelled version of this modified DNA-based elastase inhibitor was recently used for inflammatory *in vivo* imaging in a mouse model [41••]. Remarkably, a much clearer signal was achieved by the aptamer in less time, compared to the signal obtained by technetium (^{99m}Tc)-labelled rat anti-elastase antibody used in the clinic for anti-inflammatory *in vivo* imaging (Figure 1). This effect was attributed to its faster clearance from the peripheral circulation compared to the antibody.

In vitro selection was also used to obtain RNA aptamers directed against the Syrian golden hamster cellular prion protein PrP^{c} [42•]. Radiolabelled aptamers were shown to recognize authentic PrP^{C} in crude brain homogenates from various species such as wild type mice, hamster and cattle (Figure 2). The isolated aptamers are specific for the amino terminus of PrP^{C}, which is missing in the infectious isoform $PrP_{27–30}$ and consequently is not recognized by the aptamers. Thus, the RNA aptamers may provide a

Figure 1

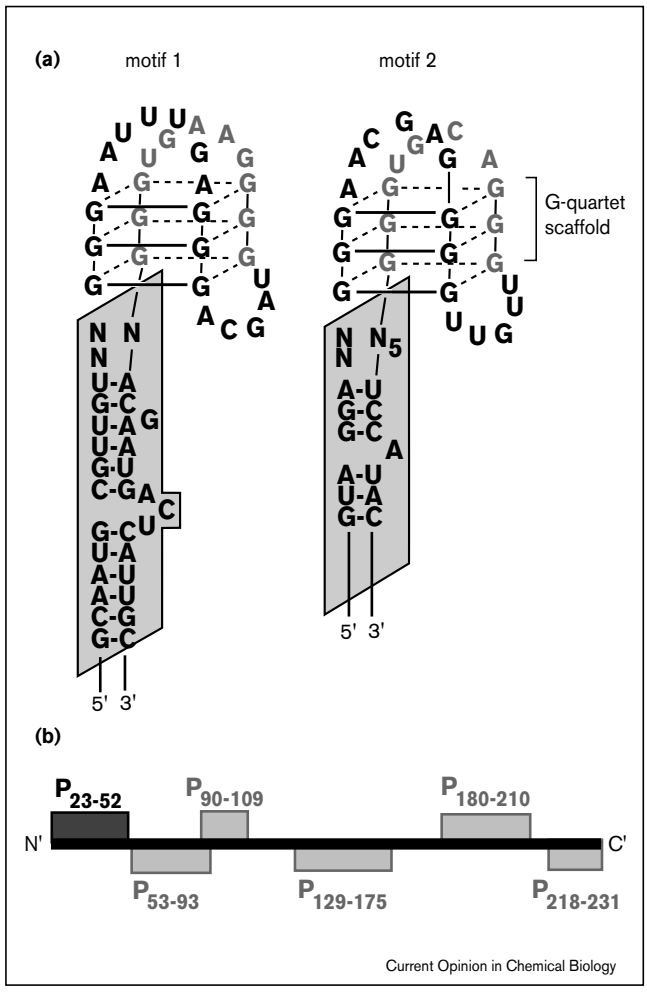


An aptamer and its application in *in vivo* imaging. **(a)** DNA aptamer that binds to neutrophil elastase and acts as an irreversible inhibitor via the attached valine derivative. **(b)** Inflammation imaging by aptamer NX21909 (right) and IgG (left) after ten minutes. cpm, counts per minute. Reproduced with permission from [41••].

first milestone in the development of a diagnostic assay in which one would be able to determine the amount of PrP^{C} in scrapie-infected versus uninfected biological materials.

An interesting example of an aptamer that may quickly find its way into molecular biological laboratories was provided by Dang and Jayasena [43]. They selected ssDNA aptamers that bind and inhibit several DNA polymerases. Various aptamers affecting different polymerases were combined to form heterodimeric aptamers that inhibit some of the most commonly used DNA polymerases, such as those from *Thermus thermophilus* or *Thermus aquaticus* [44]. When added to PCR reactions, the engineered aptamer simulates the so-called 'hot start' conditions. Hot

Figure 2



PrP^{C}-specific RNA aptamer motifs. **(a)** Secondary structure for two cellular prion protein (PrP^{C})-binding aptamers (motifs 1 and 2). Both structures are likely to contain a three-layered G-quartet scaffold, based on sequence conservation. The helical regions shown in the gray plane (i.e. to the back) can be deleted without significant loss of binding activity. **(b)** The recombinant GST–peptide fusion constructs used for mapping the aptamer-binding site on the prion protein. In binding assays, the fusion peptide construct encompassing the extreme amino terminus of PrP^{C} ($P_{23–52}$) was the only one that showed a defined and specific gel-shift in the presence of radiolabeled aptamer motifs 1 and 2 in nondenaturing agarose gels, indicating that the aptamer binds the protein at this position. Gray boxes indicate sites of the protein that are not recognized by the aptamer. Numbers indicate residue positions; the black box represents the region of the protein required for aptamer binding.

start PCR conditions are normally used to avoid the generation of undesirable amplification products resulting from the extension of nonspecifically annealed primers that are present at temperatures below the optimum for specific annealing (for example, during the heating period). This can be a serious obstacle in the amplification of low copy number targets in a background of nonspecific nucleic acids. In hot start PCR this problem can be eliminated by adding the polymerase when the sufficient reaction temperature has been reached; however, this process is prone to contamination and can become costly when handling large numbers of samples. The aptamer might overcome these limitations because it only inhibits the polymerase at temperatures below 40°C. At higher temperatures, the aptamer can no longer bind and inhibit the polymerase; the enzyme becomes active *in situ* and DNA polymerization commences.

In vitro selected ribozymes

Pioneers of the *in vitro* selection of novel ribozymes and deoxyribozymes from combinatorial nucleic acid libraries were joined by other groups who presented an impressive number of high quality studies in this field within the past twelve months.

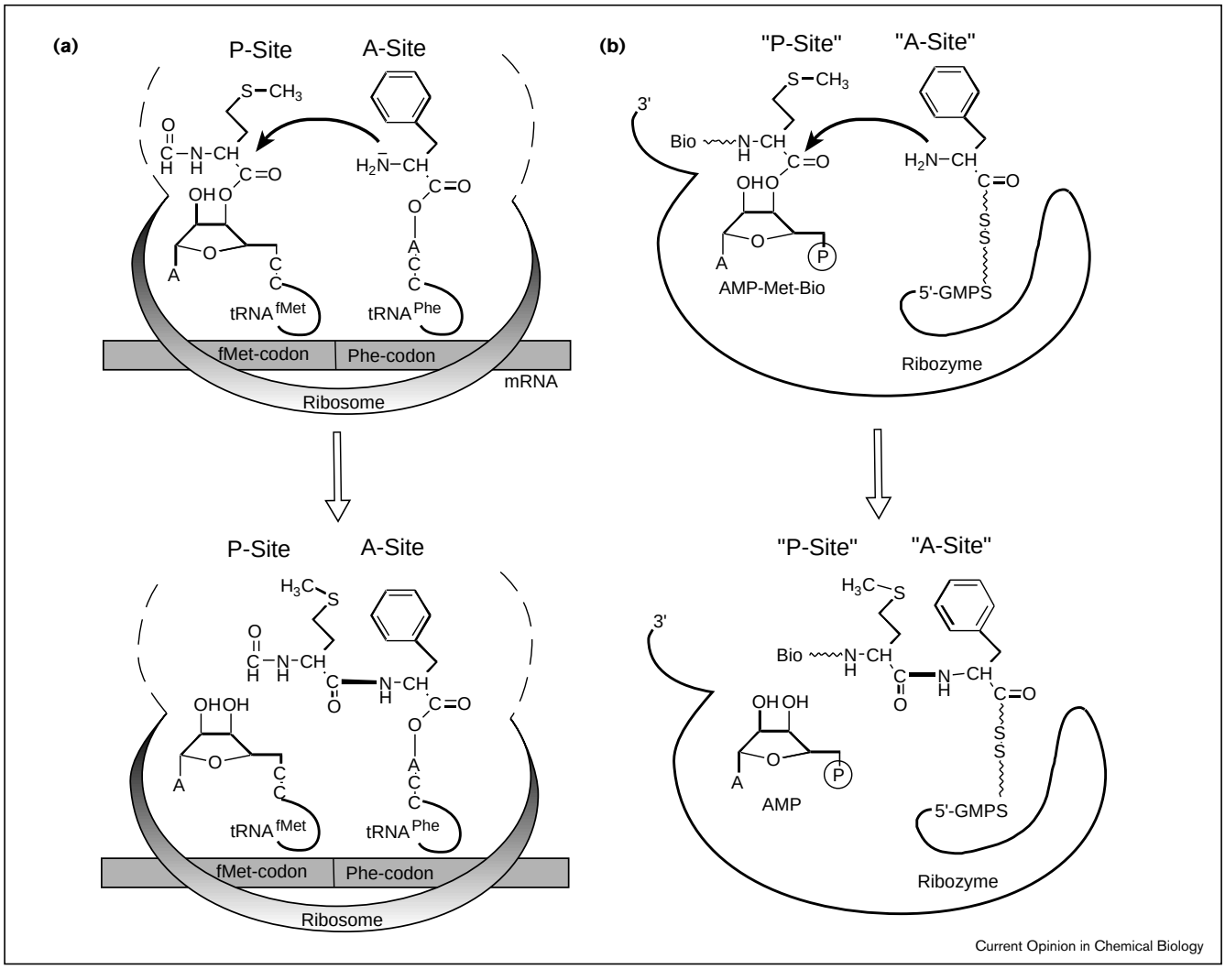
The latest results in this field demonstrate that ribozymes or deoxyribozymes are able to catalyze a wide range of chemical reactions. They are summarized in various comprehensive reviews [3,4,45–47,48•,49]. The most recent advances include ribozymes for the Diels–Alder reaction [50•], amide bond formation [51,52•], peptide bond formation [53••], aminoacyl transfer [51,54], novel oligonucleotide ligation activity [55], the formation of 5′-5′-diphosphate bonds [56] and RNA cleavage at low pH [57]. Vaish *et al.* [58] used a partially randomized version of the hammerhead ribozyme (HHR) to isolate HHR variants with altered core sequences. Of great importance for evolutionary RNA biotechnology and molecular evolution is a novel technique that allows the continuous evolution of ribozymes [59••]. This process allows for the amplification, mutation and selection of many ribozyme generations within a very short period of time. In some cases, new techniques for the selective chemical derivatization of RNA libraries have been developed and applied [60].

Zhang and Cech [53••] isolated a ribozyme that catalyzes the joining of two amino acids to form a peptide bond similar to the reaction catalyzed by the ribosome (Figure 3). They obtained two different sequence families of ribozymes and characterized the more active one in detail. Compared to an uncatalyzed model reaction in which the *N*-acetylated amino acid of an *N*-acetyl aminoacyl-tRNA is transferred onto glycine ethylester, the ribozyme achieves a rate enhancement of peptide bond formation of 1×10^{6}.

The scope of deoxyribozyme-catalyzed reactions [47] has also been enhanced in recent studies. Burmeister *et al.* [61] selected a phosphoramidate-cleaving catalytic DNA that requires a trinucleotide cofactor for the cleavage reaction. Other previously described deoxyribozymes were investigated mechanistically or characterized for requirements such as dependence on metal ions or pH [62–65].

Santoro and Joyce [66••] added a new deoxyribozyme to the various DNA enzymes previously selected in the Joyce laboratory. The new deoxyribozyme is able to hybridize to and cleave any RNA sequence under

Figure 3



Comparison of peptidyl transfer reactions in **(a)** a ribosome and **(b)** a selected ribozyme. The ribosome contains one binding site for the *N*-biotinylated aminoacyl donor substrate AMP–Met–Bio (the 'P-site') and one for the aminoacyl tRNA (the 'A-site'). **(b)** The binding site for the AMP–Met–Bio substrate is analogous to the ribosomal P-site. The attacking α-amino group, which is located in the ribosomal A-site, is covalently attached to the 5′ end of the ribozyme. Catalytically active RNAs provide themselves with a covalently attached biotin group and can thus be separated from inactive RNAs by streptavidin–biotin binding using streptavidin chromatography.

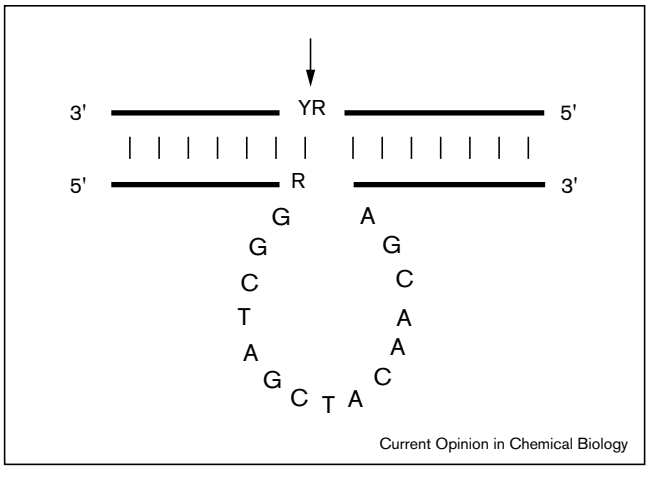
physiological conditions. The enzyme consists of a 15-mer catalytic domain that is flanked by sequences that can hybridize to the RNA target through Watson–Crick pairing (Figure 4). These flanking sequences can be designed *ad libitum*, making the deoxyribozyme a general purpose RNA-cleaving sequence. This catalytic DNA might be particularly useful in antisense technology for incorporation into antisense oligonucleotides to facilitate cleavage of the targeted mRNA.

By incorporating the ATP- or theophyllin-binding aptamer sequences [9•] into the HHR an allosterically regulatable HHR was engineered that carried out the phosphodiester cleavage of RNA only after the respective ligands had been added to the cleavage buffer [67•,68]. This study established that natural and *in vitro* selected ribozymes can be further engineered to respond to tight regulatory control. Considering the many different aptamers and ribozymes that are available, this approach considerably increases the potential for RNA to direct or control sophisticated metabolic processes.

Conclusions

Since the first examples of the use of combinatorial nucleic acid libraries for the *in vitro* selection of specific ligand-binding RNAs in 1990, considerable progress has been achieved in this field. Nucleic acid aptamers for more than a hundred different targets have been described, showing that aptamers can now routinely be obtained for almost every desired target. The international interest in RNA technologies is rapidly increasing. Various recent examples impressively illustrate the wide range

Figure 4



Cleavage of RNA by deoxyribozyme. The DNA enzyme (bottom strand) binds the RNA substrate (top strand) through Watson–Crick pairing. Cleavage occurs at the position indicated by the arrow. R represents adenine or guanine; Y represents uracil or cytosine. The Watson–Crick pairing parts of the enzyme can be chosen *ad libitum*, so that any desired RNA sequence can be targeted by the DNAzyme.

of applications for aptamers. The same also holds for catalytic nucleic acids. We expect to soon see examples of ribozymes and deoxyribozymes evolved for the catalysis of complex chemical transformations. There is enough reason to assume that such synthetic enzymes will be used as catalysts in organic syntheses. The novel catalysts not only support theories of an 'RNA world', in which the metabolism and replication of primitive organisms were controlled by RNA enzymes [69], but there is also promising potential for biotechnological, synthetic or diagnostic applications. Increasing numbers of examples appear in the literature that show that combinatorial nucleic acid libraries can be applied to facilitate our understanding of molecular mechanisms of all kinds of cellular processes, or of factors that are disease-related.

Acknowledgements

We thank E Weiss and G Mayer for critical reading of the manuscript and E-L Winnacker for support.

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