Lanthanide Cofactors Accelerate DNA-Catalyzed Synthesis of Branched RNA

Fatemeh Javadi-Zarnaghi and Claudia Höbartner*

Research Group Nucleic Acid Chemistry, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

Supporting Information

ABSTRACT: Most deoxyribozymes (DNA catalysts) require metal ions as cofactors for catalytic activity, with Mg2+, Mn2+, and Zn2+ being the most represented activators. Trivalent transition-metal ions have been less frequently considered. Rare earth ions offer attractive properties for studying metal ion binding by biochemical and spectroscopic methods. Here we report the effect of lanthanide cofactors, in particular terbium (Tb3+), for DNA-catalyzed synthesis of 2′,5′-branched RNA. We found up to 104-fold increased ligation rates for the 9F7 deoxyribozyme using 100 μM Tb3+ and 7 mM Mg2+, compared to performing the reaction with 7 mM Mg2+ alone. Combinatorial mutation interference analysis (CoMA) was used to identify nucleotides in the catalytic region of 9F7 that are essential for ligation activity with different metal ion combinations. A minimized version of the DNA enzyme sustained high levels of Tb3+-assisted activity. Sensitized luminescence of Tb3+ bound to DNA in combination with DMS probing and DNase I footprinting results supported the CoMA data. The accelerating effect of Tb3+ was confirmed for related RNA-ligating deoxyribozymes, pointing toward favorable activation of internal 2′-OH nucleophiles. The results of this study offer fundamental insights into nucleotide requirements for DNA-catalyzed RNA ligation and will be beneficial for practical applications that utilize 2′,5′-branched RNA.

INTRODUCTION

Deoxyribozymes (DNA enzymes) are catalytically active synthetic DNAs that are of interest in fundamental research and have found practical use as components of biosensors and functional devices in DNA nanotechnology.1–3 In the field of nucleic acids research, DNA enzymes are established catalysts for preparative applications, including the site-specific cleavage and ligation of RNA substrates.3–5 Several deoxyribozymes have been described that enable the efficient synthesis of 2′,5′-branched RNA,6 a class of nucleic acid structures related to lariat RNA, which is produced during RNA splicing by the group II intron and the spliceosome. DNA enzymes that synthesize 2′,5′-branched RNA activate the 2′-hydroxyl group of the branch-site nucleotide in the acceptor strand for the nucleophilic attack onto the 5′-triphosphate of the donor RNA (Figure 1a).6 Upon release of pyrophosphate as the leaving group, a new 2′,5′-phosphodiester bond between donor and acceptor is formed. For this reaction to occur, the RNA substrates hybridize to the binding arms of the deoxyribozymes, and form active RNA–DNA complexes in different topologies. In the 7S11 DNA enzyme family,7 the branch-site nucleotide is selected by Watson–Crick base pairing directly upstream and downstream of the reaction site and is located in the center of a three-helix junction architecture.8 In the 9F7 DNA enzyme family,9 the branch-site nucleotide is recognized in an as-yet unknown structural context, in which the nucleotides 3′ of the branch site are not part of a standard double helix, but their interaction partners in the deoxyribozyme core have not yet been identified.10

The 9F7 deoxyribozyme (Figure 1a) was identified by in vitro selection from a DNA pool containing 40 random nucleotides1 and has been well characterized with respect to substrate scope.10 However, at the outset of this study it was not known which of the 40 nucleotides in the catalytic region are essential for formation of the active site and are potentially involved in the catalytic mechanism. Based on secondary structure predictions, the presence of a stem-loop structure in the core region of 9F7 is conceivable.6 It can be expected that several of the nucleotides in the large stem-loop and the upstream single-stranded region participate in tertiary structure formation and may play crucial roles in catalysis.

In contrast to nucleolytic ribozymes and RNA-cleaving deoxyribozymes, the catalytic mechanisms of RNA-ligating DNA enzymes have not been studied in detail. Like most nucleic acid catalysts, deoxyribozymes that synthesize 2′,5′-branched RNA use divalent metal ions, such as Mg2+, Mn2+, or Zn2+ as cofactors for efficient catalysis. It is poorly understood how metal ions and/or nucleobases interact with the substrates along the reaction coordinate, which involves in-line attack of the 2′-OH group on the α phosphate and release of pyrophosphate. In analogy to ribozyme mechanisms, the
reaction partners and the transition state are likely candidates for metal coordination. In addition, metal ions can also play essential roles outside of the catalytic core via stabilization of critical tertiary structure elements.11

Several methods are available to study metal ion interactions and to delineate critical nucleobase contacts in nucleic acid enzymes.12 Lanthanide ions have proven useful for probing metal ion binding in ribozymes.13 The high affinity to Mg2+ binding sites and the similar pKₐ of hydrated Ln3+ ions compared to Mg2+ make rare earth ions good candidates for biochemical probing experiments, such as Tb3+ footprinting.14 Moreover, the useful luminescent properties of lanthanides, such as Tb3+ and Eu3+, have been harnessed in studies of metal ion binding to RNA.15−17 Tb3+ has been found to inhibit RNA cleavage by the small nucleolytic ribozymes, including the hammerhead and hairpin RNA enzymes.14,18 The effect of lanthanides on the activity of artificial RNA-cleaving ribozymes and deoxyribozymes has also been investigated. The first known deoxyribozyme, a lead-dependent phosphodiester-cleaving DNA19 showed activity with the small Lu3+ ion as known deoxyribozyme, a lead-dependent phosphodiesterase. Lanthanides on the activity of artificial RNA-cleaving ribozymes and deoxyribozymes has also been investigated. The high affinity to Mg2+ binding sites and the similar pKₐ of hydrated Ln3+ ions compared to Mg2+ make rare earth ions good candidates for biochemical probing experiments, such as Tb3+ footprinting.14

Moreover, the useful luminescent properties of lanthanides, such as Tb3+ and Eu3+, have been harnessed in studies of metal ion binding to RNA.15−17 Tb3+ has been found to inhibit RNA cleavage by the small nucleolytic ribozymes, including the hammerhead and hairpin RNA enzymes.14,18 The effect of lanthanides on the activity of artificial RNA-cleaving ribozymes and deoxyribozymes has also been investigated. The first known deoxyribozyme, a lead-dependent phosphodiester-cleaving DNA19 showed activity with the small Lu3+ ion as the sole metal ion.20 A related ribozyme, the leadzyme,21 was activated when rare earth ions (in particular Nd3+) were present in addition to Pb2+ and Pb2+.22 In contrast, the RNA-cleaving 8-17 deoxyribozyme23 was strongly inhibited by Tb3+.24 Recently, lanthanides have been reported as cofactors for DNA-catalyzed hydrolysis of DNA.25 In this case, Ce3+, Eu3+, or Yb3+ were included with Zn2+ as cofactors during in vitro selection and were found to be required for activity of the isolated deoxyribozymes.

Besides achieving a better understanding of the mechanism of DNA-catalyzed RNA ligation, one of our research goals is to develop DNA enzymes for practical utility. Successful engineering of DNA catalysts requires knowledge of structural and functional principles of deoxyribozymes. In this respect, we explore DNA catalysts that have proven useful for the manipulation of RNA, such as linear ligation, branched ligation, and site-specific modification. We have developed combinatorial probing methods to identify nucleotides and to pinpoint the essential functional groups that are required for catalytic activity.26,27 Here, we report on functional studies of 2,5-branched RNA-forming deoxyribozymes and discuss the interesting finding of lanthanide-mediated acceleration of DNA-catalyzed RNA ligation by 9F7 and related DNA enzymes. We focus on Tb3+ due to its particular properties as a structural probing agent and promising candidate for spectroscopic studies, and we demonstrate that several other lanthanides have similar accelerating effects on 9F7 catalysis. We employed combinatorial mutation interference analysis (CoMA)26 under various conditions to identify functional nucleotides, devised an efficient minimized version of the DNA, and examined metal ion binding and DNA folding by luminescence and footprinting experiments.

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**RESULTS AND DISCUSSION**

**Metal Ion Cofactors for 9F7 DNA-Catalyzed RNA Ligation.** The 9F7 deoxyribozyme (Figure 1a) was identified together with related active DNA sequences by in vitro selection, using Mg2+ as cofactor for RNA ligation.9 The DNA-catalyzed synthesis of 2,5-branched RNA with 9F7 occurred with a rather slow ligation rate of 0.003 min−1 with 80 mM Mg2+ at pH 7.5 and 37 °C. We were intrigued by our unexpected observation that the addition of Tb3+ at 1 μM concentrations impressively accelerated the DNA-catalyzed ligation reaction. In the presence of 100 μM Tb3+ and 80 mM Mg2+, the kobs reached 0.35 min−1, which was ca. 120-fold faster than in the absence of Tb3+. In a chase experiment, Tb3+ was added to the reaction mixture to a final concentration of 100 μM after 60 min of ligation with only Mg2+ (Figure 1b), demonstrating that the acceleration of product formation was caused by Tb3+.

Earlier reports demonstrated considerably faster ligation rates for 9F7 using Mn2+ instead of Mg2+ as divalent metal ion cofactor.9,10 At 20 mM Mn2+, kobs reached 0.3 min−1, but a 10-fold reduced [Mn2+] of 2 mM supported only similar ligation rates as 80 mM Mg2+ (Figure S1). Addition of Tb3+ to Mn2+-mediated reactions resulted in 500-fold faster ligation rates than in the absence of Tb3+ (see below).

Variation of metal ion conditions for ribozyme-catalyzed reactions can potentially activate or repress alternative reaction sites.28 Therefore, the ligation products generated by 9F7 under various metal ion conditions were investigated for homogeneity. Alkaline hydrolysis of isolated branched RNAs confirmed that the presence of Tb3+ did not change the site of reaction (data not shown).

The finding of Tb3+-mediated acceleration of the 9F7-catalyzed RNA ligation reaction is significant, particularly in the context of earlier studies of lanthanide effects on ribozyme and deoxyribozyme activities that reported inhibition of catalysis.14,24 Only under special conditions, small acceleration effects were found for nucleolytic ribozymes. For example, when tested in the presence of high concentrations of Na+ or spermidine (>1 M), low amounts of Tb3+ (<25 μM) accelerated the hairpin ribozyme-catalyzed RNA cleavage up to 2.5-fold, while higher Tb3+ (>30 μM) inhibited the ribozyme.14 With an optimal combination of Nd3+ and Pb2+, the leadzyme catalyzed RNA cleavage about 20-fold faster than...
with Pb\(^{2+}\) alone.\(^\text{22}\) The effects for the 9F7 deoxyribozyme are much larger, but rate and yield of the ligation are also strongly dependent on the Tb\(^{3+}\) concentration. While 100 \(\mu\)M Tb\(^{3+}\) activated the DNA enzyme 120-fold in combination with 80 mM Mg\(^{2+}\), 50 \(\mu\)M Tb\(^{3+}\) led to almost no acceleration, and a higher concentration of 150 \(\mu\)M Tb\(^{3+}\) caused fast reaction of only a fraction of the available RNA substrate, resulting in a reduced amplitude with retained high rate constant (Figure 1c). Comparable effects were observed for Eu\(^{3+}\) and Gd\(^{3+}\) (Figures 1d and S2), which have similar ionic radii (1.04–1.07 Å) and \(pK_a\) values (7.8–8.0) of bound water molecules compared to Tb\(^{3+}\).\(^\text{29}\) Early lanthanide ions (La\(^{3+}\) and Ce\(^{3+}\)) also accelerated the DNA-catalyzed ligation efficiently, but the effect was less pronounced with Yb\(^{3+}\), which is the last f-block element in the lanthanide row (Figure S2). Other trivalent nonlanthanide metal ions neither accelerated nor inhibited 9F7-catalyzed RNA ligation (Al\(^{3+}\) or Fe\(^{3+}\) were tested up to 100 \(\mu\)M in presence of 80 mM Mg\(^{2+}\)).

A more detailed analysis of the concentration-dependent lanthanide effect is depicted in Figure 2. Plotting the yield of ligated RNA product as a function of [Tb\(^{3+}\)], in the presence of 100 \(\mu\)M Tb\(^{3+}\) (when no divalent metal ions were present). Increasing the concentration of monovalent ions from 150 mM Na\(^+\) and 2 mM K\(^+\) up to 3 M Na\(^+\) or 2 M K\(^+\) (again in the absence of divalent metal ions) eliminated Tb\(^{3+}\)-mediated hydrolysis but did not support 9F7 catalysis.

The presence of optimal concentrations of Tb\(^{3+}\) increased the affinity for the divalent metal ions (Figure 3). The \(K_{d, app}\) for Mg\(^{2+}\) decreased from >200 to 57 mM, and \(K_{d, app}\) for Mn\(^{2+}\) decreased ca. 3-fold from 7.6 to 2.6 mM. The impressive effect of Tb\(^{3+}\) is especially visible at low divalent metal ion concentrations. At 2 mM Mn\(^{2+}\), addition of 10 \(\mu\)M Tb\(^{3+}\) caused almost 500-fold acceleration. The lowest [Mg\(^{2+}\)], which led to detectable ligation product (1%) after 24 h, was 7 mM Mg\(^{2+}\). Addition of 100 \(\mu\)M Tb\(^{3+}\) to 7 mM Mg\(^{2+}\) caused 1000-fold faster ligation to yield >95% product in 1 h, thereby reducing \(t_{1/2}\) from 1600 h to 10 min (Table 1).

All kinetic assays of the ligation reactions were performed in 50 mM HEPES buffer pH 7.5, with 150 mM NaCl and 2 mM KCl. Addition of up to 100 \(\mu\)M trivalent ions did not significantly influence the ionic strength of the solution in comparison to the same divalent metal ion concentration in the absence of Tb\(^{3+}\). Therefore the effect of Tb\(^{3+}\)-mediated acceleration is not easily explained by changes in ionic strength that could lead to stabilization of the preligation complex. In contrast, more specific interactions must be responsible for the

![Figure 2.](image)

**Figure 2.** (a) Ligation yield at different [Tb\(^{3+}\)] with 80 mM Mg\(^{2+}\). Gel images show yield after 5 min (top) and 5 h (bottom) reaction at 37 °C. (b) Plot of data in (a) shows optimal Tb\(^{3+}\) concentration at 100 \(\mu\)M. (c) Gel image of terbium titration; ligation yields at 1 min (top) and 1 h (bottom) reaction time in presence of 20 mM Mn\(^{2+}\). (d) Plot of data in (c) shows optimal Tb\(^{3+}\) concentration at 10–20 \(\mu\)M. For curve fit description see Supporting Information (analogous plots for Eu\(^{3+}\) and Gd\(^{3+}\) are in Figure S3).
observed effects. A likely candidate for metal ion interaction in the ligation reaction is the triphosphate of the donor RNA. Hydroxo complexes of transition-metal ions are known to accelerate the hydrolysis of activated phosphate esters, \(^{30}\) as, for example, the triphosphate of ATP. \(^{31}\) It is conceivable that the synergistic effect of \(M^{2+}\) and \(Tb^{3+}\) is related to increased electrophilicity of the \(\alpha\) phosphate in RNA 2 upon metal ion coordination. The reduced ligation amplitude could potentially be rationalized by accelerated competing hydrolysis of the triphosphate at higher \(Tb^{3+}\) concentrations. This hypothesis was tested by HPLC analysis of the fate of donor RNA upon incubation under different metal ion combinations but was not substantiated by our experimental results. RNA 2 was hybridized to a complementary DNA oligonucleotide resembling the deoxyribozyme binding arm and was incubated under various conditions. More than 80% of triphosphate was still intact after 1 h in presence of 100 \(\mu\)M \(Tb^{3+}\) and 80 mM \(Mg^{2+}\); the triphosphate content decreased only slightly with 200 \(\mu\)M \(Tb^{3+}\) (Figure S4). In contrast, the ligation yield was strongly reduced to 30% with 200 \(\mu\)M \(Tb^{3+}\) (compared to 89% yield with 100 \(\mu\)M \(Tb^{3+}\)), although the donor substrate 2 was supplied in 6-fold excess over acceptor RNA 1. These results indicated that the competing triphosphate hydrolysis was not responsible for the reduced ligation amplitude, but higher \(Tb^{3+}\) likely caused misfolding of the deoxyribozyme into an inactive conformation. When the donor, acceptor, and deoxyribozyme were supplied in 6-fold excess over acceptor RNA 1, the ligation reaction was slower (Figure S5), likely due to increased unspecific binding of \(Tb^{3+}\) to the nucleic acids, causing reduced availability of “active” \(Tb^{3+}\) ions.

To further explore the specific synergistic effects of divalent \(Mg^{2+}/Mn^{2+}\) ions and trivalent rare earth ions on activation of the RNA substrates for 9F7-catalyzed ligation, we investigated phosphorothioate-modified donor substrates in which one oxygen atom at either the \(\alpha\) or \(\gamma\) phosphatase was replaced by sulfur. Phosphorothioate (PS) interference and metal ion rescue experiments have the potential to reveal crucial information about metal ion interactions in the active site. \(^{32}\) Installation of \(\gamma\)-PS can be easily achieved by in vitro transcription using \(\gamma\)-S-GTP. In contrast, transcription with \(\alpha\)-S-GTP results in incorporation of PS at the \(\alpha\)-terminal \(\alpha\)-phosphate and at all other guanosines in the strand. To avoid cumulative effects of more than one PS in the substrate, we designed an alternative donor substrate (2a), that contained only one guanosine at the \(\gamma\)-end and otherwise consisted of only A, C, and U (sequences in Table S1).

Replacement of the \(\gamma\)-phosphate by PS in RNA 2 caused a considerably reduced ligation rate, which was about 100-fold slower than for wt RNA 2 (in presence of 80 mM \(Mg^{2+}\); 15-fold slower in presence of 20 mM \(Mn^{2+}\), Figure S6), but addition of \(Tb^{3+}\) did not change the outcome of the reaction. These results indicated that the larger and softer sulfur atom was less accommodated than oxygen at the \(\gamma\) position and lent support to the hypothesis of preferred interaction of the terminal phosphate with hard lanthanides. Softer and more thiophilic metal ions, such as \(Cd^{2+}\) and \(Mn^{2+}\), could potentially rescue PS effects. Addition of 1 mM \(Cd^{2+}\) did not significantly improve the ligation rate (Figure S6c), while addition of 2 mM \(Mn^{2+}\) accelerated ligations of both PO and PS RNAs by a factor of ca. 10–20, which indicated no specific rescue effect for the \(\gamma\) PS (Figure S6a). In the presence of higher \(Mn^{2+}\) concentration (20 mM \(Mn^{2+}\) with 80 mM \(Mg^{2+}\)), the ligation of the PS substrate
was accelerated 250-fold, while the reaction of the PO substrate was only ca. 70-fold faster than with Mg$^{2+}$ alone.

The alternative substrate 2a was ligated less efficiently than 2 under the standard reaction conditions ($k_{\text{obs}}$, ca. 10-fold slower). Replacement of the α phosphate with PS resulted in comparable ligation rates using Mn$^{2+}$ as cofactor, while PS at the γ position again severely inhibited the reaction (Figure S7). Upon addition of 1 mM Cd$^{2+}$, the $k_{\text{obs}}$ for the α-PS analog of 2a increased ca. 3-fold, while the ligation rate of the unmodified substrate remained unaffected (Figure S7a). Presence of 10 μM Tb$^{3+}$ had a comparable 3-fold effect for the α-PS substrate, while ligation of the unmodified RNA 2a was ca. 10-fold faster than in the absence of Tb$^{3+}$ (Figure S7b). Collectively, these results point toward important interactions at the α- and γ-phosphates of the donor RNA, although the PS and rescue effects were less pronounced than for other metalloribozymes.\textsuperscript{33,34}

**Combinatorial Mutation Interference Analysis.** Besides the nucleophile and electrophile involved in the reaction, nucleotides of the catalytic core are also likely to participate in metal ion interactions. Therefore, we investigated if all 40 nucleotides of the original 9F7 core region were necessary for catalysis and if any candidate nucleotides were responsive to different metal ions.

We capitalized on our recently developed approach for combinatorial mutation interference analysis (CoMA)\textsuperscript{26} to further examine the 9F7 deoxyribozyme. In this method, four combinatorial DNA libraries are prepared by solid-phase synthesis, using mixtures of phosphoramidites that yield on average one mutation per DNA molecule, statistically distributed over the length of the DNA of interest. Incorporation of mutations as ribonucleotides enables analysis of interference effects by alkaline hydrolysis followed by denaturing PAGE analysis. A separate library is synthesized with each of the four standard ribonucleotides. A key step in the analysis is the separation of active from inactive library members. The DNA-catalyzed ligation reaction is performed in a bimolecular format, with the donor substrate covalently attached to the DNA library (Figure 4a). Upon incubation with the acceptor RNA under desired reaction conditions, only those library variants yield ligation products, in which the mutations do not interfere with catalysis. On the sequencing gels for analysis, the detrimental mutations are identified as missing bands (Figure 4b). Interference effects are quantified as the ratio of band intensities in the active fraction and the unseparated reference library and clearly distinguish essential from mutable nucleotides.

Here, CoMA was performed for 9F7 under five different ligation conditions. To compare nucleotide requirements of 9F7 at low and high bivalent metal ion concentrations, we chose 2 mM Mn$^{2+}$, 20 mM Mn$^{2+}$, and 80 mM Mg$^{2+}$. To examine the influence of Tb$^{3+}$, we also included the analysis in the presence of 10 μM Tb$^{3+}$ with 2 mM Mn$^{2+}$ as well as 100 μM Tb$^{3+}$ with 80 mM Mg$^{2+}$ (note that 10 and 100 μM Tb$^{3+}$ were the optimum concentrations in presence of Mn$^{2+}$ and Mg$^{2+}$, respectively). The different ligation kinetics observed under these different metal ion conditions were taken into account for the ligation reaction before the separation step. Too short incubation times would exclude the slow mutants present in the library and therefore increase the interference values for positions where mutations reduce the ribozyme activity but are not detrimental. On the other hand, too long incubation would enrich very slow mutants in the ligated fraction, and therefore the mutation effects could be hidden in the bulk of low interference values. We chose the reaction times according to the results of the trimolecular 9F7 ligation kinetics in the respective conditions, i.e., reaction times that yield ~90% of the maximum yield (that is, 10 h with 2 mM Mn$^{2+}$, 8 h with 80 mM Mg$^{2+}$, 2 h with 100 μM Tb$^{3+}$ + 80 mM Mg$^{2+}$, 1 h with 10 μM Tb$^{3+}$ + 2 mM Mn$^{2+}$, and 20 min with 20 mM Mn$^{2+}$). For all five reactions, these incubation times reproducibly resulted in formation of ca. 30% ligation product (i.e., ca. one-third of the library was in the active fraction). Figure 4b shows a representative hydrolysis gel for the active fractions of all four libraries (after ligation with 20 mM Mn$^{2+}$) in comparison with an unseparated reference lane. The full gel images for all five conditions are shown in Figure S8. The interference values were calculated and plotted in the contour plot in Figure 4c; green indicates low interference (i.e., the mutation is tolerated), and red indicates strong interference, (i.e., the mutation inhibits catalytic activity).

The most striking result from this analysis is the large green region between nucleotides 18 and 35 where all mutations are tolerated under all tested conditions, suggesting that this stretch of nucleotides does not play any essential role in catalysis. All 18 nucleotides could indeed be removed, resulting in only 2-fold slower ligation kinetics. When 1, 2, or 3 nucleotide(s) of the 18 dispensable nucleotides were retained (i.e., only 17, 16, or 15 nucleotides were deleted), the catalytic activity improved and reached 80% of the wt $k_{\text{obs}}$ in 20 mM Mn$^{2+}$ for 9F7 min (Figure S9). Importantly, the acceleration of the ligation reaction with Tb$^{3+}$ was as effective for the minimized variant as for the wt (Figure 5 and Figure S10).

![Figure 5. Minimized 9F7 deoxyribozyme and comparison of ligation kinetics of wt and 9F7 min under optimal conditions. The green arrow indicates the deletion of 15 nt with respect to wt. Results for other metal ion combinations in Figure S10.](image-url)
potentially associated with changes in nucleobase orientation, could negatively impact correct folding of the DNA.

The second group of results concerns the impact of Tb³⁺ on reduced interference values for several mutations. For example, A5G or C13T appeared in the active fractions when Tb³⁺ was present, but the corresponding hydrolysis bands were strongly reduced in the absence of Tb³⁺. For C38T, this effect was only visible for reactions with Mn²⁺ but not with Mg²⁺. Several other nucleotides tolerated mutations only with one of the two divalent metal ions (independent of absence or presence of Tb³⁺), such as AST and C8G, which showed high interference with Mg²⁺ but lower values with Mn²⁺. Those nucleotides which cannot be mutated to any other nucleotide under any of the tested conditions are the most critical ones for retaining ligation activity of 9F7, and are found at A1, A2, G4, from G7 to G12, and at G39. The CoMA data also revealed nucleotides that tolerated only certain types of mutations, such as only transitions or only transversions. Examples include G15 and G16, at which transversions were tolerated only in the presence of transitions or only transversions. Examples include G15 and G16, at which transversions were tolerated only in the presence of Tb³⁺, but transitions inhibited activity under all conditions. These mutation results are graphically summarized in Figure S12.

Nucleotides G41 and T42 were included in the mutation analysis as “positive control positions”, for which the nucleotide requirements were known from previous studies. These two nucleotides interact with the acceptor substrate upstream of the branch-site nucleotide. Disruption of the DNA–RNA Watson–Crick base pairs inhibited the reaction. The CoMA results confirmed these interactions, since only the parent (ribo)-nucleotides were tolerated, but all mutations showed large interference effects under all assayed conditions. This result supports the interpretation of the interference effects for other nucleotides discussed above.

**Tb³⁺ Interactions with the Minimal 9F7 Deoxyribozyme.** Having identified a minimal 9F7 deoxyribozyme and potential nucleotide candidates for metal ion interactions by CoMA, we explored sensitized Tb³⁺ luminescence to probe Tb³⁺ interactions with the DNA enzyme and the RNA substrates. To study binding of Tb³⁺ to the deoxyribozyme–substrate complex, we chose a preligation complex formed with the reactive triphosphate in the enzyme complex that lacks the 2’-deoxyadenosine at the branch site. Addition of Tb³⁺ to annealed complexes under various divalent metal ion conditions resulted in an increase in Tb³⁺ luminescence upon excitation at 284 nm. The four distinct emission peaks at 488, 543, 584, and 620 nm (Figure S13) are typical for Tb³⁺ luminescence. Quantitative information on Tb³⁺ binding was obtained by titration experiments up to 200 μM Tb³⁺, in the presence of 1–100 mM Mg²⁺ or 1–10 mM Mn²⁺, and measuring the intensity of the corresponding luminescence signal at 543 nm (Figure 7a,b). It is noteworthy that different binding kinetics of Tb³⁺ was observed depending on type and concentration of the divalent metal ions. In the presence of Mn²⁺, a fast increase in emission was followed by a slow decrease of characteristic signature within 10 min. In the presence of Mg²⁺, three different binding modes were observed (Figure S13). At low concentrations of Tb³⁺ (<20 μM), an initial increase was followed by an exponential decay to almost the initial value within 1–3 min. At slightly elevated Tb³⁺ concentrations (between 20 and 60 μM), the signal intensity increased rapidly and stayed at the elevated level for at least 10 min. Higher Tb³⁺ concentrations (>60 μM), resulted in a slower response of the emission intensity. For consistency, the plots in Figure 7 display the emission intensity after an equilibration time of 10 min for each data point. As anticipated for competitive M²⁺ and Tb³⁺ binding, the maximal emission intensities and the apparent affinity for Tb³⁺ (expressed as increasing [Tb³⁺]₁/₂) decreased with rising divalent ion concentrations. The fit values for binding curves in Figure 7 are summarized in Table S3.

Comparable Tb³⁺ titration experiments were performed with an alternative inactive substrate–enzyme complex that contained a 5’-monophosphate at the donor RNA (2i) instead of the reactive triphosphate in 2 (Figure S14). This complex resulted in qualitatively comparable luminescence results as depicted in Figure 7 for the complex with 2i that lacks the 2’-OH nucleophile, which is a possible site for direct or indirect interaction with metal ions.

Based on the CoMA results, G4 is one potential candidate nucleotide for coordination of metal ions; the sensitivity to the 2’-OH ribose modification is relieved with Tb³⁺; no mutations are allowed with Mg²⁺, and a purine nucleotide must be retained for activity with Mn²⁺ (Table S4). A comparison of the luminescence response upon Tb³⁺ titration for 9F7 min (G4 wt) and the inactive G4T mutant in presence of 20 mM Mg²⁺ indicated a slight left-shift of the mutant titration curve upon addition of Tb³⁺ up to 200 μM (Figure 7c). Significant differences were observed at low [Tb³⁺] (Figure 7d). While a luminescence increase for the wt deoxyribozyme was detected only above 25 μM Tb³⁺, all three G4 mutants (in which G4 was...
changed to A, C, or T) showed a left-shifted binding curve, indicating facilitated binding of Tb³⁺ when the active site is not intact. These results underline the finding that coordination of a divalent metal ion in the active site is essential for activity and further support the interpretation that Tb³⁺ first binds to peripheral sites before displacing catalytic metal ions and/or causing inactive conformations. However, since the luminescence data were all recorded with inactivated substrates and reported on the sum of specific and unspecific binding of Tb³⁺ to the nucleic acid complex, the luminescence binding curves cannot be directly correlated to the kinetic results.

Although the luminescence data are not sufficient to fully describe all details of Tb³⁺ binding and the mechanism of activation, the results support our kinetic data which suggested that the 9F7 deoxyribozyme provides specific binding sites for Tb³⁺ ions. We hypothesize that acceleration of the ligation activity results from Tb³⁺ binding to structurally important sites which causes an increased affinity for divalent metal ions at catalytically critical M²⁺ binding sites. Studies are underway to gain additional insights into Tb³⁺ coordination from luminescence resonance energy transfer (LRET) using a combination of Tb³⁺ with site-specifically installed fluorescent dyes (e.g., Cy3). In combination with sensitized luminescence, nucleotide analogue interference mapping of DNA (DNAIM) is expected to identify nucleotides that tolerate substitution by 4-thiouridine, known as a good sensitizer for Ln³⁺ luminescence. Luminescence lifetime analyses in water and D₂O as well as Tb³⁺-induced paramagnetic shifting of ¹H resonances should reveal mechanistic details of Tb³⁺-assisted acceleration of the 9F7-catalyzed RNA ligation.

Metal-ion mediated folding of the minimized 9F7 deoxyribozyme was further studied by chemical dimethyl sulfate (DMS) probing and enzymatic DNase I footprinting experiments (Figure 8). The accessibility of nucleotides in the catalytic region of 9F7 min was assayed in buffer without divalent metal ions and in the presence of 20 mM Mn²⁺. Three different sample compositions were used to compare different folding states of the DNA. First, the DNA enzyme was analyzed as the only nucleic acid component in the sample (a, 9F7 min alone), second in complex with inactivated substrates (b, standard acceptor RNA (1) and 5ʿp donor (2i)), and third in complex with the 2ʿ,5ʿ-branched RNA product (c). Methylation at N7 of guanosines by DMS, followed by piperidine-mediated cleavage of the DNA backbone at modified positions, reveals the accessibility of the Hoogsteen face of guanosines. For the 9F7 min deoxyribozyme, reduced cleavage intensity was observed for G4, G6, G7, G11, G12, and G15 in the folded sample b (open complex; analysis of cleavage intensities is shown in Figure S15). G16 and G17 remained accessible in folded sample b (open complex; analysis of cleavage intensities is shown in Figure S15). G16 and G17 remained accessible in folded DNA−enzyme product complex, while reduced cleavage was observed in the presence of 20 mM Mn²⁺. These results underline the involvement of the critically important guanosine nucleotides in formation of the folded DNA structure.

**Figure 8. DMS and DNase I probing of 9F7 min.** Sample description: (a) 9F7 min alone; (b) 9F7 min deoxyribozyme in complex with (inactive) substrates 1 + 2i (open complex); (c) 9F7 min deoxyribozyme in complex with 2ʿ,5ʿ-branched RNA product (closed complex). M is the guanosine-specific marker lane.

A18 and C34). Several other positions are also worth highlighting. For example, digestion at G7 was blocked upon hybridization of the binding arms to the RNA substrates/product, even in the absence of Mn²⁺, while cleavage at position T9 was unaffected. The DNase-mediated strand break at G17 was enhanced in the product−DNA complex, while reduced intensity was observed at C21. Strikingly, cleavage at all DNase-cleavable positions appearing in the absence of Mn²⁺ was strongly diminished in the presence of Mn²⁺, especially for the folded complex c, although Mn²⁺ is known to increase the activity of DNaseI. This observation relates to the metal ion-induced formation of a tight catalytic core that is inaccessible to DNase cleavage. Overall, the footprinting results confirmed the involvement of the critically important guanosine nucleotides in formation of the folded DNA structure.

**Tb³⁺-Mediated Acceleration of RNA Ligation by Other Deoxyribozymes.** To investigate the broader applicability of lanthanide-assisted DNA-catalyzed RNA ligation, we studied the effect of Tb³⁺ on the ligation activity of additional deoxyribozymes related to 9F7. The 9F13 DNA enzyme⁹ synthesizes 2ʿ,5ʿ-branched RNA also with 5ʿ-triphosphorylated RNA as donor substrate but activates the 2ʿ-OH group of a branch-site uridine instead of adenosine (Figure S16). Tb³⁺ in the presence of 20 mM Mn²⁺ was found to slightly activate the 9F13 DNA-catalyzed reaction at an optimal Tb³⁺ concentration of 10 μM (Figure S17). Higher concentrations up to 50 μM Tb³⁺ caused reduced ligation yields. CoMA of 9F13 revealed numerous nucleotides that were dispensable for activity (Figure S18); the minimized 9F13 variant with 15 nucleotides less in the core region (23 versus 38 nt) ligated the RNA substrates 4-fold faster than the wt sequence (Figure S19). The effect of Tb³⁺ on the minimized 9F13 needs to be further explored to identify the structural features of the DNA core that are
**CONCLUSIONS**

In this study we have shown that lanthanides act as highly effective cofactors for DNA-catalyzed RNA ligation, thus significantly reducing the concentrations of divalent metal ions required for reaching high rate and yield. For example, with 100 μM Tb³⁺ and 7 mM Mg²⁺, the 9F7-catalyzed synthesis of 2′,S′-branched RNA was accelerated up to 10 000-fold. The intrinsically faster ligation rates with Mn²⁺ were further increased by the addition of Tb³⁺. These significant findings open new avenues for creative use of lanthanides in nucleic acid chemistry beyond established functions as structural and mechanistic probing agents and as cofactors for DNA-catalyzed hydrolysis of DNA.

Combinatorial mutation interference analysis under various metal ion conditions guided partial minimization of the DNA catalysts and revealed novel insights into the mutation tolerance of individual nucleotides. We found that the 2′-OH group was better accommodated in the catalytic core of the deoxyribozyme when Tb³⁺ was provided as additional cofactor. The nucleotides at which Tb³⁺ could relieve the 2′-OH effect highlight the importance of the ribose conformation for structure formation and suggest that conformationally sensitive nucleotides may directly interact with metal ions. The mutation interference results also point toward nucleotides involved in tertiary interactions that could be stabilized by metal ions.

Furthermore, we demonstrated that the observed effects of Tb³⁺ acceleration were not restricted to the specific case of 9F7. The related 9F13 deoxyribozyme that uses uridine as branch-site nucleotide (rather than adenosine) was activated by μM concentration of Tb³⁺. CoMFA also guided minimization of 9F13, resulting in a faster variant of the DNA enzyme. In addition, Tb³⁺ accelerated the synthesis of branched RNA in the three-helix junction format of 7S11. The comparison of Tb³⁺-mediated activation of all studied branched RNA-forming deoxyribozymes revealed an optimal concentration of ca. 100 μM Tb³⁺ in combination with Mg²⁺ but of only 10 μM Tb³⁺ in combination with Mn²⁺.

Strong concentration dependence and narrow optima for metal ion concentrations have been observed for other DNA enzymes. For example, a DNA-hydrolyzing deoxyribozyme was reported to be most effective with a combination of Zn²⁺ and Mn²⁺ in a precise concentration range, but only two nucleotide mutations enabled the DNA to be active with Zn²⁺ as sole metal ion cofactor. Different types of metal ions can enable catalysis of the same reaction to occur via different mechanisms, as has been proposed for DNA-catalyzed RNA cleavage with Zn²⁺, Mg²⁺, or Pb²⁺, respectively, based on studies of deoxyribozyme folding and catalysis by bulk and single-molecule FRET experiments.

For the deoxyribozymes in the center of this study, the structural organization around the active site and the three-dimensional folding of the catalyst are not yet known. Nevertheless, our mutation data, supported by luminescence and footprinting results, suggest interaction networks that will be further refined in combination with future structural data. Recently, the solution structure of a lariat-forming ribozyme (2′,S′ AG1) was solved by NMR, and a model for the active state was built by combining data from mutation analyses and molecular modeling. The 2′,S′ AG1 ribozyme, 9F7, and 7S11 have related substrate requirements (2′-OH at a branch-site adenosine and S′-triphosphate at guanosine), but the reaction partners are presented in different secondary structure contexts. Future studies will shed light on the question whether similar hypotheses on how metal ions assist ribozyme activity (as derived from NMR structure and modeling) can also be substantiated for DNA-catalyzed synthesis of branched RNA. In this context it is also conceivable that activating metal ions interact simultaneously with the nucleophile and electrophile.

Besides advancing fundamental insights into similarities and/or differences of RNA- and DNA-catalyzed synthesis of 2′,S′-branched RNA, the results of this study have key practical implications. We conclude that the combination of Mg²⁺ or Mn²⁺ with lanthanides, in particular with Tb³⁺, constitutes a promising approach to drastically enhance the ligation rate and increase the accessibility of RNA structures with branch-site attachments. In this context, the reduced requirement for divalent metal ions in the presence of Tb³⁺ is considered an asset for future applications.

**EXPERIMENTAL SECTION**

**Assays of Ligation Kinetics.** Standard kinetic assays were performed in a trimeolecular format under single-turnover conditions. The 5′-[32P] trace-labeled acceptor RNA (1 pmol labeled and 4 pmol unlabeled) was annealed with deoxyribozyme (15 pmol) and donor RNA (20 pmol) in 6 μL annealing buffer (5 mM HEPES pH 7.5, 15 mM NaCl, 0.1 mM EDTA) at 95 °C for 2 min, followed by incubation.
were incubated at room temperature in the presence of 50 mM concentrated Tb\textsuperscript{3+} stock solution (Figure S5). The luminescence
μ0.1 DNA with 10-fold excess of branched RNA product). Samples
analysis, as described previously.\textsuperscript{26} The inactivated preligation complex was
\textit{fl}ly, mutant libraries were prepared by solid-phase
\textit{fl}er by 15\% denaturing PAGE and band intensities were quantified using a PhosphorImager. The fractions ligated versus time data were fit to the equation: fraction ligated = Y(1 − e^{-kt}), where k = k_{obs} and Y = final yield.

\textbf{Combinatorial Mutation Interference Analysis.} Combinatorial
mutation interference analysis (CoMA) was performed as described earlier.\textsuperscript{28} Briefly, mutant libraries were prepared by solid-phase synthesis, radiolelbled at the 3' end by templated extension with α-\textit{32P}-dATP using Klenow DNA polymerase, and then ligated to the donor substrate (2) using T4 RNA ligase. Separation of active mutants was performed in the presence of 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, and 5 different combinations of divalent metal ions (Mg\textsuperscript{2+} or Mn\textsuperscript{2+}) and Tb\textsuperscript{3+} in a volume of 60–70 μL. The reaction times were 20 min for 20 mM Mn\textsuperscript{2+}, 10 h for 2 mM Mn\textsuperscript{2+}, 1 h for 2 mM Mn\textsuperscript{2+} with 10 μM Tb\textsuperscript{3+}, 8 h for 80 mM Mg\textsuperscript{2+}, and 2 h for 80 mM Mg\textsuperscript{2+} with 100 μM Tb\textsuperscript{3+}. Alkaline hydrolysis was performed in presence of 10 mM NaOH by heating at 95 °C for 10 min. Denaturing sequencing gels were run at 35 W for 3–4 h for high resolution. Quantification of interference values was performed by volume or area analysis, as described previously.\textsuperscript{28}

\textbf{Tb\textsuperscript{3+} Luminescence.} The inactivated preligation complex was prepared by annealing inactivated acceptor 1a, 9F7 DNA and donor RNA 2 (1.2 nmol each) in 60 μL in presence of annealing buffer by heating for 2 min at 95 °C and incubation at room temperature for exactly 15 min. Buffer and metal ions were then added to the sample to reach the final concentrations of 2 μM preligation complex, 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, and indicated concentration of divalent metal ion in 600 μL. The samples were equilibrated at room temperature for 1 h before Tb\textsuperscript{3+} titration was initiated by addition of small volumes (1 μL) of appropriately concentrated Tb\textsuperscript{3+} stock solution (Figure S5). The luminescence experiments were performed on a Cary Eclipse fluorescence spectrophotometer, with excitation at 284 nm (slit width 10 nm). Emission intensity was collected at 545 nm (slit width: 10 nm; total exposure time: 5 s).

\textbf{DMS Probing and DNase I Footprinting.} Structural probing experiments were performed with 3-\textit{32P}-labeled-DNA. The samples contained DNA alone (0.1 μM), the preligation complex (b, 0.1 μM DNA with 10-fold excess of 1 and 2), or the postligation complex (c, 0.1 μM DNA with 10-fold excess of branched RNA product). Samples were incubated at room temperature in the presence of 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, and 20 mM MnCl\textsubscript{2} (or H\textsubscript{2}O\textsubscript{2}) for 5 min in a volume of 10 μL prior to footprinting assays.

Methylation of accessible nucleobases was initiated by adding 1 μL of a freshly prepared solution of 2% DMS in water, followed by incubation at room temperature for 10 min. The reaction was stopped by addition of 1 M β-mercaptoethanol, followed by ethanol precipitation. The pellets were suspended in 50 μL of 10% piperidine, and the DNA was cleaved at the methylated sites upon incubation at 90 °C for 30 min. Piperidine was removed by evaporation under vacuum, and the pellets were washed 2 times with 100 μL 70% ethanol.

DNase I footprinting was performed in presence of 10 mM Tris-HCl pH 7.6, 2.5 mM MgCl\textsubscript{2}, and 0.5 mM CaCl\textsubscript{2} using 0.1 unit DNase I for 10 min at 37 °C. The digestion was stopped by addition of 3 μL of stop solution (80% formamide, 1X TBE, 50 mM EDTA containing 0.025% bromophenol blue and xylene cyanol).

DMS and DNase I samples were separated by denaturing PAGE (20% acrylamide, 1X TBE, 35 W) and analyzed by PhosphorImaging.

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