DNA-catalyzed sequence-specific hydrolysis of DNA

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Deoxyribozymes (DNA catalysts) have been reported for cleavage of RNA phosphodiester linkages, but cleaving peptide or DNA phosphodiester linkages is much more challenging. Using *in vitro* selection, here we identified deoxyribozymes that sequence-specifically hydrolyze DNA with multiple turnover and with a rate enhancement of 10^8 (possibly as high as 10^{14}). The new DNA catalysts require both Mn^{2+} and Zn^{2+}, which is noteworthy because many natural DNA nucleases are bimetallic protein enzymes.

The first deoxyribozyme (DNA enzyme) was identified by *in vitro* selection in 1994 and cleaves an RNA phosphodiester bond[1]. Since that time, DNA has been shown to catalyze many reactions, including several preparatively useful transformations[2]. RNA cleavage is the most studied DNA-catalyzed reaction[3], and deoxyribozymes with practical cleavage abilities for nearly all RNA dinucleotide target sequences have been developed[4,5]. However, DNA-catalyzed hydrolysis of other biologically relevant bonds, such as amide (peptide) or DNA phosphodiester linkages, has not been reported. Non-site-selective Cu^{2+}-dependent oxidative cleavage of DNA has been described[6]. These reactions appear to proceed through a diffusible intermediate, resulting in nonhydrolytic and nonspecific DNA cleavage. Self-hydrolysis of G-quadruplex DNA has been reported, but the sequence scope appears to be limited[7]. Amide and especially DNA phosphodiester linkages are intrinsically more difficult to hydrolyze than RNA, by about a factor of 20 for amides[8] and at least 400 (possibly much more) for DNA. Estimates of the uncatalyzed half-life of DNA at near-physiological conditions range from ~4,000 years[9] to 30 million years[10] to over 10 billion years[11], which makes catalysis of DNA hydrolysis an especially challenging goal[12–14].

In this study, we initially sought to use *in vitro* selection to identify deoxyribozymes that cleave amide bonds[15] by presenting a tripeptide sequence to a random N_{40} DNA region in the structural arrangement of **Figure 1a**. The desired reaction site was held across from the initially random DNA enzyme region by Watson-Crick interactions between fixed DNA sequences of the substrate and complementary binding arm sequences of the deoxyribozyme strand. This arrangement has been used to identify deoxyribozymes with a variety of catalytic activities including RNA cleavage[2], and here it allows us to focus on the fundamental ability of DNA to catalyze cleavage of our substrate.

The selection strategy was derived from two precedents: (i) many successful selections for deoxyribozymes that cleave RNA linkages[3]; and

(ii) our own extensive efforts to identify deoxyribozymes that ligate two RNA substrates[2,16]. During the key bond-cleavage step of each selection round, the incubation conditions included 40 mM $MgCl_2$, 20 mM $MnCl_2$ and 1 mM $ZnCl_2$ in 70 mM Tris, pH 7.5, and 150 mM NaCl at 37 °C. Three divalent metal ions were included to enable emergence of a potentially wide range of catalytic mechanisms. The substrate (**Fig. 1b**) incorporated an Ala-Ser-Ala tripeptide cleavage target between two DNA sequences designed to interact with fixed DNA elements ("binding arms") that flank the N_{40} region. The C-terminal alanine of the tripeptide was connected by an amide bond to 5′-NH_2-T of DNA, and the N-terminal alanine was joined to DNA via a short γ-hydroxybutyric acid (GHB) linker. This substrate offers for cleavage four amide bonds as well as numerous DNA phosphodiester bonds. At the outset, our expectation was that peptide bond cleavage should be favored over DNA hydrolysis, due to the intrinsic reactivity difference noted above. In a parallel selection experiment, the GHB linker was omitted from the substrate, and the N-terminal alanine was connected directly to the 3′-phosphorus of the DNA binding arm via a phosphoramidate
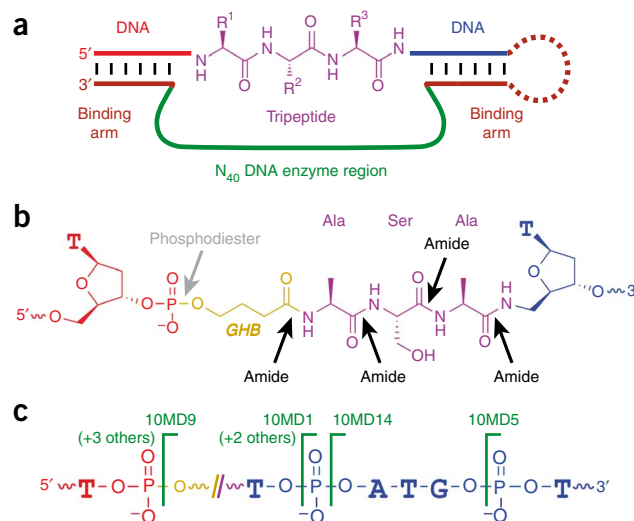

Figure 1 *In vitro* selection of deoxyribozymes that cleave a substrate with both amide and DNA phosphodiester linkages. (**a**) Key step of the selection strategy. The loop on the right enables PAGE separation during selection of the DNA sequences that cleave the substrate, but this loop is dispensable for catalysis. (**b**) Chemical composition of the substrate between the two unpaired thymidine DNA nucleotides at the ends of the binding arms. (**c**) Observed cleavage sites within the substrate for nine unique new deoxyribozymes. All four sites correspond to hydrolysis at DNA phosphodiester linkages. 10MD30, 10MD36 and 10MD41 cleave at the same site as 10MD9; 10MD4 and 10MD13 cleave at the same site as 10MD1. Sequences of all nine deoxyribozymes are provided in **Supplementary Table 1**.

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(P-N) bond. The emergent deoxyribozymes cleaved only this phosphoramidate linkage, rather than any amide or phosphodiester bond of the substrate (**Supplementary Fig. 1**). This outcome is sensible because the phosphoramidate bond is expected to be the most labile linkage within the substrate[17].

After nine selection rounds with the substrate of **Figure 1b**, the DNA pool achieved 35% substrate cleavage during the 14 h incubation period (**Supplementary Methods**). A tenth round with 2 h of incubation led to 16% substrate cleavage, which did not increase after additional rounds. Individual round 10 deoxyribozymes were screened for activity, cloned and sequenced essentially as described previously[16,18]. Of 44 clones, a total of four different cleavage sites in the substrate were used by nine unique deoxyribozymes (**Fig. 1c**). Surprisingly, all four cleavage sites corresponded to hydrolysis at specific DNA phosphodiester linkages, whereas no substrate amide bond was hydrolyzed by any deoxyribozyme. The precise location of each DNA cleavage site and the integrity of the hydrolyzed DNA termini were assigned and verified by MALDI mass spectrometry of PAGE-purified reaction products (**Supplementary Fig. 2** and **Supplementary Table 2**), by comparison with PAGE standards (**Supplementary Fig. 3**) and by biochemical ligation assays using the reaction products and T4 DNA ligase (**Supplementary Fig. 4**).

Four new deoxyribozymes, named 10MD9, 10MD1, 10MD14 and 10MD5 (one for each observed cleavage site), were characterized further. All four deoxyribozymes require both Mn^{2+} and Zn^{2+} (**Supplementary Fig. 5**). Mg^{2+} is either dispensable (10MD9 and 10MD5), supportive of twofold higher yield than with Mn^{2+} and Zn^{2+} alone (10MD1), or several-fold inhibitory (10MD14). Whereas 10MD9, 10MD1 and 10MD14 function better in Tris than in HEPES buffer, 10MD5 has higher activity in HEPES and is inhibited by high concentrations of Tris (**Supplementary Fig. 6**). Standard incubation conditions of 70 mM Tris or HEPES (as appropriate), pH 7.5, 20 mM $MnCl_2$, 1 mM $ZnCl_2$ and 150 mM NaCl at 37 °C were used in further assays.

10MD9 and 10MD1 strictly require the presence of the tripeptide region in the substrate (**Fig. 2a**). 10MD14 retains some activity with an all-DNA substrate but is ~40-fold faster when the tripeptide region is included (**Fig. 2a**). The role of the tripeptide region in catalysis has not yet been examined. All three deoxyribozymes cleave a DNA phosphodiester linkage very close to the tripeptide region (additionally, 10MD9 appears to catalyze a secondary reaction of its product, resulting in loss of the GHB moiety; **Supplementary Fig. 7**). In contrast, 10MD5 hydrolyzes a DNA linkage four nucleotides removed from the tripeptide region (**Fig. 1c**). 10MD5 cleaves an all-DNA substrate as efficiently as the original tripeptide-containing substrate ($k_{obs} = 2.7 \pm 0.3$ h^{-1} and yield = $66 \pm 4\%$; $n = 11$; **Fig. 2a**), with formation of 5′-phosphate and 3′-hydroxyl DNA termini as established above.

We evaluated the 10MD5 deoxyribozyme for its tolerance of DNA substrate sequences (**Fig. 2b**; data in **Supplementary Fig. 8**). Outside of the ATG trinucleotide segment immediately 5′ of the hydrolysis site and the thymidine immediately to the 3′ side, 10MD5 tolerates all substrate nucleotides, as long as Watson-Crick base pairing is maintained. The detailed sequence requirements within this ATG^T "recognition site" are currently under investigation.

As divalent metal ion cofactors, Cu^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} do not affect 10MD5 cleavage activity when included at either 0.1 μM or 1 μM along with Mn^{2+} and Zn^{2+}. Cu^{2+}, Fe^{2+}, Co^{2+} or Ni^{2+} at 0.1 μM through 10 mM cannot substitute for either Mn^{2+} or Zn^{2+}, whereas 1 mM Cd^{2+} can substitute for Mn^{2+} with about a 10^5 lower DNA cleavage rate. In addition, 40 mM Ca^{2+} can substitute for Mn^{2+} with about a 40-fold lower rate (**Supplementary Fig. 9**). When 10MD5 was tested with Mn^{2+} and Zn^{2+}, the $K_{d,app}$ was 5 ± 2 mM for Mn^{2+} (at 1 mM Zn^{2+}); the optimal Zn^{2+} concentration was ~1 mM (at 20 mM Mn^{2+}; **Supplementary Fig. 10**), with little activity below 0.5 mM Zn^{2+}. The importance of monovalent ions was also examined (**Supplementary Fig. 11**). When Na^+ was decreased from 150 mM to either 10 mM or 0 mM, activity was slightly suppressed, whereas increasing Na^+ to 450 mM or replacing 150 mM Na^+ with 150 mM K^+ had little effect. Finally, the pH dependence of 10MD5 was examined (**Supplementary Fig. 12**). The deoxyribozyme has a rather sharp pH optimum near 7.5 (as measured for the 1 M buffer stock solution), with substantially reduced activity when the pH was increased or decreased by merely 0.1–0.2 pH units. Changing the sulfonic acid buffer from HEPES to MOPS led to comparable activity. Both the rather sharp Zn^{2+} optimum and the strong pH dependence of 10MD5 are similar to our observations for a different Zn^{2+}-dependent deoxyribozyme that catalyzes an unrelated RNA ligation reaction[19]. Several other deoxyribozymes and ribozymes are known that either require or can use Zn^{2+} as a cofactor[20].

To validate that 10MD5 catalyzes hydrolytic rather than oxidative DNA cleavage, we performed additional experiments. When the 10MD5-catalyzed cleavage reaction was performed in ^{18}O-water ($^{18}OH_2$), we observed by mass spectrometry that ^{18}O was
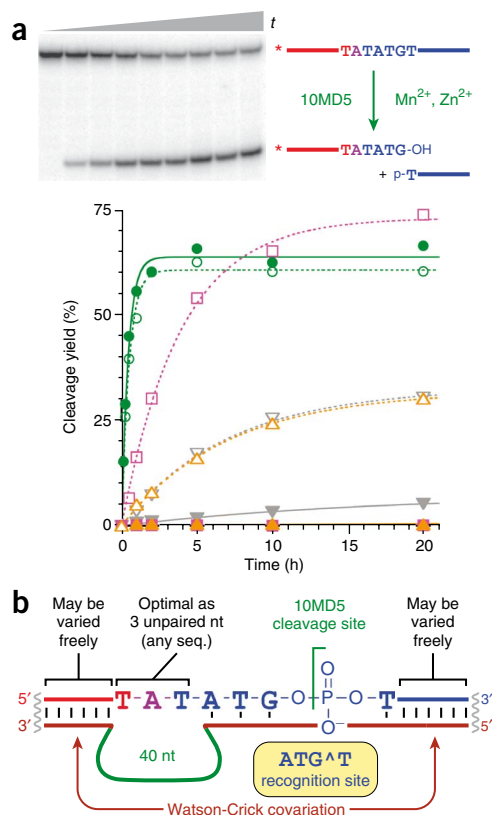

Figure 2 Hydrolysis of DNA phosphodiester bonds by the new deoxyribozymes. (**a**) Single-turnover data under standard incubation conditions (including Mn^{2+} and Zn^{2+}) for 10MD9 (■), 10MD1 (▲), 10MD14 (▼) and 10MD5 (●), with either the original tripeptide-containing substrate (open symbols) or the all-DNA analog in which the tripeptide portion is replaced by deoxyadenosine (filled symbols). k_{obs} values are in **Supplementary Table 3**. The PAGE image shows cleavage of the all-DNA substrate by 10MD5 (t = time). (**b**) Summary of DNA substrate sequence requirements for 10MD5, which efficiently cleaves an all-DNA substrate with a modest recognition site.

incorporated into the 5′-phosphate group (**Supplementary Fig. 13**). This observation is consistent with a hydrolysis reaction in which water is the source of the new oxygen atom, but it is inconsistent with oxidative cleavage, in which O_2 from the air would be the source of the oxygen atom. Of course, much work remains to investigate the mechanisms of DNA-catalyzed DNA hydrolysis, including dissection of the structural and catalytic roles of Mn^{2+} and Zn^{2+}. With unrelated inorganic catalysts, "formal hydrolysis" of DNA can result from an oxidative pathway[21]. We also evaluated the effect of including Mn^{3+}, H_2O_2 or $(NH_4)_2S_2O_8$ (ammonium persulfate) as potential oxidants, either in addition to Mn^{2+} and Zn^{2+} or in place of one of the two metal ions. In all cases, no effect of the added oxidant was observed (**Supplementary Fig. 14**). Finally, we included either catalase or super-oxide dismutase or both, and suppression of DNA cleavage was not observed (**Supplementary Fig. 14**). Taken together, these experiments validate the conclusion that 10MD5 catalyzes DNA phosphodiester cleavage via a hydrolytic rather than an oxidative mechanism. In sharp contrast, the Cu^{2+}-dependent deoxyribozymes reported previously involve diffusible intermediates, resulting in relatively nonselective DNA cleavage by "oxidative destruction" of a nucleotide[6].

The new DNA-hydrolyzing deoxyribozymes are capable of multiple turnover. When the "binding arms" of 10MD5 were shortened, which is anticipated to promote product release[4], at least 40 turnovers were observed (**Supplementary Fig. 15**). Similar results were found for the other three deoxyribozymes. When the 10MD5 deoxyribozyme was isolated after a multiple-turnover reaction and treated with piperidine to induce strand cleavage at any damage sites, strand-cleavage events were not observed (**Supplementary Fig. 16**), thus further validating nonparticipation of oxidizing species in 10MD5-catalyzed DNA hydrolysis.

In summary, we have identified numerous deoxyribozymes that require two different divalent metal ions, Mn^{2+} and Zn^{2+}, to catalyze sequence-specific cleavage of DNA phosphodiester linkages. A requirement by a deoxyribozyme or ribozyme for two different metal ions simultaneously is rare but precedented[20]. The DNA-catalyzed heterobimetallic DNA hydrolysis activity was identified here despite the presence in the selection substrate of four nearby amide bonds, which have a much higher uncatalyzed hydrolysis rate. The reason that the peptide bonds in the selection substrate were not cleaved is currently unknown. One focus of our ongoing efforts is to identify deoxyribozymes that function with peptide substrates[22]. These efforts include revised selection strategies specifically seeking DNA-catalyzed amide bond cleavage.

The rate enhancement of the 10MD5 deoxyribozyme, which sequence-specifically cleaves an all-DNA substrate with multiple turnover, is at least 10^8 and possibly as high as 10^{14} over the uncatalyzed DNA hydrolysis reaction, as calculated from the corresponding half-lives. Mechanistic analyses of the new deoxyribozymes are warranted, especially regarding the roles of Mn^{2+} and Zn^{2+}, because many natural enzymes are DNA or RNA nucleases that require multiple metal ion cofactors[23,24]. Considerable attention has been devoted to small model systems for DNA nuclease activity[13,14]. The identification of heterobimetallic DNA catalysts that sequence-specifically

hydrolyze DNA phosphodiester linkages suggests that deoxyribozymes have significant potential as practical sequence-specific DNA cleavage reagents[25,26].

Note: Supplementary information is available on the Nature Chemical Biology website.

AUTHOR CONTRIBUTIONS
M.C. performed *in vitro* selections using substrates synthesized by A.S. M.C. and A.S. characterized the deoxyribozymes. S.K.S. conceived the project, assisted M.C. and A.S. with analysis of experimental data and wrote the manuscript with input from M.C. and A.S.

1. Breaker, R.R. & Joyce, G.F. *Chem. Biol.* **1**, 223–229 (1994).
2. Silverman, S.K. *Chem. Commun. (Camb.)* 3467–3485 (2008).
3. Silverman, S.K. *Nucleic Acids Res.* **33**, 6151–6163 (2005).
4. Santoro, S.W. & Joyce, G.F. *Proc. Natl. Acad. Sci. USA* **94**, 4262–4266 (1997).
5. Schlosser, K., Gu, J., Lam, J.C. & Li, Y. *Nucleic Acids Res.* **36**, 4768–4777 (2008).
6. Carmi, N. & Breaker, R.R. *Bioorg. Med. Chem.* **9**, 2589–2600 (2001).
7. Liu, X. *et al. Chem. Commun. (Camb.)* 380–382 (2008).
8. Radzicka, A. & Wolfenden, R. *J. Am. Chem. Soc.* **118**, 6105–6109 (1996).
9. Radzicka, A. & Wolfenden, R. *Science* **267**, 90–93 (1995).
10. Schroeder, G.K., Lad, C., Wyman, P., Williams, N.H. & Wolfenden, R. *Proc. Natl. Acad. Sci. USA* **103**, 4052–4055 (2006).
11. Williams, N.H., Takasaki, B., Wall, M. & Chin, J. *Acc. Chem. Res.* **32**, 485–493 (1999).
12. Robertson, D.L. & Joyce, G.F. *Nature* **344**, 467–468 (1990).
13. Mancin, F., Scrimin, P., Tecilla, P. & Tonellato, U. *Chem. Commun. (Camb.)* 2540–2548 (2005).
14. Mancin, F. & Tecilla, P. *New J. Chem.* **31**, 800–817 (2007).
15. Dai, X., De Mesmaeker, A. & Joyce, G.F. *Science* **267**, 237–240 (1995).
16. Flynn-Charlebois, A. *et al. J. Am. Chem. Soc.* **125**, 2444–2454 (2003).
17. Wada, T., Moriguchi, T. & Sekine, M. *J. Am. Chem. Soc.* **116**, 9901–9911 (1994).
18. Kost, D.M., Gerdt, J.P., Pradeepkumar, P.I. & Silverman, S.K. *Org. Biomol. Chem.* **6**, 4391–4398 (2008).
19. Hoadley, K.A., Purtha, W.E., Wolf, A.C., Flynn-Charlebois, A. & Silverman, S.K. *Biochemistry* **44**, 9217–9231 (2005).
20. Silverman, S.K. in *Wiley Encyclopedia of Chemical Biology* (ed. Begley, T.P.) (John Wiley and Sons, Hoboken, New Jersey, 2009).
21. Shell, T.A., Glass, J.E., Mackey, M.A., Layman, K.A. & Mohler, D.L. *Inorg. Chem.* **46**, 8120–8122 (2007).
22. Pradeepkumar, P.I., Höbartner, C., Baum, D.A. & Silverman, S.K. *Angew. Chem. Int. Ed.* **47**, 1753–1757 (2008).
23. Wilcox, D.E. *Chem. Rev.* **96**, 2435–2458 (1996).
24. Shan, S.O., Kravchuk, A.V., Piccirilli, J.A. & Herschlag, D. *Biochemistry* **40**, 5161–5171 (2001).
25. Kim, Y.G., Cha, J. & Chandrasegaran, S. *Proc. Natl. Acad. Sci. USA* **93**, 1156–1160 (1996).
26. Komiyama, M., Aiba, Y., Yamamoto, Y. & Sumaoka, J. *Nat. Protoc.* **3**, 655–662 (2008).