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# A DNAzyme requiring two different metal ions at two distinct sites

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## ABSTRACT

**Most previously reported RNA-cleaving DNAzymes require only a single divalent metal ion for catalysis. We recently reported a general trivalent lanthanide-dependent DNAzyme named Ce13d. This work shows that Ce13d requires both $Na^+$ and a trivalent lanthanide (e.g. $Ce^{3+}$), simultaneously. This discovery is facilitated by the sequence similarity between Ce13d and a recently reported $Na^+$-specific DNAzyme, NaA43. The Ce13d cleavage rate linearly depends on the concentration of both metal ions. Sensitized $Tb^{3+}$ luminescence and DMS footprinting experiments indicate that the guanines in the enzyme loop are important for $Na^+$-binding. The $Na^+$ dissociation constants of Ce13d measured from the cleavage activity assay, $Tb^{3+}$ luminescence and DMS footprinting are 24.6, 16.3 and 47 mM, respectively. Mutation studies indicate that the role of $Ce^{3+}$ might be replaced by $G_{23}$ in NaA43. $Ce^{3+}$ functions by stabilizing the transition state phosphorane, thus promoting cleavage. $G_{23}$ competes favorably with low concentration $Ce^{3+}$ (below 1 μM). The $G_{23}$-to-hypoxanthine mutation suggests the N1 position of the guanine as a hydrogen bond donor. Together, Ce13d has two distinct metal binding sites, each fulfilling a different role. DNAzymes can be quite sophisticated in utilizing metal ions for catalysis and molecular recognition, similar to protein metalloenzymes.**

## INTRODUCTION

Metal ions play critical roles in biocatalysis. Protein metalloenzymes use a diverse range of metal ions for structural, general acid/base and redox functions (1,2). In comparison, metal ions in ribozyme catalysis are much less versatile; most ribozymes use only $Mg^{2+}$ (3–5). Since 1994 (6), DNA has also been evolved for catalysis (so called DNAzymes) (7–10). DNA is much less susceptible to non-specific metal-induced cleavage, allowing many transition metal ions to be used as their cofactors (11), including $Pb^{2+}$ (6), $Zn^{2+}$ (12), $Cu^{2+}$ (13,14), $UO_2^{2+}$ (15), $Cd^{2+}$ (16) and $Hg^{2+}$ (17). Owing to their high catalytic efficiency, programmability and stability, DNAzymes have found important application in biosensor development, viral and cancer RNA cleavage, and nanotechnology (18–21).

While divalent metals have been the main focus of study in the past two decades, recently progress has also been made on mono and trivalent metal ions. For example, we isolated three trivalent lanthanide ion dependent DNAzymes (22–24). Lu *et al.* reported a new sodium-specific DNAzyme named NaA43 (25). Quite intriguingly, a close examination of the NaA43 sequence revealed a stretch of 16 nucleotides identical to one of our lanthanide-dependent DNAzymes, named Ce13d (24). Since NaA43 clearly requires only $Na^+$ while Ce13d requires trivalent lanthanides, we are interested in exploring their differences and identifying the role of metal ions in this work.

Our results indicate that Ce13d requires both $Na^+$ and lanthanide ions for catalysis; it is inactive with either metal alone. Most RNA-cleaving ribozymes and DNAzymes use only single metal ions. Multiple metal binding has been reported only in a few systems. For example, the leadzyme can be accelerated by lanthanides, but lanthanides are not required (26,27). The Tm7 DNAzyme requires three lanthanide ions working cooperatively, but they are the same type of ion (22). Multiple $Mg^{2+}$ ions are involved in large ribozymes performing more complex reactions such as RNA splicing (28,29). The group I intron ribozyme contains multiple $Mg^{2+}$ and $K^+$ ions in its crystal, and these metals stabilize the ribozyme folding and enhance the activity (28,30,31). Multiple metal requirements for DNA-cleaving enzymes are known (32,33), but those enzymes are less characterized for metal binding, while RNA cleavage is a mechanistically better defined reaction. To the best of our knowledge, this is the first RNA-cleavage DNAzyme with two different *required* metal ions for catalysis. More importantly, as will be described, each metal has a distinct binding site and chemical role. It shows the possibility of evolving more complex metalloDNAzymes.

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## MATERIALS AND METHODS

### Chemicals

All of the DNA samples were purchased from Eurofins (Huntsville, AL). Their sequences and modifications are listed in Supplementary Table S1. All the metal salts, dimethyl sulfate (DMS), β-mercaptoethanol and piperidine were from Sigma-Aldrich. Tris(hydroxymethyl)-aminomethane (Tris) was from Mandel Scientific Inc. (Guelph, Ontario, Canada).

### Activity assays

For a typical gel-based activity assay, a final concentration of 0.85 μM FAM-labeled substrate strand and 2 μM enzyme strand were added to 50 mM Tris buffer (pH 7.5) containing 100 mM monovalent metal ion. The DNAzyme complex was formed by incubating at 80°C for 2 min and then slowly cooling to 4°C. At room temperature, target metal ions were added to initiate the cleavage reaction. The reaction was quenched by transferring 2.5 μl sample to 14 μl urea (8 M) at designate time points. The cleaved products were separated using 15% denaturing polyacrylamide (PAGE) gel and analyzed by a Bio-Rad ChemDoc MP imaging system.

### $Tb^{3+}$ luminescence

The DNAzyme complex was prepared by mixing the non-cleavable substrate strand (named Sub-dA) and enzyme strand (each 5 μM) in 50 mM Tris buffer (pH 7.5) containing 25 mM of various types of monovalent metal ions. After annealing to form DNAzyme complex, a final concentration of 5 μM $Tb^{3+}$ was added, followed by 5 min incubation. The $Tb^{3+}$ luminescence studies were carried out using a 96-well plate and the sensitized $Tb^{3+}$ emission was monitored with a SpectraMax M3 microplate reader. The spectra were recorded in the range from 520 to 570 nm by exciting at 290 nm.

### DMS footprinting

A 3′-end FAM labeled enzyme strand (Ce13d-FP-FAM) and the non-cleavable substrate strands were used for DMS footprinting. The DNAzyme complex ([Ce13d-FP-FAM] = 4 μM, [Sub-dA] = 20 μM) was formed by annealing in 50 mM Tris buffer (pH 7.5) containing 600 mM various types of monovalent metal ions. To measure $K_d$, the DNAzyme complex was prepared in water without additional buffer or salt, followed by adding different concentrations of $Na^+$ and annealing to form their respective $Na^+$-binding DNAzyme complexes. To methylate the guanosine, 1 μl of freshly prepared 4% DMS was added to 10 μl DNAzyme complex, followed by 15 min incubation in dark. Then, the reaction was quenched by adding a solution (200 μl) containing β-mercaptoethanol (1 M) and NaOAc (600 mM, pH 5.2), followed by ethanol precipitation. The pellets were washed with 150 μl ethanol (70%) and suspended in 50 μl of freshly prepared piperidine (10%). The DNA was cleaved at the methylated sites after heating at 90°C for 30 min, followed by vacuum drying at 60°C for 30 min to remove piperidine. The products were suspended in 8 M urea and the cleaved DNAs were separated by 15% PAGE.

## RESULTS AND DISCUSSION

### Structure comparison

The secondary structures of the Ce13d and NaA43 DNAzymes are shown in Figure 1A and B, respectively. Both enzymes cleave their substrate strand at the rAG junction, where rA represents an adenine ribonucleotide. While at a first glance these two enzymes are quite different, a careful comparison indicates that the nucleotides marked in red are identical. The main difference lies in the few nucleotides on the left side of the enzyme loop in blue. Our biochemical studies indicate that the nucleotides in red are highly important for Ce13d (24,34); truncation and most mutations completely inhibit the Ce13d activity. Therefore, this similarity between Ce13d and NaA43 should not be a coincidence.

These two DNAzymes were selected from different DNA libraries under different conditions with different metal binding properties. For example, Ce13d was selected using $Ce^{4+}$ as the intended metal ion cofactor, although it is much more active with $Ce^{3+}$ and other trivalent lanthanide ions (24). The selection buffer contained 25 mM NaCl and MES (pH 6). In this buffer, Ce13d has a cleavage rate of ∼0.2 $min^{-1}$ with 10 μM $Ce^{3+}$. Without $Ce^{3+}$, no cleavage is detectable. Therefore, Ce13d requires a lanthanide ion for activity. On the other hand, NaA43 was selected in citrate buffer with 400 mM $Na^+$. It is highly specific for $Na^+$ and requires only $Na^+$ for catalysis. With 10 mM $Na^+$ alone, the rate is 0.02 $min^{-1}$; with 400 mM $Na^+$, the rate reaches 0.11 $min^{-1}$. In their reaction buffer (50 mM Bis-Tris, pH 7.0, 90 mM LiCl), lanthanide ions did not produce cleavage (25). Note that LiCl was added to maintain a high ionic strength for increasing the stability of the DNAzyme complex.

To understand this difference, a side-by-side comparison was designed. Both DNAzymes contain a hairpin, but with different sequences. Our previous Ce13d assays indicated that the hairpin is mainly for structure stabilization and its sequence is unimportant for activity (24). For better comparison, we grafted the Ce13d hairpin to NaA43 (Figure 1C, named NaA43T). To confirm activity, NaA43 and NaA43T were tested in 100 mM $Na^+$ (no other metal ions), and both were active (Figure 1F). Since Ce13d and NaA43T differ only in the nucleotides marked in blue, they are the subjects of comparison in this study.

### Ce13d is also a $Na^+$-dependent DNAzyme

Since NaA43 requires only $Na^+$ for activity, we first studied the effect of monovalent metal ions on Ce13d. All the group 1A metal ions plus $NH_4^+$ (100 mM each) were tested, and 10 μM $Ce^{3+}$ was added to each sample. Interestingly, cleavage was observed only in the presence of $Na^+$ (Figure 2A). Our previously studies showed that the metal preference of Ce13d can be shifted by introducing a phosphorothioate (PS) modification at the cleavage site (34,35). Using the PS-modified substrate, cleavage was observed when Ce13d was incubated with $Cd^{2+}$ plus $Na^+$ but not with $K^+$ (Figure 2B). Therefore, Ce13d is also a $Na^+$-dependent DNAzyme. Since

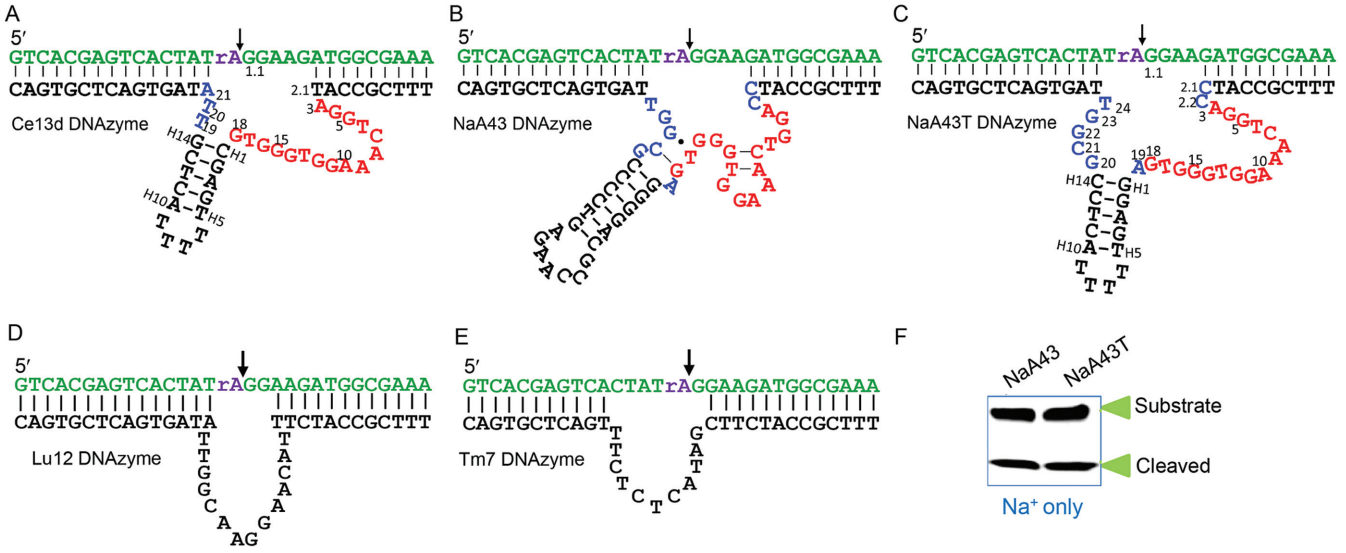


**Figure 1.** The secondary structures of the (**A**) Ce13d; (**B**) NaA43; (**C**) Na43T; (**D**) Lu12; and (**E**) Tm7 DNAzymes. The identical nucleotides in loop region are highlight in red in (**A**–**C**). (B) and (C) differ in the hairpin sequence. The arrowheads indicate the cleavage sites. (**F**) Activity test of NaA43 and NaA43T in presence of 100 mM $Na^+$ alone for 1 h reaction. Changing the hairpin sequence does not change its $Na^+$-dependent activity.

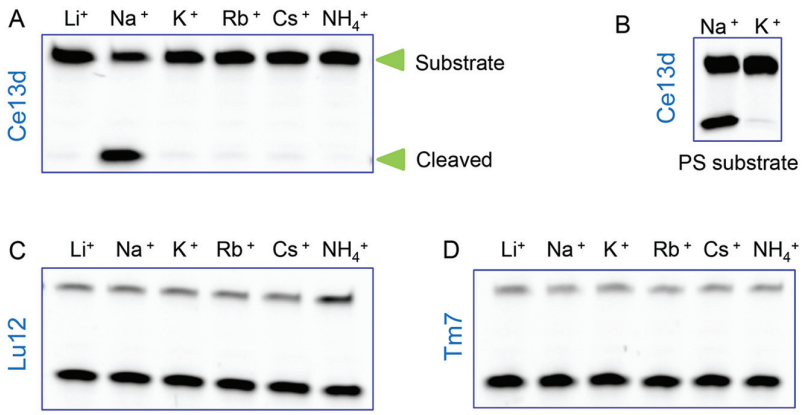


**Figure 2.** (**A**) Gel image showing the activity of Ce13d with 10 μM $Ce^{3+}$ in present of 100 mM of various monovalent metal ions. Only the $Na^+$ containing sample produced cleavage. (**B**) The Ce13d DNAzyme activity using the PS-modified substrate assayed with 50 μM $Cd^{2+}$ in the presence of 100 mM $Na^+$ or $K^+$. Gel images showing the activity of (**C**) the Lu 12 DNAzyme with 1 μM $Ce^{3+}$, and (**D**) the Tm7 DNAzyme with 10 μM $Tm^{3+}$ in present of 100 mM monovalent metal ions.

we already know that $Ce^{3+}$ is required (for the normal phosphodiester substrate), Ce13d requires both $Na^+$ and $Ce^{3+}$ for activity.

In our previous *in vitro* selection efforts, two other lanthanide-dependent DNAzymes were identified: Lu12 (Figure 1D) (23) and Tm7 (Figure 1E) (22). Lu12 also works with all trivalent lanthanides, while Tm7 works only with the seven heavy ones. To test whether the $Na^+$ requirement is unique to Ce13d, we repeated the assay with these two DNAzymes (Figure 2C and D), and cleavage occurred with all the monovalent salts. Therefore, the $Na^+$-specificity is unique to Ce13d.

### $Na^+$ and $Ce^{3+}$ work additively to activate Ce13d

Since both $Na^+$ and $Ce^{3+}$ are required, we hope to find out their relationship in activating Ce13d. We first fixed the $Ce^{3+}$ concentration at 5 μM, and found that the cleavage rate was higher with higher $Na^+$ concentrations (Figure 3A). This $Na^+$ effect was further measured at different $Ce^{3+}$ concentrations, and similar trends were observed at each $Ce^{3+}$ concentration (Figure 3B). For quantitative understanding, the slopes of the lines in Figure 3B are plotted as a function of $[Ce^{3+}]$, and a linear relationship is obtained (Figure 3C). Similarly, we fixed $Na^+$ at a few concentrations and the rate also increased linearly with $Ce^{3+}$ concentration (Figure 3D). The slopes of the curves can also fit to a straight line (Figure 3E). This indicates that the rate law of Ce13d can be written as $rate = k[Na^+][Ce^{3+}]$, where $k$ is the rate constant including the effects from the non-metal factors. If this rate law reflects an elementary reaction, at the rate limiting step, the reaction requires one $Na^+$ and one $Ce^{3+}$ (i.e. first order with respective to each metal).

This rate law also suggests that Ce13d has two independent metal binding sites. If the two metal binding sites were

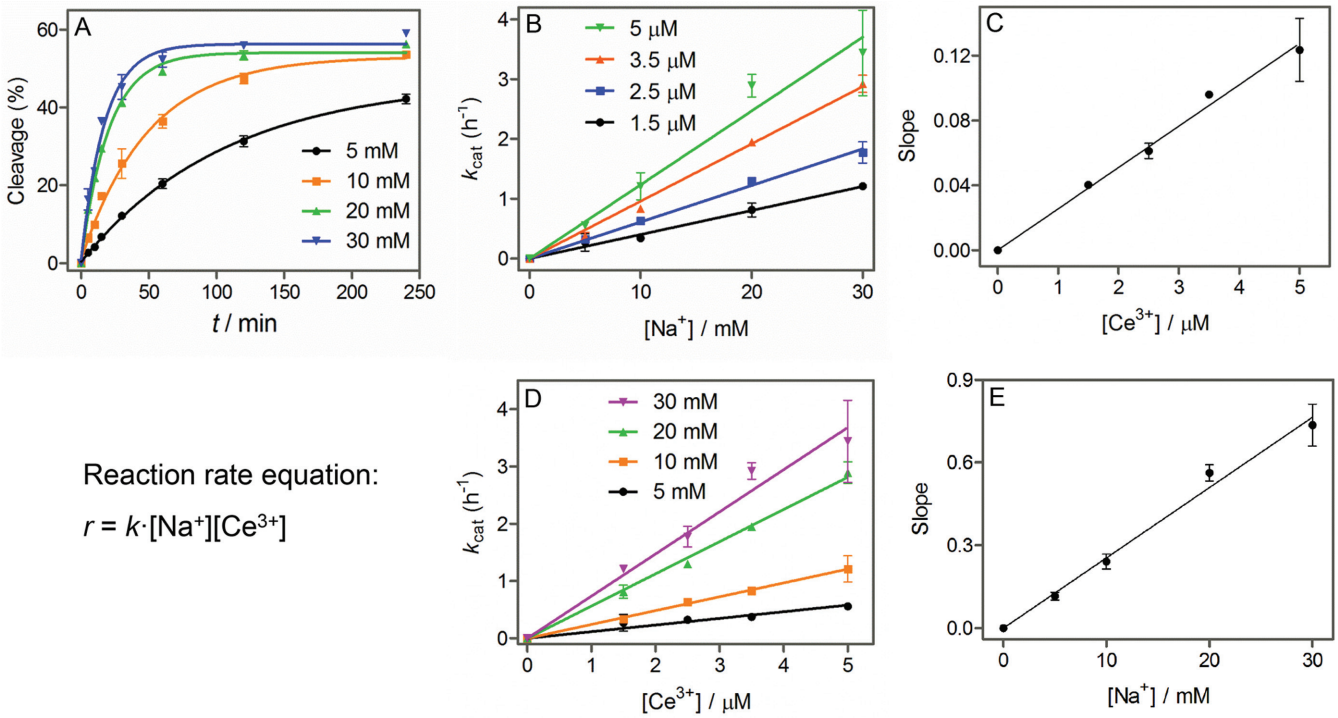


**Figure 3.** (**A**) Kinetics of substrate cleavage by Ce13d with various concentrations of $Na^+$ in the presence of 5 μM $Ce^{3+}$. Buffer: 80 mM $K^+$, 50 mM Tris (pH 7.5). (**B**) Cleavage rate of Ce13d as a function $Na^+$ concentration in the presence of various concentrations of $Ce^{3+}$. (**C**) The slopes of the lines in (B) at each $Ce^{3+}$ concentration. (**D**) Cleavage rate of Ce13d as a function of $Ce^{3+}$ concentration in the presence of various concentrations of $Na^+$. (**E**) The slopes of the lines in (D) at each $Na^+$ concentration.

dependent on each other, it is unlikely to yield this linear relationship. For example, if they work synergistically, the lines in Figure 3C and E should curve up.

### NaA43 can be accelerated by $Ce^{3+}$

After knowing that Ce13d requires both $Na^+$ and $Ce^{3+}$, the next logical question is the effect of $Ce^{3+}$ on NaA43T. Lanthanide ions including $La^{3+}$, $Eu^{3+}$, $Sm^{3+}$, $Yb^{3+}$ were previously tested on NaA43 and they showed no activity. However, those assays were performed in a buffer without $Na^+$. Herein, we measured the NaA43T activity in our $Na^+$ containing buffer with increasing of $Ce^{3+}$ concentration (Figure 4A), and the rate constants are plotted in Figure 4B (black dots). Since NaA43T does not require $Ce^{3+}$, even in the absence of $Ce^{3+}$, a rate of ∼0.5 $h^{-1}$ was observed. This rate remained unchanged with up to 1 μM $Ce^{3+}$. At higher $Ce^{3+}$ concentrations, accelerated cleavage was achieved. The rate enhanced by ∼5-fold with 5 μM $Ce^{3+}$. In contrast, Ce13d is completely inactive without $Ce^{3+}$, but it is activated linearly at low $Ce^{3+}$ concentrations (from 0.1 μM $Ce^{3+}$ or lower). This difference is highlighted in the inset of Figure 4B. We reason that NaA43T may have two distinct mechanisms. The $Na^+$ alone mechanism dominates when the $Ce^{3+}$ concentration is below 1 μM. At higher $Ce^{3+}$ concentrations, the $Ce^{3+}$-involved mechanism dominates.

### Sequence evolution from Ce13d to NaA43T

The above studies indicate that Ce13d and NaA43T are highly related, both in secondary structure and activity. The main difference is that NaA43T works with $Na^+$ alone, while Ce13d requires both $Na^+$ and $Ce^{3+}$. NaA43T can also be accelerated by $Ce^{3+}$. Therefore, for both enzymes, $Na^+$ is a common requirement. We suspect that some nucleotides in NaA43T can fulfill the role of $Ce^{3+}$. These nucleotides are likely to reside in the small loop marked in blue in Figure 1C, since this loop is the main difference between these two enzymes. Yet at high $Ce^{3+}$ concentrations, $Ce^{3+}$ takes over and further accelerates the enzyme via a different mechanism. The involvement of nucleobases in RNA cleavage reactions has been reported in a number of ribozymes, and good examples include the HDV, hairpin and hammerhead ribozymes (3,36,37).

Following this logic, we might evolve one enzyme from the other by rational mutations, and identify the critical nucleotides along the way. As the first step, we designed seven mutants based on Ce13d. Ce13d is shorter in three different regions, and we gradually inserted nucleotides to fill in the gaps to evolve it to NaA43T (Figure 5A). The activity of these mutants was first measured in 100 mM $Na^+$ alone (no $Ce^{3+}$), and only the MS7 mutant was moderately active (Figure 5B). In contrast, all the mutants were active with an additional 10 μM $Ce^{3+}$. Therefore, in the presence of $Ce^{3+}$, mutations are tolerated. With $Na^+$ alone, the requirement on the nucleotide sequence is very stringent.

With $Na^+$ alone, only MS7 and NaA43T can cleave the substrate, while MS6 is completely inactive. MS6, MS7 and NaA43T each differ by only one nucleotide at the 23rd and 24th positions, indicating that $G_{23}$ might be highly important for NaA43T. To further confirm this, a more system-

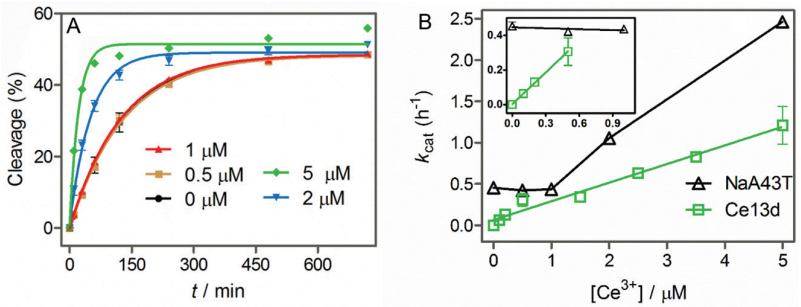


**Figure 4.** (**A**) The kinetics of substrate cleavage by NaA43T in the presence of various concentrations of $Ce^{3+}$ (all with 10 mM $Na^+$). (**B**) The cleavage rate of Ce13d and NaA43T as a function of $Ce^{3+}$ concentration in 10 mM $Na^+$. Inset: cleavage rates at the low $Ce^{3+}$ concentrations.

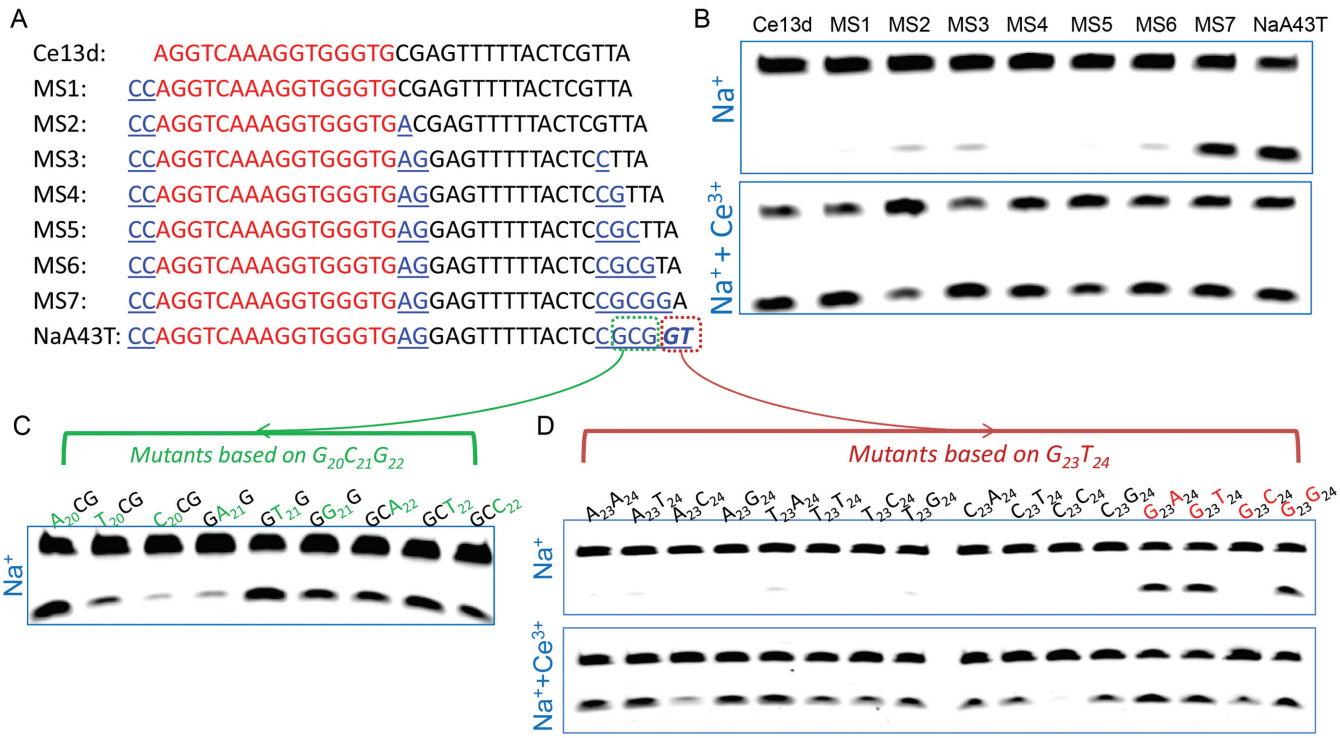


**Figure 5.** (**A**) Sequence evolution from Ce13d to NaA43T. Only the enzyme catalytic core sequences are drawn. See Figure 1 for their full sequences. The color scheme matches with that in Figure 1A and C. (**B**) Gel images showing the activity of each sequence in the present of 100 mM $Na^+$ alone (upper panel) and with additional 10 μM $Ce^{3+}$ (lower panel). (**C**) Gel images showing the effects of mutating $G_{20}C_{21}G_{22}$ in NaA43T with 100 mM $Na^+$ alone. (**D**) The effect of mutating NaA43T at its $G_{23}T_{24}$ positions in 100 mM $Na^+$ (upper panel) and with additional 10 μM $Ce^{3+}$ (lower panel).

atic mutation study was carried out on these two positions (Figure 5D). All the 16 possible combinations were tested and they were all active (except for the $C_{23}C_{24}$ mutant) in the presence of 100 mM $Na^+$ plus 10 μM $Ce^{3+}$. While with 100 mM $Na^+$ alone, cleavage was observed only when the $G_{23}$ nucleotide was retained; any mutation to $G_{23}$ abolished the activity. We also carefully examined some other nucleotides in this loop (Figure 5C). For example, the activity was maintained when $C_{21}$ was mutated to all the other three nucleotides, and the highest activity was seen when the C was mutated to T. This suggests that $C_{21}$ is likely to play a structural role and can tolerate pyrimidine-to-pyrimidine replacement. $G_{20}$ and $G_{22}$ can both tolerate mutations as well. Taken together, $G_{23}$ is the only immutable nucleotide in this part of the DNAzyme. Therefore, $G_{23}$ is the nucleotide critical for catalysis, and it might fulfill the role of $Ce^{3+}$. Guanine is often used in ribozyme catalysis as a general base (3,37). The role of $Ce^{3+}$ is to stabilize the phosphorane negative charge during the pentavalent transition state through inner sphere coordination (i.e. Lewis acid catalyst), and this is supported by our PS-substitution studies (34,35).

While guanine is unlikely to be a Lewis acid catalyst, it is well capable of being a hydrogen bond donor to fulfill the same role, e.g. in the hairpin ribozyme (3,38). In a crystal structure of the hairpin ribozyme transition state with vanadate analog, the $G_8$ guanine serves as a hydrogen bond donor using its exocyclic amine and the N1 amine protons (39). The same guanine also contributes to both precursor and product complex stabilization. To further understand the role of $G_{23}$ in NaA43T, we mutated it to a hypoxanthine

(HX). The enzyme still retained its activity (Supplementary Figure S1), indicating that the exocyclic amine group is unimportant and leaving the N1 proton as the hydrogen bond donor for the catalysis.

We also thought about the opposite evolution starting from NaA43T to see the essential mutations for the $Ce^{3+}$-dependent activity in Ce13d. Since nearly all the mutants are active with $Ce^{3+}$ and combined with our previous mutation studies (24) and PS probing (34), we conclude that as long as the conserved loop structure ($A_3$ to $G_{18}$) is maintained, the DNAzyme is active with $Ce^{3+}$.

### $Tb^{3+}$ luminescence probing $Na^+$ binding

So far, we focused our attention mainly on the $Ce^{3+}$ binding site (namely, the phosphate at the cleavage junction) and its role may be replaced by $G_{23}$. The other part of the story is $Na^+$. Since Ce13d and NaA43T share a common loop sequence and they both require $Na^+$, we suspect that $Na^+$ is associated with the DNAzymes through this loop region. A close examination reveals quite a few guanines in this loop and previous activity assays indicate that most of these guanines cannot be mutated, except that $G_{14}$ can be replaced by an adenine (34). To test for $Na^+$ binding, we first employed sensitized $Tb^{3+}$ luminescence. $Tb^{3+}$ is a widely probe for nucleic acids due to its luminescence properties (40,41). When excited at 290 nm, free $Tb^{3+}$ has almost no emission. While after bind with DNA, the complex exhibits a strong emission peak at 543 nm due to DNA transferring energy to $Tb^{3+}$. More importantly, it has been previously reported that guanine is the most effective nucleotide in enhancing $Tb^{3+}$ luminescence (42,43). Therefore, $Tb^{3+}$ is an ideal probe in this system.

To prevent cleavage, the cleavage site rA was replaced by its DNA analog (dA). We added $Tb^{3+}$ to the non-cleavable DNAzyme complex in the presence of various monovalent metal ions. In both Ce13d (Figure 6A) and NaA43T (Figure 6B), the lowest $Tb^{3+}$ luminescence was observed with $Na^+$, while all the other monovalent cations produced a similar emission intensity. It is likely that $Na^+$ can fold the loop into a more compact structure, thus impeding $Tb^{3+}$ binding. A more careful comparison shows that NaA43T produced higher emission intensity than Ce13d did, and this is attributable to that NaA43T contains three extra guanines (Figure 1C). As a control, we also studied the same reaction using the Lu12 DNAzyme (Figure 1D). Lu12 is also a general lanthanide-dependent DNAzyme, but it lacks the loop sequence common to Ce13d and NaA43T. In addition, it has no selectivity for $Na^+$ in terms of enzyme activity. Since it has the least number of guanines, its overall intensity is the lowest (Figure 6C). In addition, $Na^+$ showed the same intensity as the rest of the cations, indicating that it has no specific binding for $Na^+$. Therefore, the common loop sequence in Ce13d and NaA43T are likely to be the reason for their $Na^+$ requirement.

To quantitatively correlate $Na^+$ binding with catalytic activity, we titrated $Na^+$ to the Ce13d DNAzyme monitored by $Tb^{3+}$ luminescence in the presence of 5 μM $Tb^{3+}$. Normalized luminescence at 543 nm is plotted and we obtained a $K_d$ of 16.3 mM $Na^+$ (Figure 6D, blue trace). Since Ce13d is also active with $Tb^{3+}$, we next measured the cleavage rate at different $Na^+$ concentrations by using also 5 μM $Tb^{3+}$ (Figure 6D, red trace), and a $K_d$ of 24.6 mM $Na^+$ was obtained. These two $K_d$ values are comparable, further confirming that the $Na^+$ binding is directly responsible for catalysis.

### DMS footprinting probing $Na^+$ binding

The $Tb^{3+}$ luminescence data strongly indicate a well-defined $Na^+$ binding site in these two DNAzymes, and the binding site is likely to be associated with the guanine-rich loop. To gain further insights, we probed $Na^+$ binding using the dimethyl sulfate (DMS) footprinting assay, which specifically probes guanine. The N7 methylated guanosine (by DMS) can be cleaved by piperidine. The relative cleavage yield at each guanosine reflects its accessibility. To avoid substrate cleavage, the non-cleavable all-DNA substrate was again used (Figure 7A). A FAM (carboxyfluorescein) was labeled on the enzyme strand and the substrate was non-labeled. We also replaced the guanines in the substrate binding arms of the enzyme by cytosine to simplify gel interpretation (Figure 7A, nucleotides in orange).

As shown in Figure 7D, disappeared or reduced cleavage intensity was observed for $G_4$, $G_5$, $G_{11}$, $G_{12}$, $G_{15}$ and $G_{16}$ in the buffer containing $Na^+$ (600 mM) compared to those in other monovalent ions, suggesting these nucleotides are involved in $Na^+$-specific tertiary folding that prevent methylation by DMS. It is interesting to note that $G_{14}$ was not affected, and this indeed agrees with our mutant assay that $G_{14}$ can be replaced by other nucleotides (34). In the presence of $Na^+$, these conserved guanosines (likely along with other important nucleotides) are folded to assist catalysis. Combined with the $Tb^{3+}$ luminescence data, we conclude that the conserved loop in red (Figure 1A–C) is responsible for $Na^+$ specificity.

Next, we carried out the experiment in the presence of various concentrations of $Na^+$ (Figure 7E). For quantification, we compared each guanine band intensity and normalized based on the one in the absence of any $Na^+$ (Figure 7B). The above-mentioned six guanines showed similar $Na^+$-dependent protection, yield $K_d$ values ranging from 36 mM to 60 mM and the average value is 47 mM $Na^+$. This is just slightly higher than the $K_d$ from the above activity-based measurement (24.6 mM), and this small difference might be attributed to the different experimental conditions (e.g. $Tb^{3+}$ for the activity assay and DMS for footprinting). Overall, the $K_d$ values from all the three measurements agree with each other quite well.

Next, we further probed the nucleotides in the substrate strand. The FAM fluorophore is still labeled on the enzyme as shown in Figure 7A. The difference is that the substrate cleavage junction is switched to a few other base compositions based on the original junction of AGGA (Figure 7A, highlight in blue). As shown in Figure 7F, only the original AGGA junction showed the normal guanine protection pattern, while the mutants all cleaved at these guanine sites. This suggests that the substrate strand is also involved to form the binding pocket for $Na^+$. The molecular mechanism of $Na^+$ binding for catalysis is a topic of further studies, but this part is likely to be related to the activation of the 2′-OH (since the stabilization of the transition-state phos-

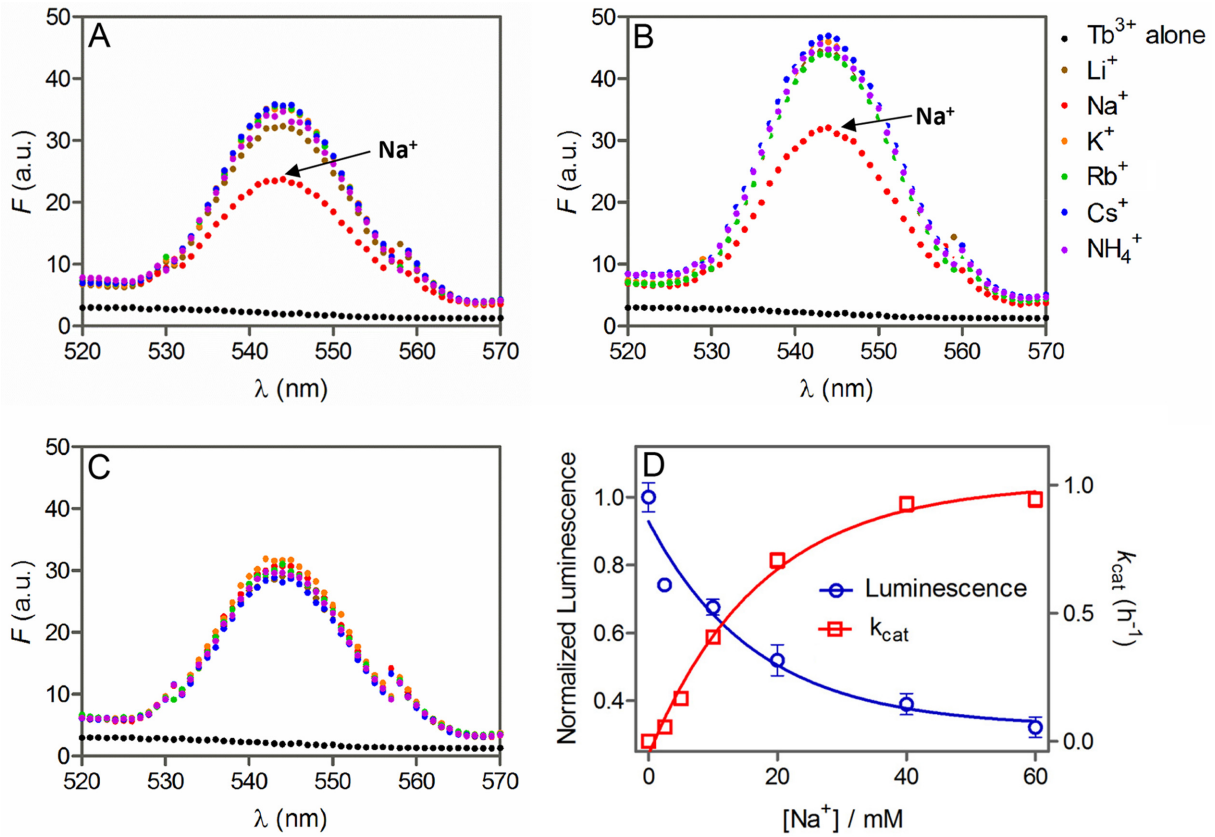


**Figure 6.** $Tb^{3+}$ luminescence spectra of its free ion (5 μM) and after mixing $Tb^3$ with 5 μM of (**A**) Ce13d, (**B**) NaA43T and (**C**) Lu12 DNAzyme complexes in the presence of various monovalent metal ions (25 mM each). The lower emission in (A) and (B) with $Na^+$ indicates that both DNAzymes have a well-defined binding site for $Na^+$. (**D**) Comparison of cleavage rate and $Tb^{3+}$ luminescence intensity as a function of $Na^+$ concentration using Ce13d. For the $Tb^{3+}$ luminescence experiment, the non-cleavable dA-substrate was used; for the cleavage assay, the rA-substrate was used. The DNAzyme was 1 μM, $Tb^{3+}$ was 5 μM and the buffer was 50 mM Tris, pH 7.5, 25 mM $Li^+$ for both experiments.

phorane has already been shown to be related to $Ce^{3+}$ binding or $G_{23}$).

## Further discussions

Taken together, we proposed a model in Figure 7C to unify our findings. Ce13d contains two metal ion binding sites, which is the origin of its dual metal ion dependency. The first site is related to the scissile phosphate. The role of $Ce^{3+}$ is likely to bind the non-bridging oxygen atoms and neutralize the negative charged built up in the transition state. The second site is the well-defined $Na^+$ binding loop formed together with a few nucleotides in the substrate strand. This structure has given significant tolerance for variations on the left side of the enzyme loop (e.g. from $T_{19}$ to $A_{21}$ in Figure 1A and from $G_{20}$ to $T_{24}$ in Figure 1C); mutations and deletions to these have little effect on the activity as long as $Ce^{3+}$ is added. The fact that $Ce^{3+}$ binding to the scissile phosphate is from PS probing (34). The tolerance to mutation suggests that $Ce^{3+}$ binding to the scissile phosphate independent of the other bases. Only under a special condition, where $G_{23}$ is present, the requirement of $Ce^{3+}$ can be relaxed, and this is the origin of the $Na^+$-specific NaA43T DNAzyme. It is likely that the $Na^+$ binding pocket has brought the enzyme to a ready-to-go state, as long as $Ce^{3+}$ is present.

*Probing metal binding.* Most previous DNAzymes showed metal-dependent activity, but they often lack well-defined metal binding sites. So far, no active DNAzyme crystal structures or even NMR structures have been obtained, reflecting flexibility in DNAzyme conformation. This $Na^+$ binding loop appears to be a well-defined aptamer for $Na^+$. Therefore, we can study metal binding in DNAzyme with this scaffold. In particular, this metal binding is directly linked to catalysis.

Despite the fact that the $Na^+$ binding loop has eight conserved guanines (six from the enzyme and two in the substrate), we have ruled out the possibility of G-quadruplex structure based on our previous base mutation studies (34). At this moment, it is unclear how $Na^+$ is bound by the enzyme, but this molecule could be a good candidate for further structural biology studies.

*Functional motifs from independent in vitro selections.* *In vitro* selection of $Na^+$-dependent RNA-cleaving DNAzymes has been attempted previously intentionally or unintentionally (even many failed selections had $Na^+$ in the selection buffer) (25,44,45). However, this particular $Na^+$ binding motif emerged very recently from independent selections (24,25). The chance of obtaining Ce13d is much higher since the sequence requirement is relatively lower; it only requires the conserved 16 nucleotides. While for

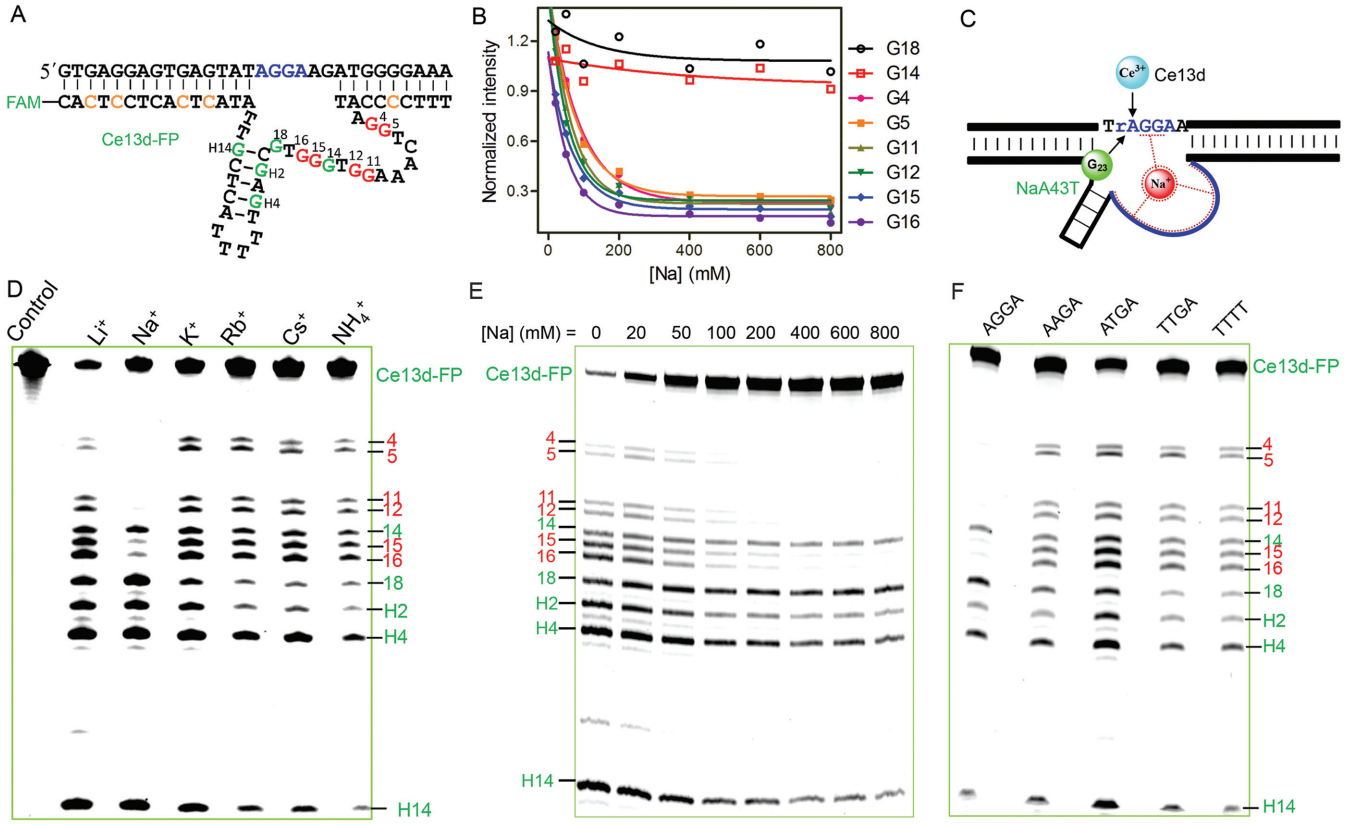


**Figure 7.** (**A**) The secondary structure of the Ce13d DNAzyme used for DMS footprinting. The guanine nucleotides are numbered and highlight in red (protected by $Na^+$)/green (unprotected). The substrate is uncleavable since the rA is now replaced by A. The guanines in the enzyme arms are replaced by cytosines (in orange). The FAM is labeled on the enzyme strand instead of the substrate. (**B**) Quantification of $Na^+$-dependent protection of the guanines from the gel in (E). (**C**) A model summarizing the metal binding sites of Ce13d and NaA43T. $Ce^{3+}$ (for Ce13d) or $G_{23}$ (for NaA43T) work at the scissile phosphate. Meanwhile, the enzyme loop region together with the substrate junction forms a selective binding pocket for $Na^+$. DMS footprinting gel of Ce13d with (**D**) 600 mM various monovalent metal ions, and (**E**) various concentrations $Na^+$. (**F**) DMS footprinting of Ce13d using various substrates with 600 mM $Na^+$. The numbering of each band in (**D**–**F**) corresponds to the guanines in (A).

NaA43, the sequence requirement is even more stringent. If all the nucleotides in blue and red need to be conserved (Figure 1B), there are 24 of them plus a hairpin structure, and the chance of have such a sequence is one in $4^{24} = 2.8 \times 10^{14}$. While there likely to have some tolerable mutations, this size is already approaching the physical size of a typical library used in *in vitro* selection. So, it is understandable that the emergence of this sequence is statistically challenging. For our lanthanide-dependent selection, the chance is much larger (1 in $4^{16}$). Indeed, we have observed this sequence in most of our lanthanide-dependent selections.

Only a few cases are known that functional nucleic acid motifs emerged from independent selections, such as the adenosine aptamer (46,47), the hammerhead ribozyme (48,49), and the 8–17 DNAzyme (7,50). This adds another example of it. Compared to the previous examples, this $Na^+$-binding motif is quite long, containing 16 consecutive nucleotides with very few tolerable mutations.

## CONCLUSIONS

In summary, we have demonstrated that Ce13d is a unique DNAzyme that simultaneously requires two different metal ions. The role of each metal and their positions have been studied through a comparison with the recently reported $Na^+$-specific NaA43 DNAzyme. $Na^+$ and $Ce^{3+}$ work additively for the Ce13d activity, and the overall rate depends on each metal's concentration linearly. In particular, through rational sequence evolution, along with our prior work on the substrate PS modification study, $Ce^{3+}$ is found to involve in binding the scissile phosphate, and the role of $Ce^{3+}$ might be replaced by a specific guanine nucleotide. We also took advantage of sensitized $Tb^{3+}$ luminescence for probing the general role of $Na^+$, which suggests a $Na^+$ aptamer motif. DNA footprinting was used to directly map the important nucleotides responsible for $Na^+$ binding. $G_4$, $G_5$, $G_{11}$, $G_{12}$, $G_{15}$ and $G_{16}$ in the enzyme together with the substrate junction are particularly important. While most previously reported RNA-cleaving DNAzymes use only a single metal ion for catalysis, this is the first example of dual metal requirement. This DNAzyme scaffold can be used as a model system for studying metal binding sites in DNA, DNAzyme catalysis and biosensors. This study suggests that we may have only explored a small part of what DNA can do in terms of molecular recognition, metal binding and catalysis. For example, using longer libraries may allow the evolution of more new DNAzymes. At the proofing stage of

this paper, Torabi and Lu also reported the $Na^+$-dependent activity of Ce13d (51).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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