DNA Catalysis: Potential, Limitations, Open Questions

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Introduction

The "RNA world" hypothesis, initially formulated in the 1960s, states that life evolved from some protobiotic system in which RNA molecules were capable of self-replication and of a rudimentary form of metabolism.[1] The hypothesis is consistent with circumstantial evidence, and it was strongly supported by the discovery, in the early 1980s, of catalytic RNA molecules (ribozymes). In fact, it is now known that catalytic RNAs play key roles even in extant organisms, where several crucial processes—including RNA splicing and protein synthesis—are carried out by ribozymes.[1,2] RNA appears to be a plausible candidate as the progenitor biopolymer, since it can both carry genetic information and assume a great variety of tertiary structures and, hence, of functions.

However, other RNA-like polymers could, in principle, play the same dual role, most notably DNA, which also is a fundamental component of modern living organisms. Is it then possible to hypothesize that a "DNA world" existed or that it could exist? Or, if RNA (and not DNA) is really at the origin of life, does this mirror simply an accident of evolution or does it hint at some more profound, inherent differences between the two polynucleotides? These kinds of questions have begun to be addressed experimentally in recent years, during which time a large amount of research has shown that single-stranded DNA, much like single-stranded RNA, can fold into structures capable of molecular recognition and catalysis.[3–6]

This Minireview summarizes our current understanding of the catalytic capabilities of DNA, highlighting the theoretical and practical implications of this topic and stressing some of the questions that remain open in the field.

Known Catalytic DNAs Come from in vitro Selection

The catalytic potential of DNA has been revealed and explored through use of a combinatorial technique known as "in vitro selection", which has allowed the isolation of many DNA enzymes (deoxyribozymes) that carry out a variety of chemical transformations.[3–9] In vitro selection is an approach that mimics natural selection in a chemical setting.[10,11] It exploits the creation of large libraries of DNA sequences, together with the possibility of amplifying a tiny subset of selected molecules through the polymerase chain reaction (PCR). An example of in vitro selection strategy is sketched in Figure 1.

The first step of an in vitro selection experiment is the synthesis of a pool of semirandomized DNA molecules. Such molecules typically contain two fixed sequences at the 5′ and 3′ ends (required for amplification) bracketing a random stretch of 20 to >200 nucleotides. Initial libraries containing up to 10^{16} individual DNA molecules can be synthesized; this large size should offer a reasonable chance that sequences possessing the ability to catalyze a given reaction will be represented in the pool.

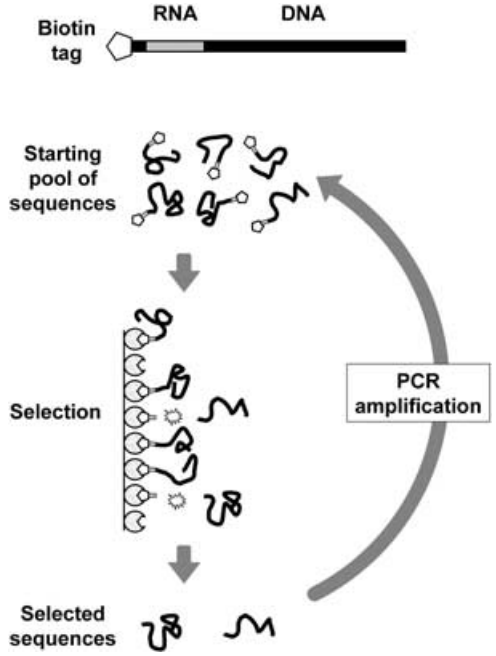

Figure 1. A schematic example of an in vitro selection experiment, designed to search for RNA-cleaving DNA motifs.[16] An initial population of single-stranded oligonucleotides is produced by solid-phase synthesis. The oligonucleotides contain DNA (in black; the sequence of the DNA region is mostly random) and a short stretch of ribonucleotides (in gray) near to the 5′ end, which bears a biotin tag (white pentagon). This population is loaded onto a streptavidin-coated matrix, to which the oligonucleotides become stably attached. The matrix is incubated under specific ionic conditions, and the rare molecules that can cleave the RNA region detach themselves from the matrix and can be eluted. These molecules are amplified by PCR and subjected to a new round of selection. The cycle is repeated until catalytic activity can be detected through biochemical assay in the pool of selected molecules. Many of these molecules can then be cloned, sequenced, individually tested for function, and further optimized.

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Subsequently, the library is challenged to select those molecules that perform the desired reaction. This selection step must be very carefully devised, as it requires effective means for the physical separation of functional molecules from the overwhelming majority of nonfunctional ones. Once such separation is achieved, the selected molecules are amplified by PCR, and the product DNAs can be subjected to a new round of selection–amplification. The cycle is repeated a number of times, while often making the selection conditions more and more demanding. In a successful experiment, the final population is composed of oligonucleotides that are particularly proficient in the desired catalytic function.

The strategy shown in Figure 1 is designed to select for deoxyribozymes with RNA-cleavage activity. Indeed, many in vitro selection studies have focused on the identification of DNA motifs that cut RNA phosphodiester linkages, mainly because RNA-cleaving catalysts are especially interesting from an applications viewpoint (see below).[6,9] The first deoxyribozyme ever described, in 1994, was a lead-dependent catalyst capable of cleaving a single RNA phosphodiester linkage embedded in a DNA molecule.[12] In the following years, further searches led to the identification of deoxyribozymes that cleave RNA phosphodiester bonds in the presence of Mg^{2+} or Ca^{2+}[13–19] or even in the absence of divalent metal ions.[20,21] Some of these catalytic DNAs are shown in Scheme 1.

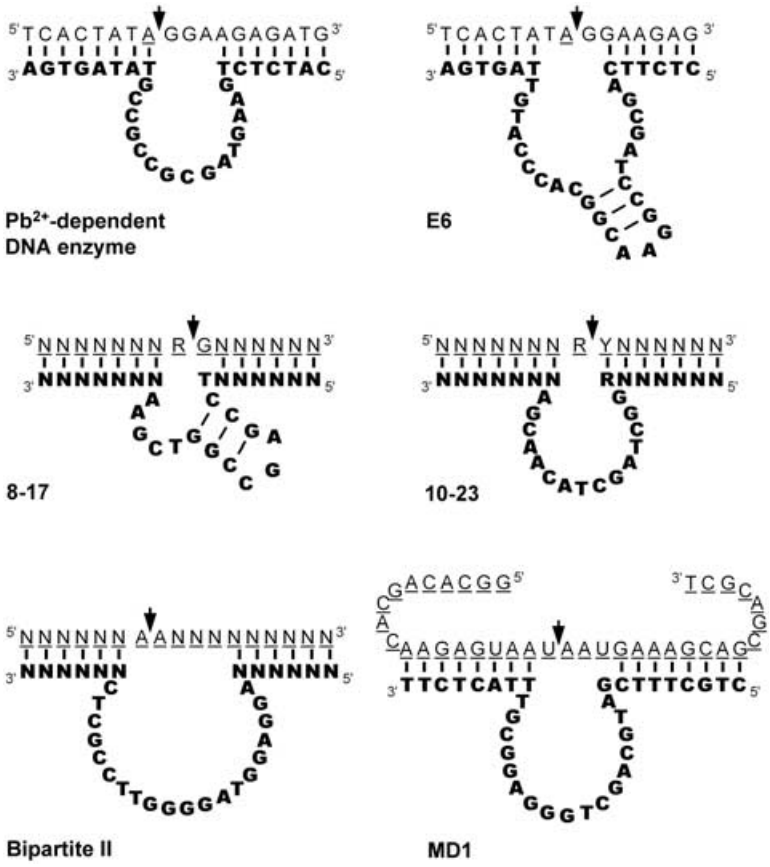
In addition to RNA-cleaving DNAs, many other types of deoxyribozymes have been isolated and described. A representative list is provided in Table 1.[22–32] All this research has clearly shown that DNA can be a versatile catalyst, in spite of its limited chemical arsenal (for example DNA, like RNA but in contrast to proteins, does not contain thiolic groups or side chains with an unperturbed $pK_a \approx 7$). In turn, the ability of DNA to carry out many mechanistically diverse reaction types suggests that deoxyribozymes have access to a variety of effective catalytic strategies.

Limits of Known Catalytic DNAs and Structural Differences between DNA and RNA

Even though the catalytic versatility of DNA is well established, no deoxyribozyme has ever been isolated from a living organism, in striking contrast to the widespread occurrence of ribozymes in nature. Furthermore, the catalytic DNAs obtained by in vitro selection appear generally less efficient than the corresponding "natural" ribozymes. Perhaps the most pertinent example is provided by the "10–23" deoxyribozyme (Scheme 1), which catalyzes an RNA-cleavage reaction analogous to those performed by several natural ribozymes, including the so-called hammerhead. 10–23 is considered the most efficient RNA-cleaving deoxyribozyme and was purposely selected to function under "physiological" conditions (pH 7.5, 37 °C, Mg^{2+} concentration in the low millimolar range).[16] Nevertheless, when assayed under such conditions, the deoxyribozyme shows a $k_{cat} \approx 0.1\ min^{-1}$, whereas natural hammerhead isolates exhibit turnover numbers about 100 times larger.[22,33,34]

Are these observations simply accidental, or do they imply that DNA is intrinsically less apt than RNA for catalysis? One possibility is that the small chemical differences between the two polynucleotides might disfavor DNA enzymes with respect to RNA enzymes. These differences are universally known: in DNA, 2-deoxyribose replaces ribose, and the thymine base replaces uracil. While the latter feature is not expected to have any momentous functional implication, the absence of the 2′-OH groups seems much more consequential. It has an impact on reactivity, since the hydroxyl could act directly (e.g., as an intrinsic nucleophile) in specific reaction mechanisms. Moreover, the lack of 2′-OH group affects the conformation of DNA helices (Figure 2) and could limit their assemblage into functional structures.

The ability of RNA to catalyze chemical reactions relies on its capacity to fold into three-dimensional structures, in which helical segments assemble through tertiary interactions usually provided by



Scheme 1. Some representative deoxyribozymes with RNA-cleavage activity.[12,13,16–18] These enzymes catalyze a transesterification reaction in which a specific 2′-hydroxyl of the substrate attacks the adjacent phosphodiester bond; this leads to formation of two products, one of which contains a 2′,3′ cyclic phosphate. The DNA enzymes are depicted in bold letters, and their substrates are in thin letters; ribonucleotides are underlined. N represents any nucleotide, while R indicates a purine and Y a pyrimidine. The arrows show the sites of cleavage.

Table 1. Examples of reactions catalyzed by in vitro-selected deoxyribozymes.

Reaction type	Deoxyribozyme name[a]	Size [nt]	Reaction conditions	k_{cat} or k_{obs} [min^{-1}]	Ref.
RNA cleavage (intramolecular transesterification)	10–23	31	2 mM Mg^{2+}, pH 7.5, 37 °C	0.1	[22]
RNA ligation (5′-triphosphate substrate)	8AY13	50	160 mM Mg^{2+}, pH 9.0, 37 °C	0.027	[23]
2′,5′ RNA branch formation (5′-triphosphate substrate)	7S11	47	40 mM Mg^{2+}, pH 9.0, 37 °C	0.54	[24]
Porphyrin metallation (Cu^{2+} and protoporphyrin IX substrates)	PS5.M[b]	24	40 mM K^{+}, 50 mM Tris, pH 6.2, 15 °C	1.3	[25]
Oxidative DNA cleavage	class II DNAzyme	28	30 μM Cu^{2+}, pH 7.0, 23 °C	0.2	[26]
DNA phosphorylation	DK1	69	30 mM Mn^{2+}, pH 7.0, 23 °C	2.8	[27]
DNA ligation (3′-phosphoimidazole substrate)	E47	47	4 mM Zn^{2+}, pH 7.2, 25 °C	0.07	[28]
DNA adenylation (capping)	class I adenylating DNAzyme	41	10 mM Mg^{2+}, 10 μM Cu^{2+}, 400 mM Na^{+}, pH 7.0, 23 °C	0.005	[29]
DNA deglycosylation (depurination)	10–28	93	2 mM Ca^{2+}, pH 5.2, 25 °C	0.018	[30]
thymine dimer photoreversion	UV1C	42	240 mM Na^{+}, pH 7.0, RT[c]	4.5	[31]

[a] The list includes only deoxyribozymes that do not use organic cofactors or contain "unnatural" nucleotides. For each reaction, only one well-characterized motif is cited. [b] The same deoxyribozyme, complexed with a Fe^{III}-protoporphyrin derivative, was shown to possess a modest but significant peroxidase activity.[32] [c] The reaction is a photochemical process and was measured under 305 nm light (3.4×10^{-9} einsteins min^{-1}).

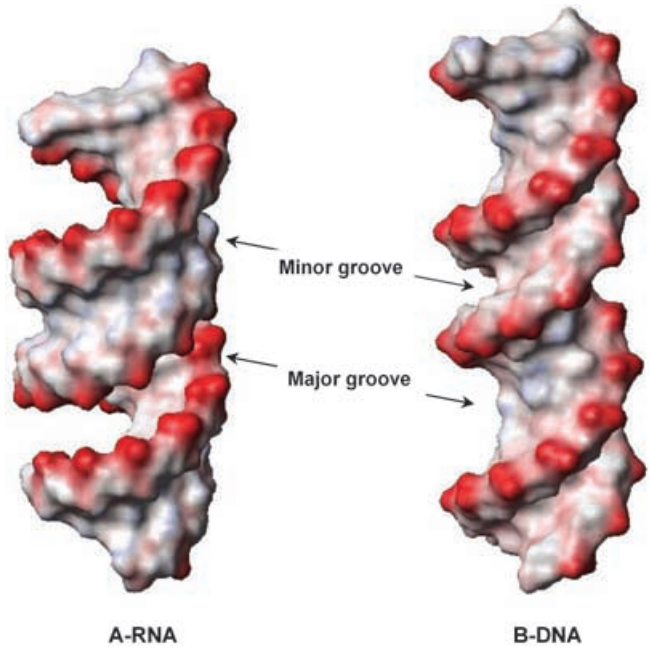


Figure 2. The structures of two double-stranded nucleic acids with identical sequences: A-RNA and B-DNA. The picture shows the water-accessible surface of the two helices; atoms contained in the surface are colored according to their partial charges. The typical B-form double helix of DNA is thinner than the A-form helix seen in RNA, it shows a much wider major groove, a narrower and deeper minor groove, and a reduced distance between adjacent phosphates.

loops or unpaired nucleotides,[35,36] so as to create well-defined binding and active sites. For DNA, the lack of a 2′-OH group will affect the formation of such tertiary structures in at least two ways. First, DNA cannot employ this group to form stabilizing interactions, such as in the so-called "ribose zippers", that are recurring motifs in RNA tertiary structures.[37] Second, the reduced accessibility of the B-DNA minor groove (Figure 2) could impede the formation of other specific interactions common in folded RNAs, such as the docking into the minor groove of unpaired adenine bases.[38]

Yet these differences simply mean that the tertiary structures accessible to DNA (and the underlying interconnecting motifs) must be different from those observed in RNA. Although our knowledge of tertiary DNA structures is currently very limited compared to our knowledge of RNA structures, there is no compelling reason to assume that the two polynucleotides differ significantly in terms of structural versatility.[39] For example the major groove of B-DNA is more accessible to ligands than the major groove of A-RNA and might thus represent a preferred environment for tertiary interactions. Note also that DNA can assume, in addition to the canonical B-helix, a variety of other regular and irregular secondary conformations, including triple-stranded and quadruple-stranded forms.[40] Indeed, some deoxyribozymes appear to contain triple-stranded regions and stacked G quartets.[25,26,29,31]

Size Matters: Limits of the in vitro Selection Technique

While the catalytic efficiency of known deoxyribozymes seems significantly lower than that of natural ribozymes, it is generally comparable to the efficiency of other catalytic RNAs discovered by in vitro selection.[18] This raises the possibility that in vitro selection is not as effective as natural selection in identifying good catalysts. The technique has several limits: for example, during selection the allowed reaction time cannot usually be shortened below a few seconds; furthermore, selected catalysts are optimized for single-turnover (rather than multiple-turnover) performance.[9] One limit that might be even more relevant, however, is the practical difficulty in selecting catalytic DNAs of large size.

It is commonly assumed that the more efficient or more difficult catalytic tasks demand extended catalytic domains. Large structures, held together by extensive networks of stabilizing interconnections, might help to form highly refined active sites, allowing an optimal positioning of reacting groups and cofactors. While the most efficient natural ribozymes (e.g., the self-splicing group I introns) comprise hundreds of nucleotides, nearly all catalytic DNAs isolated to date are much smaller

(Table 1) and may lack the structural robustness required to achieve a compact and fully catalytic structure.[41,42]

Unfortunately, when it comes to long molecules, in vitro selection experiments cannot exhaustively explore sequence space (i.e., the set of all possible sequences of a given length). For example, there are $\sim 10^{60}$ possible variants of a random 100-nucleotide-long DNA sequence. To include all these variants, the starting library for an in vitro selection experiment would have a total mass approaching that of a galaxy. Instead, within the practically achievable size of a starting pool ($\leq 10^{16}$ molecules), and even when such pool is formed by relatively long molecules, sequence space will be explored to a significant extent only for short sequences and only small catalytic domains will have a high probability of being selected and amplified.[7,11] This phenomenon is sometimes termed the "tyranny of the small motif".[11]

A case in point is provided by the RNA-cleaving 8–17 deoxyribozyme (Scheme 1). This motif is small (the catalytic "core" is constituted by just 14 or 15 residues) and relatively tolerant to mutations, so that a high number of copies of this motif are likely to exist in a starting population. Moreover, 8–17 activity can be supported by a variety of different divalent metal ion cofactors. All these factors explain why this motif has been fished out several times in independent in vitro selection procedures.[14,16,43–45]

In contrast to 8–17 stands a DNA enzyme termed 10–28 that catalyzes the depurination of a DNA substrate (Table 1). This is the largest deoxyribozyme identified to date, being composed of 93 nucleotides, and characterized by a complex predicted secondary structure.[30] The discovery of such an extended catalytic motif is surprising especially considering that the random sequence contained in the initial pool was just 85 nucleotides long,[30] but indicates that large catalytic DNAs do exist and that they can be identified in the laboratory.

Are there practical ways of specifically searching for such large deoxyribozymes, which, similar to the naturally occurring large ribozymes, might possess more efficient or more complex activities? One possible approach would be to design an initial library in which a preexisting catalytic motif is linked to a random-sequence region, thereby searching for auxiliary domains or elements that support an improved performance. A similar strategy has already been exploited for the in vitro evolution of novel catalytic RNAs.[46]

If compatibility of the catalysts with physiological systems is not a constraint, there are other shortcuts that researchers can use to identify more active DNA enzymes. One of them is providing the DNA with cofactors that expand its chemical functionalities and favor its structural stability. Since deoxyribozymes are often active only in the presence of metal ions, a simple option is performing selection in the presence of appropriate metal ion cofactors. This point is illustrated by a study conducted to analyze the effect of different divalent metal ions on the selection of deoxyribozymes with a DNA kinase activity.[27] The authors found that motifs selected in the presence of transition metal ions (Mn^{2+}, Cu^{2+}) were more diverse and more catalytically proficient than motifs selected in the presence of alkali metal ions (Mg^{2+}, Ca^{2+}).[27] This observation could partially reflect the inherent ability of transition metals to coordinate strongly with DNA and stabilize tertiary interactions.

The Basis of DNA Catalysis: Open Questions

The two previous sections took their start from a parallel between catalytic DNAs and catalytic RNAs. Such a comparative approach might offer insights into nucleic acid catalysis by illustrating the functional similarities and differences between the two polynucleotides. The similarities are likely to represent fundamental features of catalytic nucleic acids (and possibly of biocatalysts in general) while the differences might provide better comprehension of the nature of DNA and RNA as macromolecules. However, drawing a meaningful comparison between catalytic DNAs and RNAs will require a more complete picture of the structural and mechanistic basis of DNA catalysis, since, in these respects, the deoxyribozyme field lags substantially behind the field of ribozyme studies.[9]

In fact, no three-dimensional structure of an active catalytic DNA is currently known. Only one X-ray structure of a RNA-cleaving deoxyribozyme has been reported,[47] but the complex formed in the crystal (a tetramer containing two molecules of deoxyribozyme and two molecules of its RNA substrate) did not reflect the active structure of the catalyst.[47] In another instance, deoxyribozyme crystals were described,[48] but these crystals, too, did not yield any three-dimensional structure, presumably because they failed to diffract to good resolution. These frustrating results might be correlated to the flexibility of these small catalysts and their propensity to adopt alternative structures.

With respect to catalytic mechanisms, most questions also remain unanswered. The best-characterized deoxyribozyme is arguably the small PS5.M motif, which carries out the insertion of a Cu^{2+} ion into the ring of protoporphyrin IX (Table 1). This motif had been selected to bind a transition-state analogue for the metalation reaction, that is, a porphyrin derivative with distorted ring geometry. A series of experiments indicated that PS5.M would also bind protoporphyrin IX in a distorted form, so as to change its basicity and favor copper insertion.[25,49,50] This supported the notion that PS5.M can employ part of the interaction energy available from substrate binding to activate the substrate itself and enhance its reactivity.[50]

Our understanding of the catalytic mechanisms adopted by other DNAs is very poor. For example, Breaker and co-workers have outlined schematically four strategies that RNA-cleaving deoxyribozymes could employ for catalysis (including positioning and activation of the 2′-OH nucleophile and stabilization of the leaving group) and convincingly argued that two or more of these strategies must be operative in the reactions catalyzed by several representative RNA-cleaving motifs.[18,51] However, it remains to be established which combinations of catalytic strategies are actually exploited by individual deoxyribozymes, and by which means such strategies are enforced.

As noted above, most RNA-cleaving motifs require metal ion cofactors for activity, and might employ such metals in their catalytic mechanisms.[17,22,52] Yet distinguishing between chemi-

cal and structural roles is problematic, for example because transition metal ions, which are expected to function more efficiently in the activation of nucleophiles or in the stabilization of leaving groups, are also better suited to stabilizing DNA tertiary structures.[53] These uncertainties are compounded by our current ignorance of the binding sites and binding modes of these activating ions in deoxyribozyme cores.

Another catalytic strategy that RNA-cleaving ribozymes (as well as other deoxyribozyme classes) might adopt is general acid–base catalysis, operated by amino groups on specific base rings. Several natural ribozymes have been proposed to employ this type of mechanism, even though the standard RNA bases, due to their pK_a values being far from neutral, do not seem well suited to catalytic proton exchanges.[54] In deoxyribozyme studies, the possibility of general acid–base catalysis has been only marginally touched upon[52] and might deserve to be further investigated, for example in those RNA-cleaving motifs that do not require divalent metal ions for function.[20,21]

The questions about the mechanisms accessible to deoxyribozymes are tightly linked to the more general issue of defining the boundaries of DNA catalysis. There are myriad catalytic tasks for which DNA has yet to show competence, such as the formation of carbon–carbon bonds or the direct hydrolysis of DNA or peptides. Reactions involving small, anionic substrates might be particularly difficult to perform, as they entail the formation of active sites that efficiently bind and position these reactants. Some other chemical processes could be simply too challenging for plain DNA, but might be tackled by deoxyribozymes that (not unlike many protein enzymes) recruit organic cofactors. The use of organic cofactors was illustrated by an RNA-cleaving deoxyribozyme that needs histidine in order to function,[55] although it remains to be shown that such cofactors can broaden the scope of DNA catalysis beyond that seen with metal ions.

Catalytic DNAs as a Biomolecular Tools

The interest in deoxyribozymes' structures and mechanisms is not just academic, as these molecules are being used as tools in various applicative disciplines. Catalytic DNAs are chemically stable, easy to produce, biocompatible, and amenable to rational design—a series of advantageous features that make them particularly suited for many biotechnological and pharmaceutical tasks. The applications of deoxyribozymes have been reviewed in detail in a number of recent publications.[6,8,9,56] The examples below give a sense of the continuing research in this area, while further underscoring the functional versatility of DNA.

RNA-cleaving deoxyribozymes (in particular, the 10–23 deoxyribozyme) can be designed to cleave RNA substrates in a sequence-specific manner, and have been largely studied as potential chemotherapeutics to target the disruption of pathogenic mRNAs.[8,57] Meanwhile, these deoxyribozymes have also found use in laboratory applications that range from the analysis and quantification of nucleic acids[58,59] to the preparation of homogeneous RNA transcripts[60,61] and from the development of molecular-scale computational devices[62] to the biosensing of metal ions.[63,64]

The use of DNA catalysts as advanced biosensors seems especially promising, due in particular to the introduction of allosteric deoxyribozymes (aptazymes).[65,66] These molecules have been produced through rational design, by fusing a catalytic domain with a DNA motif (aptamer) capable of specifically interacting with an exogenous ligand.[65,66] Analogous to allosteric protein enzymes, aptazymes possess catalytic activities that are modulated by effector molecules, and can be used to detect analytes in kinetic assays.

For example, one recently described biosensor exploits an ATP-activated deoxyribozyme ligase that is able to circularize a linear DNA substrate.[67] The circular substrate, in turn, can be amplified by DNA polymerase through a rolling-circle mechanism and this amplification can be revealed by using fluorescent probes. The system, adapted to a chip format, responded to ATP concentrations in the 10–100 μM range and showed a signal-to-background ratio of ~100.[67] Another study described a colorimetric adenosine biosensor based on an RNA-cleaving deoxyribozyme, whose substrate acts as a linker between DNA-functionalized gold nanoparticles.[68] In the absence of adenosine, the allosteric deoxyribozyme is inactive and the nanoparticles aggregate, yielding a blue color. In the presence of adenosine, the aptazyme cleaves its substrate and prevents the formation of aggregates; the dispersed gold nanoparticles result in a red color.[68]

An RNA-cleaving deoxyribozyme has also been used to engineer an autonomous nanomotor, that is, a nanodevice capable of continuous mechanical motions without intervention by the experimenter.[69] The design and operation principle of the nanomotor are shown in Figure 3. The device contains the 10–23 motif and, in the absence of the deoxyribozyme substrate, sits in a closed (compact) state. Upon binding of the 10–23 substrate, the device opens. When the substrate is cleaved, the cleavage products dissociate from the device and the device closes. Another molecule of the substrate (present in solution) can then bind, and the system can go through another round.[69] The system represents a crude but effective functional homologue of cellular protein motors, in that it exploits chemical energy (stored in the RNA phosphodiester linkages, rather than in ATP) to conduct iterative movements.

Conclusion

There are convincing arguments suggesting that DNA emerged as a biopolymer much later than RNA during evolution and that an all-DNA world never occurred during the history of our form of life.[70] According to this view, DNA represents an evolutionary improvement on RNA solely as a medium for storing genetic information, since the lack of the 2′-OH group strongly reduces the chemical instability of the nucleic acid.[7]

Such a late start of DNA might explain why deoxyribozymes have not been found in living systems (although their existence remains possible), but it does not imply a reduced functional versatility of DNA itself. Contrary to earlier preconcep-

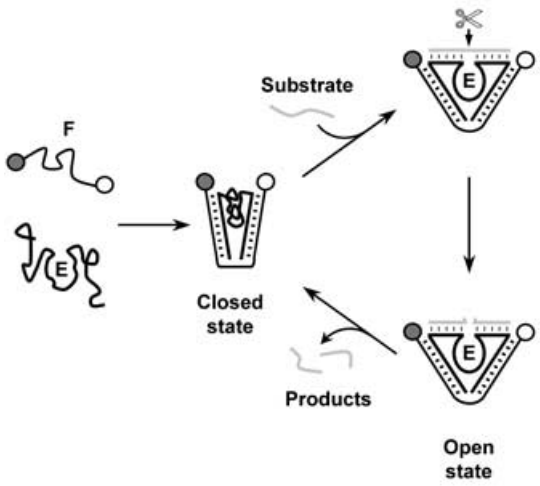


Figure 3. A DNA nanomotor based on an extended 10–23 deoxyribozyme.[69] The 10–23 motif is embedded in a DNA molecule (E) whose extremities are complementary to another, double-dye-labeled, DNA strand (F). Association of E and F, in the presence of divalent metal ions, leads to the formation of a relatively compact "closed state" (which presumably includes a distribution of conformational isomers). When the 10–23 substrate binds, the system switches to an "open state". Following substrate cleavage, the products are sufficiently short that they dissociate, and the device reverts to the closed state. In the presence of excess substrate, the cycle can be repeated several times. The motions associated with the catalytic cycle can be monitored by fluorescence resonance energy transfer (FRET) by exploiting the dyes bound at the ends of the F strand (white and gray circles).

tions, catalytic DNAs and RNAs show similar ranges of activities and efficiencies, while the limits of the presently known deoxyribozymes might simply reflect the inherent limits of the strategies that have led to their identification. Thus a "DNA world", albeit unlikely in terms of natural history, does not appear a practical impossibility. Some researchers today are considering the creation of sophisticated molecular systems in which all major functional roles are played by DNA, de facto mimicking the complexity of the hypothetical RNA world for engineering purposes.[71]

On a more immediate perspective, deoxyribozymes 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.

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Keywords: DNA · enzymes · in vitro selection · nucleic acid catalysis · structure

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