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Assemblage of Signaling DNA Enzymes with Intriguing Metal-Ion Specificities and pH Dependences

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Abstract: We report a group of new DNA enzymes that possess a synchronized RNA-cleavage/fluorescence-signaling ability and exhibit wide-ranging metal-ion and pH dependences. These DNA catalysts were derived from a random-sequence DNA pool in a two-stage process: (1) establishment of a catalytic DNA population through repetitive rounds of in vitro selection at pH 4.0, and (2) sequence-diversification and catalytic-activity optimization through five parallel paths of in vitro evolution conducted at pH 3.0, 4.0, 5.0, 6.0, and 7.0, respectively. The deoxyribozymes were evolved to cleave the phosphodiester bond of a single ribonucleotide embedded in DNA and flanked immediately by two deoxyribonucleotides modified with a fluorophore and a quencher, respectively—a setting that synchronizes catalysis with fluorescence signaling. The most dominant catalyst from each pool was examined for metal-ion specificity, catalytic efficiency, pH dependence, and fluorescence-signaling capability. Individual catalysts have different metal-ion requirements and can generate as much as a 12-fold fluorescence enhancement upon RNA cleavage. Most of the DNA enzymes have a pH optimum coinciding with the selection pH and exhibit a rate constant approximating 1 min^{-1} under optimal reaction conditions. The demonstration of DNA enzymes that are functional under extremely high acidity (such as pH 3 and 4) indicates that DNA has the ability to perform efficient catalysis even under harsh reaction conditions. The isolation of many new signaling DNA enzymes with broad pH optima and metal-ion specificities should facilitate the development of diverse deoxyribozyme-based biosensors.

Introduction

Although double-stranded DNA is best known as the material that stores genetic information for living organisms, many single-stranded DNA molecules have been shown to have catalytic or binding capabilities.[1−6] Despite very limited chemical functionalities, many catalytic DNAs (also known as DNA enzymes, DNAzymes, or deoxyribozymes) have been isolated from random-sequence DNA pools by in vitro selection[7,8] and have been shown to be capable of catalyzing a number of chemical transformations,[9−19] some with k_{cat} values above 1 min^{-1}.[20−24] A variety of DNA enzymes have also been evolved to take advantage of metal-ion[4,6] and small-molecule cofactors,[25] providing new platform technologies for the detection of such species.[26]

The lack of a 2′-hydroxyl group in DNA relative to RNA does not appear to reduce the catalytic performance, but rather provides advantages that include ease of preparation and better resistance to chemical and enzymatic degradation. Therefore, properly engineered and catalytically efficient DNA enzymes are particularly attractive molecules for practical applications. The extraordinary chemical stability, in particular, should make DNA suitable for the development of robust catalysts to operate in chemically demanding situations such as under conditions of high pH, low pH, or high temperature.

As all known deoxyribozymes have been created to function at or near physiological conditions, the first goal of this study was to determine whether DNA has the ability to carry out catalysis under more demanding reaction conditions. More specifically, we sought to create efficient DNA enzymes from

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a random-sequence DNA pool that could perform catalysis in a highly acidic solution. Jayasena and Gold have demonstrated that self-cleaving ribozymes that are functional at low pH can be isolated from a random-sequence RNA pool,[27] thereby establishing a precedent for low-pH compatible nucleic acid-based enzymes. Given the fact that DNA and RNA have very similar chemical structures and that DNA is inherently stable, we hypothesized that it should also be feasible to create DNA enzymes that are functional at low pH.

The second goal of this study was aimed at creating fluorescence-signaling DNA enzymes with a broad range of pH optima to allow biosensing applications to be done in solutions of varying pH. We have recently shown that a catalytic event involving the cleavage of an RNA linkage embedded in DNA by a DNA enzyme can be directly coupled to a fluorescence-signaling event.[24] In that study, we created an efficient DNA enzyme, DEC22−18, using selection and evolution at pH 7.0, which was capable of cleaving a single RNA linkage immediately flanked by a fluorophore-containing nucleotide and a quencher-bearing nucleotide. Such a unique setting permitted the synchronization of the cleavage with fluorescence signal generation. However, further examination of DEC22−18 led to the finding that this catalytic DNA had a rather narrow pH optimum near pH 7.0 and a change of 1 pH unit in either direction resulted in a serious decrease in catalytic efficiency (unpublished data). Herein, we report the isolation of many fluorescence-signaling DNA enzymes with a wide range of pH optima and metal ion specificities. These deoxyribozymes were created using a two-stage selection and evolution strategy that involved an initial series of selection rounds at pH 4.0 followed by further selection and evolution at pH values ranging from 3.0 to 7.0. The implications of these finding for the development of robust reporters that can function under extreme conditions are discussed.

Experimental Section

Materials. Standard and modified oligonucleotides were prepared and purified using previously described procedures.[24] Nucleoside 5′-triphosphates, [γ-^{32}P]ATP and [α-^{32}P]dGTP, were purchased from Amersham Pharmacia. *Taq* DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase (PNK) were purchased from MBI Fermentas. All other chemical reagents were purchased from Sigma and used without further purification.

General Protocols. The cleavage reaction during in vitro selection and subsequent kinetic analysis was carried out at room temperature (23 °C) in the presence of the following metal ions if not otherwise specified: 400 mM NaCl, 100 mM KCl, 8.5 mM $MgCl_2$, 5 mM $MnCl_2$, 1.25 mM $CdCl_2$ and 0.25 mM $NiCl_2$. The total DNA concentration in each reaction was between 0.1 and 0.3 μM. The solution pH was controlled with the following buffering reagents (each used at 50 mM): citrate for pH 2.5−5.5, MES for pH 5.5−6.5, HEPES for pH 6.5−8.0.

Each full-length, *cis*-acting DNA catalyst used in PAGE-based kinetic analyses was pieced together by ligation of the substrate A1 with a 100-nt synthetic deoxyribozyme using DNA template T1 and T4 DNA ligase (all DNA sequences are shown in Figure 2A). Prior to DNA ligation, each deoxyribozyme was phosphorylated with PNK in the presence of [γ-^{32}P]ATP using a previously described protocol.[24] Each ligated DNA catalyst was further purified by 10% denaturing PAGE. The cleavage reaction was stopped by the addition of 30 mM EDTA and 8 M urea and the mixture was analyzed using denaturing 10% 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.

DNA molecules used in fluorescence experiments were produced in a similar way except that standard ATP was used to replace [γ-^{32}P]-ATP in the phosphorylation step. Fluorescence measurements were made on a Cary Eclipse Fluorescence Spectrophotometer (Varian) using a microvolume cuvette containing 50 μL of a 100 nM DNA solution. The excitation was set at 490 nm and emission was monitored at 520 nm over the time course of an experiment.

As described in more detail below, the optimal metal ions and pH for each catalytic DNA were as follows: 500 mM Na^+ and pH 3.0 for pH3DZ1 (k_{obs} = 0.023 min^{-1}); 400 mM Na^+, 10 mM Cd^{2+} and pH 3.8 for pH4DZ1 (k_{obs} = 1.1 min^{-1}); 250 mM Na^+, 25 mM Mn^{2+} and pH 4.8 for pH5DZ1 (k_{obs} = 0.72 min^{-1}); 800 mM Na^+, 8 mM Mn^{2+}, 2 mM Ni^{2+} and pH 6.0 for pH6DZ1 (k_{obs} = 0.25 min^{-1}); 100 mM K^+, 14 mM Mn^{2+} and pH 8.0 for pH7DZ1 (k_{obs} = 1.3 min^{-1}).

In Vitro Selection. Catalytic DNAs were isolated using the selection scheme shown in Figure 1A that was adapted from a previously established protocol[24] with the changes specified below. (1) 275 pmol of DNAs each containing a 70-nt random-domain (see Figure 2A) were used as the initial pool. (2) The RNA cleavage reaction in the first 8 rounds (G0−G7) was allowed to proceed for 5 h at pH 4.0. (3) G8 DNA was split into 5 pools with which 5 parallel paths of selection were carried out at pH 3.0, 4.0, 5.0, 6.0, and 7.0, respectively (denoted pH3 path, pH4 path and so on). A hyper-mutagenic PCR protocol[23,28] was used to introduce a high rate of mutations in each path for five consecutive rounds following the pool splitting (i.e., G8−G12). The cleavage time was progressively reduced from the initial 5 h (G7) to 30 min (G8−G10, all paths), to 5 min (G11−G16, pH3 path; G11−G13, all other paths), to 30 s (G14−G16, pH4−7 paths), to 5 s (G17−G24, pH6−7 paths; G17−G21, pH5 path), and finally to 1 s (G22−G24, pH5 path). Each selection was discontinued if no significant increase of cleavage activity was observed over at least 3 consecutive rounds at a given cleavage time. DNA sequences from each terminal round (G16 for pH3−4 paths; G24 for pH5−7 paths) were amplified by PCR, cloned and sequenced using previously described protocols.[24]

Sequence Truncation. Full-length DNA catalysts and their shortened versions (with one or several nucleotides truncated from the 3′-end of each deoxyribozyme each time) were compared for cleavage activity under the original selection conditions.

Metal Specificity. Each catalyst was studied for metal ion requirements in a 10-min cleavage reaction except for pH3DZ1, for which a 40-min reaction was conducted. The metal ion specificity was determined by monitoring the ability of the DNA enzyme 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 Figure 3.

pH Profiles. Each catalyst was allowed to undergo the RNA cleavage reaction under the optimal metal ion conditions (as well as under selection metal ion conditions; data not shown) at several different pH values. Aliquots of a reaction mixture were collected at various time points within 15% completion, and the resulting DNA fragments were analyzed by 10% denaturing PAGE. The rate constant was determined as the negative slope of a plot of the natural logarithm of the fraction of DNA that remained unreacted vs the reaction time. Experiments were run in duplicate (with less than 20% variation) and the average rate constants at each pH value were determined for the dominant DNAzyme from each selection.

Real-Time Signaling. Each catalyst was first incubated in the absence of metal cofactors for 120 s, followed by the addition of metal ions and a further incubation for 2000 s. The fluorescence intensity was recorded every 2 s for the first 600 s and then every 10s afterward.

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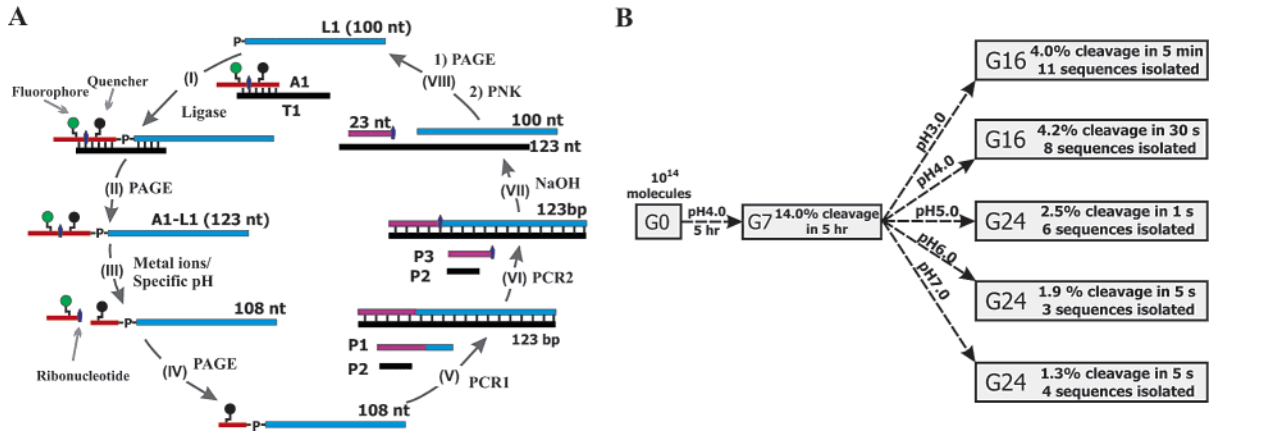

Figure 1. Selection of signaling catalytic DNAs. **(A)** Selection scheme. Each selection cycle consists of steps I−VIII. **(I)** 100-nt DNA L1 is ligated to acceptor DNA A1. **(II)** Ligated 123-nt DNA is isolated by PAGE. **(III)** Purified DNA is incubated with divalent metal ions for RNA cleavage. **(IV)** 108-nt cleavage fragment is isolated by PAGE. **(V)** The recovered 108-nt DNA is amplified by PCR using primers P1 and P2. **(VI)** 123-bp PCR product in (V) is further amplified by PCR using primers P2 and P3 to introduce a ribonucleotide linkage embedded within DNA. **(VII)** The resulting double-stranded DNAs are treated with NaOH to cleave the RNA link. **(VIII)** 100-nt cleavage fragment is purified by PAGE, phosphorylated at 5′-end, and used to initiate the next round. **(B)** Selection progress. During the first eight rounds of selection (G0 to G7), DNA was incubated at pH 4.0 for 5 h for RNA cleavage. G8 DNA was split into 5 pools with which 5 different selections were carried out each under a given pH (i.e., pH 3.0, pH 4.0, pH 5.0, pH 6.0, and pH 7.0). Detailed procedures are given in "Material and Methods". The percentage of RNA cleavage in a given reaction time is indicated for the listed selection rounds. The number of unique DNA sequences revealed from the cloning and sequencing experiments is also shown for each terminal pool.

A control sample was also examined over the same timespan in which A1 was used to replace the deoxyribozyme. Fluorescence enhancement was calculated as F/F_0, where F is the fluorescence intensity of the deoxyribozyme solution at any given time point and F_0 is the initial intensity. The data were obtained under optimal metal ion and optimal solution pH values.

Results

In Vitro Selection. An established protocol[24] was used to isolate the DNA enzymes reported herein (see Figure 1A). In addition to Mg^{2+}, Na^+, and K^+, three transition metal ions—Mn^{2+}, Cd^{2+}, Ni^{2+}—were also used as potential metal-ion cofactors based on a finding that divalent transition metal ions are able to improve the catalytic efficiency of deoxyribozymes.[23] These metal ions were used along with Co^{2+} in our previous study that led to the isolation of an efficient signaling DNA enzyme named DEC22−18.[24] Because DEC22−18 showed a preference for Co^{2+} as the metal ion cofactor, we deliberately omitted this metal ion in the present study, with the intention of isolating new signaling deoxyribozymes that utilized different divalent metal ions (such versatile metallodeoxyribozymes might be quite useful as selective metal ion sensors[26]).

In vitro evolution has previously been used to alter the properties of ribozymes, including metal ion specificities[29,30] and substrate recognition patterns.[31] We were interested in determining whether a similar technique could be utilized to alter the pH dependence of signaling deoxyribozymes. The general selection strategy began with a single-path selection at pH 4.0 to produce a population of DNAzymes from which pH-dependent species could be generated. A starting pH of 4 was chosen since this value was in the acidic range (the range in which we desired to create our new signaling DNA enzymes) but was slightly greater than the pK_a values of N1 of adenine and N7 of guanine[32] so that the DNA would still be anionic and thus bind divalent metal ions. A long reaction time (5 h) was used in these initial rounds of selection to facilitate the establishment of a diverse catalytic DNA pool for subsequent evolution experiments. A catalytic population was obtained after eight rounds (G0−G7) of selection and amplification at pH 4.0.

The DNA population obtained at pH 4 was split into five individual pools for carrying out five parallel selections each conducted at a defined pH (pH 3, 4, 5, 6, and 7). An error-prone PCR protocol with up to 10% mutation rate per cycle[28] was used during the first five rounds (G8−G12) in each selection to create diversity.[16,23] Such a high level of mutagenesis allows catalytic DNA molecules to acquire the essential mutations necessary to respond to the pH change. The reaction time was reduced to 10 min during these mutagenic rounds and then progressively decreased to as little as 1 s, provided that a relevant catalytic population was responding positively by producing an increase in the cleavage activity. The decrease in reaction time was done so that only the most efficient catalysts in each round would proceed to the next. If there was no noticeable increase in activity for at least three consecutive rounds at a given reaction time, then the selection was discontinued. For pH3 and pH4 pools, 8 more rounds were conducted after pool splitting, whereas for pH5−7 pools, 16 more rounds were performed. In the end, we derived five catalytic DNA populations that underwent efficient cleavage under the selection pH settings (the selection progress in each case is summarized in Figure 1, Panel B).

Sequence Diversity. 11, 8, 6, 3, and 4 unique sequences were revealed from the pH3−7 pools, respectively, after ∼20 clones were sequenced from each terminal pool (the sequences of all DNA catalysts are given in Supporting Figure 1). Most DNA catalysts appeared in a single pool and only five deoxyribozymes were observed in two or more pools, suggesting that we isolated diverse deoxyribozymes with wide-ranging pH dependences. In total, our efforts led to the identification of 22 different deoxyribozymes from ∼90 sequenced clones.

As to the five deoxyribozymes seen in two or more pools, one appeared in four pools (as pH3DZ11, pH4DZ7, pH5DZ5,

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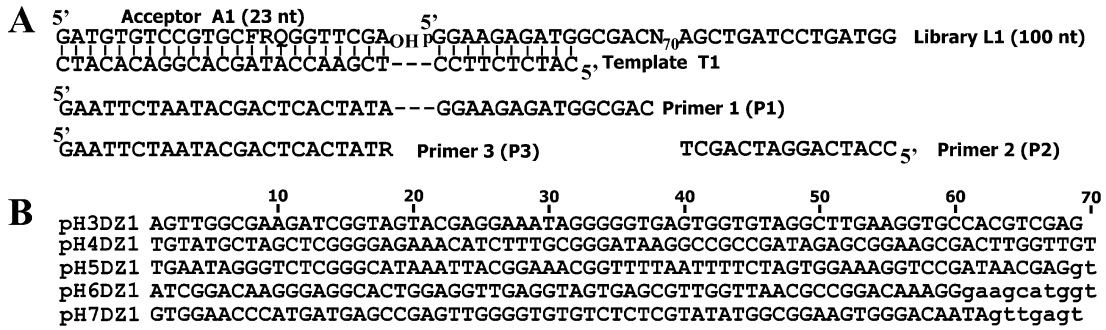

Figure 2. **(A)** DNA molecules used for in vitro selection. L1: random-sequence DNA pool; A1: modified substrate; T1: template for ligation; P1−3: primers for PCR. N70 is the random domain. **(B)** Sequences of the dominant deoxyribozymes (only the random domain is shown). Each catalyst also contains GATGT GTCC GTGCF RQGGT TCGAG GAAGA GATGG CGAC (F: fluorescein-dT; R: ribo-A; Q: DABCYL-dT) at the 5′-end and AGCTG ATCCT GATGG at the 3′-end. Nucleotides shown in small letters (as well as nucleotides after them) in pH5DZ1, pH6DZ1 and pH7DZ1 are not required for catalysis. All of the 15 nucleotides in the fixed 3′ region of pH3DZ1 are not needed for catalytic activity while the first 6 nucleotides (AGCTGA) in the same region are required by pH4DZ1.

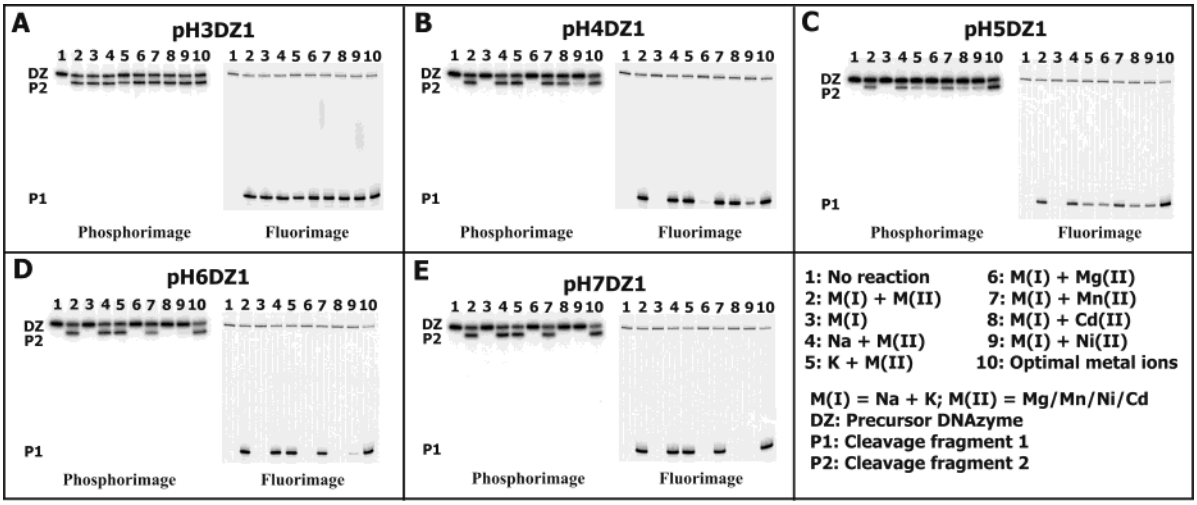

Figure 3. Metal-ion specificities of the optimal deoxyribozymes. Each DNA catalyst contained a ^{32}P-phosphodiester bond linking the 23rd and 24th nt and was tested for RNA cleavage under various salt conditions. Reaction products were analyzed on 10% denaturing PAGE, which was scanned for both radioactivity (left image) and fluorescence (right image). DZ stands for the full-length DNA, P1 and P2 for the 5′ and 3′ cleavage products, respectively. 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); the optimal metal ions and pH listed in the "General Protocols" (lane 10).

and pH6DZ3), another turned up in three pools (as pH3DZ10, pH4DZ2, and pH5DZ1), and the remaining three were seen in two neighboring pH pools (pH3DZ9 and pH4DZ8, pH5DZ6 and pH6DZ2, pH6DZ1 and pH7DZ2). No single deoxyribozyme was observed in all DNA pools. For each of these DNA catalysts, nucleotide variations were observed at many sequence locations. For example, for pH3DZ11 and its variants in the other 3 DNA pools, base variations were observed in a total of 13 positions throughout the original random-sequence domain (data not shown). It remains to be determined whether all or only some of these mutations bear any structural or catalytic significance.

Sequence Truncation. The dominating sequence from each selection (Figure 2, panel B) was chosen for further characterization. To determine whether these deoxyribozymes could be shortened from the 3′-end, various truncated mutants were produced by chemical synthesis and tested for catalytic activity (data not shown). All of the dominant deoxyribozymes could be shortened to a certain degree without causing a significant reduction in their catalytic ability (the nonessential nucleotides are shown in small letters in Figure 2B). pH3DZ1 and pH4DZ1 appeared to require more 3′ nucleotides for catalytic activity, suggesting that these two low-pH deoxyribozymes might require more complex tertiary structures to achieve catalytic activity.

Metal-Ion Specificity. The dominant deoxyribozyme from each selection was labeled with ^{32}P at the phosphodiester bond linking the 23rd and 24th nucleotides in addition to the fluorescein-dT (14th nt), ribo-A (15th nt), and DABCYL-dT (16th nt). This labeling pattern made the uncleaved deoxyribozyme (denoted DZ) both fluorescent and radioactive. Upon RNA cleavage, two products were expected: a large DNA fragment (P2) that was only radioactive and a small DNA fragment (P1) that was only fluorescent. Moreover, for a given reaction R_{P2}/R_{DZ} (R stands for radioactivity) should be significantly smaller than F_{P1}/F_{DZ} (F stands for fluorescence), since 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) 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′-fragment.

The data shown in Figure 3 indicate that the five deoxyribozymes (one per pool) exhibited broad metal-ion specificities. All the deoxyribozymes except pH3DZ1 required divalent metal ion cofactors (lane 1: no metal ions; lane 2: full set of divalent metal ions and monovalent metal ions; lane 3: only monovalent metal ions). pH7DZ1 was extremely specific for Mn^{2+} (lane 7). In contrast, pH5DZ1 was a nonselective metalloenzyme with only a slight preference for Mn^{2+}. pH4DZ1 also appeared to be nonmetal-selective; it had a high catalytic activity with Mn^{2+} and Cd^{2+} and a reduced activity with Ni^{2+} but was inactive in the presence of only Mg^{2+}. pH6DZ1 is perhaps most intriguing as it appeared to require both Mn^{2+} and Ni^{2+} for optimal activity and was incapable of using Mg^{2+} and Cd^{2+}. It is noteworthy that all four divalent metal ion-dependent deoxyribozymes were capable of performing efficient catalysis in the presence of Mn^{2+}. Many other deoxyribozymes have been reported that either are extremely specific for Mn^{2+} or have a significantly enhanced catalytic activity in the presence of Mn^{2+}.[15,21−23] The existence of such a variety of Mn^{2+}-utilizing deoxyribozymes seems to suggest that this metal ion may play a special structural or electronic role that is important for supporting catalysis by DNA enzymes.

We performed additional experiments to establish the metal ion concentrations that supported the optimal catalysis for each deoxyribozyme (see discussion below; the optimized conditions are given in Material and Methods section). Lane 10 of each gel in Figure 3 shows the maximum catalytic efficiency for each of the dominant deoxyribozymes under the optimized metal ion levels.

pH Profiles. A cursory assessment of the pH dependence of the above five dominant deoxyribozymes under the salt conditions used for in vitro selection revealed that nearly all the deoxyribozymes had the highest catalytic activity at or near the selection pH. The only exception was pH7DZ1 which had a significantly enhanced activity when the solution pH was raised from 7 to 8 (data not shown). On the basis of this finding and our desire to establish an optimal salt condition for each catalyst for subsequent fluorescence-signaling experiments (see discussion below), we carried out a series of experiments to optimize salt concentrations for each deoxyribozyme at the selection pH. First, a specific metal ion (or a combination of metal ions) was chosen for concentration optimization based on the results shown in Figure 3. For example, Na^+ and K^+ were chosen for optimization for pH3DZ1 because this deoxyribozyme does not require any divalent metal ion for catalytic activity. Second, the cleavage activities of a particular deoxyribozyme were compared in the presence of different amounts of chosen metal ions. For example, different concentrations of Na^+ and K^+ (alone and in combination) were tested for the support of the cleavage ability of pH3DZ1. This led to the finding that 500 mM Na^+ supported the most efficient catalysis for pH3DZ1. Similar procedures were used for the selection and optimization of metal ions for other DNA catalysts (the optimized salt conditions are given in the Material and Methods section).

Following the establishment of optimal metal ion concentrations, we carried out another series of experiments to establish a pH profile for each deoxyribozyme under optimal metal concentrations (Figure 4). Several features merit special attention. First, enzymes selected at pH values of 3−6 show corresponding maximum catalytic rate constants at pH values

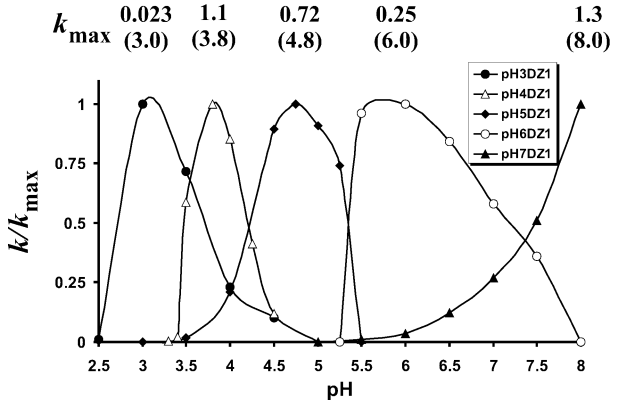

Figure 4. Normalized catalytic rates vs pH for the five chosen deoxyribozymes. k is the rate constant at a given pH and k_{max} is the maximum rate observed in each data series. k_{max} (min^{-1}) values are listed above the graph (the number in parentheses is the pH where the k_{max} value was observed for each deoxyribozyme).

that are in all cases at or near the selection pH. Second, pH 3−6 systems show relatively narrow pH windows, bracketing 1.5 to 2.8 pH units. The only system that does not show a pH maximum is pH7DZ1, whose catalytic rate rose with pH up to the highest tested pH of 8.0. These findings clearly indicate that the pH dependence of the final DNA enzyme was controlled to a large degree by the pH used during the selection step, indicating that the setting of solution conditions is important in directing the evolution of DNA enzymes. A third feature of the pH dependence is the variation in maximum catalytic rate constants. In most cases (pH4−7 deoxyribozymes) the enzymes showed fairly large rate constants (k_{obs} values ranged from 0.2 to 1.3 min^{-1}). However, pH3DZ1 was a factor of 10 less efficient, likely because many bases would be expected to be protonated and thus the DNA molecule might have experienced a reduced ability to fold into catalytically active structures. This speculation draws support from the fact that pH3DZ1 did not require a divalent cation for catalytic activity (see Figure 3 and discussion above).

To examine whether the pH profile obtained above for each deoxyribozyme holds true only for the optimal metal ion conditions, we also determined the pH profile for each deoxyribozyme under the metal ion setting used for the in-vitro-selection (which is quite different from each optimal metal ion condition in terms of metal identities and concentrations). We found that the normalized pH profiles obtained under the two metal ion settings are essentially identical for each deoxyribozyme (data not shown), suggesting that the pH profiles shown in Figure 4 largely reflect the intrinsic pH dependence of each deoxyribozyme.

Signaling Properties. We next examined the real-time fluorescence signaling capabilities of the deoxyribozymes under conditions at which optimal catalytic rate constants were observed (see Materials and Methods). Each deoxyribozyme was incubated alone for 120 s before the essential metal ions were added to initiate catalysis. Figure 5 plots the fluorescence ratio F/F_0, where F_0 is the initial fluorescence intensity and F is the intensity measured at different points throughout the incubation. A striking feature of the signal response is that the net increase in fluorescence intensity is dependent on the pH of the solution utilized for the analysis, with increased signal intensity being observed at higher pH values. This can be rationalized by

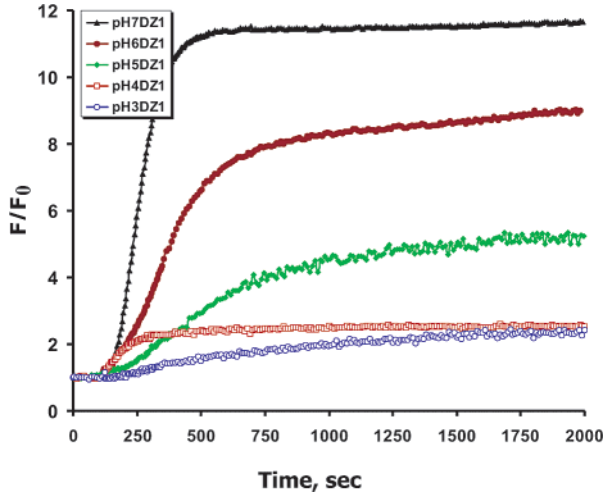

Figure 5. Real-time signaling capability of the deoxyribozymes. Each deoxyribozyme was incubated without the required metal ions for 120 s, followed by the addition of metal ions and a continued incubation for 2000 s. F is the fluorescence intensity of deoxyribozyme mediated reaction at any given time and F_0 is the initial reading.

considering the inherent pH response of fluorescein. In cases where analysis is done at pH 7, fluorescein exists predominantly in the dianionic form, and as such has a large emission yield ($\Phi = 0.93$). At pH 5, fluorescein exists predominantly as a monoanion, and thus has a quantum yield that is 2.5-fold lower than the dianion ($\Phi = 0.37$).[33] At pH values of 3 and 4 the probe exists predominantly as a nonfluorescent neutral species, which is able to undergo deprotonation in the excited state to elicit monoanion emission. Because quenching by energy transfer to DABCYL must compete with all other forms of quenching (including internal conversion, which is enhanced for the monoanionic and neutral forms relative to the dianionic form[33]), the degree of quenching by the energy transfer mechanism is reduced at lower pH values, leading to a reduction in the overall intensity enhancement upon cleavage of the RNA linkage. Even so, the >2-fold intensity enhancement at lower pH values is sufficient to provide a useful pH-dependent signal.

To determine whether the rate of the fluorescence response reflected the catalytic rate constant revealed by PAGE analysis for each deoxyribozyme (k_{max} in Figure 4), we estimated the signaling rate constant, k_{obs}, for each deoxyribozyme using the data taken from the linear signaling range in Figure 5. The following procedures were used: (1) we assume that the reaction was completed at 2000 s; (2) each data series was normalized using the equation $x = (F - F_0)/(F - F_{2000})$, where F_{2000} is the fluorescence intensity at 2000 s, F and F_0 are defined above, and x is equivalent to the fraction of cleaved DNA; (3) the natural logarithm of the fraction of DNA that remained unreacted [i.e., $\ln(1 - x)$] was plotted versus the reaction time and the negative slope was taken as the k_{obs} for each deoxyribozyme. From the above data manipulation, we determined the signaling k_{obs} values for pH 3−7 deoxyribozymes to be 0.061, 0.56, 0.19, 0.13, and 0.65 min^{-1}, respectively. These rate constants overall reflected the relative catalytic efficiency of the deoxyribozyme. In comparison to the rate constants determined by PAGE, the estimated signaling rate constants for pH 4−7 deoxyribozymes are smaller by a factor of 2 or more. We attribute this discrepancy to the use of an inefficient manual sample mixing method when the metal ions were introduced into the sample cells (we noticed a small lag in signal response in the initial 20 s). The larger k_{obs} for pH3DZ1 likely results from the assumption that the reaction was completed at 2000 s (this assumption is less true because pH3DZ1 is a relatively inefficient catalyst).

Secondary Structures. Several secondary structures were predicted for each deoxyribozyme by the mfold program (data not shown; the mfold program can be accessed at **http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi**). Various synthetic DNA molecules were used to test two key aspects of the predicted structures: (1) the effect of changing the identities of selected base pairs in the predicted stems and (2) the effect of replacing large loops with 3- or 4-nt loops. Although most of the altered DNA molecules were no longer catalytically active, one of the predicted secondary structures for pH7DZ1, as shown in Figure 6A (a structure with two stem-loop motifs; stem 1/loop 1 and stem 2/loop 2), was indeed supported by our experimental data, as shown in Figure 6B and 6C. A significantly shortened version of pH7DZ1, denoted pH7DZ1S, in which a 19-nt original loop 1 was replaced by a GAA triloop and a 13-nt loop 2 by a TTTT tetraloop along with the deletion of 20 nucleotides from the 3′-end, maintained full catalytic activity (Figure 6B, lanes 3 and 4).

The existence of stem 1 was confirmed through the use of an engineered *trans*-acting DNA enzyme denoted E1 that was shown to cleave the matching external substrate S1 (lanes 5 and 6). Similarly, the existence of stem 2 was verified through the use of a bipartite deoxyribozyme assembly, E2A/E2B, that was able to cleave S1 (lanes 7−9). Finally, the two *trans*-acting systems were examined for fluorescence-signaling capability (Figure 6C). Each system exhibited the expected signaling behavior: for E1/S1, a rapidly increasing fluorescence signal was observed upon the addition of E1 to a S1-containing solution (diamonds, E1: S1 = 10:1; circles, E1:S1 = 1:10); for the E2A/E2B/S1 system, fluorescence signaling could only be achieved when both E2A and E2B were added to the S1-containing solution (triangles, E2A:S1:E2B = 1:10:10).

Several previously identified RNA-cleaving deoxyribozymes bind their substrates through the formation of two long stretches of Watson−Crick base pairs that flank the cleavage site.[20−22] For example, 10−23 deoxyribozyme uses two single-stranded arms to grab its all-RNA substrate while placing the catalytic core across the cleavage site.[20] Although the mfold program correctly identifies two stem regions within pH7DZ1 one of which links the substrate S1 to the deoxyribozyme E1, it does not predict any helical interactions that involve the nucleotides in the two single-stranded regions in E1 and unpaired nucleotides in S1. It is quite possible that E1 uses some tertiary interactions to engage the other half of S1. The complete elucidation of the secondary structure of pH7DZ1 may require the creation of mutant deoxyribozymes and will be the subject of our future research efforts.

Discussion

Catalytic DNAs are not known to exist in nature and the interest in studying DNA enzymes mainly stems from their potential practical applications, particularly considering the fact that DNA has exceptional chemical stability and DNA enzymes are easy to obtain through in vitro selection. Although many deoxyribozymes have been reported to catalyze chemical

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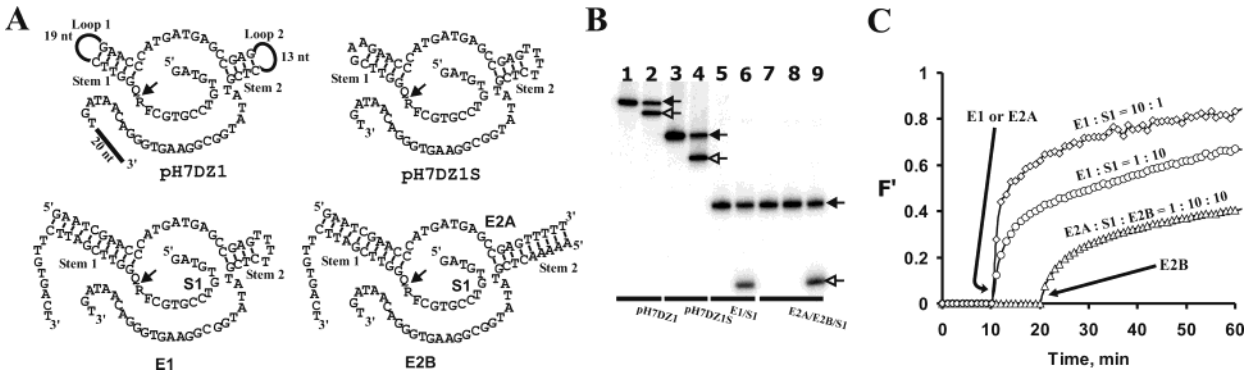

Figure 6. Proposed Secondary Structure of pH7DZ1. **(A)** DNA constructs tested. pH7DZ1S is a shortened *cis*-acting deoxyribozyme where the original loops 1 and 2 in pHD7Z1 were replaced with two small loops. E1/S1 is a *trans*-acting system in which E1 binds S1 through 10-bp stem 1. E2A/E2B/S1 is another *trans*-acting system in which E2B binds E2A through 9-bp stem 2 which in turn binds S1 through 10-bp stem 1. Arrows indicate the cleavage site. **(B)** Cleavage products. pH7DZ1 (1 μM) was treated in the reaction buffer without (lane 1) and with Mn(II) (lane 2); pH7DZ1S (1 μM) was treated in the reaction buffer without (lane 3) and with Mn(II) (lane 4); S1 (1 μM) was incubated in the Mn(II)-containing buffer in the absence of E1 (lane 5) and in the presence of 2.5 μM of E1 (lane 6); S1 (1 μM) was incubated in the Mn(II)-containing buffer in the absence of E2A and E2B (lane 7) and in the presence of 2.5 μM of E2A (lane 8) and in the presence of 2.5 μM of E2A and 5 μM of E2B (lane 9). Unfilled arrowhead indicates cleavage product and filled arrowhead indicates precursor DNA. **(C)** Real-time signaling capability of E1/S1 and E2A/E2B/S1 systems. For E1/S1 (circles), the substrate S1 (1 μM) was incubated at room temperature in the absence of E1 for 10 min, followed by the addition of E1 to 0.1 μM and a further incubation for 3000 more minutes (only the first 60 min are shown); a similar experiment was conducted with S1 at 0.1 μM and E1 at 1 μM (diamonds). For E2A/E2B/S1 (triangles), S1 (1 μM) was incubated at room temperature in the absence of both E2A and E2B for 10 min, followed by the addition of E2A to 0.1 μM and a 10-min incubation, followed by the addition of E2B to 1 μM and a 3000-min incubation (again only the first 60 min are shown). $F' = (F - F_0)/(F_{3000} - F_0)$, where F_0 and F_{3000} are the fluorescence readings taken at the beginning and the end of each reaction and F is the reading at any given time. The reaction solution contained 50 mM HEPES (pH 8.0, at 23 °C), 100 mM K^+ and 14 mM Mn^{2+}.

transformations under relatively mild reaction conditions, no DNA enzymes have been generated that can function under harsh reaction conditions, such as high acidity, high basicity, or high temperature. This void has now been partially filled by the DNA enzymes described in this report. As we have shown above, DNA enzymes can be selected to perform efficient catalysis even at pH values as low as 3. This has significant implications because it shows that DNA has the ability to catalyze reactions under extreme pH conditions and suggests that "extremophile" DNA enzymes (which are akin to the proteins that are produced by organisms that exist under extreme temperature, pressure, pH or ionic strength conditions) can be created for unique applications which demand acidic pH values.

The successful creation of a large number of DNA enzymes with pH optima covering 5 pH units (pH 3−8) from a single catalytic pool originally selected at pH 4 indicates that the combined in vitro selection and in vitro evolution approach used herein is very powerful when used for fine-tuning and altering a given property of a pool of DNA catalysts.

A particularly interesting finding from this work was that metal ion specificity was dependent on the selection pH. Although divalent metal ions are required by most of the deoxyribozymes that we examined, pH3DZ1 does not require divalent metal ions for catalysis. We speculate that since the bases in DNA are significantly protonated at pH 3 (protonation is expected to occur at N1 of adenine, N3 of cytosine, and N7 of guanine, which have pK_a values of 3.9, 4.6, and 3.6, respectively[32]), the DNA molecule may have enough internal cations (positively charged bases) for structural folding or catalysis, and thus divalent metal ions become unnecessary.

The isolation of a variety of signaling DNA enzymes with broad pH optima and metal ion dependences could lead to several potential applications. First, many of the deoxyribozymes obtained exhibit catalytic activity only in the presence of selected divalent metal ions, such as Mn^{2+}, Ni^{2+}, or Cd^{2+}. With further optimization for more stringent metal-ion selectivity and tighter metal-ion binding affinity, these DNA enzymes could be developed into sensitive signaling probes to detect specific divalent metal ions at a given pH. Second, the DNA enzymes could be used as unique pH-reporting probes, either in solution or after immobilization onto a surface. Another potentially interesting application is the use of these signaling probes as pH-dependent fluorogenic reagents to follow chemical or enzymatic reactions that alter the pH of a reaction mixture.

Many recent studies have shown that ribozymes[34] and deoxyribozymes[24,35] can be designed into allosteric nucleic acid enzymes and used as effective reporters for the detection of important biological targets. It is conceivable that the signaling DNA enzymes described above can be further engineered into signaling allosteric deoxyribozymes and used as catalytic and real-time reporters in a variety of detection-directed applications over a range of pH values. A significant advantage of using these signaling DNA enzymes is that both the catalytic and signaling components are present in a single molecule. Thus, the potential exists for the development of "reagentless" sensors based on immobilization of the DNAzyme onto a suitable surface such as that of an optical fiber or a microarray.

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Supporting Information Available: DNA sequences from all five pH pools (Supporting Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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