

**SECONDARY STRUCTURE CHARACTERIZATION OF pH6DZ1, A
FLUORESCENCE SIGNALING AND RNA CLEAVING DNA
ENZYME**

**SECONDARY STRUCTURE CHARACTERIZATION OF pH6DZ1, A
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ENZYME**

By

YUTU SHEN, B.Sc.

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**TITLE: Secondary Structure Characterization of pH6DZ1, a Fluorescence
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AUTHOR: Yutu Shen, B.Sc. (Nanchang University)

SUPERVISOR: Professors Yingfu Li and John D. Brennan

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ABSTRACT

pH6DZ1 is a synthetic deoxyribozyme that is able to couple catalysis with fluorescence signal generation. This deoxyribozyme has the ability to cleave itself at a lone ribonucleotide that is present between a pair of deoxyribothymidines, one modified with a fluorophore (fluorescein) and the other with a quencher (DABCYL). Herein we report on the sequence truncation and secondary structure characterization of pH6DZ1 as well as the identification of functionally important nucleotides within this deoxyribozyme. Our data indicate that pH6DZ1 has a four-way junction-like secondary structure comprised of four short duplexes, three hairpin loops, and three inter-helical unpaired elements. Ten nucleotides, all located in two separate single-stranded regions, were identified as functionally indispensable nucleotides. Nine nucleotides, most of which are also distributed in three single-stranded DNA elements, were identified as functionally vital nucleotides. Our study has shown that pH6DZ1 has a secondary structure that is more complex than those reported for other RNA-cleaving deoxyribozymes. A *trans*-acting DNA enzyme was also developed from the minimized version of pH6DZ1, which behaves as a true enzyme with a k_{cat} value of $\sim 1 \text{ min}^{-1}$ and generates a large fluorescence signal upon catalysis. This study should facilitate the future exploration of this unique DNAzyme for the development of DNAzyme-based biosensors.

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LIST OF ABBREVIATIONS

ATP: adenosine 5'-triphosphate

DABCYL: 4-(4-dimethylaminophenylazo)benzoic acid

DMS: dimethyl sulfate

DTT: dithiothreitol

EDTA: ethylenediamine tetraacetic acid

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

PAGE: polyacrylamide gel electrophoresis

T4 PNK: T4 polynucleotide kinase

TOM: triisopropylsilyloxymethyl

CHAPTER 1: INTRODUCTION

1.1 DNA enzyme

Proteins are the dominant enzymes in nature because of their twenty distinct amino acid building blocks offering a large diversity of chemical functional groups that are useful for catalysis. Compared to proteins, nucleic acids are made of only four chemically similar building blocks. It was logical to think that Mother Nature might not utilize nucleic acids as catalysts. However, since the early 1980s, when the Nobel Prize-winning discovery was made that certain RNA molecules possess catalytic activity (1, 2), more and more RNA catalysts (ribozymes) have been found, including naturally occurring ribozymes (3, 4) and man-made ribozymes that were developed in laboratories (5, 6, 7, 8, 9, 10). This demonstrated that RNA has the capacity to fold into intricate tertiary structures that can catalyze chemical reactions. Thus the question arose as to whether DNA, which differs from RNA in the 2' hydroxyl group, could serve as a catalyst.

DNA is always associated with the famous Watson-Crick duplex model and also with its important role as the repository of genetic information. It is certain that double-stranded DNA (dsDNA) cannot act as a biological catalyst simply due to the formation of monotonic helical structure (11). However, Joyce and Breaker in 1994 postulated that single-stranded DNA (ssDNA) should have the potential to form intricate tertiary structures that are indispensable for enzymatic function (12). They

confirmed this speculation through an elegant experiment that led to the creation of the first artificial DNA enzyme (also known as deoxyribozyme, DNAzyme, or catalytic DNA) (12). Since then, a large number of deoxyribozymes have been created in many research laboratories around the world (13, 14), even though no naturally occurring catalytic DNA has been discovered.

All deoxyribozymes reported to date have been created through “*in vitro* selection”—a powerful screening technique that allows scientists to search vast DNA libraries for rare sequences that support catalysis (15). The general *in vitro* selection cycle begins with a large pool of random-sequence oligonucleotides. These molecules are given a task to perform, such as binding to a target or performing chemical catalysis. Only the oligonucleotides that are successful in completing this task are selected and amplified by the polymerase chain reaction (PCR). The cycle is repeated, each time enriching the population of successful sequences, until significant activity is detected. During the selection process, stringency is usually increased by either decreasing the time given to complete the task and/or providing a less favorable environment to perform these tasks in order to eliminate the oligonucleotides that do not perform the tasks as optimally as others. Once sufficient activity is achieved, the enriched population is then cloned and sequenced to test each individual sequence separately.

Up to the present, a large array of deoxyribozymes have been created to perform diverse chemical transformations, involving RNA cleavage, RNA ligation, DNA cleavage, DNA ligation, DNA phosphorylation, DNA adenylation, porphyrin metallation and N-glycosylation (15). The proficiency of some of the deoxyribozymes is remarkable, with the rate enhancements between four to ten orders of magnitude over the corresponding uncatalyzed reactions (15). For example, a small-sized RNA-cleaving DNA enzyme coined the 10-23 exhibits a k_{cat} of $\sim 10 \text{ min}^{-1}$ and k_{cat}/K_M of $\sim 10^9 \text{ M}^{-1} \text{ min}^{-1}$ (16), which rivals traditional protein enzymes and ribozymes for the same reaction.

1.2 RNA-cleaving DNA enzyme

Most deoxyribozymes generated to date promote RNA cleavage. The mechanism of this catalytic reaction is thought to go through the deprotonation of the 2' hydroxyl adjacent to the cleavage site, resulting in cleaved products that bear a 2', 3' cyclic phosphate and 5' hydroxyl group (17). In addition, most deoxyribozymes require divalent metal cofactors for the purpose of maximizing catalytic efficiency (18).

The first DNA enzyme reported by Breaker and Joyce is the RNA-cleaving deoxyribozyme that catalyzes the cleavage of a single RNA phosphodiester embedded within an otherwise all-DNA substrate in the presence of Pb^{2+} (12). One year later, they created a Mg^{2+} -dependent deoxyribozyme that cleaved the same RNA junction (19). In

1996, the Famulok's group derived another RNA-cleaving deoxyribozyme that used Ca^{2+} as a cofactor (20). Instead of utilizing divalent metal ions as cofactors, an RNA-cleaving deoxyribozyme that required histidine as a co-factor was isolated the following year (21). One assumption is that the imidazole group of histidine acts as a general base to assist in deprotonation of the 2' hydroxyl adjacent to the cleavage site.

Acquisition of the two crucial RNA-cleaving deoxyribozymes named '10-23' and '8-17' showed promise for the application of DNA enzymes in the diagnostic and therapeutic fields. 10-23 and 8-17 (Figure 1.1) are extremely small and can cleave an all-RNA substrate under physiological conditions with high catalytic efficiency and high substrate sequence specificity (16). There are many published examples describing the use of the 10-23 DNA enzyme to reduce the amount of mRNA molecules *in vivo* (22, 23, 24, 25, 26, 27). 10-23 also has been applied in a diagnostic context as part of the 'DzyNA-PCR' method for quantitative PCR (28). Although its sibling 8-17 has not been shown to be as useful as 10-23 *in vivo*, it has been exploited as a biosensor for Pb^{2+} ions with practical application for the measurement of the lead content in paint chips (29, 30). Owing to their utilization in broad fields ranging from gene regulation to biosensing, investigation of RNA-cleaving deoxyribozyme is currently one of the most active areas of catalytic DNA research.

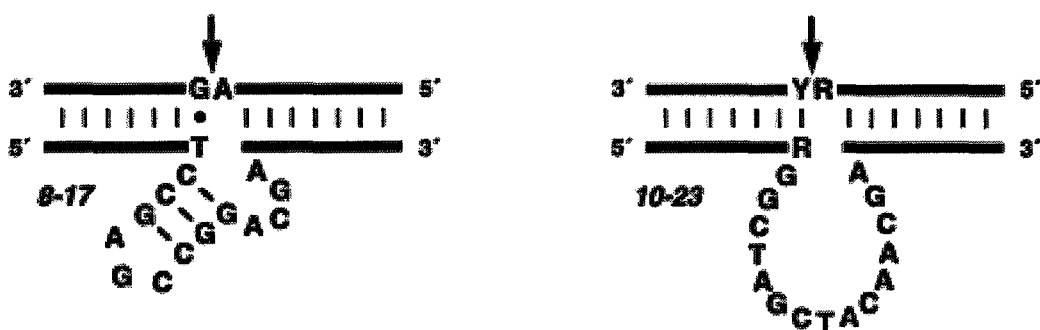


Figure 1.1 Two representative RNA-cleaving deoxyribozymes, 8-17 and 10-23, created by Santoro SW and Joyce GF. Both enzymes are extremely small and can bind to RNA substrate through Watson-Crick pairing regions that surround the cleavage site. 8-17 cleaves an A-G junction with a catalytic core of 14-15 nt, while 10-23 is capable of cleaving any RNA molecule at an R-Y junction (R = purine; Y = pyrimidine).

1.3 Fluorescence signaling DNA enzyme

Deoxyribozymes possess many unique properties that have led to these species becoming desirable alternatives to traditional protein enzymes and ribozymes for applications such as fluorescence-based biosensing. First of all, deoxyribozymes have extraordinary chemical stability. The phosphodiester bond of DNA is nearly 1000-fold more resistant to hydrolytic degradation than the peptide bond of a protein, and about 100,000-fold more stable than RNA under physiological conditions (31). Second, the *in vitro* selection technique makes it easy to either generate deoxyribozymes *de novo* or to improve existing deoxyribozymes for particular applications such as low pH fluorescence signaling or cofactor dependent signaling (14). Third, deoxyribozymes are amenable to *in vitro* selection, which provides enormous versatility through mixing and matching of functional compositions (32). Fourth, most deoxyribozymes can

withstand many cycles of denaturation and renaturation without losing activity (14). Finally, when compared to ribozymes, deoxyribozymes are easier to handle and cheaper to produce (14).

In recent years, considerable efforts have been undertaken to develop deoxyribozymes as reporter molecules for biosensing applications, one of which is the fluorescence signaling deoxyribozyme biosensor. Fluorescence signaling is one of the most desirable bioanalytical methods owing to the convenience of detection, the multidimensional nature of the fluorescence signal (i.e., signals can be based on changes in intensity, wavelength, decay time or polarization), and the availability of a great many fluorophores and quenchers for nucleic acid modification (33). Up to now, almost all the fluorescence-signaling catalytic DNA systems have been engineered by adopting the concept of FRET (fluorescence resonance energy transfer). Fluorescence resonance energy transfer (FRET) is an electronic interaction involving two dye molecules when the emission spectrum of a donor molecule overlaps with the adsorption spectrum of an acceptor molecule. The distance between the two molecules determines the extent of energy transfer.

A special deoxyribozyme termed 17E (an 8-17 variant) was reported to possess Pb^{2+} -dependent RNA cleavage activity by Liu's group (29). The 3' end of the enzyme strand was modified with a quencher and the 5' end of the substrate strand was modified with a fluorophore in order to achieve maximal fluorescence quenching due

to the proximal position of the labels. Upon addition of Pb^{2+} ions, the enzyme was activated and cleaved the single ribonucleotide linkage in the substrate strand, resulting in the separation of the fluorophore and quencher, which generated a fluorescence enhancement. The creation of a colorimetric lead-ion sensor has also been described that is capable of being monitored visually (30).

The other typical example for illustrating the use of dequenching as a signal evolution mode for deoxyribozymes is the catalytic molecular beacon, which uses a molecular beacon module to transduce the molecular recognition of an oligonucleotide target to a change in fluorescence intensity through deoxyribozyme-mediated cleavage of a dual-chromophore labeled substrate (34). In this case, the fluorophore and quencher modify the 5' end and 3' end of the substrate. Stojanovic's group have used such systems to create deoxyribozyme networks that can receive inputs and make decisions, aiming to put it into practice in molecular computing (35, 36).

While there are many exciting advancements in the development of fluorescence-signaling deoxyribozymes, there are also inherent flaws in the aforementioned systems. Incomplete annealing between the substrate strands and enzyme strands might give rise to a false-positive signal in the catalytic DNA sensor for lead ions. In the case of catalytic molecular beacons, inefficient quenching is obtained owing to the comparatively long distance between the fluorophore and quencher pair. In addition, the fluorescence enhancement and the catalytic activity of

the above DNA sensors were difficult to optimize because these species were not generated specifically for signaling applications (37).

1.4 Creating fluorescence-signaling RNA-cleaving DNA enzyme de novo

To overcome some of the problems outlined above, our laboratory has developed an efficient RNA-cleaving deoxyribozyme that synchronizes catalysis with fluorescence signaling (38). Following this development, a series of deoxyribozymes with similar signaling capabilities but with broad pH dependencies and metal ion specificities were isolated (39). The key element in this catalytic signaling system is a novel fluorophore/quencher-containing substrate, which offers a high intensity of fluorescent signaling while maintaining a low level of fluorescence background in the absence of RNA-cleavage. The unique substrate comprises a single ribonucleotide linkage embedded in a DNA chain and sandwiched between a fluorophore-labeled deoxyribonucleotide and a quencher-modified deoxyribonucleotide (38). Such a chimeric setting permits the synchronization of the cleavage with fluorescence signal generation and overcomes the drawbacks of the previous fluorescent signaling reporter systems.

To obtain a deoxyribozyme that could efficiently cleave this unique RNA junction, an *in vitro* selection scheme (Figure 1.2) was employed to isolate *cis*-acting deoxyribozymes that could perform autocatalysis. There are a total of eight steps of the

selection strategy. In step 1, an 86-nt randomized library was ligated to the specific substrate that is 23-nt long. The ligated 109-nt DNA product was isolated by denaturing polyacrylamide gel electrophoresis (PAGE) in step 2. In step 3, the purified DNA was incubated with a reaction buffer containing divalent metal ions. The RNA linkage was cleaved to produce a 94-nt fragment that was subsequently separated from the 109-nt precursor by PAGE in step 4. The amplification of recovered 94-nt DNA was carried out by PCR using primers P1 and P2 in step 5. A second PCR was performed in step 6 to introduce the ribonucleotide linkage embedded within DNA by using a ribonucleotide-terminated primer P3. In step 7, the DNA product was treated with sodium hydroxide to cleave the RNA linkage. Finally, the isolated 86-nt cleavage fragment was phosphorylated at the 5' end for the next round of selection (38).

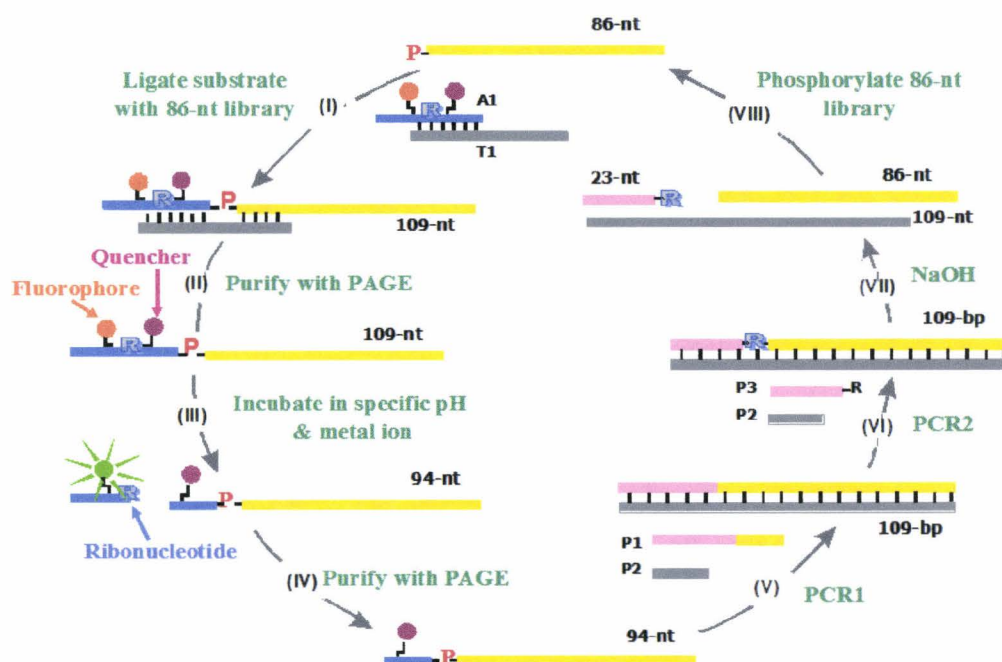


Figure 1.2 *In vitro* selection scheme used by Mei *et al.* for the creation of *cis*-acting fluorescence-signaling deoxyribozymes.

Using this selection method, no detectable cleavage activity was seen on the PAGE gel until the ninth round. Following detection of cleavage, the stringency of the selection was increased by progressively decreasing the reaction time. In total, twenty-two rounds of *in vitro* selection were carried out and the cleavage product obtained using a 1-min incubation time from the last round was used for DNA cloning. A single class of deoxyribozyme named ‘DEC22-18’ was discovered in this selection after sequencing over 20 clones (38). Truncation studies on the original 109-nt deoxyribozyme led to the discovery of another more efficient 83-nt DNA enzyme termed ‘DEC22-18A’, which had a k_{obs} of more than 10 min^{-1} under optimal conditions.

Based on the prototype of this highly proficient DNA enzyme, a true *trans*-acting deoxyribozyme entitled ‘DET22-18’ was developed that could perform multiple turnover catalysis in an intermolecular format with a k_{cat} of nearly 7 min^{-1} , making it rank the second fastest DNA enzyme reported to date (38).

To take advantage of the above deoxyribozyme for biosensor applications, a further modification was done to produce an allosterically regulated, fluorescence-signaling catalytic reporter. In designing an allosteric nucleic acid enzyme (ribozyme or deoxyribozyme), the ligand-binding site of the allosteric enzyme is spatially separated but allosterically linked to the active site of the catalyst. A conformational change in the structure of the catalytic domain is induced by binding of ligand, which can lead to either increased or decreased enzymatic function of the catalyst (40). Several allosteric ribozymes have been logically built mainly in Breaker’s group by coupling pre-existing ligand-binding and catalytic RNA motifs (40). Based on this rational design strategy, an ATP-binding DNA aptamer (aptamer refers to a ssDNA that is capable of specific recognition of a target of interest) was coupled to the catalytic domain of DET22-18A through a weakened stem (Figure 1.3). When introducing ATP, the target interacts with the aptamer, inducing the aptamer to undergo a conformational change that stabilizes the communication module. The catalytic activity increases due to the optimal folding of the enzymatic domain promoted by the stabilization of that communication module. It is conceivable that

without ATP, the aptamer could not fold tightly to strengthen the weakened stem, leading to a poor catalytic activity. This reporter exhibits a nearly 20-fold rate enhancement in the presence of ATP over GTP (38). Those valuable findings provide a platform for DNA catalysts as controllable sensors for their cognate ligand compounds.

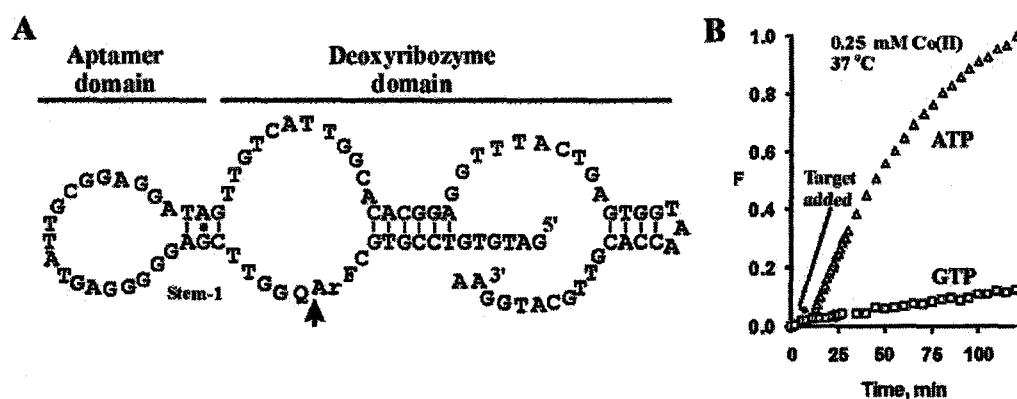


Figure 1.3 An ATP-dependent signaling allosteric deoxyribozyme engineered by our groups. (A) DEC22-18A has been transformed into allosteric deoxyribozyme by appending an aptamer domain that specifically binds ATP. (B) Fluorescence measurement upon adding target ATP and GTP.

1.5 More RNA-cleaving fluorescence-signaling deoxyribozyme

In addition to DEC22-18, a series of new fluorescence-signaling deoxyribozymes that could operate over a large range of pH values were isolated by our groups using the same *in vitro* selection scheme as was employed to derive DEC22-18 (39). The same dual-chromophore-labeled substrate linked to a longer sequence containing a 70-nt random domain was applied in this study. A pool of 10^{14}

DNA molecules underwent seven repetitive rounds of selection at pH 4 to establish a catalytic DNA population from which pH-dependent species could be generated (39). From the 8th round onward, the DNA pool was split into five individual pools for conducting five parallel selections at pH 3, 4, 5, 6 and 7, respectively. An error-prone PCR (up to 10% mutation rate per cycle) was used during amplification to create diversity (39). The reaction time was stepped down from the initial 5 hours to as short as 1 second to search for the most efficient catalysts. The continuance of selection was dependent on whether there was a noticeable increase in the activity for three successive rounds (39). For the pH3 and pH4 pools, eight more rounds were carried out after dividing the initial population. For pH5-7 pools, 17 more rounds were conducted. After significant cleavage was found in each of the five pools, cloning and sequencing were performed. The primary species from each pH pool was denoted as pH3DZ1, pH4DZ1, pH5DZ1, pH6DZ1, and pH7DZ1 (39). The sequence, metal ion dependence, pH dependence and signaling profile for pH6DZ1 (which is the focus of my thesis) are shown in Figure 1.4. Intriguingly, pH6DZ1 is catalytically active in the presence of Mn^{2+} or Ni^{2+} but inactive with Mg^{2+} or Cd^{2+} (39). Equally interesting is the fact that this deoxyribozyme exhibits the fairly tight functional pH range with optimal activity centered at pH 6 (39).

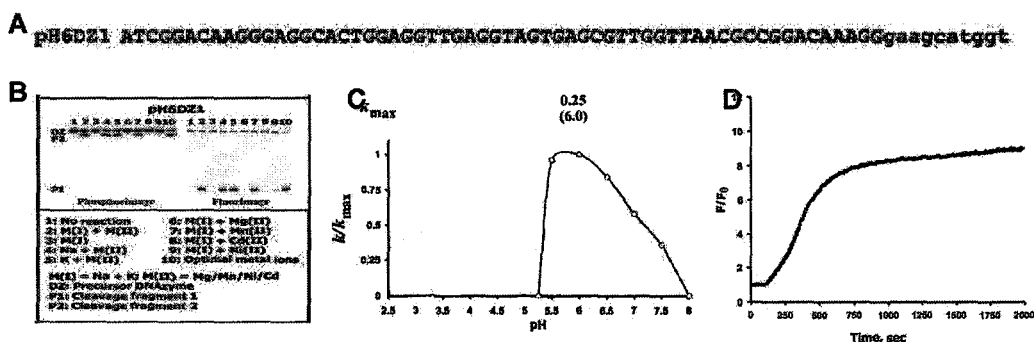


Figure 1.4 pH6DZ1 isolated by our groups. (A) Sequence of pH6DZ1. (B) Metal-ion dependence of pH6DZ1. (C) Measurement of catalytic rate at different pH for pH6DZ1. (D) Real-time signaling profile for pH6DZ1.

Due to the uniquely linked catalysis-signaling capability, these special RNA-cleaving deoxyribozymes offer an excellent opportunity for designing deoxyribozyme-based biosensors (29, 38). The most desirable biosensing application for these unique deoxyribozymes is the design of allosteric signaling systems for the real-time detection of chemical and biological ligands (38). However, engineering such allosteric signaling deoxyribozymes requires detailed knowledge of the secondary structures of these single-stranded DNA molecules. Therefore, elucidating their secondary structures is imperative to the realization of their full biosensing potential. Moreover, the elucidation of their secondary structures will allow us to determine whether these RNA-cleaving DNA catalysts share secondary-structural features that are similar to those exhibited by several known deoxyribozymes that cleave unmodified RNA substrates (12, 16, 19, 41, 42). This in turn will address the question

of whether single-stranded DNA can use diverse structures to perform a similar catalytic task and such information is significant to the future catalytic DNA research. Finally, the knowledge of the secondary structure can provide much needed insight into the potential for designing a *trans*-acting deoxyribozyme that is capable of multiple substrate turnovers, and thus amplification of ligand binding into multiple dequenching events.

1.6 Research objective

The research in this thesis is mainly focused on a detailed examination of the secondary-structural properties of pH6DZ1 (39). As noted above, this autocatalytic DNA exhibits a fairly robust catalytic activity (k_{obs} of $\sim 0.2 \text{ min}^{-1}$), a large signaling magnitude (~ 10 -fold fluorescence enhancement), a unique metal-ion dependence (requiring both Mn^{2+} and Ni^{2+} for optimal activity), and an intriguing pH profile (a bell curve with an optimal pH of 6) (39). These phenotypic properties hint at the possibility that pH6DZ1 may have a unique secondary structure that is worth probing. Comprehensive sequence truncations and nucleotide alterations were performed to achieve the following three goals: (i) minimizing the size of pH6DZ1, (ii) establishing its secondary structure, and (iii) categorizing its composite nucleotides according to their functional importance. While the first two objectives were explored to obtain crucial information for potential biosensor engineering, the third goal was critical to

laying the foundation for future mechanistic studies on this deoxyribozyme. The remaining part of this thesis aimed to use the information gained about the structural properties of the shortened pH6DZ1 deoxyribozyme to design a *trans*-acting, “true” catalyst that can process external substrates with turnovers. Both the multiple-turnover ability and fluorescence-generating properties of the *trans*-acting deoxyribozyme were determined and compared to those of the original *cis*-acting deoxyribozyme.

CHAPTER 2: MATERIALS AND MATHODS

2.1 Oligonucleotides and other materials

Standard and modified DNAs were prepared by automated chemical synthesis (HHMI-Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University) using standard phosphoramidite chemistry. DNA oligonucleotides were purified by 10% preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE), and quantified using standard spectroscopic methods (38). Nucleoside 5'-triphosphates and [γ - 32 P]ATP were purchased from Amersham Pharmacia. T4 DNA ligase and T4 polynucleotide kinase (T4 PNK) were obtained from MBI Fermentas. All other chemical reagents were acquired from Sigma and used without further purification.

Fluorescein and DABCYL [4-(4-dimethylaminophenylazo)benzoic acid] labels were incorporated into the oligonucleotide sequences during automated DNA synthesis using fluorescein-dT amidite and DABCYL-dT amidite (Glen Research, Sterling, Virginia). The adenine ribonucleotide linkage was also introduced during solid-state synthesis using A-TOM-CE Phosphoramidite (Glen Research). The TOM (triisopropylsilyloxymethyl) protecting group on the 2'-hydroxyl group of the RNA linkage was removed by incubation with 150 μ l of 1M tetrabutylammonium fluoride (TBAF) in THF at 60 °C with shaking for 6 h, followed by the addition of 250 μ l of 100 mM Tris (pH 8.3) and further incubation with shaking for 30 min at 37 °C. The DNA

was recovered using ethanol precipitation, dissolved in water containing 0.01% SDS, and the tetrabutylammonium salt was removed by centrifugation using a spin column (Nanosep 3K Omega, Pall Corp., Ann Arbor, Michigan).

The reaction buffer used for all catalytic assays contained a final concentration of 800 mM Na⁺, 8 mM Mn²⁺, 2 mM Ni²⁺ and 50 mM HEPES, pH 6.0 (produced from a 2× stock). The stop solution contained 40 mM EDTA, 8 M urea, 90 mM Tris, 90 mM boric acid, 10% sucrose (w/v), 0.025% xylene cyanol, and 0.025% bromophenol blue. DNA phosphorylation reactions were performed in a 50 mM Tris-HCl (pH 7.6, 25 °C), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA solution containing 0.1 units/μL of PNK. DNA ligation reactions were carried out in solution containing 40 mM Tris-HCl (pH 7.8, 25 °C), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP and 0.05 units/μL of T4 DNA ligase.

2.2 Deoxyribozyme activity assays

All the *cis*-acting pH6DZ1 variants required for the sequence truncation and nucleotide mutation studies were constructed in the following way: a relevant catalytic domain was 5'-labeled with 10 μCi of [γ-³²P]ATP using PNK. After a 60-min incubation, the 5'-phosphorylated catalyst was ligated to a relevant substrate sequence in the presence of an appropriate DNA template using T4 DNA ligase for 1 h at room temperature. The ligated DNA construct was purified by 10% denaturing PAGE and

isolated by the crush and soak method (43), followed by ethanol precipitation (repeated twice). The resulting precipitate was redissolved in deionized water. After heating at 90 °C for 1 min and cooling to room temperature for 5 min, the RNA cleavage reaction was initiated by adding the required reaction buffer (used as a 2× stock). The final DNA concentration was set at ~0.1 μM. At 0.1, 1, 10, 100, and 1000 min, an aliquot of the solution was quenched with the stop solution, followed by freezing at -20 °C. Cleavage products were separated by 10% denaturing PAGE and product yields were determined by quantitating product bands using a PhosphoImager and ImageQuant software (Molecular Dynamics). The catalytic ability of the deoxyribozyme was scored using the following scheme: any deoxyribozyme construct exhibiting ≥10% cleavage in 1 min was given the highest score at “+++++”; mutant deoxyribozymes with ≥10% cleavage in 10 min, 100 min and 1000 min were scored “++++”, “+++” and “++”, respectively; those with less than 10% cleavage in 1000 min were given a “+”; molecules that produced no cleavage bands after incubation for 1000 min were denoted by a “-”. At least two independent assays were conducted for each deoxyribozyme construct to ensure that the scoring was accurate. The kinetic assay of all the trans-acting pH6DZ1 constructs was conducted using the same method described above. The data are fitted according to a mathematical equation to derive relevant parameters such as rate constants.

2.3 DMS methylation interference assays

EC93 was used for this experiment and its sequence will be given in Chapter 3. The method to be described below was adapted from a previously published protocol (44). The relevant deoxyribozyme construct was made as follows: 500 pmol of donor DNA (a relevant catalyst domain) was phosphorylated with a trace amount of [γ - 32 P]ATP using PNK (0.1 units/ μ L, final concentration) for 20 min at 37 °C, and was then supplemented with non-radioactive ATP to a final concentration of 1 mM and incubated at 37 °C for another 20 min to ensure complete phosphorylation. PNK was inactivated by heating the reaction mixture at 90 °C for 5 min. The phosphorylation reaction mixture was directly added to a ligation reaction mixture containing 600 pmol DNA template and 550 pmol acceptor DNA (a required substrate domain). The ligation reaction was initiated by adding T4 DNA ligase and allowed to proceed at room temperature for 5 h. After ethanol precipitation, the ligated DNA was isolated by 10% denaturing PAGE.

One hundred pmol of the above DNA construct in water was heated at 90 °C for 30 s and cooled to room temperature for 5 min. The self-cleavage reaction was initiated by adding the 2 $\times$ reaction buffer and the resultant mixture was incubated for 30 min at room temperature. The reaction was quenched by adding 30 mM EDTA (final concentration). The DNA recovered by ethanol precipitation was resuspended in 500 μ L of water, heated at 90 °C for 1 min, and quickly cooled to room temperature. An

equal volume of 0.4% (v/v) DMS (dimethyl sulphate, freshly made) was added and the mixture was incubated at room temperature for 40 min. Methylated DNA was recovered by ethanol precipitation, followed by two washes with 70% cold ethanol. This sample was labelled as the “control”. Another 100 pmol deoxyribozyme construct was also methylated with DMS using the protocol described above. This methylated DNA was then allowed to self-cleave in the reaction buffer for 30 min, using a similar procedure to that described for the unmethylated DNA. After the self-cleavage reaction, the DNA was recovered by ethanol precipitation, followed by two washes with 70% ethanol. This sample was labelled as the “test”. Both the control and test DNA samples were 5'-labeled with 20 μCi [γ - ^{32}P]ATP using PNK at 37 °C for 20 min. After ethanol precipitation, the 5'-labeled 3'-cleavage fragment (containing the catalytic domain) was purified by 10% denaturing PAGE. These modified oligonucleotides were dissolved in 10% (v/v) piperidine (100 μL , freshly made) and heated at 90 °C for 30 min. The resultant cleaved products were dried under vacuum. The dried pellet was resuspended in 25 μL deionised water and evaporated to dryness under vacuum (twice). The cleaved products were then analyzed by denaturing 10% PAGE.

Densitometry on bands from both the test and control lanes was performed in order to score the degree of methylation interference observed. The difference in background-corrected band intensity between equivalent cleavage fragments from each

lane was normalized using the difference observed in a pair of cleavage fragments originating from a guanine located in the confirmed catalytically unimportant stem-loop region of the deoxyribozyme construct. The ratio of intensities for each pair of bands (R) was determined by dividing the corrected band intensity in lane T (sample lane) by the corrected band intensity from lane C (control lane). These ratios were then normalized on a scale from 0 to 100, where 0 indicates the least interference and 100 the most, using the following equation: $R_{\text{normalized}} = [(R-R_0)/(R_{100}-R_0)] \times 100$.

2.4 Metal specificity

The metal ion specificity of pH6DZ1 and EC56 (a minimized version of pH6DZ1) was determined by monitoring the ability of each deoxyribozyme (containing an internal ^{32}P -labeled phosphodiester linkage, see main text) to undergo self-cleavage, as demonstrated by the presence of cleavage product on a 10% denaturing PAGE gel. Specific metal ion concentrations that were tested are listed in the caption to corresponding figure. Each *cis* deoxyribozyme construct was allowed to cleave for 10 min prior to the stopping of the reaction and analyzing of the reaction mixture by PAGE. Both a phosphorimage (taken on a Storm 820 Phosphorimager, Molecular Dynamics) and a fluorimage (taken on a Typhoon 9200, Molecular Dynamics) were obtained following gel electrophoresis to examine for radioactivity and fluorescence in the DNA bands.

2.5 pH dependence

pH6DZ1 and EC56 were used for this experiment and their sequences are listed in Chapter 3. Both deoxyribozymes (containing an internal ^{32}P -labeled phosphodiester linkage after the cleavage site) were allowed to undergo the RNA cleavage reaction for 10 min under the optimal metal ion conditions at several different pH values. The reaction was then stopped and analyzed by 10% denaturing PAGE, and scanned for both radioactivity and fluorescence in the DNA bands.

2.6 Fluorescence measurement

The trans acting deoxyribozyme ET1 and the matching substrate were used for this experiment and their sequences are listed in Chapter 4. Fluorescence measurements were conducted in 50- μl solutions on a Cary Eclipse Fluorescence Spectrophotometer (Varian). The excitation was set at 490 nm and emission at 520 nm. The concentrations of DNA molecules and other details are given in the legends of relevant figures.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Characterizing the secondary structure and identifying functionally essential nucleotides of pH6DZ1, a fluorescence-signaling and RNA-cleaving DNzyme

3.1.1 Scoring catalytic activity of mutant deoxyribozyme constructs

The primary sequence of pH6DZ1, shown in Figure 3.1, is divided into two domains labelled “substrate” (italicized letters) and “catalyst” (normal letters) to reflect the fact that this deoxyribozyme was originally isolated from a library of 100-nt single-stranded DNA molecules (ssDNAs) to which the 23-nt substrate was ligated. For convenience, we numbered the nucleotides in the substrate domain from –1 to –23 in the 3'-5' direction and those in the catalytic domain from 1 to 100 in the 5'-3' direction. The specific numeral given to each nucleotide remains unchanged throughout this report, even though many mutant deoxyribozyme constructs had one or more internal nucleotides removed. It should be noted that the substrate domain contains three special nucleotides: the fluorescein-containing deoxyribothymidine (F; Fluorescein-dT) at the –10th position, the DABCYL-containing deoxyribothymidine (Q; DABCYL-dT; DABCYL: 4-(4-dimethylaminophenylazo)benzoic acid) at the –8th nucleotide, and an adenosine ribonucleotide (A) located between F and Q. The ribonucleotide acts as the lone RNA cleavage site while F and Q provide the required situation for fluorescence signaling upon catalysis, allowing for future biosensor engineering.

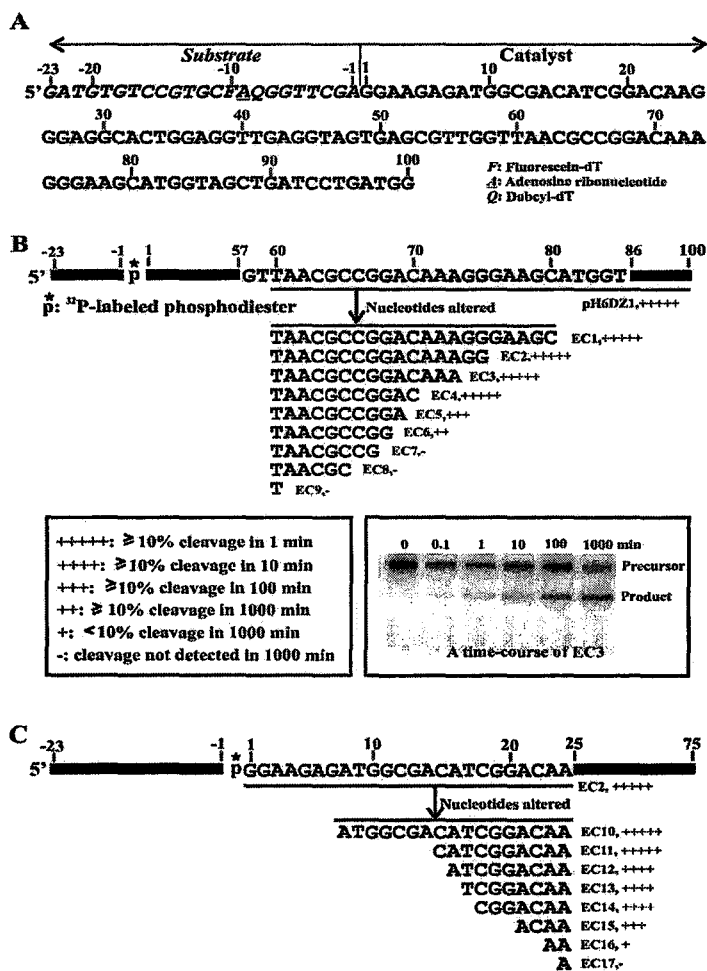


Figure 3.1 The nucleotide sequence of pH6DZ1 and the activity of various truncated deoxyribozymes. (A) The primary sequence of pH6DZ1. The self-cleaving deoxyribozyme is divided into the “substrate” domain (italicized letters; numbered –1 to –23 in the 3’-5’ direction) and the “catalyst” domain (normal letters only; numbered 1 to 100 in the 5’-3’ direction). (B) Results of truncating nucleotides from the 3’ end of the catalytic domain. The reference sequence is pH6DZ1. (C) Results of truncating nucleotides from the 5’ end of the catalytic domain. The reference sequence is EC2. Underlined nucleotides are the region under investigation, with the vertical arrow pointing to all mutated sequences. The identities of the nucleotides represented by black bars are omitted for clarity. The name of each mutant deoxyribozyme is listed next to its altered sequence. The activity of each construct in this figure (as well as in all remaining figures) is scored using the activity map shown as the left insert in (B). The right insert in (C) is a representative PAGE gel obtained with deoxyribozyme construct EC3.

The activity of all deoxyribozymes, analyzed using synthetic DNA oligonucleotides containing an internal ^{32}P -labeled phosphodiester, was scored as described in “Materials and Methods” (see the first insert in Figure 3.1B as well). Generally speaking, a mutant deoxyribozyme with a score of +++++ or ++++ is regarded as an efficient catalyst (with estimated $k_{\text{obs}} \geq 0.01 \text{ min}^{-1}$) while a sequence scored +++ or lower is treated as a poor catalyst. It should also be noted that this scoring method was adopted simply to provide a convenient and experimentally manageable way to compare the relative activities of a large number of mutant deoxyribozymes employed in this study.

3.1.2 Truncating nucleotides in the catalyst domain

Progressive removal of nucleotides at the 3' end of the catalyst domain was carried out first and the data are presented in Figure 3.1B. Truncating as many as thirty nucleotides from the 3' terminus had no apparent effect on the catalytic activity, as the mutant deoxyribozymes EC1-EC4 (E: enzyme; C: *cis*-acting) retained full catalytic activity. Removing one (EC5, +++) and two (EC6, ++) additional nucleotides from the same end resulted in a reduction in activity by two and three orders of magnitude, respectively. Further deletion of even one more nucleotide (EC7, -) completely abolished the RNA-cleaving activity. C₇₀ and G₆₈ are therefore the

3'-terminal nucleotides defining the fully active sequence and the minimally active sequence within the catalytic domain, respectively.

The effects of deleting nucleotides from the 5' end of the catalyst domain were assessed next. The first fourteen nucleotides in this region were completely dispensable, as the deletion of either seven nucleotides (EC10, +++++) or fourteen nucleotides (EC11, +++++) had no effect on the catalytic activity. Removing the next three nucleotides one at a time (EC12-14, all +++) all caused about a 10-fold reduction in activity. Removing three (EC15, +++) and five (EC16, +) more nucleotides significantly reduced the catalytic activity, by approximately 100 and 10000-fold, respectively. When the deleted residues were expanded to include A₂₃ (EC17, -), the RNA-cleaving activity was completely eradicated. Therefore C₁₅ and A₂₃ are the 5'-terminal nucleotides that define the fully active sequence and the minimally active sequence within the catalyst domain, respectively.

3.1.3 Truncating nucleotides in the substrate domain

Several deoxyribozyme constructs with sequential nucleotide truncations from either the 5' or 3' end of the substrate domain were examined using EC10 as the reference deoxyribozyme; the data are provided in Figure 3.2. Removing the first three 5'-terminal nucleotides (EC18, +++++) had no effect on the catalytic activity. Constructs with one (EC19, +++) and three (EC20, +++) more nucleotides deleted

from the same end both exhibited an activity ~10-fold less than EC10. Another 10-fold reduction in activity was observed when two more nucleotides were removed from the substrate chain (EC21, +++). The RNA-cleaving activity was completely lost with the further removal of three (EC22, -) or five (EC23, -) nucleotides.

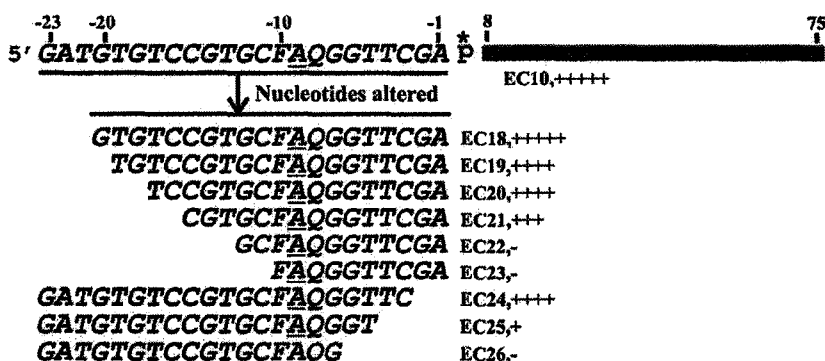


Figure 3.2 Nucleotide truncation in the substrate domain. The reference sequence is EC10. Underlined nucleotides are the region examined, with the vertical arrow pointing to all mutated sequences. The name of each mutant deoxyribozyme is listed next to each altered sequence. The activity of each deoxyribozyme construct is scored using the activity map shown in Figure 3.1B.

Interestingly, all nucleotides downstream of the cleavage site in the substrate domain were required for the optimal function of the deoxyribozymes, as deletion of even two nucleotides from the 3' end of the substrate domain (EC24, +++) lowered the catalytic activity by ~10 fold. Removal of two more nucleotides from the same end (EC25, +) led to a new construct whose activity was weakened by ~10,000 fold. Not surprisingly, the RNA cleavage activity was abolished upon deletion of two more

nucleotides (EC26, -). These results indicate that a majority of nucleotides in the substrate domain are required for the catalytic function of the deoxyribozyme. It is possible that these nucleotides are engaged in extensive secondary or tertiary interactions that are responsible for substrate recognition.

3.1.4 A putative secondary structure

Nucleotide truncation experiments not only established a significantly shortened *cis*-acting deoxyribozyme sequence but also implicated the functional involvement of up to 20 nucleotides in the original 23-nt substrate domain. Based on the fact that all known RNA-cleaving deoxyribozymes have used extensive Watson-Crick base-pairing interactions to engage substrate nucleotides both upstream and downstream of the cleavage site for binding (12, 16, 21, 41, 45, 46), we hypothesized that pH6DZ1 may adopt the same strategy for substrate recognition. Careful inspection of the sequence of the shortened deoxyribozyme EC10 indeed led to the identification of two short DNA duplexes, one on each end of the cleavage site (Figure 3.3A). The first duplex, denoted P1, is a 6-bp helix formed between G₋₁₄-T₋₁₉ and C₆₆-A₇₁. The second one, denoted P2, is also a 6-bp helix formed between A₋₁-G₋₆ and T₁₇-C₂₂.

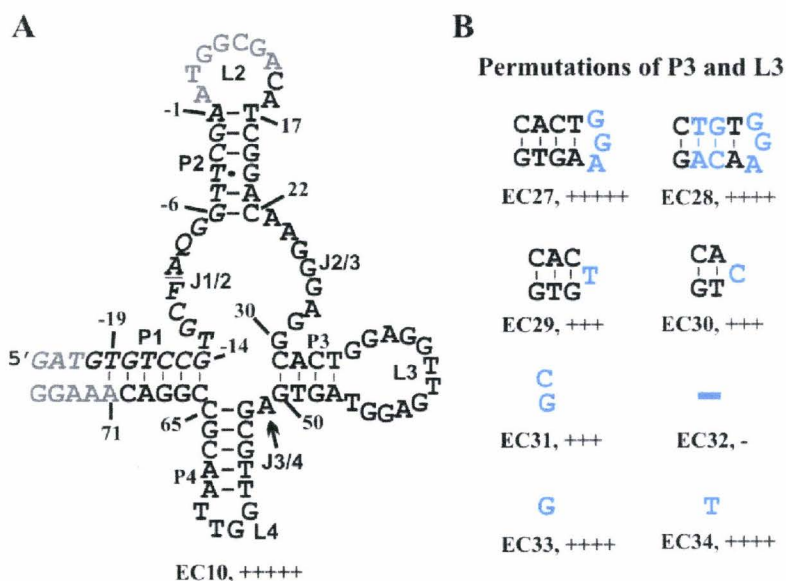


Figure 3.3 Proposed secondary structure of EC10 and role of nucleotides in P3 and L3. (A) The putative secondary structure of EC10, a truncated version of pH6DZ1. Individual elements are marked as P (pairing region), L (loop) and J (junction; single-stranded region between two pairing regions). Nucleotides in grey are dispensable nucleotides that can be removed without any loss of catalytic activity. Nucleotides in black are required for full activity up to the previous experiment (at least 10-fold activity reduction upon deletion or mutation). (B) Mutant deoxyribozymes with permuted P3 and/or L3 elements. The name of each mutant deoxyribozyme is given underneath each altered motif. The activity of each construct is scored using the activity map shown in Figure 1B. Nucleotides in blue are nucleotides altered in comparison to EC10. The thick short blue line denotes the deletion of entire P3-L3 motif.

Searching the remainder of the sequence for additional Watson-Crick base pairs led to the discovery of two more putative short DNA duplexes named P3 and P4 (Figure 3.3A). P3 is a 4-bp helix formed between C₃₁-T₃₄ and A₄₇-G₅₀, while P4 is a 6-bp helical region involving G₅₂-T₅₆ and A₆₁-C₆₅. These four short duplexes create six single-stranded DNA elements referred to as L2 (L: loop; L2: the DNA loop

attached to P2), L3, L4, J1/2 (J: junction; J1/2: the single-stranded sequence linking P1 and P2), J2/3 and J3/4 throughout this report. Most significantly, the multiple helical and unstructured elements give pH6DZ1 a unique secondary structure that is different from those described for all known RNA-cleaving deoxyribozymes (12, 16, 21, 41, 45, 46).

For the remaining figures in the structural studies, the following nucleotide-colouring scheme will be used: nucleotides in grey can be safely removed without the loss of activity; nucleotides in black are required for full activity (mutating or deleting each of these nucleotides will result in a reduction in activity from +++++ to +++++), based on the results obtained from previous mutations; nucleotides in green are absolutely essential for catalysis (mutating any of these nucleotides will completely inactivate the deoxyribozyme); nucleotides in red play an important, although nonessential, role in the catalytic function of the deoxyribozyme (mutating any of these nucleotides will result in a reduction in activity by at least two orders of magnitude, from +++++ to +++ or lower).

3.1.5 Permutations of P3 and L3

The first mutant deoxyribozyme in Figure 3.3B, EC27, was designed to test the dispensability of L3. In this deoxyribozyme construct, the original 12-nt L3 in the reference deoxyribozyme EC10 was replaced with an arbitrarily chosen GGA triloop.

The fact that EC27 was fully active demonstrates that L3 has no relevance to the catalytic function of the deoxyribozyme. The next four deoxyribozymes—EC28-32—were designed to examine the importance of P1. In EC28, the two middle base pairs of P1 were altered from A-T and C-G to T-A and G-C, respectively. These changes resulted in moderate activity reductions (+++++ to ++++). The constructs in which the stem-loop element of EC10 was progressively reduced to 7 nt (EC29, +++), 5 nt (EC30, +++) and 2 nt (EC31, ++) all exhibited an RNA-cleaving activity that was reduced by two orders of magnitude, while the deletion of the entire P3-L3 motif (EC32, -) completely deactivated the deoxyribozyme. Interestingly, when a single nucleotide (G in EC33 and T in EC34) was inserted into the location of the deleted stem loop, most of the catalytic activity was recovered (++++). These results collectively demonstrate that P3 and L3 do not contain any catalytically essential or vital nucleotides. However, P3 appears to play a structural role that is important for the optimal activity of the deoxyribozyme.

3.1.6 Permutations of P4, L4 and J3/4

These three elements together cover the nucleotides from A₅₁ to C₆₅ (Figure 3.4). Alteration of base-pairing partners of each of the five base pairs in P4 resulted in a reduction in activity by 10-1000 fold, depending on the location of the specific base pair. Conversion of the first base pair (G₅₂-C₆₅) from G-C to C-G (EC35) or the

second base pair (C₅₃-G₆₄) from C-G to G-C (EC36) caused only a 10-fold activity reduction. When the third base pair (G₅₄-C₆₃) was changed from G-C to A-T (EC38), no decrease in activity was observed; however, if the same base pair was altered to C-G pair (EC37), the activity dropped by 100-fold. Alteration of each of the last two base pairs (T₅₅-A₆₂, T₅₆-A₆₁) from T-A into any other base pair produced a much more profound change in the catalytic activity, as each mutant deoxyribozyme (EC39-43) was barely active.

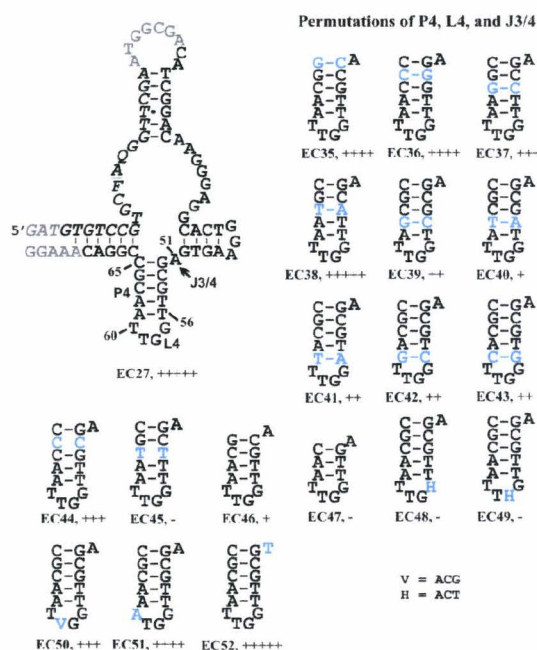


Figure 3.4 Permutations of P4, L4 and J3/4. The putative secondary structure of EC27 is shown on the left and mutant deoxyribozymes with permuted P4 (EC35-47), L3 (EC48-51) or J3/4 (EC52) are shown on the right. The name of each mutant deoxyribozyme is given underneath each altered motif. Nucleotides shown in blue are the actual altered nucleotides in each construct in comparison to EC27. Other nucleotide colouring schemes are identical to those used in the previous figure. The activity scoring scheme is the same as that given in Figure 3.1B.

Two mutant deoxyribozymes with a mismatch present at the second base pair (EC44, C-C mismatch) or third base pair (EC45, T-T mismatch) were also examined. With EC44, the catalytic activity was reduced by 100-fold, while for EC45, the enzymatic activity was completely abolished. We also examined the influence of shortening P3 by eliminating one or two base pairs (EC46 and EC47, respectively). EC46 was catalytically very weak (EC46, +) while EC47 was completely inactive (EC47, -). Low activity (++) was also observed in the mutant deoxyribozyme in which an additional G-C pair was inserted above the two T-A pairs (data not shown). The above data can be interpreted as follows: (i) P3 does exist; (ii) the identity of each base pair, particularly the last two base pairs, is important for the deoxyribozyme function; (iii) the size of P3 is also important for the function of the deoxyribozyme.

The 4-nt loop L4 was examined next. Changing G₅₇ to T (data not shown) or H (equal mixture of A/C/T; EC48) resulted in no detectable activity. The same outcome was observed when G₅₈ was changed to T (data not shown) or H (EC49). These experiments show that these two residues are absolutely required for catalytic function. In contrast, the last two nucleotides of L3—T₅₉ and T₆₀—are not as essential because the mutant deoxyribozyme with the T-to-V mutation at nucleotide 59 (EC50; V: equal mixture of A/C/G) or the T-to-A mutation at nucleotide 60 (EC51) exhibited about 1/100 and 1/10 of the activity of the reference deoxyribozyme EC27, respectively.

Lastly, the identity of the single nucleotide—A₅₁—located between P3 and P4 was investigated. Based on the observation that no decrease in activity was produced when A₅₁ was replaced with T₅₂ (EC52), it can be concluded that this nucleotide is not a functionally vital residue.

The above analyses led to the identification of two functionally essential nucleotides, G₅₇ and G₅₈ (coloured green in Figure 3.5A), and five functionally important nucleotides in T₅₅, T₅₆, T₅₉, A₆₁, A₆₂ (labelled red in Figure 3.5A).

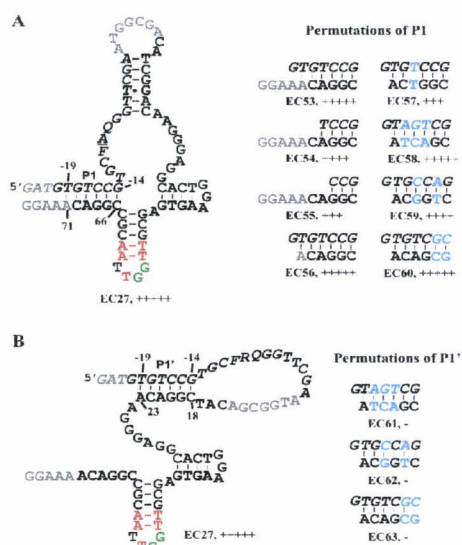


Figure 3.5 P1 and alternative P1 (P1'). (A) Deoxyribozyme constructs for examining the formation of P1. The putative secondary structure of EC27 is shown on the left and the mutant deoxyribozymes within which P1 (formed between G₁₄-T₁₉ and C₆₆-A₇₁) is permuted are shown on the right. (B) Alternative pairing between G₁₄-T₁₉ and C₁₈-A₂₃. The name of each mutant deoxyribozyme is given underneath the each altered motif. Nucleotides shown in blue are the actual altered nucleotides in each construct in comparison to EC27. Nucleotides in green are absolutely essential (no activity observed upon alteration); those in red are catalytically important (activity reduced by at least 100 fold after a base mutation). Other nucleotide colouring schemes are identical to those used in previous figures. The activity scoring scheme is same as that given in Figure 3.1B.

3.1.7 Permutations of P1

P1 is one of the two helices proposed to be responsible for substrate binding. Several deoxyribozyme constructs based on EC27 were designed to test whether P1 indeed exists and whether any nucleotide within P1 is critically involved in the catalytic function of the deoxyribozyme. The data are presented in Figure 3.5A.

Deletion of first three overhanging nucleotides at the 5' end of the substrate domain (G₋₂₃-T₋₂₁) had no influence on the catalytic activity (EC53, +++++). However, further removal of three and four additional nucleotides in the same domain, which resulted in a shortened P1 of only four and three base pairs, respectively, led to the deoxyribozyme constructs (EC54, ++++; EC55, +++) with activity weakened by 10 and 100-fold, respectively. Not surprisingly, most of the unpaired nucleotides at the 5' end of the substrate domain and the 3' end of the catalytic domain can be safely removed without any influence on the catalytic activity (EC56, +++++). However, deletion of G₋₄ resulted in a 10-fold activity reduction (data not shown). This finding, consistent with the data presented earlier (see EC19 in Figure 3.2), seems to suggest that G₋₄ may contribute to the stability of the relatively short duplex P1 or facilitate the geometric orientation of P1 in an optimally folded structure of the deoxyribozyme. The importance of a stable P1 was further manifested by the use of one additional construct, EC57: when a single mismatch pair was introduced in the middle of P1, the catalytic activity was weakened by 100-fold.

We next examined whether the identities of individual nucleotides within P1 were important for catalytic function by constructing three mutant deoxyribozymes, EC58-60, with significantly altered base pairs. EC58 has three consecutive central nucleotide co-variations, EC59 has two separate internal co-variations, while EC60 has two terminal co-variations. All these mutant deoxyribozymes exhibited either full activity (EC58 and EC60, ++++++) or near-full activity (EC59, ++++). The data given in Figure 3.5A allow us to conclude that (i) P1 does exist; (ii) a stable P1 is required for the full activity of the deoxyribozyme; (iii) P1 does not contain any catalytically crucial nucleotide.

3.1.8 Alternative P1 (P1')

EC27 has an additional CGGACA motif located between the 18th and 24th nucleotides. This raises a possibility of alternative helical formation that involves G₋₁₄-T₋₁₉ and C₁₈-T₂₃ and we term this alternative duplex P1' (Figure 3.5B). Three new mutant deoxyribozymes, EC61-63, were designed to rule out the existence of P1'. EC61-63 were constructed with the same set of substrates used for EC58-60 but with corresponding changes in the C₁₈-A₂₄ region (rather than C₆₆-A₇₁ for EC58-60). None of these mutants exhibited any catalytic activity. This analysis convincingly demonstrates that G₋₁₄-T₋₁₉ are pairing with C₆₆-A₇₁ to form P1, and therefore not pairing with C₁₈-A₂₃ to form P1'.

10-fold. When the T₄•G₂₀ wobble pair was mutated into a T-A pair (EC68, +++++), no reduction in activity was observed; if the same wobble pair was changed to the C-G pair (EC69, ++++), the activity decreased by 10-fold. When T₅-A₂₁ was switched to A-T, a 10-fold activity decrease was obtained; in contrast, replacing the same base pair with an A-A mismatch caused a 1000-fold reduction in activity. These data collectively suggest that the content of all these four base pairs was not very important to the function of the deoxyribozymes, as long as the Watson-Crick rules were followed.

The last base pair in P2, namely the G₆-C₂₂ pair, behaved considerably differently. Changing G₆-C₂₂ either to C-G (EC72, ++) or to T-A (EC73, ++) led to a 1000-fold reduction in activity. In contrast, when G₆ was not mutated but C₂₂ was changed to T (EC74, +++) or A (EC75, +++), 10- and 100-fold reductions in activity were observed, respectively. These results could not conclusively support or rule out the formation of a proposed G₆-C₂₂ pair. However, it is certain that the identity of G₆ is vital for the enzymatic function.

3.1.10 Permutations of J2/3

J2/3 contains eight unpaired bases linking P2 and P3. Interestingly, all nucleotides in this motif are purine nucleotides. To examine the roles of these

nucleotides, many mutant deoxyribozymes were synthesized and tested and the results are shown in Figure 3.7.

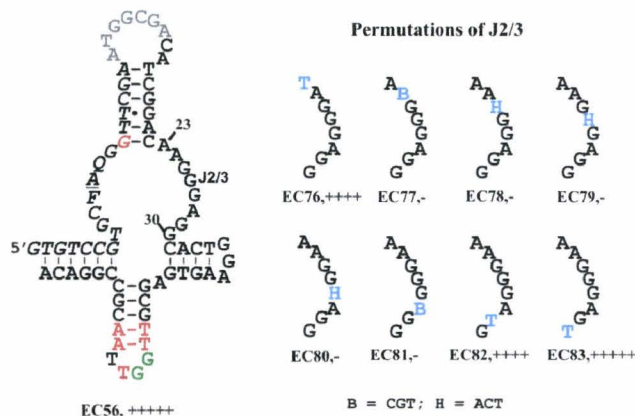


Figure 3.7 Permutations of J2/3. J2/3 is the single stranded region covering the 23rd to 30th nucleotides. The putative secondary structure of the reference deoxyribozyme EC56 is shown on the left and mutant deoxyribozymes each containing a single base mutation within J2/3 are shown on the right. The nucleotide colouring scheme and activity scoring method are identical to those used in the previous figures.

The mutant EC83 (+++++), containing a G-to-T mutation at the 30th nucleotide, exhibited full catalytic activity. The other two mutants EC76 (+++++) and EC82 (++++), which contained an A-to-T mutation at the 23rd position and a G-to-T mutation at the 29th position, respectively, showed relatively moderate (10-fold) reductions in activity. These findings indicate that none of these three purine nucleotides play critical roles in the function of the deoxyribozyme. Most importantly, the RNA-cleaving activity was completely lost in all the constructs in which A₂₄, G₂₅, G₂₆, G₂₇, or A₂₈ were mutated to T (data not shown) or to the mixture of the remaining nucleotides (B₂₄, H₂₅,

H₂₆, H₂₇, B₂₄, respectively, in EC77, EC78, EC79, EC80, and EC81; B: equal mixture of CGT; H: equal mixture of ACT). These data reveal that these five nucleotides are essential to the catalytic function of the deoxyribozyme.

3.1.11 Permutations of J1/2

J1/2 is the single-stranded region that contains two specially modified deoxyribonucleotides Q₋₈ (DABCYL-dT) and F₋₁₀ (fluorescein-dT), the lone ribonucleotide A₋₉ (ribo-A), and four standard deoxyribonucleotides G₋₇, C₋₁₁, G₋₁₂, and T₋₁₃. Deoxyribozyme constructs in which either fluorescein (EC84) or DABCYL (EC85) or both (data not shown) were removed exhibited no catalytic activity. This result indicates that both the fluorescein and DABCYL labels are crucial to the deoxyribozyme function.

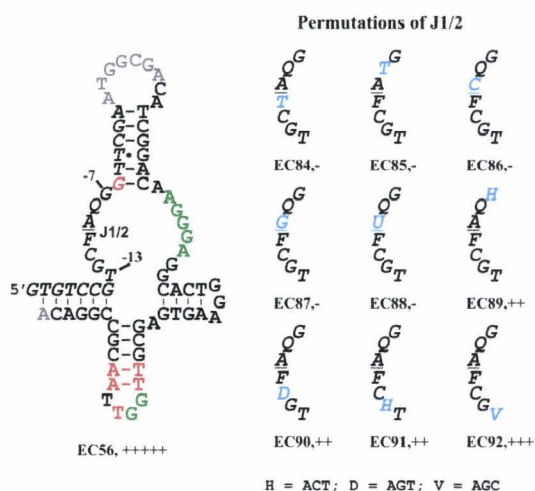


Figure 3.8 Permutations of J1/2. J1/2 is the single stranded region located between the -7 and -13 nucleotides. The putative secondary structure of EC56 is shown on the left and mutant deoxyribozymes containing a single base mutation within J1/2 are shown on the right. The nucleotide colouring schemes and activity scoring methods are identical to those used in previous figures.

The adenosine ribonucleotide is also a nucleotide specifically required for the deoxyribozyme function. This was revealed by the complete loss of activity in each of the three constructs in which the cleavage site was changed to either a cytidine ribonucleotide (EC86), a guanosine ribonucleotide (EC87) or a uridine ribonucleotide (EC89). This finding implies that the adenine attached to ribose is involved in highly specific interactions that are part of the active deoxyribozyme structure.

Three of the four remaining nucleotides within J1/2—G₇, C₁₁, and G₁₂—appear to play crucial roles in the catalytic function of EC57 as well, because mutating any of these nucleotides to the mixture of the three remaining residues (G₇ to H₇, EC89; C₁₁ to D₁₁, EC90; G₁₂ to H₁₂, EC91; H = ACT, D = AGT) weakened the RNA-cleaving activity by 1000-fold. The other nucleotide in this single-stranded

region, T₋₁₃, is only important for the optimal function of the deoxyribozyme as the T-to-V mutation (V = AGC, EC92) caused a 100-fold activity reduction.

3.1.12 DNA footprinting via methylation interference

From the extensive mutagenesis studies presented above, it is clear that the active site of the deoxyribozyme must involve some or all of the 10 functionally essential nucleotides (green nucleotides in Figure 3.9A) located in J1/2, J3/4, and L4. Noticeably, half of these nucleotides contain a guanine base. Therefore, it is certain that some of these guanine residues are critically involved in the catalytic function of the deoxyribozyme.

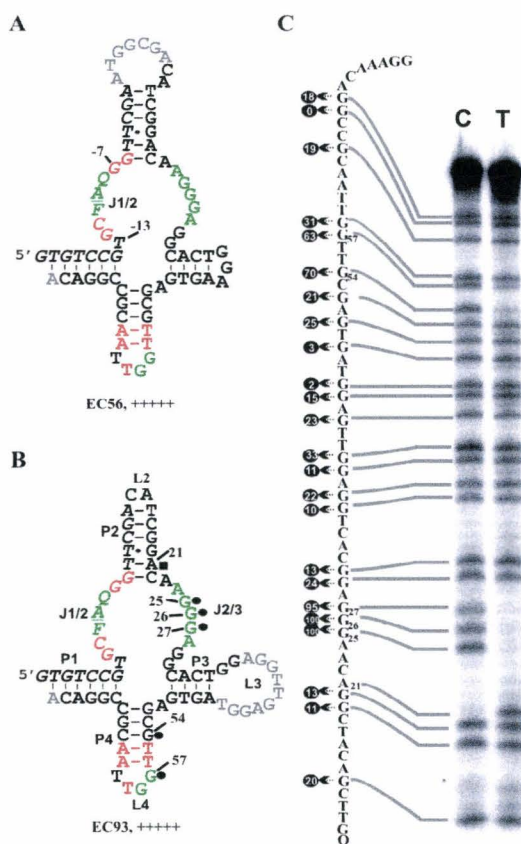


Figure 3.9 Methylation interference. (A) The confirmed secondary structure of fully functional deoxyribozyme EC56 with all nucleotides properly coloured. (B) The secondary structure of fully functional deoxyribozyme construct EC93 with labelled nucleotides that have produced a methylation interference effect. The black circles indicate the guanines that have shown substantial methylation interference effect. The black square indicates the lone adenine that is significantly methylated. (C) Methylation data. 3'-cleavage fragment obtained from EC93 treated with DMS before (Test-T) or after (Control-C) the cleavage reaction was performed and labelled at the 5' end with ^{32}P . Under our reaction condition, DMS only methylated the N7 atom of one guanine per deoxyribozyme molecule. Methylated guanines were cleaved by piperidine and cleaved fragments were resolved by denaturing 10% PAGE. Methylated guanines that disrupt deoxyribozyme activity appear lighter in lane T than lane C. The degree of interference at each guanine (each circled number) was normalized as described in "Materials and Methods" (with 0 being the minimal observable interference and 100 being the maximum). Filled black circles at the right side of the gel indicate the DNA bands with significantly reduced intensity (score of 50 and above) in T lanes while the black square labels the DNA band that had a significantly enhanced intensity in the T lane.

We sought to use the methylation interference approach to obtain additional evidence that can further implicate these guanine residues in the tertiary folding or catalytic function of the deoxyribozyme. More specifically, we wanted to determine whether the methylation of the N7 atom of each functionally essential guanine within the fully functional deoxyribozyme construct EC57 (+++++) could be tolerated. Theoretically, the methylation of N7 of a functionally essential guanine residue is prohibited if any one of the following three scenarios applies: (i) the N7 atom is directly involved in a tertiary interaction that is essential for structural folding or catalytic function, (ii) the N7 atom is located in a defined structural arrangement that can not tolerate the bulky methyl group, and (iii) the positive charge introduced by the methylation disrupts the vital electronic landscape where the guanine residue is located. In contrast, the guanine residues that have already been shown to be functionally insignificant usually should not produce a significant methylation interference effect.

For each methylation interference experiment, we used a set of two samples, which are denoted “control” and “test” (44). For the control sample, the methylation was performed on the 3'-cleavage product generated after the catalytic action of the *cis*-acting deoxyribozyme. When methylation is performed in water (where DNA exists in an unfolded random-coil state), every guanine residue should be methylated with approximately equal probability. Methylation was also carried out under a

reaction condition that only allowed the methylation of one guanine per DNA molecule. Therefore, upon cleavage by piperidine and analysis by denaturing PAGE, DNA fragments, each corresponding to the cleavage product at a given guanine location, should be observed with relatively equal intensity. For the test sample, methylation with DMS in water was carried out with the deoxyribozyme construct prior to the self-cleavage reaction. If the methylation at a particular guanine residue hindered the self-cleaving activity of the deoxyribozyme, no RNA cleavage or significantly reduced RNA cleavage should occur in all individual DNA molecules that contained the methylated guanine. As a result, these molecules should be under-represented in the cleaved product. This will eventually lead to a missing DNA band or a band with significantly reduced intensity on denaturing PAGE. In other words, methylated guanines that hindered proper enzymatic activity were identified by their absence or reduced visibility in the resultant ladder in the test lane (T lanes in Figure 3.9C) as compared to the control lane where all guanines are represented (C lanes in Figure 3.9C). The degree of interference was calculated using densitometry measurements of corresponding bands from each lane with a value of 100 indicating the most interference observed to 0 indicating the least. These values are given in the black circles shown next to their corresponding guanines within Figure 3.9C. Bases with values above 50 were considered to yield a substantial interference effect and were labelled with a filled circle next to the nucleotide within Figure 3.9B.

Among the five functionally essential guanines identified by mutagenic analysis, three (G_{25} , G_{26} , and G_{27}) exhibited extremely severe methylation interference and one (G_{57}) displayed a high level of interference. The only exception was G_{58} , which exhibited a less severe but still noticeable effect. The methylation of the N7 atom of a guanine located in standard duplexes and unstructured loops should not have a strong methylation interference effect. As expected, there is no substantial methylation interference at all for the guanines of P1, P2, P3, L3 and most guanines in P4. The only exception was G_{54} . Considering that G_{54} is located next to two functionally important T-A pairs and that the nearby L4 harbours two catalytically essential nucleotides, this finding is not very surprising. We suspect that L4 may interact with J1/2 and J2/3 to form the active site of the deoxyribozyme and this may place G_{54} in a tight spatial arrangement that could not tolerate the methyl group on its N7 atom.

Interestingly, although most of the adenines within the deoxyribozyme were weakly methylated under our methylation conditions (based on the observation that most of adenines were not cleaved after piperidine treatment), A_{21} was found to be hyper-methylated. The precise reason for this finding cannot be determined at this point; however, we suspect that the observed hyper-methylation may somehow relate to the fact that the G_{-6} - C_{22} pair adjacent to A_{21} did not tolerate any Watson-Crick co-variation. It is possible that G_{-6} and C_{22} either do not engage themselves into a Watson-Crick base pair or form a highly unusual base pair that deviates significantly

from the geometry of a standard Watson-Crick base pair. Methylation of the N7 of the nearby A₂₁ may strongly stabilize the required geometric arrangement, and therefore, the methylated A₂₁ was significantly over-represented in the cleaved product.

The above methylation interference experiment has provided strong evidence that further implicates the functional involvement of almost all of the indispensable guanine residues identified by mutational analysis.

3.1.13 Comparison of Metal-ion specificity and pH dependence of pH6DZ1 and EC56

The original pH6DZ1 was known to function in the presence of both Mn(II) and Ni(II) but was incapable of performing catalysis in the presence of Mg(II) and Ca(II) (39). To determine whether the minimization of the catalytic DNA sequence altered the metal-ion specificity, an experiment was performed to compare the metal-utilizing abilities of the full-length pH6DZ1 and shortened EC56. The data are shown in Figure 3.10.

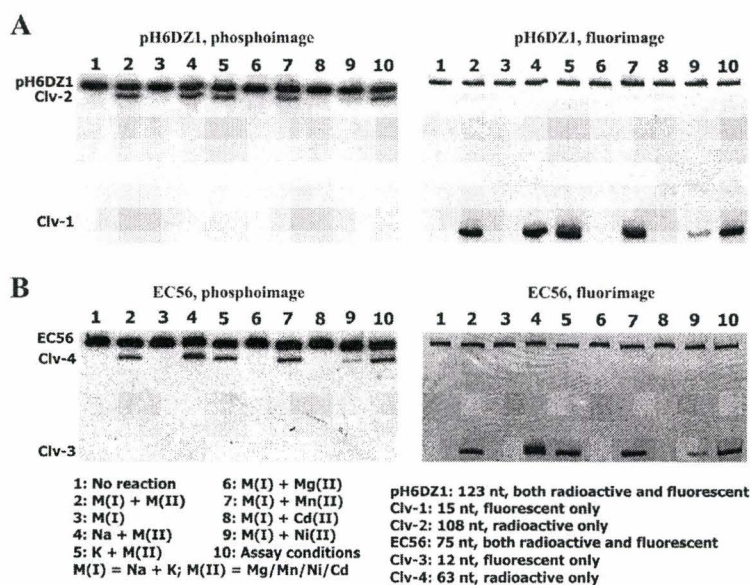


Figure 3.10 Comparison of metal-ion specificity of pH6DZ1 and EC56. Each DNA catalyst containing a ^{32}P -phosphodiester bond linking the substrate domain to the catalyst domain was tested for RNA cleavage under various salt conditions. Reaction products were analyzed on 10% denaturing PAGE, which was scanned for both radioactivity (left image) and fluorescence (right image). Clv-1 and Clv-2 denote the 5' and 3' cleavage products from pH6DZ1, and Clv-3 and Clv-4 are for the 5' and 3' cleavage products from EC56. Metal ions present are as follows (all in mM): no metal ions (lane 1); 400 Na^+ , 100 K^+ , 8.5 Mg^{2+} , 5 Mn^{2+} , 1.25 Cd^{2+} and 0.25 Ni^{2+} (lane 2); 400 Na^+ and 100 K^+ (lane 3); 400 Na^+ , 8.5 Mg^{2+} , 5 Mn^{2+} , 1.25 Cd^{2+} and 0.25 Ni^{2+} (lane 4); 100 K^+ , 8.5 Mg^{2+} , 5 Mn^{2+} , 1.25 Cd^{2+} and 0.25 Ni^{2+} (lane 5); 400 Na^+ , 100 K^+ , 15 Mg^{2+} (lane 6); 400 Na^+ , 100 K^+ , 10 Mg^{2+} , 5 Mn^{2+} (lane 7); 400 Na^+ , 100 K^+ , 13.75 Mg^{2+} and 1.25 Cd^{2+} (lane 8); 400 Na^+ , 100 K^+ , 14.75 Mg^{2+} and 0.25 Ni^{2+} (lane 9); 800 mM Na^+ , 8 mM Mn^{2+} , 2 mM Ni^{2+} (lane 10; as the optimal metal ions established in ref. 39). Each reaction mixture also contained 50 mM HEPES, pH 6.0.

For this experiment, each deoxyribozyme was labeled with ^{32}P at the phosphodiester bond linking the substrate domain to the catalytic domain, in addition to having the standard fluorescein-dT, ribo-A, and DABCYL-dT moieties. This labelling pattern made the uncleaved deoxyribozyme (the top DNA bands in Figure 10,

A and B, respectively) both fluorescent and radioactive. Upon RNA cleavage, two products were expected: a large DNA fragment (Clv-2 from pH6DZ1; Clv-4 from EC56; Figure 10) that was only radioactive and a small DNA fragment (Clv-1 from pH6DZ1; Clv-3 from EC56; Figure 3.10) that was only fluorescent. Moreover, for each reaction the ratio of fluorescence intensity of the small cleavage fragment vs. that of the uncleaved DNAzyme should be significantly larger than the ratio of radioactivity of the large fragment vs. that of the uncleaved DNAzyme, because fluorescence dequenching was expected to occur upon cleavage.

When each deoxyribozyme was assessed for the cleavage activity and scanned for both radioactivity (left image for each deoxyribozyme, Figure 3.10) and fluorescence (right image), the expected fragmentation and signaling patterns were indeed observed, indicating that each deoxyribozyme cleaved the embedded RNA linkage and produced a highly fluorescent 5'-cleavage fragment. More importantly, comparison of the cleavage bands clearly indicates that EC56 and pH6DZ1 have identical metal-ion specificity: both exhibited a strong activity with Mn^{2+} and a reduced activity with Ni^{2+} , but were incapable of using Mg^{2+} or Cd^{2+} .

We also conducted an experiment to compare the pH dependence of the same full-length and shortened DNAzymes, using the same procedure described above for the metal-ion specificity comparison. The data are presented in Figure 3.11. Not surprisingly, both pH6DZ1 and EC56 have identical pH dependences: the highest

activity occurs at pH 6.0, complete loss of activity is observed when the acidity of the solution increased to pH 5.2, and a progressive decrease in activity resulted when the solution pH was raised from pH 6.0 to 8.0.

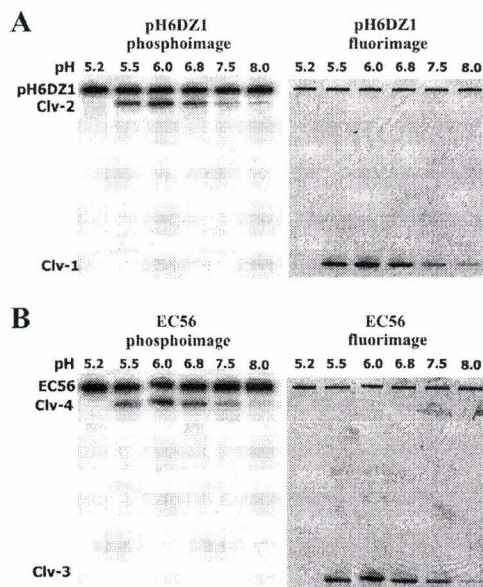


Figure 3.11 Comparison of pH dependence of pH6DZ1 and EC56. Each DNA catalyst containing a ^{32}P -phosphodiester bond linking the substrate domain to the catalyst domain was tested for RNA cleavage under various pH settings listed in the figure. Reaction products were analyzed on 10% denaturing PAGE, which was scanned for both radioactivity (left image) and fluorescence (right image). Clv-1 and Clv-2 denote the 5' and 3' cleavage products from pH6DZ1, and Clv-3 and Clv-4 are for the 5' and 3' cleavage products from EC56. Salt conditions were: 800 mM Na^+ , 8 mM Mn^{2+} , 2 mM Ni^{2+} . The solution pH was controlled with the following buffering reagents (each used at 50 mM): MES for pH 5.2 and 6.0, HEPES for pH 6.8, 7.5 and 8.0. These pH values were chosen according to Reference 39.

The data illustrated in Figure 3.10 and 3.11 clearly indicate that the removal of the nonessential nucleotides from pH6DZ1 did not alter its metal ion binding properties

or pH sensitivity of the original construct. Therefore, we can conclude that EC56 is a true representation of minimized pH6DZ1.

3.1.14 Predicted secondary structure of pH6DZ1

Through comprehensive sequence truncations, we have reduced the size of *cis*-acting pH6DZ1 from 123 nt to ~70 nt. Forty-four of these remaining nucleotides are distributed into four short helices of 4-6 base pairs each. All these helical nucleotides appear to play only structural roles that support the optimal activity of the deoxyribozyme, based on the observation that each of these nucleotides can tolerate a base mutation to some degree. Eighteen of the twenty-two base pairs can each be changed to another Watson-Crick base pair without significant loss of the catalytic activity. The three remaining base pairs, two in P4 and one in P2, are highly identity-specific and replacing any of them with another Watson-Crick base pair can result in a loss of activity by three orders of magnitude. Interestingly, each of these three base pairs is located near a single-stranded region that contains some catalytically indispensable nucleotides. Therefore, these three base pairs may participate in some important interactions that support the formation of the active site.

Three hairpin loops also exist in the structure. Two of them, L2 and L3, do not appear to contain any functionally important nucleotide and both loops can be significantly shortened without any reduction in activity. However, the remaining

4-nt loop L4 has two guanine residues that cannot be mutated. It is possible that these two guanines are part of the active site or at least participate in the tertiary interactions that are essential for the folding of the active deoxyribozyme structure.

The remaining 15 nucleotides are distributed into two inter-helical single-stranded motifs, J1/2 and J2/3, of 7 and 8 nt, respectively. The 7-nt J1/2 possesses the ribonucleotide, the fluorophore-modified dT, and the quencher-modified dT, along with four other standard deoxyribonucleotides. The fact that neither the fluorophore nor the quencher can be removed, and that the adenine located on the ribose at the cleavage site cannot be mutated into other bases, strongly suggest that these three moieties are essential for substrate recognition, or contribute significantly to the formation of the active site. Three of the four remaining nucleotides in J1/2 were found to be functionally important, signifying that they may participate in important tertiary interactions that support the active site.

The 8-nt motif J2/3 contains five functionally essential nucleotides, all of which are purine bases. Considering that J2/3 is located in physical proximity to the cleavage-site-containing J1/2 and that most of the nucleotides in these two single-stranded regions are either indispensable or less prone to mutagenesis, we conclude that J2/3 and J1/2 must contribute the crucial nucleotides that form the active site of the deoxyribozymes.

In summary, our data indicate that pH6DZ1 has a four-way junction-like secondary structure comprised of four short duplexes, three hairpin loops, and three interhelical unpaired elements. Ten nucleotides, all located in two separate single-stranded regions, were identified as functionally indispensable nucleotides (complete loss of the catalytic function was obtained upon mutation). Nine nucleotides, most of which are also distributed in three single-stranded DNA elements, were identified as functionally vital nucleotides (at least a 1000-fold activity reduction was obtained upon mutation).

3.2 Catalytic and signaling properties of trans-acting pH6DZ1

3.2.1 Engineering and kinetic analyzing the trans-acting pH6 DNA enzyme

As noted above, we have found that pH6DZ1 has a four-way junction structure, which is more complex than all other known RNA-cleaving deoxyribozymes. Figure 3.12 shows the confirmed secondary structure of EC56—the minimized version of pH6DZ1—that contains four short duplexes (P1-P4), three hairpin loops (L2-L4), and two inter-helical unpaired elements (J1/2 and J2/3). Seven nucleotides located in J2/3 and L4 (shown in black circles) have been identified as functionally indispensable nucleotides. It should be noted that in the secondary structure of EC56, “F” and “Q” represent deoxythymidines modified respectively with a fluorescein and DABCYL, “R” is adenine ribonucleotide, and the remaining nucleotides are all

deoxyribonucleotides. The elucidation of the secondary structure of pH6DZ1 permitted the design of a “true” catalyst that can process external substrates with turnover.

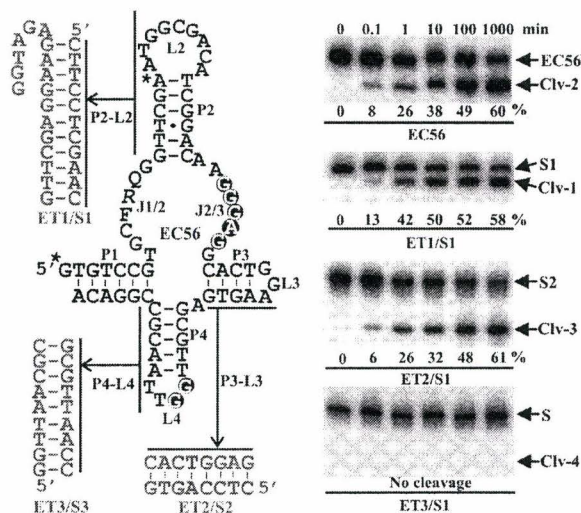


Figure 3.12 Designing trans-acting systems for pH6DZ1. EC56—a minimized version of pH6DZ1—was used as the reference cis-acting deoxyribozyme. EC56 is a 75-nt chimeric DNA/RNA sequence with the following special nucleotides: F: fluorescein-dT; Q, DABCYL-dT; R, adenosine ribonucleotide. All other nucleotides are deoxyribonucleotides. For the activity assay, EC56 was labeled with a ^{32}P -phosphodiester bond linking the 20th and 21st nucleotide. Upon RNA cleavage, a 12-nt non-radioactive fragment (denoted “clv-1”, invisible in the radioactivity scan) and a 63-nt radioactive fragment (“clv-2”, visible in the radioactivity scan) would be produced (see the first gel image in the figure). Trans-acting constructs were made by sequence manipulations at P2-L2, P3-L3, and P4-L4 locations; the resulting systems are denoted ET1/S1, ET2/S2 and ET3/S3, respectively. For the activity assay of ET1/S1 (second image), the 31-nt S1 was labeled with a ^{32}P -phosphate at the 5'-end; upon RNA cleavage, the visible 12-nt clv-1 and invisible 19-nt fragment are produced. For the activity assays of ET2/S2 (third image) and ET3/S3 (fourth image), 51-nt S2 and 64-nt S3 were both labeled with a ^{32}P -phosphodiester bond linking the 20th and 21st nucleotides (as with EC56). As a result, visible 39-nt clv-3 and 52-nt clv-4 are produced respectively. EC56 was used at 100 nM. ET1, ET2 and ET3 were used at 500 nM while each matching substrate was used at 10 nM.

The unique four-way junction structure of EC56 allows the design of several trans-acting systems simply by separating the cis-acting EC56 into two separate entities at L2, L3 or L4. The resulting bimolecular systems are denoted in this thesis as ET1/S1 (E: enzyme; T: trans; S: substrate), ET2/S2 and ET3/S3, accordingly. We deliberately extended the number of base pairs of P2, P3 or P4 in these trans constructs to create stable duplexes for strong interactions between the two partners of each relevant bimolecular construct. The substrate cleavage activity of each bimolecular construct was first assessed under single-turnover conditions ($E:S = 50:1$) using a qualitative scoring scheme described in “Materials and Methods”. As a comparison, the activity of EC56 was also examined by the same method. The results from these experiments are illustrated in the gel images given on the right side of Figure 3.12.

ET1/S1, derived from the sequence manipulation at P2 and L2, exhibited an RNA cleavage activity that was slightly better than EC56 (more substrate cleavage observed at both 0.1, 1 and 10 min). ET2/S2, as a result of sequence manipulation at P3 and L3, exhibited an almost identical time-course profile relative to EC56. On the other hand, ET3/S3, from the sequence manipulation at P4 and L4, was found to be completely inactive (no detectable cleavage even at 1000-min time point). These findings are not surprising because our prior work has shown that L2 and L3 do not contain any functionally important nucleotides (circled residues in the secondary structure of EC56) while two such nucleotides reside in L4. In other words, L4 plays

an indispensable role in the formation of the active structure of pH6DZ1 and cannot tolerate the sequence manipulation required for the design of a trans system at this location. In contrast, L2 and L3 are not part of the active site and can be manipulated with ease.

We chose to focus on ET1/S1 for multiple turnover analysis based on the consideration that this trans construct is not only more catalytically active than ET2 but also represents more of a classic trans system in which all catalytically important nucleotides are located in the deoxyribozyme strand. We first determined the initial velocity (v_0) at substrate concentrations of 50, 100, 200, 300, 400, 700 and 1000 nM and a fixed enzyme concentration of 10 nM. Figure 3.13 plots the v_0 values vs. the substrate concentrations obtained from three independent measurements. We found that the data fitted well to the Michaelis-Menten equation, resulting in a k_{cat} of $0.81 \pm 0.05 \text{ min}^{-1}$ and a K_M of $218 \pm 41 \text{ nM}$.

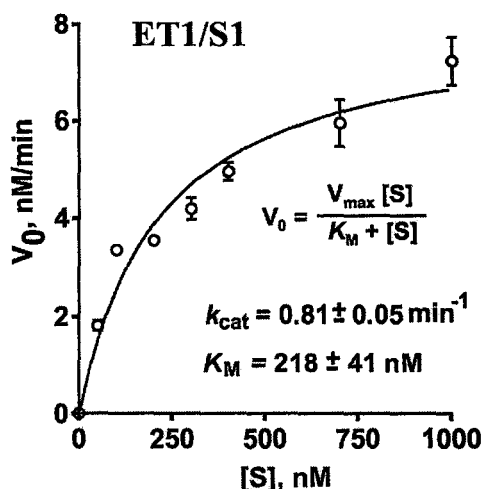


Figure 3.13 Examining the multiple turnover capability of ET1/S1. The substrate S1 was labeled at the 5'-end-with [γ - ^{32}P]ATP and PNK. 10 nM of ET1 was incubated with S1 at 50, 100, 200, 300, 400, 700 and 1000 nM in the reaction buffer. Aliquots were collected at different time points for determining the fraction of S1 that remained uncleaved. The initial velocity (v_0) was then determined for each substrate concentration by plotting $\text{Ln}(\text{fraction of S1 uncleaved})$ vs. time at each substrate concentration. Experiments were conducted in triplicate and the average velocity at each substrate concentration is shown in the graph. The curve shows the fitting of the initial velocity according to the Michaelis-Menten equation written in the graph.

3.2.2 Comparison of the enzymatic behaviour between *cis* and *trans* enzymes

To quantitatively compare the catalytic performance of ET1/S1 to its *cis* sibling EC56, we next carried out kinetic analysis of the latter deoxyribozyme under the same reaction conditions. Once again three separate measurements were performed and Figure 3.14 plots the fraction of substrate cleavage vs. reaction time measured up to 100 min (for simplicity, only data points up to 30 min are shown). Interestingly, the data did not fit well to the monophasic kinetic equation $Y = Y_{\max} \cdot (1 - e^{-kt})$ but rather

required fitting to the biphasic kinetic equation $Y = Y_{\max 1} \bullet (1 - e^{-k_1 \bullet t}) + Y_{\max 2} \bullet (1 - e^{-k_2 \bullet t})$.

The biphasic fitting resulted in a k_1 of $1.52 \pm 0.17 \text{ min}^{-1}$ and a k_2 of $0.018 \pm 0.006 \text{ min}^{-1}$.

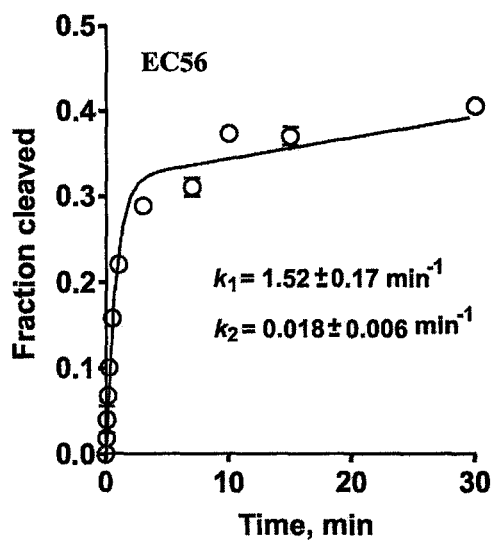


Figure 3.14 Determining the catalytic rate constant of EC56. Radioactively labeled EC56 (see Figure 3.12 for the labeling site) was allowed to self-cleave in the reaction buffer for designated lengths of time before each reaction was stopped with EDTA (pH 8.0, 23 °C). The reaction mixtures were then analyzed by denaturing PAGE and quantitated using ImageQuant software to calculate the fraction of cleaved EC56 at each time point. The obtained data were then fitted to the following equation: $Y = Y_{\max 1} \bullet (1 - e^{-k_1 \bullet t}) + Y_{\max 2} \bullet (1 - e^{-k_2 \bullet t})$, using GraphPad Prism software to derive rate constants. Three independent experiments were conducted and the average value for each time point is shown, along with the error bars.

The two different rate constants seen with EC56 could be explained by at least three possible scenarios. One is that the deoxyribozyme could fold into two catalytically active stable structures with vastly different catalytic activities reflected

by k_1 and k_2 . The second possibility is that the deoxyribozyme could adopt both a catalytically active structure (indicated by k_1) and a less stable catalytically inactive structure that could slowly convert into the active structure (with k_2 as the structure-switching rate constant). The third possibility is that the intact deoxyribozyme is capable of folding into one structure only, but there is a significant fraction of synthetic deoxyribozyme population suffered various forms of chemical damage (such as depurination, hydrolytic cleavage of the amide bonds linking the fluorophore and the quencher to the relevant nucleobases, and the oxidation of the fluorophore and the quencher) during automated DNA synthesis and subsequent purification steps. Such chemical damage may have made the deoxyribozyme either less active or completely inactive. We suspect that chemical damage in the substrate and/or enzyme sequence may be the key reason for the presence of the slower k_2 value based on the observation that as much as 40% of the EC56 remained uncleaved even after a long incubation time at 1000 min (see gels in Figure 3.12).

To provide experimental evidence for the above speculation, we conducted an experiment to compare the cleavage activity of freshly made EC56 to that of the uncleaved EC56 (isolated by denaturing PAGE) after 1000-min incubation under permissive reaction conditions; the results are shown in Figure 3.15. The freshly made deoxyribozyme (Figure 3.15A) exhibited the expected cleavage kinetics depicted in Figure 3.14. In stark contrast, the re-isolated EC56 (Figure 3.15B) failed to cleave itself

at all. We further subjected the re-isolated EC56 to alkaline-promoted RNA cleavage (at 90 °C for 10 min) and found that such a treatment led to the full cleavage of EC56 (data not shown). This finding indicates that all EC56 molecules contained the fully cleavable RNA linkage. Taken together, we provide the following explanation for the biphasic enzymatic kinetics and substrate cleavability: (i) synthetic deoxyribozyme molecules with no (or non-crucial) chemical damage are able to cleave the attached substrate with a k_{obs} of about 1.5 min^{-1} ; (ii) molecules that suffer one or more mild forms of chemical damage are still active but less catalytically potent, exhibiting a k_{obs} of only $\sim 0.02 \text{ min}^{-1}$; (iii) molecules with crucial chemical damage, which appear to account for about 40% of the molecular population, are completely inactive. Our findings above allude to the importance of chemical integrity of the signaling deoxyribozymes and the need for extreme care in the synthesis and preparation of the synthetic substrate and deoxyribozyme to minimize any damage to the DNA.

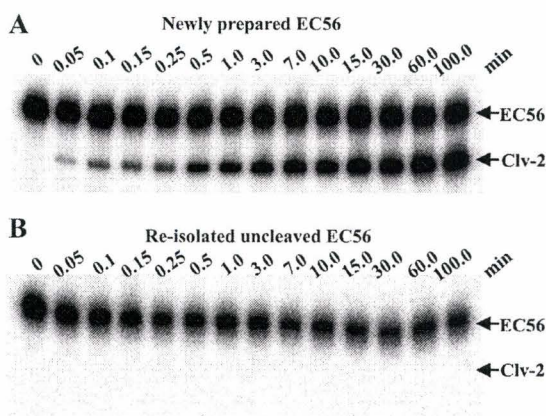


Figure 3.15 Substrate cleavability. (A) A time-course study of the cleavage reaction of freshly prepared EC56. Radioactively labeled EC56 (see Figure 3.12 for the labeling site) was allowed to self-cleave in the reaction buffer for designated lengths of time as indicated. The reaction mixtures were analyzed on denaturing PAGE. (B) A similar time-course study using the DNA re-isolated as uncleaved EC56 after 1000-min incubation in the reaction buffer.

3.2.3 Real-time signalling of ET1/S1

Finally, we determined the fluorescence-generating capability of ET1/S1 system in two different assays: a PAGE based assay involving the separation of the product from uncleaved substrate (Figure 3.16A) and the solution-based assay that monitored the fluorescence generation in real time (Figure 3.16B). For the gel-based assay, we labeled the substrate S1 with ^{32}P -phosphate at its 5'-end to provide a way of calibrating the fluorescence enhancement upon RNA cleavage. With the labels of ^{32}P , fluorescein and DABCYL, the uncleaved S1 is radioactive but weakly fluorescent (since the Q moiety is still present). The cleavage of S1 by ET1 should produce a 5'-cleavage fragment (12-nt; clv-1) that is radioactive and strongly fluorescent upon

PAGE separation. We found that the ratio of fluorescence intensity of clv-1 over that of S1 was approximately 6-fold higher than the ratio of radioactivity for these species at each time point, indicating that PAGE-based assay can generate substantial fluorescence enhancement that is consistent with the coupled catalysis-signaling mechanism.

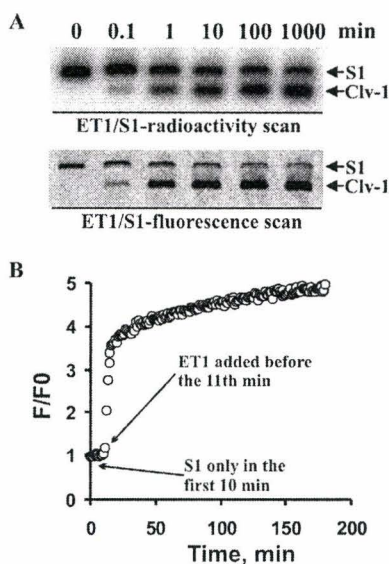


Figure 3.16 Signaling properties of ET1/S1. (A) Comparison of the radioactivity scan (top image) and the fluorescence scan (bottom image) of the same gel from PAGE analysis of a time-course study of the cleavage reaction of ET1/S1. The substrate concentration was set at 10 nM and the deoxyribozyme at 1 μ M. In addition to the fluorescein and DABCYL labels flanking the adenosine ribonucleotide, the substrate S1 (see Figure 3.12 for its sequence) was 32 P-labeled at its 5'-end. This labeling pattern makes the substrate and its 5'-cleavage fragment (denoted "clv-1",) both radioactive and fluorescent. Due to fluorescence dequenching, clv-1 is significantly more fluorescent than S1. (B) Examination of the real-time signaling capability of ET1/S1. The substrate was incubated at room temperature in the absence of the deoxyribozyme for 10 min, followed by the addition of ET1 at 11th min and a further incubation at the same temperature for an additional 170 min. The fluorescence intensity was recorded every minute. The substrate concentration was set at 0.1 μ M and the deoxyribozyme at 5 μ M.

The cleavage reaction of S1 by ET1 was then monitored in solution in real time, with a 50-fold excess of ET1 over S1 (the concentration of S1 was 100 nM). When S1 was incubated alone in the reaction buffer (first 10 minutes of the plot), a constant fluorescence was observed. When ET1 was introduced, the fluorescence intensity of the solution increased sharply in the first a few minutes and then slowed down drastically. This observation was consistent with the biphasic kinetics described above. The maximal fluorescence enhancement that can be generated by the ET1/S1 system was about 5-fold, which was similar to the maximal signaling magnitude observed above in the PAGE-based assay.

CHAPTER 4: CONCLUSIONS

I have characterized more fully the catalytic properties of pH6DZ1, one of several previously isolated fluorescence-signaling deoxyribozymes (39). This deoxyribozyme is a pH-dependent catalytic DNA that cleaves a phosphoester bond of a single ribonucleotide embedded in DNA and flanked immediately by two deoxyribonucleotides modified with a fluorophore and quencher, respectively. Sequence truncation and nucleotide alteration experiments resulted in revelation of several residues critical for catalysis as well as establishment of a putative four-way junction-like secondary structure comprising four short duplexes, three hairpin loops, and three inter-helical unpaired elements, which is more complicated than other RNA-cleaving deoxyribozymes reported before.

Several RNA-cleaving deoxyribozymes that cleave a standard RNA substrate or an unmodified RNA/DNA chimeric substrate have been reported and characterized (12, 15, 16, 19-21, 41, 42, 47-50). All of these deoxyribozymes possess similar secondary structures. The elucidation of the secondary structure of pH6DZ1 allows us to make a comparison of the secondary structure features exhibited by pH6DZ1 with those seen with these RNA-cleaving deoxyribozymes. Figure 4.1 illustrates the secondary structures of the two most well-studied RNA-cleaving deoxyribozymes known as 10-23 and 8-17 (16, 47) as the two representative RNA-cleaving

substrate-binding duplexes. This distinction may relate to the fact that pH6DZ1 has bulky fluorescein and DABCYL moieties placed on the two bases immediately surrounding the cleavage site, with both labels being absolutely required for the catalytic function. The participation of the bulky fluorescein and DABCYL groups in the tertiary folding may require that the nearby nucleotides adopt an unpaired configuration, resulting in a larger unpaired region near the cleavage site in pH6DZ1, as compared to that seen with 10-23 and 8-17.

Second, while 8-17 and 10-23 have a catalytic core of about 15 nucleotides, the shortened pH6DZ1 still uses more than 30 nucleotides to compose its catalytic core. The larger size of pH6DZ1 also results in the third difference: pH6DZ1 has a more complex secondary structure than the other two deoxyribozymes. The catalytic core of pH6DZ1 is comprised of two stem loops and one single-stranded region. By comparison, the catalytic core of 8-17 has one stem loop and a single stranded region, while 10-23 has a catalytic core of 15 unpaired nucleotides. Interestingly, both pH6DZ1 and 8-17 distribute indispensable nucleotides into two single-stranded regions that are separated by a short duplex. The rigidity of the duplex and limited number of nucleotides in these single stranded regions seems to suggest that it is difficult for these indispensable nucleotides to make direct contacts. Therefore, the precise functional roles of these essential but well-separated nucleotides remain to be deciphered. It is important to note that the catalytic core of each deoxyribozyme shares no apparent

sequence conservation to any other DNAzyme, strongly indicating that each deoxyribozyme is a distinct class of RNA-cleaving deoxyribozyme.

Based on the minimized structure of PH6DZ1, we have rationally engineered a trans-acting signaling deoxyribozyme and determined both its multiple turnover ability and fluorescence signaling properties. We found that the trans-acting deoxyribozyme behaved like a true enzyme: it followed the classic Michaelis-Menten kinetics with a k_{cat} value of $\sim 1 \text{ min}^{-1}$ and a K_M of $\sim 200 \text{ nM}$. The trans deoxyribozyme was also capable of generating 5-fold fluorescence enhancement due to catalytic action.

There are two unique properties of pH6DZ1 that should make it an excellent candidate for biosensing applications. First, it has a permanently incorporated fluorophore (F) and quencher (Q) pair, where such a molecular setting will allow for effective signaling in both cis and trans formats. This is because the fluorophore and quencher labels are all placed on the substrate strand while the deoxyribozyme strand does not contain any label that is required by the signaling mechanism. The signal generation setting within pH6DZ1 can also minimize the possibility of false signaling (such as the denaturation of the deoxyribozyme/substrate pair by an unknown chemical agent) because the signal generation is directly linked to the cleavage of the RNA moiety. Second, the existence of multiple helical elements in the secondary structure of pH6DZ1 makes this unique deoxyribozyme versatile for the design of allosteric deoxyribozymes either by rational design using existing aptamers or by in vitro

selection. This can be achieved using several principles established for the design and the in vitro selection of a large number of allosteric ribozymes and a few allosteric deoxyribozymes (40, 51-56). With its synchronized catalysis-signaling capability, we expect that pH6DZ1 will find use in the design of fluorescence-signaling allosteric deoxyribozyme biosensors for the real-time detection of any chemical or biological target for which a DNA aptamer can be isolated.

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