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# Sequence Diversity, Metal Specificity, and Catalytic Proficiency of Metal-Dependent Phosphorylating DNA Enzymes

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## Summary

**Although DNA has not been found responsible for biological catalysis, many artificial DNA enzymes have been created by "in vitro selection." Here we describe a new selection approach to assess the influence of four common divalent metal ions ($Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$) on sequence diversity, metal specificity, and catalytic proficiency of self-phosphorylating deoxyribozymes. Numerous autocatalytic DNA sequences were isolated, a majority of which were selected using $Cu^{2+}$ or $Mn^{2+}$ as the divalent metal cofactor. We found that $Cu^{2+}$- and $Mn^{2+}$-derived deoxyribozymes were strictly metal specific, while those selected by $Ca^{2+}$ and $Mg^{2+}$ were less specific. Further optimization by in vitro evolution resulted in a $Mn^{2+}$-dependent deoxyribozyme with a $k_{cat}$ of 2.8 $min^{-1}$. Our findings suggest that DNA has sufficient structural diversity to facilitate efficient catalysis using a broad scope of metal cofactor utilizing mechanisms.**

## Introduction

Although DNA enzymes (deoxyribozymes) are not known to exist in nature, they can be made in research laboratories by "in vitro selection" [1, 2]. Considering the many sequences generated for catalyzing an increasing list of chemical transformations over a short span of less than ten years [3–6], it is evident that DNA has great capability for enzymatic function [7, 8]. Deoxyribozymes have been made to catalyze DNA phosphorylation [9] and DNA adenylylation [10] with rate enhancement of $\sim 10^9$-fold over the spontaneous hydrolysis of ATP. A small RNA-cleaving DNA enzyme known as "10-23" performs site-specific RNA cleavage with a $k_{cat}$ of $\sim$10 $min^{-1}$ and $k_{cat}/K_M$ of $\sim 10^9$ $M^{-1}$ $min^{-1}$ [11, 12], values on par with those observed for natural RNA-cleaving ribozymes [13].

Among all the deoxyribozymes made to date, the most efficient ones are metalloenzymes. The known metal-dependent deoxyribozymes have vastly different cation specificities. Some require a particular divalent metal ion for function, while others can make use of a broad range of metal ions. The above-mentioned phosphorylating deoxyribozyme [9] and adenylylating deoxyribozyme [10] are two examples of highly metal-specific DNA enzymes: the former is active only in the presence of $Ca^{2+}$ and the latter requires $Cu^{2+}$ for activity. The DNA enzyme 10-23, on the other hand, can perform efficient catalysis using many different divalent metal ions [12].

We sought to create and study new deoxyribozymes that have similar catalytic abilities but different metal-ion specificities. A parallel in vitro selection approach was used to assess the influence of $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ on sequence diversity, metal specificity, and catalytic proficiency of self-phosphorylating deoxyribozymes that use either ATP or GTP as the source of activated phosphate. These four ions have been frequently used for many other in vitro selection experiments to create various metal-dependent ribozymes and deoxyribozymes. In addition, these four divalent metal ions, having divergent physical properties such as ionic radii, charge densities, and wide-ranging chemical abilities to form coordination bonds with electronegative oxygen and nitrogen atoms, are broadly representative of divalent metal ions.

In this study, we have isolated numerous catalytic DNA sequences from a random-sequence DNA pool. The most efficient deoxyribozyme exhibits a catalytic rate constant ($k_{cat}$) of 2.8 $min^{-1}$, comparable to many of the natural ribozymes and the in vitro-selected catalytic RNAs. Our findings suggest that DNA has sufficient structural diversity to facilitate efficient catalysis using a broad range of metal cofactor utilizing mechanisms.

## Results

### In Vitro Selection

We employed the selection scheme shown in Figure 1A [9]. A pool of random-sequence DNA molecules were allowed to react with both ATP and GTP (Figure 1A, Step I) in the presence of divalent metal ion cofactors. Self-phosphorylating DNAs that acquired a $\gamma$-phosphate from either ATP or GTP were selectively joined to the acceptor DNA by T4 DNA ligase (Step II). The DNA ligation facilitated the separation of the catalytic DNAs by PAGE (Step III) and their selective amplification by PCR (Step IV). A ribo-terminated primer (P1) and a normal primer (P2) were used for PCR so that single-stranded deoxyribozyme sequences could be regenerated by alkaline hydrolysis (Step V). The resulting DNA mixture was subjected to PAGE (Step VI) and the purified 100 nt DNA was used to initiate the next round of selection.

Our first objective was to assess the relative abundance of self-phosphorylating DNA sequences capable of using each of $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ as the divalent metal cofactor. In the first round, a pool of approximately $10^{15}$ individual single-stranded DNAs (G0, sequence shown in Figure 1B) were incubated for self-phosphorylation. A divalent metal ion mixture containing $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ (5 mM each) and $Cu^{2+}$ (50 $\mu$M) was used. The DNA generated at the end of the first selection cycle (Figure 1A, Step VI) was divided into four equivalent pools and used to initiate four parallel selections, each making use of a designated divalent

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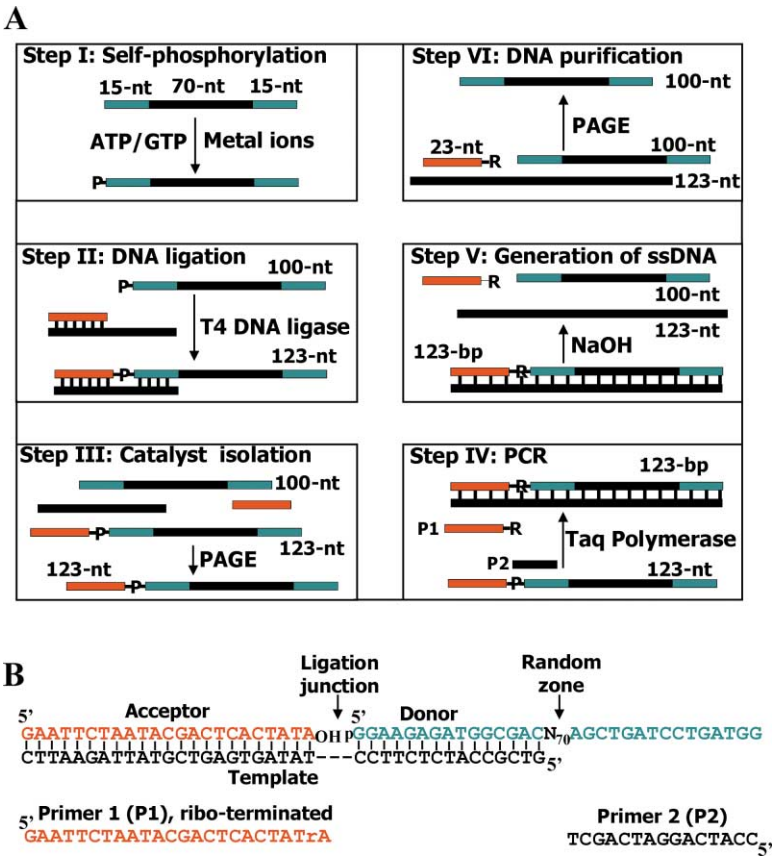



Figure 1. Selection of Self-Phosphorylating DNAs

(A) Each selection cycle consists of steps I–VI. (I) DNA molecules are incubated with both ATP and GTP and metal cofactors. (II) After incubation, the DNA mixture is combined with acceptor and template oligonucleotides and subjected to DNA ligation. (III) The ligated 123 nt DNAs are isolated by PAGE. (IV) The recovered 123 nt DNAs are selectively amplified by PCR using primers P1 and P2. (V) The resulting double-stranded DNAs are treated with NaOH to cleave the single RNA link embedded within DNA. (VI) The 100 nt DNAs are purified by PAGE and used to initiate the next round.

(B) Sequences of the DNA pool complexed with the acceptor and template oligonucleotides. N70 is the random domain. Primer sequences are also shown.

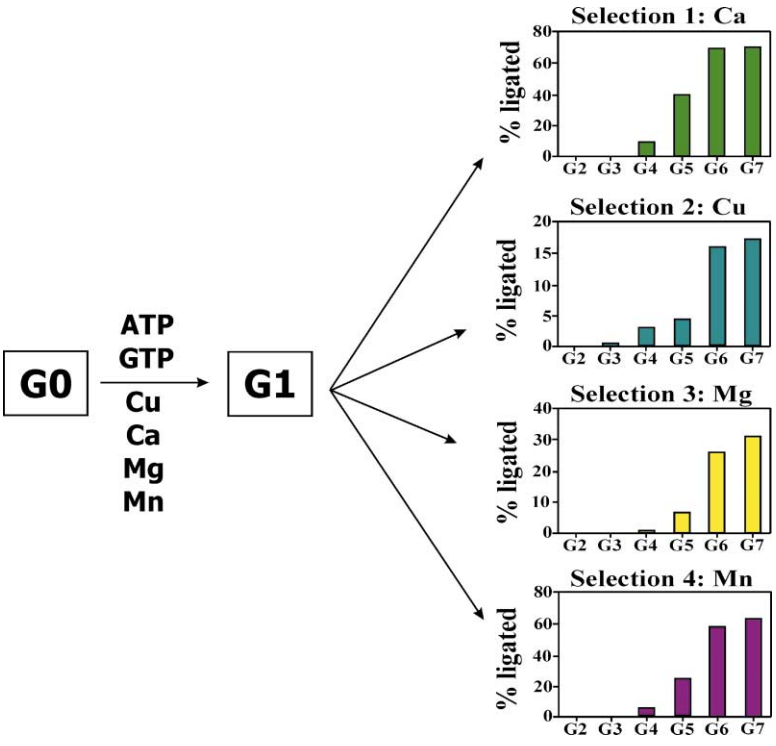



Figure 2. Selection Progress

Four selections were conducted using the same original random pool. In the first round, ~$10^{15}$ different DNAs were incubated with 1 mM each of ATP and GTP in the presence of all four divalent metal ions. At the end of step VI of round 1, the DNAs were split into four pools for individual selections using $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ as designated divalent cofactors. The *y* axis in each plot indicates the percentage of DNA that was ligated in each round.

metal ion as the metal cofactor. Hence, all selected catalytic DNAs should have originated from the same ancestral pool. $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ were used at 15 mM for each relevant selection, while 50 μM $Cu^{2+}$ and 15 mM $Mg^{2+}$ were used for the copper selection. $Cu^{2+}$ is known to be a denaturant for nucleic acid structures at concentrations above 100 μM [14, 15]. Thus, a low concentration of $Cu^{2+}$ had to be used to minimize its denaturation effect. The total divalent metal ion concentration was maintained by the inclusion of 15 mM $MgCl_2$.

The increase of catalytic activity, measured as percentage of ligation, is plotted in Figure 2. No detectable self-phosphorylating activity was observed for all G1 and G2 DNAs and the DNAs from most G3s. However, all DNA populations responded positively to the selection, and 20%–70% of the DNAs from relevant selections underwent ligation after seven iterations of selective amplification.

### Verification of Catalytic Activity

For all G7 DNAs, no 123 nt ligated product was obtained when 100 nt DNA was incubated in a reaction mixture lacking both ATP and GTP (Figure 3A). Ligated DNA appeared when ATP and GTP were supplied. If the DNA was first allowed to react with ATP and GTP and then treated with alkaline phosphatase, no ligated DNA was seen. This result is consistent with the assumption that the selected populations underwent self-phosphorylation in the presence of ATP and GTP.

To provide further evidence for DNA phosphorylation, unlabeled G0 and all G7 DNAs were allowed to react with a mixture of [γ-$^{32}$P]ATP and [γ-$^{32}$P]GTP (Figure 3B). The four deoxyribozyme populations were able to acquire radioactivity in a time-dependent manner, and phosphorylated DNA comigrates on PAGE with the authentic 5′-phosphorylated DNA produced by PNK. These findings are consistent with the transfer of γ-phosphate from ATP or GTP to the 5′-hydroxyl group of self-phosphorylating DNAs.

The G7 DNAs were assessed for the capability of using ATP or GTP separately (Figure 3C). DNA populations derived by $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ were able to use both ATP and GTP for self-phosphorylation, while Cu G7 DNA required only GTP for catalytic function. Despite the fact that this population was dominated by GTP-utilizing molecules, we speculated that individuals exhibiting specificity for ATP or for both ATP and GTP existed in the pool, perhaps in lower abundance. A new lineage of selection beginning with Cu G3 DNA using ATP as the sole phosphorylating reagent gave rise to a new G7 population that was active with ATP (data not shown).

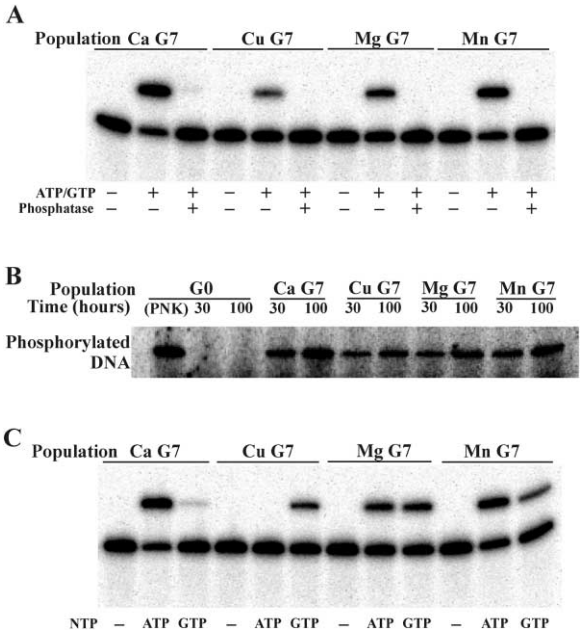



Figure 3. Self-Phosphorylating Activity by Selected DNA Populations

(A) Confirmation of catalytic activity by phosphorylation-ligation-PAGE method. G7 DNA from each selection was first incubated with or without ATP and GTP in a buffer containing the matching divalent metal ions and then treated with or without alkaline phosphatase. Self-phosphorylation reaction: DNA concentration at 0.2 μM, incubation time at 20 hr, and reaction temperature at 23°C, ATP and GTP at 1 mM each. Assay buffers contained 50 mM HEPES (7.0 at 23°C), 100 mM KCl, 400 mM NaCl, and 15 mM relevant divalent metal ions (for Cu, 50 μM $CuCl_2$ and 15 mM $MgCl_2$ were used). DNA ligation reaction: each phosphorylation was followed by the standard DNA ligation reaction in the presence of the acceptor and template oligonucleotides shown in Figure 1B. The top DNA band is ligated DNAs (123-mer) and bottom band is unligated precursor DNAs (100-mer).

(B) Acquisition of $^{32}$P by the selected populations. The first lane shows G0 DNA phosphorylated by polynucleotide kinase and the other lanes show the self-phosphorylating activity of DNA from G0, Ca G7, Cu G7, Mg G7, and Mn G7 for either 30 hr or 100 hr incubation. DNA concentrations were at ~1 μM, γ-$^{32}$P[ATP] and γ-$^{32}$P[GTP] was at ~1 μM each, and ATP and GTP were used at 200 μM each. The other conditions were same as in (A).

(C) ATP and GTP dependence of the G7 populations. Each DNA pool was incubated in the relevant metal-containing buffer in the absence of ATP and GTP and in presence of ATP and GTP. The reaction conditions were same as in (A).

### Metal Specificities of Selected Populations

G7 DNAs were assessed for metal dependences (Figure 4). Ca G7 DNA was active in the presence of either $Ca^{2+}$ or $Mn^{2+}$. However, it had no self-phosphorylating activity when $Cu^{2+}$ and $Mg^{2+}$ or $Mg^{2+}$ alone was used. The $Cu^{2+}$/$Mg^{2+}$-selected population was dependent on $Cu^{2+}$ but not on $Mg^{2+}$, even though the $Mg^{2+}$ concentration was 300× higher than that of $Cu^{2+}$. When $Cu^{2+}$ was absent and $Mg^{2+}$ was used alone, no DNA phosphorylation occurred. $Cu^{2+}$ in combination with $Mg^{2+}$, $Ca^{2+}$, or $Mn^{2+}$ induced equal levels of activity. However, $Cu^{2+}$ alone at 50 μM was not sufficient to support the catalytic activity of the deoxyribozymes, suggesting that $Mg^{2+}$ was used for a supporting role.

DNAs in Mg G7 pool also demonstrated metalloenzyme characteristics, as no catalytic activity was observed in the absence of any divalent metal ions. However, this DNA population was least specific with regard to the divalent metal identities. It had virtually equal catalytic activity in the presence of $Mg^{2+}$, $Cu^{2+}$/$Mg^{2+}$, or $Mn^{2+}$. The DNA pool could also use $Ca^{2+}$, although it had a slightly reduced activity. In sharp contrast, Mn G7 DNA appeared to contain metalloenzymes that were highly metal selective. The DNA pool had robust cata-

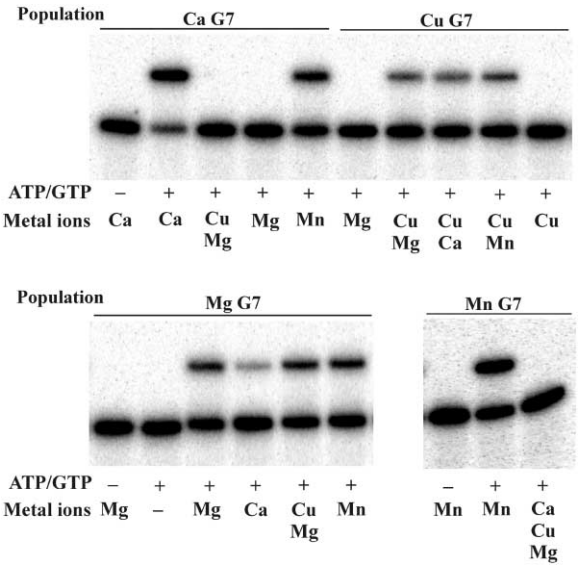



Figure 4. Metal Dependence of Selected DNA Populations

Self-phosphorylation reaction: all the conditions were the same as in Figure 3A except for divalent metal ion identities and concentrations, which were used as follows: 15 mM $CaCl_2$, 15 mM $MgCl_2$, 15 mM $MnCl_2$ for the lanes indicated with Ca, Mg, and Mn, respectively; 50 μM $CuCl_2$ for the lanes indicated with Cu; 50 μM $CuCl_2$ along with 15 mM $CaCl_2$, 15 mM $MgCl_2$, or 15 mM $MnCl_2$ for the lanes indicated with Cu/Ca, Cu/Mg, and Cu/Mn, respectively; 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 50 μM $CuCl_2$ for the lane indicated with Ca/Cu/Mg; (−) indicates no divalent metal ions. (+): ATP and GTP at 1 mM each; (−): no ATP and GTP. Ligation reaction: same as in Figure 3A.

lytic activity when $Mn^{2+}$ was used, but it failed to self-phosphorylate in the presence of the other three metal ions.

### Sequence Diversity

The four G7 populations were cloned and 25 clones from each pool were sequenced to reveal the complexity of each DNA pool (Figure 5A). Since the same initial pool was used to derive all catalytic DNA sequences, it is possible that identical sequences might have appeared in different DNA pools. Comparison of all 44 sequences listed in Figure 5A revealed that Mg2 was identical to Cu10, and Ca1 was identical to Mn11. The appearance of Mg2 in the copper pool could easily be justified, since the selection buffers used for both the copper selection and the magnesium selection contained $Mg^{2+}$. The fact that Ca1 also appeared in the Mn pool (as Mn11) is consistent with the result obtained in the above metal specificity assay (Figure 4) showing that Ca G7 population was active with either $Ca^{2+}$ or $Mn^{2+}$ as the divalent metal cofactor.

DNA pools derived by $Cu^{2+}$ and $Mn^{2+}$ contained enormously diverse catalytic DNA sequences: 22 and 18 different DNA molecules were discovered. There were no apparent dominating sequences in the collection of 25 clones, and most deoxyribozymes appeared only once, while a selective few occurred 2–3 times. Three unique sequences, Mg1, Mg2, and Mg3, were discovered from the collection of 25 clones isolated from the Mg G7 pool. Mg1 was the most dominating sequence in the pool (65%) and Mg3 occurred at the lowest frequency (10%).

The most surprising finding was that only a single sequence (Ca1) was recovered when 25 clones were sequenced from the G7 Ca pool (Ca1, Figure 5A), which appeared to be a mutated version of a previously isolated $Ca^{2+}$-dependent self-phosphorylating DNA molecule NTP-A2 [9] (Figure 5B). There are two possibilities with regard to the appearance of Ca1. One is that Ca1 existed in the initial random-sequence pool used for this study and has no direct relation with NTP-A2. Previous studies have demonstrated that similar or identical sequences can arise from different origins. For example, a cofactorless catalytic DNA motif that is capable of cleaving an embedded RNA within a DNA molecule has been discovered independently by two research groups [16–18]. Similarly, a metal-dependent deoxyribozyme motif for RNA cleavage was found separately in three research laboratories [11, 16–20]. The second possibility is that one of the many NTP-A2 variants somehow has contaminated the Ca selection.

To determine whether we could isolate new $Ca^{2+}$-utilizing DNA molecules, a new selection was conducted beginning with Ca G3 DNA (which had no detectable phosphorylating activity). To eliminate Ca1 from the population, a restriction digestion step was incorporated between step IV and step V (Figure 1A). The PCR product in every round was digested with two restriction enzymes, AclI and BsmI. *Pfu* DNA polymerase was used for the PCR instead of DNA *Taq* polymerase to avoid spontaneous mutations within AclI and BsmI sites in Ca1 that may occur during PCR. A low level of DNA phosphorylation (~5%) was observed in G6. Further selection did not significantly improve the phosphorylation activity. Analyzing 34 clones from the G10 population revealed an almost equal distribution of two different DNA sequences with the complete elimination of Ca1 from the pool (Data not shown). Upon comparison with the sequences from the other metal selections, we found that the two "new" sequences were identical to Mg2 and Mg3. This seems to suggest that Mg2 and Mg3 might be able to use both Ca and Mg (this speculation was confirmed in later assays, see Figure 6). Therefore, the revised selection effort failed to reveal the existence of abundant $Ca^{2+}$-dependent DNAs in the initial pool.

### Metal Specificity of Representative Deoxyribozymes

A number of catalytic sequences in Figure 5 were randomly chosen for further analysis (Figure 6). Each molecule was assessed for metal specificity (lanes 2–5) and ATP/GTP dependence (lanes 6 and 7) under the same conditions used for in vitro selection. As expected, the single $Ca^{2+}$-derived deoxyribozyme, Ca1, was active with both $Ca^{2+}$ and $Mn^{2+}$ as its metal cofactor, since the same sequence was identified in both $Ca^{2+}$- and $Mn^{2+}$-derived pools. This self-phosphorylating molecule was able to use both ATP and GTP as a phosphorylating substrate, although it was significantly more active with ATP than with GTP (~15-fold selectivity). The three $Cu^{2+}$-derived catalytic sequences, Cu1, Cu4, and Cu7, were all remarkably specific for $Cu^{2+}$, and no self-phosphorylating activity was detected with the use of the other three

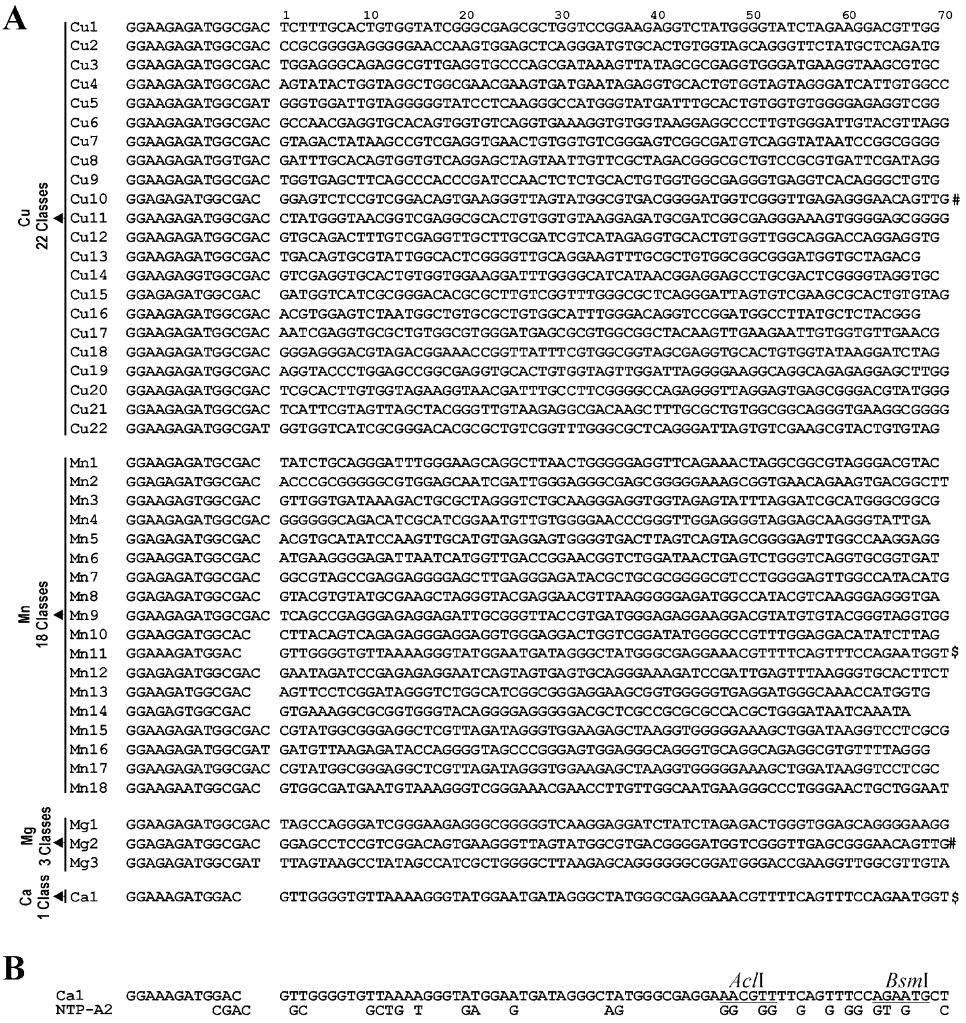



Figure 5. Deoxyribozyme Sequences

(A) Forty-four sequences identified from 100 clones. Each self-phosphorylating deoxyribozyme is named as MX, where M stands for the metal ion with which the deoxyribozyme was derived and X is the number given to a unique sequence found in a specific metal pool. Sequences that appeared in two different pools are labeled by # or $. Nucleotides in the original random domain are numbered. The 5′-unnumbered domain for each deoxyribozyme may deviate from the original sequence (GGAAGAGATGGCGAC) either due to errors in the chemical synthesis of the DNA library or mutations during in vitro selection. The 3′-constant region with a sequence of AGCTGATCCTGATGG is not shown.

(B) Sequence alignment of Ca1 with a previously reported phosphorylating deoxyribozyme NTP-A2. The nucleotides not shown are conserved in both sequences. Two restriction sites in Ca1, AclI and BsmI, are indicated.

metal ions, even after an extended incubation time (several days). These molecules were GTP-utilizing and had no self-phosphorylating activity in the presence of ATP.

The three $Mg^{2+}$-derived DNAs, Mg1, Mg2, and Mg3, exhibited the most intriguing properties. For metal ion specificity, Mg2 and Mg3 are quite receptive toward all four metal ions. Mg1 showed similar catalytic ability with $Mg^{2+}$ or $Mn^{2+}$ but demonstrated weaker activity using $Ca^{2+}$. For ATP and GTP utilization, Mg1 was capable of reacting with both ATP and GTP; in contrast, Mg2 and Mg3 were both GTP dependent.

Three $Mn^{2+}$-derived sequences, Mn1, Mn4, and Mn7, were all strictly $Mn^{2+}$ dependent. There was no detectable activity with the use of the other three metal ions, even at an extended incubation time. Mn4 and Mn7 were both ATP-dependent while Mn1 was GTP specific. Our data show that self-phosphorylating deoxyribozymes derived by $Mn^{2+}$ or $Cu^{2+}$ have strict dependency on the divalent metal ion used for the selection, while the ones derived using $Ca^{2+}$ or $Mg^{2+}$ tend to be less selective.

## Two Proficient Deoxyribozymes

Deoxyribozymes selected for the purpose of sequence-diversity comparison exhibited a $k_{obs}$ of $\sim 10^{-4}$ $min^{-1}$, corresponding to an estimated rate enhancement of $\sim 10^5$-fold over the spontaneous hydrolysis of ATP [9, 24]. Although this level of activity is quite substantial, these autocatalytic DNA molecules are not highly proficient. However, the establishment of catalytic populations containing numerous self-phosphorylating deoxyribozyme sequences has provided us with an opportunity to seek out extremely proficient autocatalytic DNAs through further optimization by in vitro evolution.

A DNA pool (G′0) made by combining all four G7 DNA

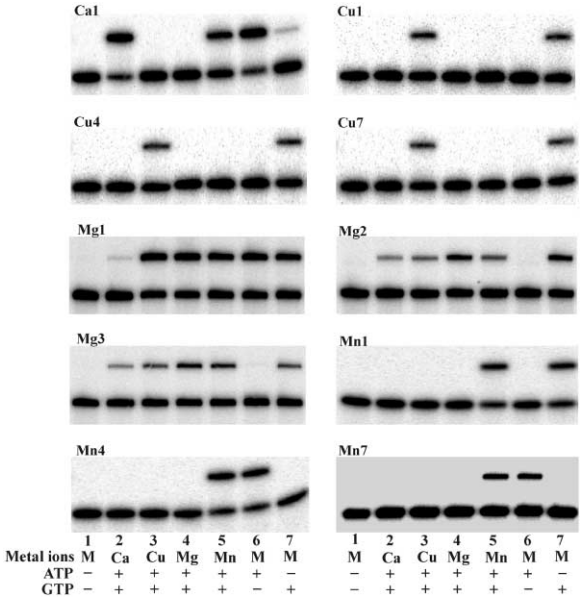



Figure 6. Metal and ATP/GTP Specificity of Ten Individual Deoxyribozymes

Each internally $^{32}$P-labeled deoxyribozyme was incubated for self-phosphorylation in the reaction buffers containing 15 mM $CaCl_2$ (lane 2), 50 μM $CuCl_2$ and 15 mM $MgCl_2$ (lane 3), 15 mM $MgCl_2$ (lane 4), and 15 mM $MnCl_2$ (lane 5), 5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 50 μM $CuCl_2$ (lanes 1, 6, and 7). ATP and GTP: both ATP and GTP for lanes 2–5 (1 mM each), ATP for lane 6 (1 mM), and GTP for lane 7 (1 mM), no ATP and GTP for lane 1. M stands for the mixture of all four metal ions. Other conditions for phosphorylation reaction were same as in Figure 3A.

populations was used to begin the optimization process. A total of 16 rounds of reselection were conducted. DNA used for each generation between G′1 and G′9 consisted of about 5% wild-type sequences (radioactive) made by standard PCR protocol and ~95% heavily mutagenized variants (nonradioactive) produced by a hypermutagenic version of PCR [21, 22] that is reported to generate a mutation rate as high as 10% per base per completed PCR reaction (50 cycles) [21]. Phosphorylation time was progressively shortened from 90 min (G′0 to G′2) to 15 min (G′3 to G′5), to 2 min (G′6 to G′9), to 20 s (G′10 to G′12), and finally to 5 s (G′13 to G′15), and the end population (G′15) exhibited a self-phosphorylation rate of ~1 $min^{-1}$ (i.e., more than 35% phosphorylation was observed in 20 s; Figure 7), suggesting that we have evolved a highly proficient deoxyribozyme population. It is noteworthy that although the G′0 population was made intentionally to use all four divalent metal cofactors at equal efficiency and had no significant bias toward either ATP or GTP, G′15 population was mainly $Mn^{2+}$ dependent and ATP utilizing (Figure 7).

Two deoxyribozymes were identified from the G′15 population and their sequences are given in Figure 8A. Dk1 was the dominating class: 17 out of 20 clones sequenced are Dk1 variants. Within the central 69 nt random core of Dk1 sequence, G, C, A, and T nucleotides occur at a frequency of 40%, 12%, 25%, and 23%, respectively, making Dk1 a guanine-rich sequence. Dk2, on the other hand, is not guanine rich (Figure 8), and all four nucleotides occur at approximately equal frequency within its 70 nt random core. Since both Dk1 and Dk2 contain quite a few stretches of consecutive G residues, they may make use of guanine quartets in their tertiary structure. Guanine quartets have been recognized as the essential tertiary element of several guanine-rich deoxyribozymes [9, 10, 23]. Detailed structural features of both Dk1 and Dk2 are under further investigation.

Dk1 was strictly dependent on $Mn^{2+}$ (data not shown). The $Mn^{2+}$ concentration that supports half-maximal enzymatic activity, defined as $K_M$(Mn), was found to be 12.5 mM (Figure 8B), close to the total divalent metal concentration (15 mM) used for the selection. Dk1 was extremely selective for ATP, showing no detectable activity with GTP (data not shown). The apparent $K_M$ for ATP (the ATP concentration for half-maximal catalytic activity) was determined to be 0.85 mM, near the ATP concentration (1 mM) used in the selection. The catalytic rate of Dk1 was not affected by the change of pH within the range of 6.0–8.0. Above pH 8.0, however, the catalytic rate dropped sharply (data not shown).

A representative kinetic assay is shown in Figure 8C. The deoxyribozyme was found to undergo self-phosphorylation to ~80% completion in 2 hr. The catalytic reaction exhibited first-order kinetics at phosphorylation below ~50% completion (see the graph in Figure 8C) with a rate constant ($k_{cat}$) of 2.8 $min^{-1}$, making Dk1 a very proficient deoxyribozyme. In comparison to the ATP-dependent self-phosphorylating deoxyribozyme NTP-A2.1 [9], Dk1 demonstrates a gain of ~500-fold in $k_{cat}$ but has a drop of ~250-fold in ATP affinity. Therefore, Dk1 and NTP-A2.1 have almost identical $k_{cat}/K_M$ at ~$3 \times 10^3$ $min^{-1}$ $M^{-1}$. Such a catalytic efficiency corresponds to a rate enhancement of ~$10^9$-fold over the spontaneous hydrolysis of ATP [9, 24]. However, Dk1 has never been optimized for ATP binding throughout the entire selection process, therefore we expect that further optimization by in vitro selection will considerably improve its ATP affinity.

Dk2 was also metal dependent. It was highly selective for $Mn^{2+}$ with a $K_M$(Mn) of 5.6 mM (Figure 8B). It was able to use the other three metal ions but at a much reduced activity (data not shown). Dk2 was a GTP-dependent catalyst and had a $k_{cat}$ of 0.8 $min^{-1}$ (Figure 8B). The lower catalytic rate may help explain why Dk2 occurred at lower abundance than Dk1 in the G′15 pool.

## Discussion

### Parallel In Vitro Selection

Divalent metal ions play important roles in the catalysis of nucleic acid enzymes [25–27] as almost all known ribozymes and deoxyribozymes are either dependent on divalent metal ions for function or use metal cofactors to enhance their catalytic activities. Since divalent metal ions have variable physical properties and chemical abilities to form coordination bonds with electron-donating atoms [28, 29], the use of different divalent metal ions could drastically alter the outcome of deoxyribozymes or ribozyme creation by in vitro selection. In this report, we have performed a series of parallel in vitro selection experiments to assess the possible impact imposed by

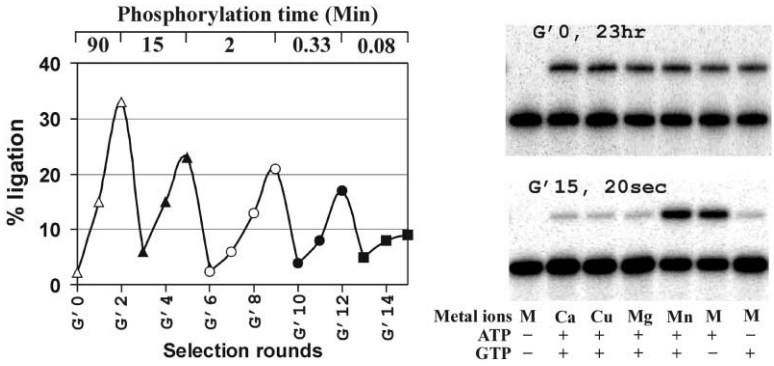
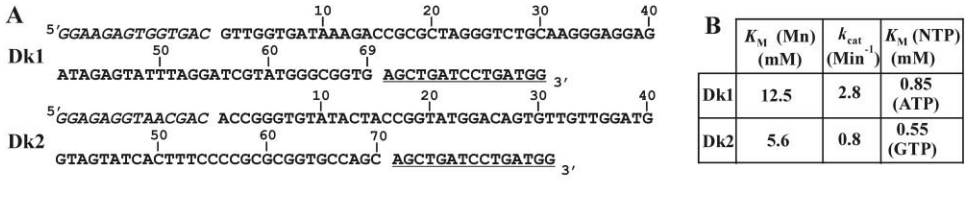



Figure 7. In Vitro Evolution of a Combined Deoxyribozyme Pool

Left graph: the evolution progress is illustrated by plotting the percentage of ligated DNA in each selection round. The phosphorylation time (in minutes) for each round is indicated on the top of the graph. Right: metal and ATP/GTP specificity analysis for the DNA populations in the first and sixteenth generations (G′0 and G′15). The experiment was conducted in the same way as shown in Figure 6 except for the phosphorylation time (23 hr for G′0 and 20 s for G′15).

$Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ on the isolation of self-phosphorylating DNA. In particular, we have attempted to determine how these metal ions affect the sequence diversity as well as the metal specificities of self-phosphorylating DNAs derived by in vitro selection. We also sought to evolve very efficient deoxyribozymes that could eventually lead to the engineering of phosphorylating DNA enzymes for practical applications.

A DNA library containing $10^{15}$ different molecules was used as the starting pool with which a single first round was carried out using all four divalent metal ions. The DNA product was then split into four populations to derive individual pools that performed self-phosphorylation using designated divalent metal cofactors. The use of the same initial pool was intended to reduce the probability that different DNA pools might have uneven distributions of DNA sequences capable of using different divalent metal ions. The pool splitting was made right after the first round to minimize the possible domination of one or a few deoxyribozymes in the selection. A relatively long phosphorylation time (20 hr) was used so as to obtain as many self-phosphorylating DNAs as possible for sequence diversity comparison. The same selection conditions were applied to all four selections. In addition, each selection was only allowed to progress to the early saturation, described as a plateau in self-phosphorylation activity. All these efforts were made with the intention to both isolate self-phosphorylating




B

| | $K_M$ (Mn) (mM) | $k_{cat}$ ($Min^{-1}$) | $K_M$ (NTP) (mM) |
|---|---|---|---|
| Dk1 | 12.5 | 2.8 | 0.85 (ATP) |
| Dk2 | 5.6 | 0.8 | 0.55 (GTP) |

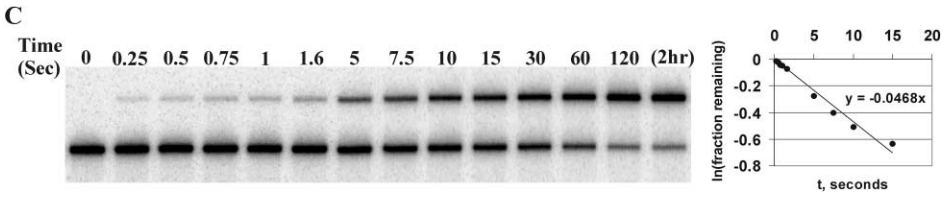



Figure 8. Two Proficient Deoxyribozymes

(A) The sequences of the two deoxyribozymes (Dk1 and Dk2) isolated from G′15. Letters in italic are the nucleotides in the original 5′-constant domain; numbered nucleotides are the original random core; the underlined nucleotides are the PCR primer P2 binding site.

(B) $K_M$ for $Mn^{2+}$ (in mM), $K_{cat}$ (in $min^{-1}$) and $K_M$ for NTP (in mM) determined for Dk1 and Dk2. The $K_M$ values for $Mn^{2+}$ was determined by plotting the initial catalytic rates versus $Mn^{2+}$ concentration varied between 1 to 50 mM. Phosphorylation reaction conditions: DNA concentration at 0.05 μM, reaction temperature at 23°C, 2.5 mM ATP (for Dk1) or 2.0 mM GTP (for Dk2). The assay buffer contained 50 mM HEPES (7.0 at 23°C), 400 mM NaCl, 100 mM KCl, and relevant $Mn^{2+}$ concentrations. The apparent $K_M$ for ATP (Dk1) or GTP (Dk2) was determined in a similar way by plotting the initial rates obtained at ATP or GTP concentrations between 0.1–5 mM and $Mn^{2+}$ concentration fixed at 30 mM. The self-phosphorylating rate constants ($K_{cat}$) were determined at 30 mM $Mn^{2+}$, 2.5 mM ATP for Dk1, or 2.0 mM GTP (7.0 at 23°C) for Dk2, with the same set of conditions used for $K_M$ experiment except that 50 mM Tris (pH 7.6) was used to replace HEPES. A typical run is shown in (C).

(C) A time-course examination for Dk1. The rate constant was determined by plotting the natural logarithm of the fraction of DNA that remains unligated versus phosphorylation reaction time. The negative slope of the line produced by a least-squares fit to the data is taken as the rate constant (see the graph on the right).

DNAs with similar catalytic performance and to minimize the possible domination of certain DNA species in each selection.

### Sequence Diversity

Sequence analysis revealed variable sequence diversities within the G7 populations. Initial sequencing of 25 clones from Cu G7 DNA identified 22 unique sequences (Figure 5A) and further sequencing of 75 additional Cu clones resulted in 29 more distinct sequences (data not shown), indicating that $Cu^{2+}$-assisted deoxyribozymes were extremely abundant in the random-sequence library. Cu G7 DNA had a noticeably lower activity than the other G7 populations (i.e., ~15% for Cu G7 DNA, 30% for Mg G7 DNA, and ~60% for Ca G7 and Mn G7 populations, see Figure 2) despite the fact that all these populations were selected using the same selection criteria. The lower phosphorylation activity in Cu G7 DNA raised concerns about its large sequence diversity. Perhaps this diversity was the result of somewhat ineffective autophosphorylation caused by the absence of any proficient species that could dominate the sequence pool. However, three lines of evidence suggest that the reduced autophosphorylation activity was not the cause for the large sequence diversity. First, although the Cu G7 DNA as a whole had about ~15% phosphorylation activity, the individual catalysts that were randomly chosen from the G7 pool all exhibited nearly 30% phosphorylation, matching the activity observed for Mg2, Mg3, and Mn7 (Figure 6). Second, it is well documented that $Cu^{2+}$-dependent DNA enzymes usually have an optimal catalytic activity within a narrow $Cu^{2+}$ concentration range. For instance, both the $Cu^{2+}$-dependent DNA-ligating deoxyribozyme [30] and DNA-cleaving DNA enzyme [31] exhibit a maximal activity at ~10 μM of $Cu^{2+}$ [30, 32]. Similarly, another $Cu^{2+}$-dependent deoxyribozyme with DNA-capping activity has an optimal $Cu^{2+}$ concentration of 5 μM [10]. All these DNA enzymes have been shown to experience severe inhibitory effects with higher $Cu^{2+}$ concentrations [10, 30, 32]. We found that the optimal $Cu^{2+}$ concentration for Cu1 was also near 10 μM. At this concentration, Cu1 can self-phosphorylate to nearly 60% in 20 hr, while at 50 μM $Cu^{2+}$ the activity drops to 30% (data not shown), suggesting that the inhibition by $Cu^{2+}$ may indeed be responsible for the low activity of the Cu G7 DNA. The third piece of evidence was the sheer domination by $Cu^{2+}$-utilizing sequences in Cu G7 DNA itself. Despite the fact that both $Cu^{2+}$ (50 μM) and $Mg^{2+}$ (15 mM) were included in the selection buffer used for the copper selection, Figure 4 shows that Cu G7 DNA had no detectable activity in the absence of $Cu^{2+}$ but in the presence of $Mg^{2+}$. The activity assay was further supported by sequence analysis (Figure 5A), which revealed that only a single sequence (Cu10 or Mg2, which are identical) out of the initially sequenced 25 clones from Cu G7 DNA utilized $Mg^{2+}$. The near absence of $Mg^{2+}$-utilizing sequences in the Cu G7 DNA pool could not have been due to $Cu^{2+}$ inhibition, as the Mg G7 DNA demonstrated no diminished activity in the presence of both $Cu^{2+}$ and $Mg^{2+}$, as compared to $Mg^{2+}$ alone (Figure 4). Taken together, the data indicate that a diverse collection of $Cu^{2+}$-dependent self-phosphorylating DNAs with robust activity was present in the initial pool.

Similarly, $Mn^{2+}$-promoted deoxyribozymes also existed in large quantity in the initial random-sequence DNA library. Eighteen unique sequences were found from 25 clones sequenced from Mn G7 DNA (Figure 5A). In stark contrast, only three unique sequences were found within the initial 25 clones sequenced from Mg G7 pool (Figure 5A). Sequencing of 25 additional clones did not identify any new sequences. These data, along with the evidence that the Cu selection was dominated by $Cu^{2+}$-utilizing sequences despite the presence of $Mg^{2+}$ in the selection buffer, suggest that $Mg^{2+}$-assisted sequences occurred in a frequency that is much smaller than $Mn^{2+}$- and $Cu^{2+}$-dependent deoxyribozymes in the initial pool.

Ca G7 DNA was found to contain only a single sequence (Ca1) that was suspiciously similar to NTP-A2, a self-phosphorylating DNA that had been previously selected [9]. It is possible that Ca1 was a mutated version of NTP-A2 and its existence may have prevented the selection of other possible $Ca^{2+}$-utilizing sequences. A revised selection was therefore conducted with the use of restriction enzymes to eliminate Ca1. However, the new effort failed to isolate any new $Ca^{2+}$-dependent DNA sequence with robust phosphorylating activity. This new evidence seems to suggest that $Ca^{2+}$-assisted deoxyribozymes were not particularly abundant in the original pool. Additional evidence consistent with the low abundance of $Ca^{2+}$-utilizing sequences came from the sequencing analysis of Mn G7 pool (Figure 5A) and the metal specificity data shown in Figure 4 and Figure 6. Although Ca1 exhibited robust activity with $Mn^{2+}$ (Figure 6) and had indeed been selected from the Mn pool as Mn11 (Figure 5A), it apparently did not dominate the Mn selection, as Ca1 was only one out of 18 sequences identified from a small collection of 25 clones and Mn G7 DNA demonstrated no detectable activity in buffer containing $Ca^{2+}$ but not $Mn^{2+}$.

In summary, our data appear to indicate that there were more $Cu^{2+}$- and $Mn^{2+}$-utilizing self-phosphorylating DNA sequences in the initial DNA library than those using $Ca^{2+}$ and $Mg^{2+}$. The propensity for DNA to make use of $Cu^{2+}$ or $Mn^{2+}$ as divalent metal cofactors is supported by several other in vitro selection studies [10, 30] (unpublished data) where $Cu^{2+}$ or $Mn^{2+}$ (but not $Mg^{2+}$ or $Ca^{2+}$) has emerged as the winning metal ion. Hypothetically, it is quite possible that the "soft" transition metal ions such as $Cu^{2+}$ and $Mn^{2+}$ have better ability than the "hard" alkali metal ions at creating diverse deoxyribozyme active sites, because the former have better ability to engage in bonding interactions of more covalent character to form strong bonds and facilitate long-range interactions. However, having studied only a small group of divalent metal ions, there is not enough experimental evidence for us to rationalize the observed sequence-diversity patterns on the basis of differences of any particular metal ion property. Furthermore, it remains to be determined whether the sequence distribution pattern that we have observed with the particular group of metal ions for DNA phosphorylation is generally observable regardless of the use of any DNA (or RNA) library for any chemical transformation.

### Metal-Ion Specificity and Covalent and Ionic Indices of Metal Ions

We have observed that $Cu^{2+}$- and $Mn^{2+}$-promoted DNAs had very specific metal dependency, while $Ca^{2+}$- and $Mg^{2+}$-derived deoxyribozymes were less specific or nonspecific in metal requirements. While the basis for these differences is likely multifactorial, we speculate that this experimental observation may be explained with the use of ionic or covalent indices. Ionic index (defined as $Z^2/r$ where Z and r are the charge and radius of a metal ion) and covalent index ($X_m^2 r$ where $X_m$ is the Pauling electronegativity) are used to measure the ability of a divalent metal ion to form ionic and covalent bonds with electron-rich atoms [28, 33, 34]. The ionic indices of $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, and $Cu^{2+}$ are 4.0, 5.6, 4.8, and 5.5, respectively. The comparable ionic index values suggest that these metal ions have similar ability to engage in ionic interactions. By contrast, the covalent indices vary considerably within the group: $Cu^{2+}$ (3.0) and $Mn^{2+}$ (2.0) have covalent indices that are larger than those of $Mg^{2+}$ (1.2) and $Ca^{2+}$ (1.0). A metal ion that forms coordination bonds of high covalent character at a catalysis-essential metal binding site is expected to be replaced only by divalent metal ions that have identical (or very similar) covalent index and coordination geometry. Conversely, a metal binding site constructed by ionic interaction should be more receptive toward the substitution by metal ions with similar ionic index. This is because ionic interaction involves electrostatic attraction between oppositely charged entities and has little geometry requirement. $Cu^{2+}$ has a large covalent index and its bonding interaction with its supported deoxyribozymes may be of very high covalency. Under this hypothesis, it may be difficult to replace the $Cu^{2+}$ ion with any of the other three metal ions due to large differences in covalent index. Similarly, $Mn^{2+}$ has tendency of forming coordination bonds of significant covalent content and hence cannot be replaced by metal ions with very different covalent index. Compared to $Cu^{2+}$ and $Mn^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ have comparable ionic indices but much smaller covalent indices. Therefore, the coordination bonds formed by these two metal ions may show a highly ionic character. As a result, metal ions with similar ionic indices should be able replace these two metal ions. This is perhaps the reason for the reduced metal specificities observed for the DNA molecules derived by $Ca^{2+}$ and $Mg^{2+}$, as the ionic index values are generally more comparable than the covalent index values. More specifically, this reasoning gives a possible explanation to the peculiar metal specificities observed for Ca1 and Mg1. As seen in Figure 6, $Ca^{2+}$-derived Ca1 was incapable of using $Mg^{2+}$ and $Mg^{2+}$-derived Mg1 had very weak activity with $Ca^{2+}$, while both deoxyribozymes were functional in the presence of $Mn^{2+}$. Since the difference of the ionic indices of $Ca^{2+}$ (4.0) and $Mg^{2+}$ (5.6) is quite substantial, the catalytic metal binding site built around $Mg^{2+}$ may not be able to accommodate $Ca^{2+}$ and vice versa. $Mn^{2+}$, however, has an intermediate ionic index at 4.8. The small differences in ionic index between $Mn^{2+}$ and $Ca^{2+}$ and between $Mn^{2+}$ and $Mg^{2+}$ may render $Mn^{2+}$ the special ability to substitute either metal in the binding site of Ca1 or Mg1. However, our hypothesis needs to be validated using more divalent metal cofactors and perhaps more self-phosphorylating DNAs that are to be created with different sets of divalent metal ions.

### Catalytic Proficiency

In total, 71 unique sequences have been identified from the four G7 populations. It is certain that more unique sequences could have been revealed if more clones had been isolated and sequenced. Although these DNAs were selected to perform self-phosphorylation in ~20 hr, the extreme sequence diversity offered a unique evolutionary opportunity to derive highly efficient self-phosphorylating DNA molecules. For this purpose, all the autocatalytic sequences were combined to make a new starting pool (G′0) for the catalytic rate optimization. Intensive mutagenesis and progressive reduction in phosphorylation time were employed from round to round to favor the isolation of highly efficient DNAs. Selection was continued until the phosphorylation time was shortened to 5 s. Surprisingly, the deoxyribozyme population always responded positively to the imposed time constraints. Consequently, the catalytic rate has improved by more than $10^4$ fold, from $\sim 10^{-4}$ $min^{-1}$ seen with the initial mixture to ~1 $min^{-1}$ that was observed for the terminal DNA population (G′15). With such a demanding evolution, only two deoxyribozymes, Dk1 (dominating sequence) and Dk2 were found in the final pool. Dk1, a $Mn^{2+}$-specific molecule, is a mutated version of Mn3 found in the Mn G7 pool (Figure 5). The highly $Mn^{2+}$-selective sequence Dk2, however, was not present in the sequence collection. It is highly likely that Dk2 also came from the Mn pool based on its $Mn^{2+}$ selectivity and the fact that only a noncomprehensive sequencing analysis was conducted for the Mn G7 pool.

The absence of $Cu^{2+}$-promoted DNAs in the terminal pool was a surprise considering the great sequence diversity of the original $Cu^{2+}$-dependent deoxyribozymes. One explanation was that the $Mn^{2+}$-dependent DNAs in the mixed pool had the best selective advantage because they not only possessed great sequence diversity but also had more robust self-phosphorylating activity when the evolution was started. It is also possible that the 50 μM of $Cu^{2+}$ included in the selection buffer may have been too high to make $Cu^{2+}$-dependent molecules highly competitive. Therefore, it remains to be investigated whether $Cu^{2+}$ can be as competent as $Mn^{2+}$ in providing support to the most proficient deoxyribozymes for DNA phosphorylation.

Dk1 exhibited a catalytic rate constant ($k_{cat}$) of 2.8 $min^{-1}$, second only to the 10-23 deoxyribozyme [11, 12]. This catalytic rate constant is comparable to many of the natural ribozymes and the in vitro-selected catalytic RNAs, indicating again that DNA has comparable ability for enzymatic function despite the lack of 2′-hydroxyl group. Catalytic rates seen with Dk1 and Dk2 are large enough for practical applications. With continuing optimization for the NTP affinity and better understanding about the structural properties of these two efficient catalytic DNAs, we may eventually be able to design an efficient deoxyribozyme system for DNA phosphorylation.

## Significance

**We have used DNA phosphorylation as a model reaction and $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ as representative metal ions to demonstrate that divalent metal ions can have a profound influence on the outcome of deoxyribozyme selection. We have shown that the choice of metal ions can affect the sequence diversity, metal-ion specificity, and catalytic proficiency of deoxyribozymes to be isolated and optimized from a random-sequence DNA library. We have found that $Cu^{2+}$- and $Mn^{2+}$-promoted self-phosphorylating deoxyribozymes appear to be significantly more populated than the ones assisted by $Ca^{2+}$ and $Mg^{2+}$ in a random-sequence library for precise reasons yet to be investigated. Hypothetically, however, this experimental observation may suggest that transition metal ions may have better ability at creating diverse deoxyribozyme structures. This may reflect the inherent ability of these metal ions to engage in bonding interactions of more covalent character to form strong bonds and facilitate long-range interactions. We have also found that $Cu^{2+}$- and $Mn^{2+}$-promoted molecules have very specific metal dependency while $Ca^{2+}$-and $Mg^{2+}$-derived deoxyribozymes are less specific or nonspecific in metal requirements. This may relate to the ability of transition metal ions to interact in a more covalent fashion with single-stranded DNA molecules in constructing catalytic metal binding sites. Furthermore, we have shown that highly efficient deoxyribozymes with $k_{cat}$ values over 1 $min^{-1}$ for DNA self-phosphorylation can be derived fairly easily by in vitro evolution of a catalytic DNA pool containing a large number of metal-dependent catalytic DNAs. Our findings not only continue to support the notion that DNA, like RNA and protein, has sufficient structural diversity for efficient catalysis, but also indicate a strong possibility of engineering very efficient metallo DNA enzymes for DNA phosphorylation.**

## Experimental Procedures

### Oligonucleotides and Nucleotides

Synthetic DNAs were prepared by automated chemical synthesis (Keck Biotechnology Resource Laboratory, Yale University). Random-sequence DNAs were synthesized by using an equimolar mixture of the four standard phosphoramidates. DNA oligonucleotides were purified by 10% preparative denaturing (8 M urea) PAGE. Purified oligonucleotides were dissolved in water and their concentrations were determined spectroscopically. Nucleoside 5′-triphosphates, [$\gamma$-$^{32}$P]ATP, [$\gamma$-$^{32}$P]GTP, and [$\alpha$-$^{32}$P]dGTP were purchased from Amersham Pharmacia. *Taq* DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase (PNK), and restriction enzymes were purchased from MBI Fermentas. All other chemical reagents were purchased from Sigma.

### In Vitro Selection Procedures

The basic protocol for in vitro selection is similar to the one used by Li and Breaker [9] with specifics described herein. 1500 pmol of the random-sequence DNA was used as the initial pool (G0). DNA was heated to 90°C for 1 min and cooled to room temperature. A 2× selection buffer containing relevant metal ions was used to prepare the reaction mixture. ATP and GTP were added to initiate the phosphorylation reaction. The reaction mixture for the first round of selection contained 50 mM HEPES (pH 7.0 at 23°C), 400 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM $MnCl_2$, 50 $\mu$M $CuCl_2$, 1 mM ATP, 1 mM GTP, and 0.25 $\mu$M DNA.

The DNA mixture was incubated for self-phosphorylation at 23°C for 20 hr. Phosphorylation reaction was stopped by the addition of EDTA (pH 8.0) to a final concentration of 30 mM. DNA was recovered by precipitation with ethanol, resuspended in 20 mM NaCl, and supplied with 2000 pmol of template DNA and 2500 pmol of acceptor DNA (Figure 1B). The mixture was heated to 90°C for 1 min, cooled to room temperature, and combined with 10× ligase buffer (supplied by the manufacture) and T4 DNA ligase. The ligation mixture contained 1 $\mu$M donor DNA, 1.3 $\mu$M DNA template, 1.7 $\mu$M acceptor DNA, and T4 DNA ligase at 0.1 U (Weiss) $\mu L^{-1}$. The solution was incubated at 23°C for 20 hr before the DNA was recovered by precipitation with ethanol.

The ligated DNA was separated from unligated DNA by denaturing 10% PAGE following a procedure described in detail by Li and Breaker [22]. The 123 nt ligated DNA product was amplified by PCR. Double-stranded DNA was recovered by precipitation with ethanol, resuspended in 90 $\mu$l of 0.25 M NaOH, and incubated at 90°C for 10 min to cleave the single embedded RNA linkage. The ~100 nt DNA cleavage fragments, corresponding in length to the original DNA constructs, were purified by denaturing 10% PAGE. Finally, alkaline phosphatase was used to treat the purified 100-mer DNA to remove all 5′-phosphate groups on DNA so as to prevent the appearance of certain "selfish," noncatalytic DNAs that can obtain 5′-phosphate during PCR reaction [22].

The above treated DNA was split into four equal pools to set up four parallel selections denoted Ca, Cu, Mg, and Mn selections. Self-phosphorylation in the second and subsequent rounds was conducted similarly to the first round except that (1) the reaction was reduced to 1/50$^{th}$ scale and (2) four different metal solutions (15 mM $CaCl_2$, 50 $\mu$M $CuCl_2$ along with 15 mM $MgCl_2$, 15 mM $MgCl_2$ and 15 $MnCl_2$) were used for the relevant selections. DNA ligation reactions were also reduced to one-tenth scale and were incubated for only 30 min.

For catalytic rate optimization, a combined deoxyribozyme population made of 25 pmol of DNA generated with the G7 pool from each metal selection was used as the beginning pool (G′0). A total of 16 iterations were carried out using the divalent metal ion mixtures containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM $MnCl_2$, and 50 $\mu$M $CuCl_2$. Selection procedures were similar to the one described above for the original round with the following modifications. (1) The phosphorylation reaction was at one-fiftieth and DNA ligation reaction at one-tenth scale. (2) The ssDNAs used for selection from round 2 to round 10 consisted of about 5% "wild-type" sequences made by normal PCR and ~95% mutated sequences by a hypermutagenic PCR [21, 22]. (3) The incubation time for self-phosphorylation was progressively reduced as follows: 90 min for G′0 to G′2, 15 min for G′3 to G′5, 2 min for G′6 to G′9, 20 s for G′10 to G′12, 5 s for G′13 to G′15. ATP and GTP were used at 1 mM each in all selection rounds.

### Cloning of Selected DNA Populations

DNA sequences from a relevant selection round were amplified by PCR and cloned into a vector by TA cloning method. The plasmids containing individual catalysts were prepared using Qiagen Mini-Prep Kit. DNA sequencing was performed on an LCQ2000 capillary DNA sequencer (Beckman-Coulter) following the procedures recommended by the manufacturer.

### Catalytic Assays for Individual Clones

To produce single-stranded DNA (ssDNA) for catalytic assays, the plasmid DNA of a specific clone was amplified by PCR in the presence of $\alpha$-$^{32}$P dGTP and then cleaved by NaOH under conditions described above. After gel purification and dephosphorylation with alkaline phosphatase, internally $^{32}$P-labeled ssDNAs (~100 nM) were tested for their ability to self-phosphorylate in designated buffers containing relevant metal ions using the same method described for selection experiments (see above) unless otherwise specified. Stop-quenching method was used to obtain kinetic data points for Dk1 at incubation times 1.6 s or less. The rate constants for DNA phosphorylation were determined by plotting the natural logarithm of the fraction of DNA that remains unligated versus the reaction

time. The negative slope of the line produced by a least-squares fit to the data was taken as the rate constant.

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