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Peptides, Proteins & Antisense

Catalytic DNAzymes: derivations and functions

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Background: Although catalytic RNA enzymes (CRzs) are naturally occurring in many organisms, their DNA counterparts (CDzs) were developed by *in vitro* selection/evolution from random sequence libraries. *Objective*: To provide a brief overview of how CDzs have been selected *in vitro*, and of their properties and functions, as well as their possible future utility. *Methods*: We concentrated on examples of 'direct' selection of CDzs. Many CDzs have been used in biological settings, for example downregulation of target mRNAs, while many more recent applications use CDzs in biosensor and nanotechnology settings. *Conclusions*: Although much work has concentrated on using CDzs for regulating gene expression, their potential as nucleic acid medicines has diminished substantially, supplanted by simple antisense oligonucleotides and, more recently, by small interfering RNAs (siRNAs). It seems unlikely that CDzs will have clinical utility. In contrast, they are likely to have significant potential in the sensor/nanotechnology arena.

Keywords: directed evolution, DNAzymes *in vitro* selection, Ribozymes, SELEX

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1. Introduction

A considerable body of evidence exists which can be construed as supporting the possibility that RNA may have catalyzed many of the reactions necessary for the existence of life's earliest organisms (an 'RNA World') [1,2]. Examples of catalytic RNA enzymes (CRzs) are numerous, and include: i) Natural RNA 'Ribozymes' such as, (a) self-splicing group I and group II introns [3,4]; (b) RNA component of RNase P [5]; (c) various self-cleaving RNAs, including 'hammerhead' or 'hairpin' motifs [6-9]; (d) RNA components of the large ribosomal subunit, which catalyzes the peptidyl transferase step of translation [10]; and (e) U2/U6 small nuclear (sn) RNA complexes within the eukaryotic spliceosome [11]. ii) CRzs developed by *in vitro* selection/evolution, for example, RNA-ligase CRzs [12,13]. iii) RNA-dependent RNA-polymerase CRzs, which can add nucleotides to the 3′-end of an oligonucleotide primer in a template-directed manner [14-16].

Mimicking the complexity of CRzs, catalytic DNA enzymes (CDzs) are single-stranded DNA molecules; CDzs have not been found in nature but have been created in test tubes. Due to varied conditions employed during selection *in vitro*, a substantially broader catalytic repertoire for CDzs has been found compared with natural CRzs. From the multiple nuclease CDzs (which cleave phosphodiester bonds) and ligase CDzs (which form phosphodiester bonds), the repertoire of catalytic functions has been expanded to include self-phosphorylating CDzs, self-capping CDzs, self-depurinating CDzs, peroxidase CDzs, and even T–T-repairing CDzs. Along with the expanding repertoire of catalytic capabilities, it becomes possible to envision the existence of a parallel 'DNA world'.

Aside from purely academic pursuits, scientists have often taken bits and pieces of proteins (and nucleic acids) from natural (and artificial) sources, tailored them by

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purposefully recombining them in different ways, and used them as reagents for biological studies or for therapeutic applications. In comparison with proteins, CDzs are normally smaller, more readily available, and easier to work with. Furthermore, the lack of a 2′-hydroxyl group in DNA relative to RNA does not reduce CDz enzymatic capabilities but rather provides advantages that include much easier synthesis and better resistance to chemical and enzymatic degradation. Accordingly, a great deal of work has been performed as described in several recent extensive reviews [17-22]. Here we focus mostly on CDz derivations by *in vitro* selection, and their characterizations, recognizing that direct selection approaches allow exploration of a wide range of chemical reactions, not necessarily with the aim of imitating natural enzymes but rather with the broader goal of understanding what is biochemically feasible regarding enzymatic capacities of CDzs.

2. Selection of CDzs by *in vitro* selection (vs evolution)

Directed evolution involves first establishing a heterogeneous population of macromolecules (genetic diversity) and then carrying out repeated rounds of selective amplification based on the physical or biochemical properties of those molecules [23]. With *in vitro* selection, there is a general elimination of non-catalytic sequences very early on, whereas with *in vitro* 'evolution' all sequences are initially retained but sequences are subsequently eliminated via internal competition within the pool. A critical aspect of directed selection or evolution is the application of an appropriate selection scheme to stringently distinguish molecules that have the desired properties from those that do not. Here, we have focused on what is generally referred to as 'direct' selection methodology; this method searches directly for a chosen catalytic event, and uses that activity to identify CDzs out of the random pool. A second approach is referred to as 'indirect' selection. The indirect selection approach is based upon preceding work for identification of catalytic antibodies [24,25]. Specifically, Cochran & Schultz [26] found that an antibody derived for binding a stable transition-state analog for the prophyrin metallation reaction, was able to form a hemin–antibody complex that enhanced the peroxidase catalytic activity of hemin significantly. Using the same strategy, others [27] used *in vitro* selection of aptamers, and identified an 18-nucleotide aptamer which formed aptamer–hemin complexes with peroxidase activity 250× greater than hemin alone. While this approach could have considerable value in identification of novel catalytic activities in future, it requires stable targets and has not been employed widely for *in vitro* selection in general.

2.1 Introduction of sequence diversity for beginning selections

Sequence diversity is introduced at the start of a directed selection (or evolution) protocol through the creation of a combinatorial library of semirandom macromolecules, which consist of central randomized single-stranded DNAs, generally flanked by fixed primer regions on each side. Direct selection protocols are often coupled with limited mutagenesis procedures to produce additional diversity at any round of selection by performing amplification under error-prone conditions or by carrying out separate mutagenesis or recombination procedures (see below).

2.1.1 *Random-sequence DNA libraries*

The simplest way to generate a combinatorial library of nucleic acids is to prepare random-sequence DNA molecules using an automated DNA synthesizer. The number of possible sequences for a nucleic acid of length (n) of random region is 4^n. Modern automated DNA synthesizers allow the synthesis of DNA up to about 120 nucleotides (although the yields deteriorate significantly for lengths > 100). Thus, allowing for primer binding sites of 15 – 20 nucleotides at each end of the DNA, the maximum number of random nucleotides that can easily be included within a synthetic DNA molecule is ~ 80. Two or more such DNA molecules can be assembled by overlap extension or ligation, although this usually requires a region of fixed-sequence nucleotides at the site of overlap or surrounding the ligation junction.

2.1.2 *Mutagenesis*

A starting pool in an *in vitro* selection protocol generally has a diversity of 10^{14} – 10^{15} sequences, which is only one out of 1 billion – 10 billion sequences, for a library containing 40 random nucleotides ($4^{40} = 1.2 \times 10^{24}$). Thus, many protocols include provisions to introduce mutations (or sequence diversity) during selection rounds to maintain heterogeneity in the population. The most common method for introducing sequence diversity during *in vitro* selection employs error-prone polymerase chain reaction (PCR). There are several standardized protocols that use either altered reaction conditions or a mutant thermostable DNA polymerase to promote the incorporation and extension of mismatched nucleotides. One procedure for mutagenic PCR achieves an error rate of 0.66% per nucleotide position with roughly equal probability of all possible transitions and transversions [28]. A second procedure is a 'hypermutagenic' PCR procedure that achieves an error rate of about 10% per nucleotide position with only modest sequence bias but it has a greatly increased risk of introducing amplification artifacts [29]. A third PCR-based procedure provides an error rate of up to 0.7% per nucleotide position over the course of the reactions [30]. This procedure generates a broad spectrum of mutations, although with a substantial preference for transitions over transversions. A second basic approach to expand library diversity (which may prove useful) uses terminal deoxynucleotidyl transferase [31], which extends random sequence regions by indiscriminately adding additional random nucleotides.

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2.1.3 *Recombination*

A different approach for the introduction of sequence diversity involves *in vitro* recombination, which is particularly useful for evolution of larger CDzs that are composed of multiple subdomains. Through either restriction digestion or primer extension at internal sites, the DNA can be broken down into component segments, which then can be reassembled in various combinations by either ligation or polymerase-mediated overlap extension. An important advancement in recombination technology was the development of 'sexual PCR,' which involves fragmenting the gene in a random manner, then allowing the fragments to serve as primers for each other to reassemble shuffled versions of the gene [32]. This technique does not require knowledge of the gene sequence and thus can be applied to entire populations of nucleic acid molecules rather than selected genes.

2.2 Direct selection based on catalytic function

Most of the reactions carried out with *in vitro* selection protocols involve either bond forming or bond breaking, with one reactive group attached to the population of potential catalysts and the other located on a separate substrate or product molecule. Typically, the separate molecule also contains a chemical tag, so that a bond-forming reaction results in joining of the tag to the catalyst, whereas a bond-breaking reaction results in detachment of the tag from the catalyst. A simple tag that is commonly employed in directed evolution is an oligonucleotide that can be distinguished on the basis of its size or ability to hybridize to a complementary nucleic acid. Alternatively, biotin tags also are commonly employed. For example, in a bond-forming reaction the active catalysts become biotinylated, allowing them to be captured using a streptavidin-coated support. In a bond-breaking reaction the molecules are prebound to streptavidin, and the active catalysts become detached from the biotin tag, thereby releasing them from the support.

The selected catalyst entails not just the catalytic activity but also the range of biochemical properties associated with that activity, including catalytic rate, substrate binding affinity, substrate specificity and preferred reaction conditions. All of these traits are selectable and can be refined by imposing the appropriate selection constraints. An adage in direct selection is that 'you get what you select for,' which refers to the totality of selection pressures, both intended and unintended, that are imposed on the evolving population of molecules. Some parameters of the selection conditions will almost certainly impose unintended/unrecognized consequences on the process. As selection proceeds, one may also choose to alter the reaction parameters, driving the population toward altered biochemical properties. For example, the reaction time may be decreased progressively to favor the development of molecules with faster catalytic rate (k_{obs} or k_{cat}), or substrate concentration may be reduced to favor an improvement in Michaelis constant (K_m).

2.3 DNA reamplification

Once a subset of the population has been selected, the selected molecules must be amplified to generate a progeny population that can be subjected to further rounds of selection. DNA molecules are amplified most conveniently using PCR, employing two oligonucleotide primers and a thermo-stable DNA polymerase. A substantial problem with these primer-binding sequences in the process of selection is that they may base pair with the central random fragments to interfere with the CDz functions, which can greatly limit the complexity of results from a standard selection.

To address this problem, methods have been created to reduce and/or eliminate the primer sequences from the selection-step, thus reducing (or eliminating) the non-specific binding interference caused by the primer sequences [33]. One of these methods, for example, used a specific genomic library with a number of steps (ligations, linear extensions and PCR amplifications) to add back the full primer sequences after selection [34]. We devised methods that allow either 'minimal-primer' or 'primer-free' selections; primer regeneration is performed after selection to allow for reamplification, followed by reelimination of primer sequences before the next round of selection, incorporating an efficient transcription step [33].

2.4 Analysis of the evolved populations

In practice, a direct selection experiment is considered completed when the desired catalytic properties have been attained, which involves carrying out a tracking assay to monitor the behavior of the evolving population. The sequence populations obtained are identified by molecular cloning and sequencing, and the individual sequences are tested for the desired catalytic activity. A sequence alignment usually helps to define a consensus sequence and to indicate conserved residues that are important for catalysis. Hypotheses regarding the essential composition of the catalytic motif can be tested by changing nucleotides at given positions or by deletion analysis. Most investigators carry out a reselection procedure in which a dominant clone or a molecule that corresponds to the consensus sequence is partially randomized and subjected to additional rounds of selection. The goal is generally to define more precisely the catalytic motif based on allowable sequence variation, rather than to seek further improvement in catalytic activity. Once a suitable catalytic motif has been defined, rational design approaches are often used to convert the '*cis*-acting' catalyst to a true enzyme that can operate on a separate substrate, producing multiple turnovers. It usually is straightforward to convert a self-modifying catalytic nucleic acid to one that operates on a separate substrate simply by dividing the catalyst at an appropriate phosphodiester linkage and leaving a few nucleotides attached to the reactive group.

Table 1. Nuclease DNAzymes.

Group	Name	Library	Round	Size (nt)	Cofactor	k_{cat} (min^{-1})	K_m
Embedded RNA-bond-cleaving	Pb^{2+}-dep.	N50	5	38	Pb^{2+}	1	2 μM
	E6	N40	6 + 7	36	Mg^{2+}	0.04	13 μM
	Mg5	N74	7 + 3	141	Ca^{2+}	0.1	6.4 μM
	Bipartite I	N40	12	107	Mn^{2+}	1.6	
RNA-cleaving	10 – 23	N50	10	31	Mg^{2+}	3.4	76 nM
	8 – 17	N50	8	31	Mg^{2+}		
	Bipartite II	N14	7	35	Mg^{2+}	1.4	230 nM
8 – 17 family	$Mg5_{8-17}$	N74	7 + 3	32	Mn^{2+}	> 3	
	17E	N50	12	32	Zn^{2+}	1.7	
	8 – 17-like	N20-43	7 + 3		Mn^{2+}		
UO_2^{2+}-dependent	39E	N50	10	52	UO_2^{2+}	1	
Sensor	DET22-18	N43	22	59	Co^{2+}	7	
	OAI-VI	N43	22 + 6	40	M^{2+}	0.2 – 1.6	
	pH 3 – 7	N70	24		M^{2+}	0.02 – 1.3	
	MgZ	N60	5 + 7	49	Mg^{2+}	1	
M^{2+}-independent	16b/c	N74	7 + 3		Na^+	0.0001	
	G3	N40	12		Histidine	0.002	
	Na8	N40	12 + 6		Na^+	0.007	
	HD2/3	N40	11 + 5	39	Histidine	0.2	
	614	N40	7 + 7		Na^+	0.0004	
DNA-cleaving	Class II	N50	8	34	Cu^{2+}	0.2	

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3. CDz derivations and characterizations

3.1 CDzs with nuclease activities (see Table 1)

3.1.1 RNA-cleaving (RNase) CDz

RNase CDzs catalyze a transesterification reaction in which a specific 2′-hydroxyl of the substrate attacks the adjacent phosphodiester bond, which leads to formation of two products, one of which contains a 2′,3′-cyclic phosphate; and other contains a 5′-hydroxyl group. Many different types of RNase CDzs have been reported; for convenience, here we arbitrarily group them into i) 'Embedded RNA-bond cleaving' CDzs; ii) RNA-cleaving CDzs; iii) 8 – 17 family CDzs; iv) Bipartite CDzs; v) UO_2^{2+}-dependent CDzs; vi) Sensor CDzs; and vii) divalent metal ion (M^{2+})-independent CDzs.

3.1.1.1 Embedded RNA-bond cleaving CDzs

In their seminal 1994 study, Breaker and Joyce reported the first catalytic DNA [35], in which five iterated selection rounds were used to identify a Pb^{2+}-dependent CDz that cleaved a single embedded ribonucleotide linkage within a DNA molecule. The Pb^{2+}-dependent CDz was found to interact with the single-RNA-linkage substrate using Watson–Crick binding arms on either side of the initially random enzyme region. The original Pb^{2+}-dependent CDz cleaved near its own 5′-terminus (cleavage in *cis*). reengineering it to work intermolecularly (cleavage in *trans*) was achieved in straightforward fashion by removing the covalent loop that connected the single-RNA-linkage substrate with the CDz; this CDz showed catalytic properties similar to those of the hammerhead CRz [36], suggesting that CDzs have no inherent functional limitations relative to natural CRzs. The 2′-hydroxyl group of the substrate alone appeared to contribute the catalytically relevant metal-binding site [37].

The next step taken was to select a CDz that was dependent on Mg^{2+}, an important divalent metal ion in biological systems [38]. After six rounds of selection, an initial CDz clone showed some activity with 1 mM Mg^{2+}, although it was 3-, 19- and 5-fold more active in the presence of 1 mM Mn^{2+}, Zn^{2+} and Pb^{2+}, respectively. Randomization was introduced at each nucleotide position within the catalytic core, and then seven additional selection rounds were performed. One of the optimized CDzs from the reselected pool, termed E6, showed good catalytic properties in the presence of 10 mM Mg^{2+}. In binary manner, the split E6 CDz could distinguish a DNA probe with single nucleotide mutation at 1 nM concentration [39].

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The third example of an embedded RNA-bond cleaving CDz was termed Mg5, a Ca^{2+}-dependent single RNA-linkage cleaving CDz, which was selected in the presence of Mg^{2+} and histidine [40]. Mg5 showed a k_{cat} of 0.01 min^{-1} in presence of Mg^{2+}, and a surprising k_{cat} of 0.1 min^{-1} in the presence of Ca^{2+} (even though Ca^{2+} was not in present during selection). The relative self-cleavage rate of Mg5 in presence of various divalent metal ions was $Pb^{2+} > Zn^{2+} > Cd^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$.

For the above three diavalent metal cation-dependent CDzs, the rate enhancement of k_{obs}/k_{uncat} was about 10^5, and none cleaved all-RNA-linkage substrates. These early studies clearly demonstrated that CDzs can use a variety of metal ion cofactors and can catalyze a biologically relevant reaction under conditions similar to those that exist within cells. Moreover, it was practical to create, through *in vitro* selection, RNA-cleaving CDzs that could be used in a therapeutic capacity (as an alternative to CRzs).

3.1.1.2 RNA-Cleaving CDz

In another seminal study, Santoro and Joyce [41] reported two DNA motifs, one of which has become the most commonly used RNA-cleaving CDz in both fundamental research and therapeutic applications [21]. A 12-nucleotide all-RNA substrate was presented to a random N_{50} DNA pool, and after eight and ten rounds of selection, cleavage was observed at two favored sites within the 12 nucleotide RNA region. The 8 – 17 and 10 – 23 CDzs (named for the round and clone number) were recast in an intermolecular cleavage format by separating the DNA catalytic domain from the RNA substrate. For 10 – 23 CDzs, the catalytic activity showed a k_{cat} of 3 min^{-1} and K_m of 0.8 nM ($k_{cat}/K_m = 4 \times 10^9$ M^{-1} min^{-1}) under the chosen *in vitro* conditions (50 mM Mg^{2+}). This k_{cat}/K_m value is 1 – 2 orders of magnitude higher than those for the naturally occurring hammerhead or hairpin RNA-cleaving CRzs, further demonstrating that DNA has no inherent catalytic inferiority relative to RNA. In addition, while the k_{cat} is much less favorable (by 10^4-fold) than the k_{cat} for a protein ribonuclease such as RNase A, the K_m is much more favorable (by 10^5-fold). Thus, by the k_{cat}/K_m criterion, CDzs are at least as good as, if not better than, protein enzymes at cleaving RNA.

Further study [42] showed that the 10 – 23 CDz kinetics were consistent with a chemical mechanism involving metal-assisted deprotonation of a 2′-hydroxyl of the substrate, leading to substrate cleavage [43]. Kinetic measurements revealed that the 10 – 23 strongly preferred cleavage of the substrate over ligation of the two cleavage products and that the catalytic efficiency was limited by the rate of substrate binding. The 10 – 23 CDz displayed a high level of substrate specificity, discriminating between RNAs that contained a single base mismatch within either of the two substrate-recognition domains (**Figure 1A**). With appropriate design of the substrate-recognition domains, the enzyme exhibited a potent combination of high substrate sequence specificity and selectivity, high catalytic efficiency and rapid catalytic turnover. Introduction of DNA into the binding helix of the substrate significantly increased the rate of chemical cleavage (k_{cat}) without interfering with substrate–CDz binding (K_m). Introduction of RNA into the binding helix of the CDz decreased the rate of chemical cleavage slightly but with a relatively lower rate of association [44]. Interestingly, phosphorothioate modifications at the nucleotide-positions 1 or 8 (**Figure 1A**) enhanced the catalytic efficiency. This modfication at position 5 also had the largest deleterious effect on catalytic rate in presence of Mg^{2+}, although this was reversed in presence of Mn^{2+}. In addition, it was found that the oxygen atom at C6 of position 6 (G) contributes to metal ion binding and that this interaction was essential for catalytic activity [45], although a recent report suggested that magnesium may not be directly involved in the catalytic process [46].

While downregulation of target genes via various antisense strategies is widely employed [47,48], the 10 – 23 CDz seems to be the only CDz that might find a use in clinical gene therapy [21]. This appears to reflect the relatively poor catalytic activity of the competing 8 – 17 CDz under physiological conditions, as well as the fact that nearly all preclinical data seem to have been developed with 10 – 23 CDzs.

It is recognized that efficient, targeted delivery is the major existing obstacle to successful deployment of CDz (as well as other antisense moieties). Despite the recognized (and substantial) delivery problems, much work has been directed toward finding chemical modifications in 10 – 23 CDz that might enhance its stability in a biological environment, although many other parameters must be considered. For example, a hairpin 10 – 23 CDz was developed, which showed remarkable resistance to nucleolytic degradation [49]; this incorporated stem-loop hairpins at either end of the CDz-binding arms without chemical modification. Introducing an 'intercalator' through a D-threoninol moiety to the 10 – 23 CDz, at the junction between its catalytic loop and binding arm, has been shown to improve the RNA cleavage rate by eightfold [50,51]. In addition, a 'photocaged' nucleoside has been incorporated into the 10 – 23 CDz; disruption of a single hydrogen bond at position 4 (**Figure 1A**) completely inhibited catalytic activity [52]. Restoration of CDz-activity was achieved through decaging with long-wavelength UV light, providing an excellent on/off switch for CDz activity.

3.1.1.3 The 8 – 17-family of CDz

Three subsequent studies revealed the remarkable convergence of the catalytic core motif of the 8 – 17 CDz across several selection experiments. Peracchi [53] performed a comprehensive mutagenesis analysis of 8 – 17 CDz and found that most mutants (as was the case with the parent CDz) functioned with 10- to 20-fold higher k_{obs} in the presence of Ca^{2+} instead of Mg^{2+}. In the same study, Mg5 CDz,

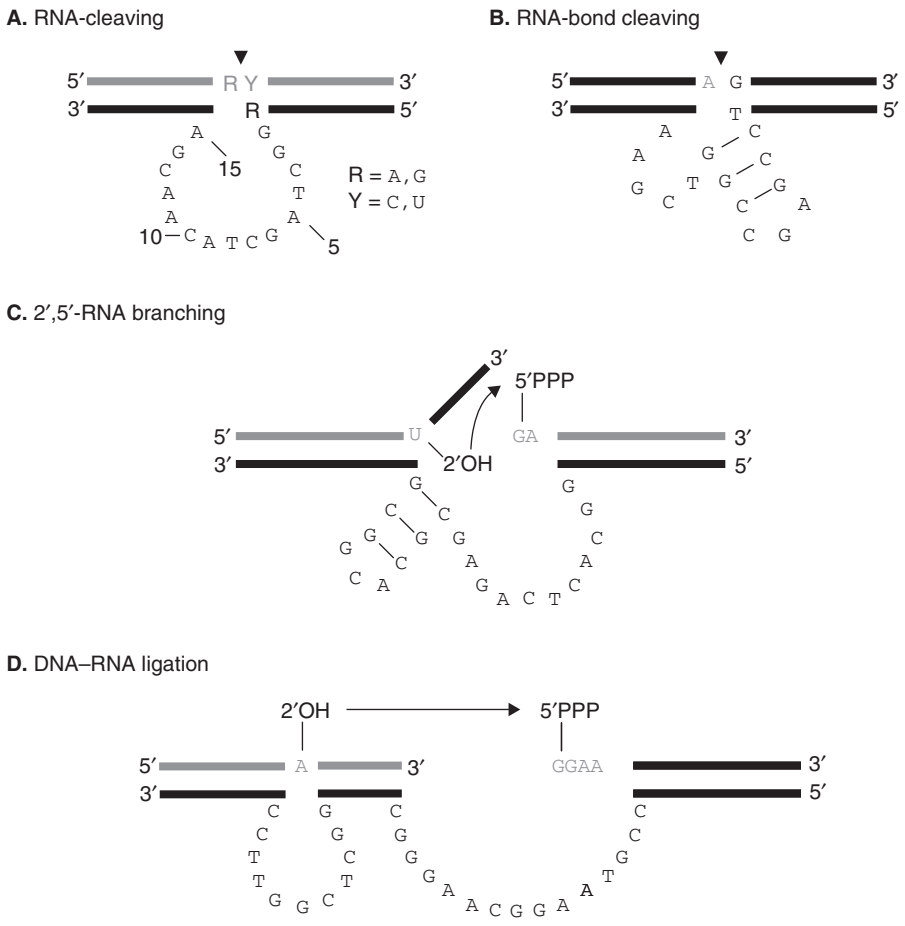

Figure 1. Some representative catalytic DNA enzymes. A. 10 – 23; **B.** 17E; **C.** 6CE8; **D.** 9FQ4. Grey letters and bars indicate RNA, and black letters and bars indicate DNA.

another Ca^{2+}-dependent CDz which had been selected in presence of Mg^{2+} [40,54], was also shown to incorporate the 8 – 17 motif within its conserved core. Secondly, Lu and co-workers [55] focused on finding CDzs that operate with transition metal ion cofactors like Zn^{2+} rather than alkaline earth metals like Mg^{2+} or Ca^{2+}. A Zn^{2+}-dependent CDz, termed 17E (**Figure 1B**), was isolated and was also found to contain an 8 – 17 like catalytic core. Although 8 – 17 cleaved only A↓G single RNA-linkages, 17E cleaved A↓G and G↓G equally well, and about an order of magnitude faster than it cleaved either U↓G or C↓G. Finally, an '8-17-like' CDz family was identified from 16 parallel *in vitro* evolution experiments. This family cleaved almost any single RNA dinucleotide junction [56]. This suggests that the 8 – 17 motif may be the simplest structure for cleaving single RNA linkages and therefore favored in DNA sequence space, just as the small hammerhead CRz motif is favored in RNA sequence space [57].

Even though the 8 – 17-like motif was obtained through *in vitro* evolution under substantially different conditions with Mg^{2+} (termed 8 – 17), Mg^{2+}/histidine (termed Mg5), Zn^{2+} (termed 17E), or Mg^{2+}/Mn^{2+} (termed 8 – 17-like), all variants were most active with Pb^{2+} [58]. The metal-binding affinity of the CDzs was $Pb^{2+} > Zn^{2+} > Mg^{2+}$, and a single deprotonation was the rate-limiting step of the reaction. While Mg^{2+} and Zn^{2+} catalyze only a transesterification reaction with formation of a product containing a 2′,3′-cyclic phosphate, Pb^{2+} catalyzes a transesterification reaction followed by hydrolysis of the 2′ 3′-cyclic phosphate bond.

Because of inefficient catalytic activity under physiological conditions, 8 – 17 CDzs have not been used to downregulate target genes, even though a photoactive 8 – 17 CDz-switch has been developed: For example, one switchable variant contained a photoactive C8-linked adenosine [59], while another utilized azobenzene-modified 8 – 17 CDzs [60]. However, 8 – 17 CDzs have been employed to generate more dynamic and controllable DNA-based nanostructures that are responsive to various chemical stimuli [20]. Lu's group [61] first reported an application of 17E CDz as a unique class of biosensors for Pb^{2+} ions, with a quantifiable detection range from 10 nM to 4 μM and a selectivity of > 80-fold for Pb^{2+} over other metal ions. Later, the 17E CDz was employed

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to obtain functional nanomaterials that were sensitive to chemical stimuli, and it was also immobilized onto carbon nanotubes or covalently attached to gold surfaces and gold-coated nanocapillary membranes (for reviews see [20,62]). Conventional methods to endow 8 – 17 CDz with fluorescent signaling properties usually involve covalent attachment of two dye molecules at nucleotide positions that are far away from the catalytic core, so that the bulky dye structures do not affect the CDz activity. However, the maximum fluorescence enhancement associated with such 8 – 17 constructs is typically ≤ 10-fold, due to a highly fluorescent background. Chiuman and Li [63] reported dyes attached to positions only one or two nucleotides away from the catalytic site were able to generate 15- to 85-fold signal enhancement within 10 min.

3.1.1.4 *Bipartite CDzs*

In 2001 Feldman & Sen [64] performed an *in vitro* selection and obtained a family of sequences containing a 22 nucleotide catalytic core (which was divalent-cation-dependent), which was referred to as Bipartite I CDz. Another *in vitro* selection was then performed for cleavage of long RNA substrates, using a mutagenized bipartite CDz pool, and this yielded another CDz which was referred to Bipartite II CDz; the catalytic core was unchanged, and the resulting CDz showed the ability to efficiently cleave long RNA targets, and had some properties analogous to those of the hepatitis delta virus CRz. Further studies have shown that bipartite CDzs represent metalloenzymes [65].

3.1.1.5 *UO_2^{2+}-dependent CDzs*

39E, a UO_2^{2+}-dependent CDz, was selected to cleave a single-RNA linkage in the presence of UO_2^{2+}. This CDz has been employed as a catalytic beacon sensor to detect uranium in water, where it showed a detection limit of 45 pM and a dynamic range up to 400 nM [66]. Furthermore, the backfilling method of immobilization was found to be most effective for preserving CDz activity, and it forms a firm basis on which practical applications of catalytic beacons can be realized, including sensors for both Pb^{2+} and UO_2^{2+} ions [67].

3.1.1.6 *Sensor CDzs*

The above-mentioned CDzs were engineered to perform fluorescence signaling by fluorescence-quenching/dequenching using existing CDzs that were not created specifically for sensing applications, and as a result the sensor molecules created were not optimized in catalytic rate or signaling properties to achieve high sensitivity. Li and co-workers [68] described the creation and characterization of a single RNA-linkage cleaving CDz that uniquely links chemical catalysis with real-time fluorescence signaling capability in the same molecule. A *trans*-acting form, DET22-18, behaved as a true enzyme with a k_{cat} of 7 min^{-1}, and cleaved a chimeric RNA/DNA substrate at the single RNA linkage; this was surrounded by a closely spaced fluorophore–quencher pair, a unique structure that permits the synchronization of the chemical cleavage with fluorescence signaling. The *cis*-acting form, DEC22-18, has a stem-loop structure and can be conjugated with DNA aptamers, such as ATP binding aptamer, to form allosteric CDz biosensors.

Notably, in an *in vitro* direct selection experiment only a handful of sequences survive the many rounds of stringent selection steps; these surviving species are nearly always the focus of interest, whereas the less competitive contenders are usually not further examined. Nevertheless, molecular species abandoned during the selection process might still represent a rich pool of catalytic motifs that are useful for examination of DNA's inherent catalytic abilities, and for design of molecular tools for practical applications. Li's group [69] reported a study of six sensor CDzs that appeared in the early rounds of selection of the CDz termed DEC 22 – 18, using the combined approaches of reselection, rational structural analysis, and reaction condition optimization. All six CDzs, designated OA I – VI ('once-abandoned'), were found to use a three-way junction as a common structural framework for catalysis. However, disparities observed in the conserved nucleotide positions and metal-ion selectivities pointed to distinct catalytic cores. The rate constants of the optimized CDzs were in the range of 0.2 – 1.6 min^{-1}, which were similar to those of other CRzs and CDzs. Three of the six OA CDzs were successfully immobilized and used for sensing of various metal ions [70]; one of them, OA-IV, was re-evolved from a low-branching catalytic module structure to high-branching nucleic acid structures; this illustrates the idea of deriving a rare structural motif by evolution of sequence variants from a given functional nucleic acid [71].

Li's group [72] also reported a group of pH-dependent CDzs that possess a synchronized single RNA-bond cleavage/fluorescence-signaling ability, while exhibiting wide-ranging metal-ion and pH dependencies. The individual CDzs had different metal-ion requirements, and were able to produce as much as a 12-fold fluorescence enhancement upon cleavage of the single RNA linkage. Most of them had a pH optimum coinciding with the selection pH and exhibited a catalytic rate constant of ~ 1 min^{-1} under optimal (selection) reaction conditions. Many of the selected CDz functioned efficiently even under harsh conditions (such as pH 3 and 4). These isolated signaling CDzs facilitated the characterization of catalytic DNAs [73,74] and the development of diverse CDz-based controllable bio-sensors [75]. In addition, a Mg^{2+}-dependent CDz, termed MgZ [76], showed a three-way junction structure, a cleavage rate of 1 min^{-1}, and 26-fold fluorescence enhancement. Two ligand-responsive sensors were rationally designed by linking an aptamer sequence to the substrate of MgZ. In the absence of the target the aptamer-linked substrate was locked into a conformation that prohibited MgZ from accessing the substrate, and in the presence

Table 2. DNAzymes with other catalytic activities.

Group	Name	Library	Round	Size (nt)	Cofactor	k_{cat} (min^{-1})	K_m
Self-phosphorylation	NTP-A2.1	N70	7		Ca^{2+}, ATP	0.01	3.3 μM
	DK1	N70	7		Mn^{2+}, ATP	2.8	13 mM
	DK2	N70	7		Mn^{2+}, GTP	0.8	5.6 mM
	DK3	N70	7		Mn^{2+}, GTP	0.2	
	DK4	N70	7		Mn^{2+}, GTP	0.3	
	DK5	N70	7		Na^{+}, GTP	0.1	
Self-capping	Class1	N70	22		Cu^{2+}, ATP	0.01	0.5 mM
Self-depurination	10FN10	N70	10	112	$NaIO_4$		
N-glycosylase	10-28	N85	10	128	Ca^{2+}	0.2	5.4 μM
T = T photo-repairing	UV1C	N40	25		UV	4.5	0.6 μM
Peroxidase	PS5.ST1	N76	12	33	Prophyrin	0.23	2.3 mM

of the target the aptamer released the substrate, which induced MgZ-mediated cleavage of the single RNA linkage.

Additional examples of sensing CDzs include a glucose oxidase/peroxidative CDz for detection of enzyme activity [77], a CDz for DNA quantitation [78], an AMP-sensing CDz-aptamer [79], use of CDzs as a bio-catalytic label for DNA detection [80], signaling CDzs which operate via quenching of fluorophores by divalent cations [81], and even CDzs 'tweezers' whose activity is followed by fluorescence resonance energy transfer [82].

3.1.1.7 *M^{2+}-independent CDzs*

To examine the roles of divalent metal ions in CDz catalysis, Faulhammer and Famulok [54] designed a series of selection conditions, and found a CDz, termed 16b/c, that showed a k_{cat} of 10^{-4} min^{-1} under Mg^{2+}/histidine free conditions. Geyer and Sen [83] also reported CDzs that functioned independently of divalent metal ions (M^{2+}). A CDz termed G3 was identified after 12 selections rounds (in 1 M NaCl, 50 mM HEPES, pH 7.0, at 23°C), which showed relatively modest catalytic activity (k_{obs} of 0.002 min^{-1}). Six additional reselection rounds were performed (without HEPES) to improve its catalytic activity. The CDz termed Na8 was identified with a higher k_{obs} of 0.007 min^{-1} (in 0.5 M NaCl). The M^{2+}-independence of Na8 CDz was convincingly demonstrated via a wide range of control measures and analytical assays: adding common divalent metal ions such as Mg^{2+}, Ca^{2+} or Zn^{2+} had no effect upon activity.

Since many protein enzymes produce dramatic rate enhancements using only the chemical groups that are furnished by their constituent amino acid, Roth and Breaker [84] selected CDz that used histidine as an active component for an RNA cleavage reaction. An optimized CDz, termed HD2, required L-histidine or a closely related analog to catalyze RNA phosphoester cleavage. Kinetic analysis indicated that a DNA-histidine complex may perform a reaction that is analogous to the first step of the proposed catalytic mechanism of RNase A, in which the imidazole group of histidine serves as a general base catalyst. In addition, a CDz termed 614, which was selected in the presence of Mg^{2+}, surprisingly displayed intermolecular catalysis which was independent of Mg^{2+} [85].

3.1.2 *DNA-cleaving (DNase) CDzs*

Breaker and co-workers [86] isolated two distinct classes of self-cleaving DNAs. Individual CDzs from class I required both Cu^{2+} and ascorbate to mediate oxidative self- cleavage, whereas individual CDzs from class II used Cu^{2+} as the sole cofactor. Further reselection of a class II catalyst yielded new catalytic DNAs that facilitated Cu^{2+}-dependent self-cleavage with rate enhancements exceeding 10^6-fold relative to the uncatalyzed rate of DNA cleavage. Thus, despite the absence of 2′-hydroxyls, single-stranded DNA can adopt structures that promote divalent-metal-dependent self-cleavage via an oxidative mechanism.

The class II CDz operated as a bimolecular complex that simultaneously made use of duplex and triplex interactions to bind and cleave separate DNA substrates with a k_{obs} of 0.2 min^{-1} [87]. The duplex and triplex recognition domains could be altered, making it possible to cleave single-stranded DNAs with different nucleotide sequences selectively. The 5′-cleavage product carried a 3′-terminal phosphate or phosphoester moiety, and maximum activity of this CDz was in 10 μM Cu^{2+} [88]. Most recently, a catalytic beacon sensor based on a DNA-cleaving CDz was described for paramagnetic Cu^{2+} ions in aqueous solution, which showed a sensitivity of 35 nM [89].

3.2 CDzs with other catalytic activities (Table 2)

3.2.1 *Self-phosphorylating CDzs*

Li and Breaker [90] isolated a series of CDzs with polynucleotide kinase-like activity, each self-phosphorylating

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CDz made use of one or more NTPs or dNTPs as a source of high-energy phosphate bonds. Although the prototypical CDzs only poorly differentiated between the ribose and deoxyribose moieties, further optimization by reselection produced variants that display up to 100-fold discrimination between related NTP and dNTP substrates. They identified one optimized ATP-dependent CDz, termed NTP-A2.1, which showed a k_{cat} of 0.012 min^{-1} in presence of 20 mM Ca^{2+}; this CDz utilized ATP > 40,000-fold more efficiently than CTP, GTP, or UTP, and operated with a rate enhancement of nearly 10^6-fold over the uncatalyzed rate of ATP hydrolysis.

Other selection approaches have been used to assess the influence of four common divalent metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}). For example kinase CDzs termed DK1 and DK2 were identified [91], which showed k_{cat} values of 2.8 and 0.8 min^{-1} respectively. Despite differences in their primary sequences and NTP requirements, ATP-dependent DK1 and GTP-dependent DK2 both possessed an analogous stem-loop element, which anchored a structural domain of substantial tertiary interactions [92]. The DK2, DK3 and DK4 kinase CDzs all utilized GTP as the source of activated phosphate, with Mn^{2+} as the divalent metal cofactor but they also shared a common secondary structure with significant sequence variations [93]. Multiple Watson–Crick helices were identified within secondary structures of these three CDzs. These sequence elements formed the catalytic core for GTP binding, and the self-phosphorylating reaction. The self-phosphorylating CDz DK5 was found to contain a unique structure consisting of a two-tiered guanine quadruplex interlinked to a Watson–Crick duplex [94].

3.2.2 *Self-capping CDzs*

A series of ATP-dependent self-capping CDzs have also been identified [95], which catalyzed the transfer of the AMP moiety of ATP to their 5′-terminal phosphate group, thereby forming a 5′,5′-pyrophosphate linkage analogous to that found in mRNAs. A class 1 CDz required Cu^{2+} as a cofactor and adopted a structure that recognized both the adenine and triphosphate moieties of ATP or dATP. The catalytic efficiency for this CDz was 0.0048 min^{-1} using either ATP or dATP.

3.2.3 *Self-depurinating CDzs*

A self-depurinating CDz has been identified that mediated site-selective depurination of its 5′-terminal guanosine nucleotide using periodate (IO_4^-) as an obligatory cofactor [96], which eventually formed an amine-reactive functional group at the 5′-end of the CDz.

3.2.4 *N-glycosidic-bond-cleaving CDzs*

Joyce and co-workers developed the 10 – 28 CDz [97], which catalyzed the site-specific depurination of DNA with a catalytic rate enhancement of about 10^6-fold. The reaction involves hydrolysis of the *N*-glycosidic bond of a particular deoxyguanosine residue, leading to DNA strand scission at the apurinic site. The 10 – 28 CDzs had an absolute requirement for a divalent metal ion (in relative order Ca^{2+} > Mg^{2+} > Mn^{2+} > Ba^{2+}) and exhibited an optimal k_{cat} of 0.2 min^{-1} in presence of Ca^{2+} (~ pH 5). Identification of the 10-28 motif demonstrated that single-stranded DNA, like RNA and proteins, can form a complex tertiary structure and catalyze an important biochemical reaction.

3.2.5 *T–T bond-cleaving CDsz*

An unexpected capability of nucleic acid enzymes is photochemistry. Sen and Chinnapen [98] reported a small, cofactor-independent CDz, termed UV1C, which was capable of repairing thymine dimers in a DNA substrate, optimally with light at a wavelength of > 300 nm Kinetic analysis yielded a k_{cat} value of 4.5 min^{-1} and a K_m of 0.58 ± 0.14 μM. Both the measured k_{cat} and K_m compared favorably with those of a catalytic antibody for thymine-dimer photo-reactivation [99]. Recognition between substrate and UV1C appeared to involve a significant degree of tertiary interaction because only modest stretches of Watson–Crick complementarity was found between them. The authors proposed that a guanine quadruplex functioned both as a light antenna and as an electron source for the repair of the substrate within the enzyme-substrate complex. Contact-crosslinking and guanosine to inosine mutational studies revealed that the thymine dimer and the guanine quadruplex were positioned close to each other in the CDz–substrate complex; this permitted refinement of the structure of the folded CDz so that it was able to repair uracil dimers as well as thymine dimers [100].

3.2.6 *Peroxidase CDzs*

Porphyrin-binding DNA aptamers [101] have been isolated to catalyze porphyrin metallation [102]. The aptamers possessed the ability to enhance peroxidative activity; the V_{obs} of the PS2.M (an 18-nucleotide oligomer)-hemin complex was 250 times greater than that of hemin alone [103]. The hemin-binding affinity of rPS2.M, an RNA version of PS2.M, was 30-fold weaker than that of PS2.M but it clearly indicated that it is possible for RNA and DNA of the same sequence to fold and form similar cofactor-binding sites and to show similar catalytic behavior [104]. The sequence studies showed that the interaction of specific guanines of PA2.M with the bound hemin cofactor might contribute to the superior peroxidative activity of the PS2.M–hemin complex [105]. Further investigations indicated that the PS2.M–hemin complex cleaved the t-butyl hydroperoxide through a hemolytic mechanism [106].

Willner and co-workers reported a study which utilized a peroxidase CDz [107] for amplified chemiluminescent surface detection of DNA and telomerase activity. The detection limit for DNA was 1×10^{-9} M, and telomerase from HeLa cells was detected with a limit of ~ 1000 cells. These authors also described a method in which proteins, modified

Table 3. Ligase DNAzymes.

Group	Name	Library	Round	Size (nt)	Cofactor	k_{cat} (min^{-1})
DNA ligase	E47	N116	9	47	Zn^{2+}, Cu^{2+}	0.07
	C14, C15	N90	11		None	0.04
2′,′-RNA ligase	9A12	N40	9		Mg^{2+}	0.07
	8BG, 12BK	N40	8, 12		Mg^{2+}	0.01
3′,5′-RNA ligase	8AY13	N40	8		Mg^{2+}	0.03
	9BX	N40	9		Mn^{2+}	
	9DB1	N40	9		Mg^{2+}	0.04
	7DE5	N40	7		Mn^{2+}	0.02
2′,5′-RNA branching	9F	N40	9		Mn^{2+}	2.2
	6BX22	N20	6	39	Mn^{2+}	0.07
	6CE8	N20	6	39	Mn^{2+}	> 5
	7S11	N37	7	51	Mg^{2+}	0.6
	10M24	N22	10	55	Mg^{2+}	0.3
RNA–DNA ligase	9FQ4	N22	9	55	Mg^{2+}	

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with the peroxidase CDz, acted as biosensors. Glucose was detected with a limit of 5×10^{-6} M, and thrombin was detected with a limit at 10^{-8} M [108]. They also linked sequence-blocked peroxidase-CDz to PCR primers [109], and were able to detect M13 phage DNA at 10^{-18} M. Most recently, they conjugated an anti-AMP/lysozme aptamers with the peroxidase-CDz, and were able to detect AMP at 10^{-6} M and lysozyme at 10^{-13} M [110].

3.3 Ligase CDzs (Table 3)

3.3.1 DNA-ligation CDzs

In another seminal report, Cuenoud and Szostak [111] described a DNA-ligase CDz, termed E47, which was selected with Mg^{2+}/Zn^{2+} from a N116 library. This catalyzed the formation of a new phosphodiester bond by the condensation of the 5′-hydroxyl of one oligodeoxynucleotide and a 3′-phosphorimidazolide on another oligodeoxynucleotide, and showed multiple turnover ligation activity with a k_{cat} of 0.07 min^{-1} in presence of Zn^{2+} or Cu^{2+}.

Ellington's group [112] described another DNA-ligase CDz, termed C14, which was identified by selection in the presence of Mg^{2+}. The C14 CDz catalyzed the chemical ligation of DNA molecules by terminal phosphorothioate displacement of a 5′-iodine in the presence of Mg^{2+}. During further selection, the pool was successively selected with five oligonucleotide substrates, each of which terminated in the same hexanucleotide sequence. This protocol identified additional ligase CDzs which could use all five substrates, albeit to different degrees, and they appeared to form secondary structures that allowed differential pairing between the CDz and each substrate. These results suggest that early replicases might have been able to bind a variety of oligonucleotide substrates while catalyzing ligation via a common junction [113]. In order to determine if CDzs can similarly be activated by effector molecules, they appended an antiadenosine aptamer to a selected ligase CDz. The resultant constructs were specifically activated by ATP, and ligation was largely via a template-specific mechanism [114].

3.3.2 RNA-ligation CDzs

Silverman and co-workers have developed all of the RNA-ligation CDzs that include: i) 2′,5′-RNA ligase CDzs termed 9A [115] and 8B [116]; ii) 3′,5′-RNA ligase CDzs termed 8AY [117], 9BX [118] and 9DB1/7DE5 [119]; and iii) 2′,5′-branching CDzs termed 9F [120,121], 6CE8 (**Figure 1C**), 6XB22, and 7S11. The characteristics and applications of these RNA-ligation CDzs have been recently reviewed [22]. It is interesting to note that 10M24 CDz (a variant of 7S11; [122]), can catalyze labeling of RNA [123], building a selective small-molecule binding site at the 5′-end [124]. Another variant of 7S11, termed 9FQ4, catalyzed ligation of DNA to RNA (**Figure 1D**), and it has been useful in studying the relationship between function and structure of hammerhead CRzs [125].

4. Conclusion

CDzs have been identified by varied *in vitro* selection/evolution protocols for catalyzing multiple biochemical reactions. Such studies began in 1994, so the relatively recent advent of catalytic DNA research might explain why CDzs have not been found in living systems, although their existence remains possible. Clearly, however, DNA has a considerable penchant for functional versatility. In

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fact, the limits to the recognized functional repertoire of the presently known CDzs probably simply reflects the inherent limits of the strategies thus far used for their identification. Thus, a 'DNA world', albeit highly unlikely in terms of natural history, is not difficult to envision for engineering purposes, and there appears to be a growing trend toward the creation of sophisticated molecular systems using CDzs.

5. Expert opinion

DNAzymes represent convenient model systems in which the structural and chemical principles of nucleic acid catalysis can be explored. Moreover, the discovery of an increasing number of these molecules, with novel activities and improved functions, is offering many uses for catalytic DNAs as reagents for molecular biology, biotechnology and nanotechnology.

With regard to their potential deployment in biological therapy, most work with CDzs has focused on use of antisense sequences flanking the catalytic core, for trans-acting cleavage of target RNAs. Such CDzs clearly can act in the same manner as antisense oligonucleotides (ASO), while offering the potential advantage of cleavage of multiple targets per CDz. On the other hand, the CDzs are larger than their ASO counterparts, increasing the difficulties in delivering them effectively (as well as in delivering equivalent quantities). While there remain some pockets of optimism regarding therapeutic potential of nucleic acid medicines [126], much of the interest in use of CDzs for therapeutic purposes has been supplanted by interest in use of small interfering RNAs (siRNAs, or their counterpart short hairpin RNAs; see [127]) or even microRNAs therapeutically. siRNA strategies offer the major advantage of targeting mRNAs specifically using cellular machinery to amplify the effects greatly. Such amplification can use cellular machinery such as the RNA-induced silencing complex for target cleavage, and/or sequestration in cytoplasmic P-bodies. Deployment of siRNAs therapeutically also faces delivery issues similar to CDzs but in addition poses other potential difficulties [128]. For example, siRNAs are much more susceptible to nucleolytic degradation; to ameliorate this problem, a number of groups are defining modifications which retain siRNA activity while affording stringent protection against degradation. siRNAs can also activate the innate immune system via interactions with Toll-like receptors, a problem that has complicated many early *in vivo* studies, although many groups are working on solutions. In our view, while a few CDzs may find their way through the early clinical trials maze, it seems unlikely that CDzs will constitute a major approach for biological therapy.

On the other hand, we envision the identification of DNA with new functionalities, including self-replicating CDz, based on selection with small molecules, proteins and other desirable targets as substrates, as well as development of a wide panoply of new biosensors based on catalytic activities of small DNAs. Such CDzs are likely to be robust (much more so than proteins, for example), enabling their use in bottom-up chip-based settings, and their selection can be performed under arbitrarily defined conditions, potentially allowing them to function in harsh environments.

Declaration of interest

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•• **A review of *in vivo* applications of small interfering RNAs.**

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