Pure Appl. Chem., Vol. 76, Nos. 7–8, pp. 1571–1577, 2004.

Selection and characterization of DNAzymes with synthetically appended functionalities: A case of a synthetic RNaseA mimic*

Richard Ting, Leonard Lermer, Jason Thomas, Yoann Roupioz, and David M. Perrin‡

Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia V6T-1Z1, Canada

Abstract: We have been interested in merging synthetic nucleotide chemistry with combinatorial selection of DNAzymes to deliver a more complete (and complex) chemical complement to the catalytic repertoire of nucleic acids. Thus we ask, what do modified dNTPs really bring to nucleic acids in terms of an increased repertoire? In asking this question, we have looked first at conditions, and more recently for reaction classes where nucleic acids are found to be catalytically inefficient, deficient, or at least to date, seemingly incapable of certain functions. A case of this is M^{2+}-independent ribophosphodiester hydrolysis at physiological pH and low ionic strength where nucleic acids exhibit especially low rate constants for self-cleavage and seem to be incapable of turnover.

INTRODUCTION

RNA cleavage has received considerable attention over the past 30 years. The construction of "biomimetic" catalysts has fundamental implications in terms of our ability to master RNA recognition and cleavage through understanding chemical reactivity. This has then led to applications in terms of diagnostics and possible antiviral therapies. Early chemical approaches to designing synthetic ribonucleases involved metal ion ligands and/or peptides containing lysines, histidines and arginines to mimic the active site of DNase and RNaseA [1–3]. In these early studies, RNA hydrolysis was observed, albeit at low rates. Sequence-specific cleavage was observed only on activated phosphate diesters and defined di/trinucleotide substrates, whereas nonspecific cleavage has been observed on radiolabeled tRNA treated with a large excess of catalyst. Sequence-specific cleavage was achieved by coupling such small catalysts to synthetic oligonucleotides that would first recognize complementary RNA [4,5]. Oligonucleotides appended with metal-chelators cleave RNA relatively efficiently and occasionally with observable catalytic turnover [6–8]. A recent report describes 70 % cleavage of *c-raf-1* RNA in 4 h by a europium-appended oligonucleotide, but only under single-turnover conditions [9]. However, the utility of such metallochelates as antivirals might be questioned since intracellular concentrations of divalent metals such as iron, copper, and zinc are negligible. In the absence of a divalent metal cation (hereafter abbreviated M^{2+}), one must properly orient imidazoles and/or cations (e.g., amines/guanidines) to cleave RNA via an RNaseA-like mechanism. Over the years, many reports have underscored the importance of imidazole/amine-appended oligonucleotides to medicine and biology [10–18]. When such synthetic conjugates hydrolyze RNA, they usually do so very slowly (hours–days). Moreover, to

*Lecture presented at the symposium "Chemistry of nucleic acids", as part of the 39th IUPAC Congress and 86th Conference of the Canadian Society for Chemistry: Chemistry at the Interfaces, Ottawa, Canada, 10–15 August 2003. Other Congress presentations are published in this issue, pp. 1295–1603.

‡Corresponding author: E-mail: dperrin@chem.ubc.ca

date, specific cleavage has been *only* stoichiometric, usually proceeding either in an intramolecular fashion or in some instances *inter*molecularly under single-turnover conditions (single-turnover being defined as reaction assayed in large excess of catalyst). The goal of developing a synthetic RNaseA mimic that hydrolyzes an RNA linkage sequence specifically, with multiple turnover, has remained elusive for the past three decades.

In contrast to rational synthetic design, combinatorial nucleic acid selection techniques, which allow for parallel sampling of ~1×10^{15} potential catalysts, provide a promising means for discovering new RNA-hydrolyzing RNAzymes and DNAzymes [19] to be used as antivirals and biosensors [20]. Such catalysts owe their catalytic activity to irregular secondary structures and their specificity for recognizing target sequences to standard base pairing [21–24]. Almost without exception, RNA-cleaving ribozymes and DNAzymes depend on M^{2+} [25–27]. Two combinatorially selected, M^{2+}-independent "DNAzymes" (40–50-nt motifs) that self-cleave in 0.25^{-1} M monovalent cations at pH 7, displayed rate constants on the order of 10^{-3} to 10^{-4} min^{-1} [28,29]. Moreover, in both cases, neither *inter*molecular catalysis nor multiple turnover was observed in the absence of M^{2+}. Together, these important studies identified the intrinsic catalytic competence of DNA. Similar results were found with the 86-nt HDV ribozyme: in the absence of Mg^{2+}, k_{cat} fell ~10^4-fold and turnover ceased [30,31]. In contrast to the HDV ribozyme, the hairpin and hammerhead ribozymes cleave intermolecularly at pH 7 in the absence of M^{2+}, but only in the presence of 1–4 M monovalent cations [32–34] or in the presence of various polyamines [35,36]. The only two reports of combinatorially selected M^{2+}-independent self-cleaving activities are: (1) a His-dependent, 30-nt DNAzyme that hydrolyzes a single ribophosphodiester linkage within an all-DNA substrate at 1 M monovalent cations [37], and (2) an all-RNA species that self-cleaves at pH 4.5, the pH at which cytosine is hemiprotonated and thus can competently mediate general acid/base catalysis [38].

This brief review of combinatorially selected catalysts demonstrates the promise that this methodology affords in imitating *natural* ribozymes, as well as in identifying other unnatural activities [39,40]. Nevertheless, combinatorial selection might seem unsuitable for discovering an RNaseA mimic as defined by a scaffold presenting a cationic functionality imitating Lys-41 and a dedicated imidazole pair imitating His-12 and His-119. The reason for this is that unmodified nucleic acids are devoid of functionalities that define the active site of RNaseA [41]. Many reports have discussed the inherent lack of chemical functionality in nucleic acids and have underscored the potential of using synthetic chemistry to append dNTPs with imidazoles, cationic amines, or other functionalities for use in a combinatorial selection [42–51]. Far fewer reports have demonstrated a successful combinatorial selection of a *modified* RNA or DNA enzyme [52–55]. Nevertheless, these studies suggest that it will be possible to discover mimics of RNaseA as well as other enzymatic activities. The use of synthetically modified nucleotides in combinatorial selection also suggests some utility for recognizing small molecules with higher affinity in the development of biosensors [56]. As chemists, we have been approaching DNAzymes from both synthetic *and* combinatorial approaches. Operating on this interface, we feel obliged to show how chemistry can be applied to biological systems to deliver enhanced activities that are, by comparison, new, interesting, and not readily observable in the absence of such synthetic endeavors.

CURRENT STATE OF RESEARCH

The long-standing goal to preparing a synthetic RNaseA mimic seems to have been achieved as described in recent publications from this laboratory and that of the late Prof. Claude Helene, where this work was initiated during a postdoctoral fellowship [44,57,58]. The first was an elaboration of combinatorial methodologies that would permit the introduction of both imidazoles and cationic amines onto DNA. This paper demonstrated that Im(NH)-dATP {8-[2-(4-Imidazolyl)ethylamino]-2′-deoxyriboadenosine triphosphate} and AA-dUTP [5-(3-Aminoallyl)-2′-deoxyribouridine triphosphate] (Fig. 1)

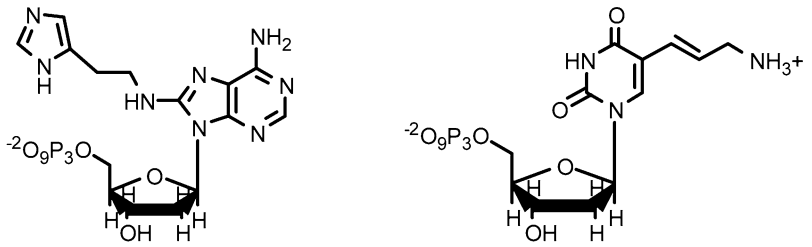

Fig. 1

are substrates for DNA polymerase and moreover are compatible with all conditions for combinatorial selection [44].

The second paper demonstrated combinatorial selection of a DNA species that accelerates the rate of *intra*molecular ribophosphodiester hydrolysis in the absence of M^{2+}. This mimic, phylogenetically denoted 9_{25}-11, presented modifications that delivered a 40–100-fold increase in activity (k_{cat} = 0.05 min^{-1}) in the *absence* of a divalent metal cation. M^{2+}-independent activity is maximal at pH 7.5—consistent with general acid/base catalysis [59]. The sequence and hypothetical 2D-structure of cloned 9_{25}-11 and its autocleavage activity is shown (Fig. 2) where the dA's present imidazoles and the dU's present cationic allylamines. Below is seen a time-dependent self-cleavage reaction that proceeds at low ionic strength and physiological pH (200 mM NaCl, 50 mM cacodylate pH 7.5, 1 mM EDTA). Beyond a fundamental interest in catalysis, there is also pharmaceutical interest in this work; the targeted ribose is contained on a DNA sequence corresponding to proviral HIV mRNA. Cleavage proceeded by 2′OH attack, depended on both cationic amines and imidazoles derived from the monomer triphosphates shown in Fig. 1, and exhibited a pH-rate-maximum at 7.5 that was consistent with general acid/base catalysis [59].

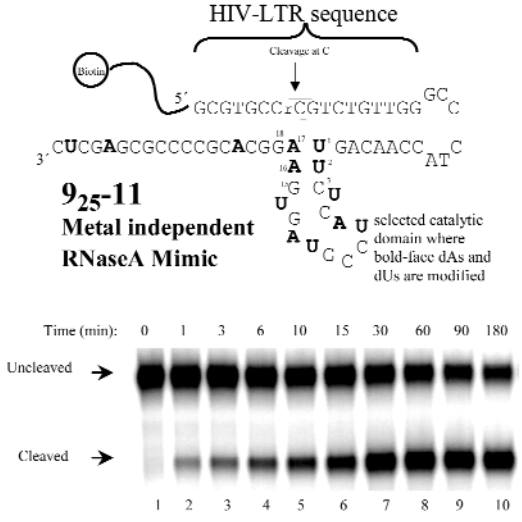

Fig. 2

The third paper demonstrated solid-phase synthetic scale-up of the catalytic motif and its conversion into a true catalyst as defined by catalytic turnover [60,61]. The catalytic motif, 9_{25}-11, which was derived from the self-cleaving sequence (Fig. 3), is one of the smallest DNA catalysts reported to date. This species hydrolyzes a single ribophosphodiester linkage embedded in an all-DNA substrate

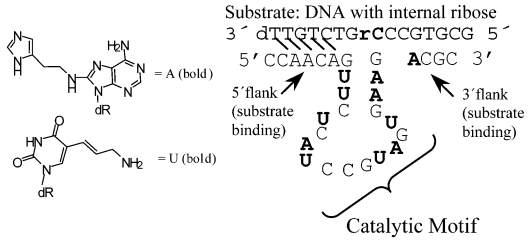

Fig. 3

d(GCGTGCC)rCd(GTCTGTT). An identical substrate, d(GCGTGCC)rNd(GTCTGTT) that was "degenerate" for ribose (implying that all four ribonucleosides were equally represented at rN) provided a quick measure of the specificity for target ribonucleoside recognition (lanes 1–5) [62]. The catalyst cleaved roughly 25 % of the degenerate substrate as expected for highly specific recognition of ribocytosine (lanes 8–13). Finally, we demonstrated multiple turnover (excess substrate). These published kinetic data are reprinted in Fig. 4 (multiple turnover in lanes 15–19). 9_{25}-11 is a unique template for structure–activity studies to characterize general acid-base RNA hydrolysis without the complicating effects of divalent metal ions [63]. Although the k_{cat} of 9_{25}-11 remains modest, 9_{25}-11 still represents the first catalyst of its kind.

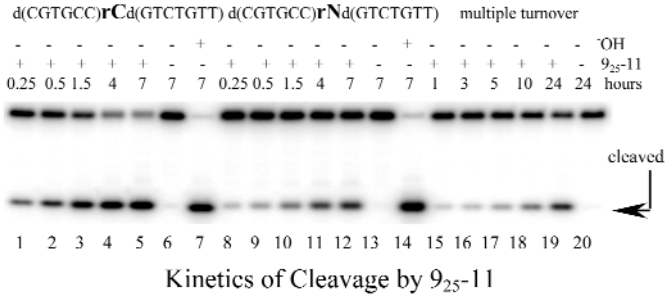

Fig. 4

Because of its relative simplicity, characterization will serve as the primary focus of future work. Investigation of its structure–function relationships should lead to a better understanding of how to improve rates and the advantage of appending such synthetic functionality. Because the physiologically abundant divalent cations Ca^{2+} and Mg^{2+} do not inhibit 9_{25}-11 [59], we suggest that this 9_{25}-11, or a related species that could be reselected in the presence of these metals might be useful for targeting mRNA within cells. It is important to note that the first M^{2+}-dependent DNAzymes exhibited k_{cat} values of only 0.0025 min^{-1} in 0.25 M NaCl and 1 mM $MgCl_2$ [64]. Changing selection conditions and increasing the number of generations resulted in 1000-fold more efficient M^{2+}-dependent DNAzymes [65]. It is also paramount to note the considerable challenge of delivering oligonucleotides within cells.

How then does this study of a catalytic DNAzyme, 9_{25}-11, which may not necessarily itself become a clinically useful antiviral, still apply to medical chemists interested in developing aptamers? The answer lies in the Pauling–Haldane antibody-enzyme analogy that would hold that DNAzymes are simply aptamers for transition states [66–69]. Nevertheless, in certain instances aptamers would seem to be inferior to antibodies and proteins, particularly in terms of recognizing anionic epitopes and transition-state intermediates [70–72]. If aptamers are the "nucleic acid equivalent" of antibodies [73], they obviously lack several functionalities found in proteins, most notably cationic amines and imidazoles. To gauge the utility of introducing these functionalities for improving catalytic properties, a synthetically modified DNAzyme was deliberately selected under conditions (no M^{2+}, pH 7.5) where unmodified

DNA is simply not capable of catalytic turnover (as previously demonstrated by two independent investigators) [74,75]. This new DNAzyme, 9_{25}-11, when resynthesized by solid phase, indeed delivers catalytic properties that cannot be selected without modified dNTPs [76] and has no comparable unmodified correlate with the exception of perhaps RNaseA itself [77]. Moreover, we have demonstrated that the functionalities on which the catalytic activity obligately depends, also react with chemically reactive substrate-mimics. As we have begun to expand the chemical potential of DNAzymes, it is now incumbent on us to define, at the molecular level, how exactly these functionalities can deliver catalytic activity and how we may, through chemistry, improve on that activity.

REFERENCES

1. D. S. Sigman, G. M. Wahl, D. J. Creighton. *Biochemistry* **11**, 2236–2242 (1972).
2. R. Breslow, J. B. Doherty, G. Guillot, C. Lipsey. *J. Am. Chem. Soc*. **100**, 3227–3229 (1978).
3. S. C. Hirst, P. Tecilla, S. J. Geib, E. Fan, A. D. Hamilton. *Israel J. Chem*. **32**, 105–111 (1992).
4. B. N. Trawick, A. T. Daniher, J. K. Bashkin. *Chem. Rev*. **98**, 939–960 (1998).
5. M. Oivanen, S. Kuusela, H. Lönnberg. *Chem. Rev*. **98**, 961–990 (1998).
6. B. N. Trawick, T. A. Osiek, J. K. Bashkin. *Bioconj. Chem*. **12**, 900–905 (2001).
7. J. Hall, D. Hüsken, R. Häner. *Nucleic Acids Res*. **24**, 3522–3526 (1996).
8. J. K. Bashkin, E. I. Frolova, U. Sampath. *J. Am. Chem. Soc*. **116**, 5981–5982 (1994).
9. L. Canaple, D. Husken, J. Hall, R. Haner. *Bioconj. Chem*. **5**, 945–951 (2002).
10. M. Komiyama and T. Inokawa. *J. Biochem*. **116**, 719–720 (1994).
11. M. A. Reynolds, T. A. Beck, P. B. Say, D. A. Schwartz, B. P. Dwyer, W. J. Daily, M. M. Vaghefi, M. D. Metzler, R. E. Klem, L. J. Arnold. *Nucleic Acids Res*. **24**, 760–765 (1996).
12. K. Ushijima, H. Gouzu, K. Hosono, M. Shirakawa, K. Kagosima, T. Kazuyuki, H. Takaku. *Biochim. Biophys. Acta* **1379**, 217–223 (1998).
13. N. G. Beloglazova, V. N. Sil'nikov, M. A. Zenkova, V. V. Vlassov. *FEBS Lett*. **481**, 277–280 (2000).
14. B. Verbeure, C. J. Lacey, M. Froeyen, J. Rozenski, P. Herdewijn. *Bioconj. Chem.* **13**, 333–350 (2002).
15. V. Vlassov, T. Abramova, T. Godovikova, R. Giege, V. Sil'nikov. *Antisense Nucl. Acid Drug Dev*. **7**, 39–42 (1997).
16. J. Hovinen, A. Guzaev, E. Azhayeva, A. Azhayev, H. Lonnberg. *J. Org. Chem*. **60**, 2205–2209 (1995).
17. J. K. Bashkin, J. K. Gard, A. S. Modak. *J. Org. Chem*. **55**, 5125–5132 (1990).
18. M. Beban and P. S. Miller. *Bioconj. Chem*. **11**, 599–603 (2000).
19. A. A. Beaudry and G. F. Joyce. *Science* **257**, 635–641 (1992).
20. B. A. Sullenger and E. Gilboa. *Nature* **418**, 252–258 (2002) and references within.
21. A. D. Ellington and J. W. Szostak. *Nature* **346**, 818–822 (1990).
22. R. R. Breaker and G. F. Joyce. *Chem. Biol.* **1**, 223–229 (1994).
23. C. Tuerk and L. Gold. *Science* **249**, 505–510 (1990).
24. A. A. Beaudry and G. F. Joyce. *Science* **257**, 635–641 (1992).
25. R. H. Symons. *Ann. Rev. Biochem*. **61**, 641–671 (1992).
26. W. G. Scott and A. Klug. *TIBS* **21**, 220–224 (1996).
27. J. Bratty, P. Chartrand, G. Ferbeyre, R. Cedergren. *Biochim. Biophys. Acta* **1216**, 345–359 (1993).
28. C. R. Geyer and D. Sen. *Chem. Biol.* **4**, 579–593 (1997).
29. D. Faulhammer and M. Famulok. *J. Mol. Biol*. **269**, 188–202 (1997).
30. T. S. Wadkins, I.-H. Shih, A. T. Perrotta, M. D. Been. *J. Mol. Biol*. **305**, 1045–1055 (2001).
31. A. T. Perrotta, I. H. Shih, M. D. Been. *Science* **286**, 123–126 (1999).
32. E. A. Curtis and D. P. Bartel *RNA* **7**, 546–552 (2001).

33. J. L. O'Rear, S. Wang, A. L. Feig, L. Beigelman, O. C. Uhlenbeck, D. Herschlag. *RNA* **7**, 537–545 (2001).
34. J. B. Murray, A. A. Seyhan, W. Ng, J. M. Burke, W. G. Scott. *Chem. Biol.* **5**, 587–595 (1998).
35. B. M. Chowrira, A. Berzal-Herranz, J. M. Burke. *Biochemistry* **32**, 1088–1095 (1993).
36. D. J. Earnshaw and M. J. Gait. *Nucleic Acids Res*. **26**, 5551–5561 (1995).
37. A. Roth and R. Breaker. *Proc. Natl. Acad. Sci. USA* **95**, 6027–6031 (1998).
38. V. K. Jayasena and L. Gold. *Proc. Natl. Acad. Sci. USA* **94**, 10612–10617 (1997).
39. R. R. Breaker. *Chem. Rev*. **97**, 371–390 (1997).
40. D. Robertson and G. F. Joyce. *Nature* **344**, 467–468 (1990).
41. G. J. Narlikar and D. Herschlag. *Annu. Rev. Biochem.* **66**, 19–59 (1997).
42. S. A. Benner et al. *Pure Appl. Chem.* **70**, 263–266 (1998).
43. K. Sakthivel and C. F. Barbas. *Angew. Chem., Int. Ed. Engl.* **37**, 2872–2875 (1998).
44. D. M. Perrin, T. Garestier, C. Helene. *Nucleosides Nucleotides* **18**, 377–391 (1999).
45. J. Matulic-Adamic, A. T. Daniher, A. Karpeisky, P. Haeberli, D. Sweedler, L. Beigelman. *Bioorg. Med. Chem. Lett*. **10**, 1299–1302 (2000).
46. N. K. Vaish, A. W. Fraley, J. W. Szostak, L. W. McLaughlin. *Nucleic Acids Res*. **28**, 3316–3322 (2001).
47. H. Sawai, A. N. Ozaki, F. Satoh, T. Ohbayashi, M. M. Masud, H. Ozaki. *Chem. Comm*. **24**, 2604–2605 (2001).
48. S. E. Lee, A. Sidorov, T. Gourlain, N. Mignet, S. J. Thorpe, J. A. Brazier, M. J. Dickman, D. P. Hornby, J. A. Grasby, D. M. Williams. *Nucleic Acids Res*. **29**, 1565–1573 (2001).
49. T. Gourlain, A. Sidorov, N. Mignet, S. J. Thorpe, S. E. Lee, J. A. Grasby, D. M. Williams. *Nucleic Acids Res*. **29**, 1898–1905 (2001).
50. O. Thum, S. Jäger, M. Famulok. *Angew. Chem., Int. Ed. Engl.* **40**, 3990–3993 (2001).
51. J. Matulic-Adamic, A. T. Daniher, A. Karpeisky, P. Haeberli, D. Sweedler, L. Beigelman. *Nucleosides Nucleotides Nucleic Acids* **20**, 1113–1115 (2001).
52. T. M. Tarasow, S. L. Tarasow, B. Eaton. *Nature* **389**, 54–57 (1997).
53. S. W. Santoro, G. F. Joyce, K. Sakthivel, S. Gramatikova, C. F. Barbas. *J. Am. Chem. Soc*. **122**, 2433–2439 (2000).
54. T. W. Wiegand, R. C. Janssen, B. Eaton. *Chem. Biol.* **4**, 675–683 (1997).
55. N. Teramoto, Y. Imanishi, Y. Ito. *Bioconj. Chem*. **11**, 744–748 (2000).
56. T. R. Battersby, D. N. Ang, P. Burgstaller, S. C. Jurczyk, M. T. Bowser, D. D. Buchanan, R. T. Kennedy, S. B. Benner. *J. Am. Chem. Soc.* **121**, 9781–9789 (1999).
57. D. M. Perrin, T. Garestier, C. Helene. *J. Am. Chem. Soc.* **123**, 1556–1563 (2001).
58. L. Lermer, Y. Roupioz, R. Ting, D. M. Perrin. *J. Am. Chem. Soc.* **124**, 9960–9961 (2002).
59. D. M. Perrin, T. Garestier, C. Helene. *J. Am. Chem. Soc.* **123**, 1556–1563 (2001).
60. L. Lermer, J. Hobbs, D. M. Perrin. *Nucleosides Nucleotides Nucleic Acids* **21**, 651–664 (2002).
61. L. Lermer, Y. Roupioz, R. Ting, D. M. Perrin. *J. Am. Chem. Soc.* **124**, 9960–9961 (2002).
62. S. Santoro and G. F. Joyce. *Biochemistry* **37**, 13330–13342 (1998).
63. S.-I. Nakano and P. C. Bevilacqua. *J. Am. Chem. Soc*. **123**, 11333–11334 (2001).
64. R. Breaker and G. F. Joyce. *Chem. Biol.* **2**, 655–660 (1995).
65. S. W. Santoro and G. F Joyce. *Proc. Natl. Acad. Sci. USA* **94**, 4262–4266 (1997).
66. L. Pauling. *Nature* **161**, 707–709 (1948).
67. This Pauling–Haldane enzyme-antibody analogy assumes that catalysis occurs by transition-state stabilization and that the transition states of the catalyzed and uncatalyzed reactions are the same.
68. J. R. Prudent, T. Uno, P. G. Schultz. *Science* **264**, 1924–1927 (1994).
69. T. Uno, J. Ku, J. R. Prudent, A. Huang, P. G. Schultz. *J. Am. Chem. Soc.* **118**, 3811–3817 (1996).
70. T. R. Battersby, D. N. Ang, P. Burgstaller, S. C. Jurczyk, M. T. Bowser, D. D. Buchanan, R. T. Kennedy, S. B. Benner. *J. Am. Chem. Soc.* **121**, 9781–9789 (1999).

71. K. N. Morris, K. B. Jensen, C. M. Julin, M. Weil, L. Gold. *Proc. Natl. Acad. Sci. USA* **95**, 2902–2907 (1998).
72. K. B. Jensen, B. L. Atkinson, M. C. Willis, T. H. Koch, L. Gold. *Proc. Natl. Acad. Sci. USA* **92**, 12220–12224 (1995).
73. L. Gold. *Nat. Biotechnol.* **20** (7), 671–672 (2002).
74. C. R. Geyer and D. Sen. *Chem. Biol.* **4**, 579–593 (1997).
75. D. Faulhammer and M. Famulok. *J. Mol. Biol*. **269**, 188–202 (1997).
76. A good example where modified and unmodified selections gave virtually the same activity is to compare the following: Joyce and coworkers selected a zinc-dependent RNase activity by incorporating an imidazole-modified deoxyuridine monomer *J. Am. Chem. Soc*. **122** (11), 2433–2439 (2000). Although micromolar (5–30 μM) divalent zinc (in the presence of 1 mM divalent magnesium) was required to give a kcat of 4 min^{-1}, no significant catalytic boost was observed over antecedent, *unmodified* catalysts selected in 2 mM magnesium by Santoro and Joyce, *Proc. Natl. Acad. Sci. USA* **94**, 4262–4266 (1997), or over *unmodified* catalysts that have recently been selected to cleave in 100 μM zinc absent of magnesium, as reported by Lu and coworkers, *Nucleic Acids Res.* **28** (2), 481–488 (2000).
77. Depending on how the comparison is made, the k_{cat} of 9_{25}-11 represents an improvement of 1–3 orders of magnitude over unmodified M^{2+}-independent RNA cleavers. Despite a rather modest overall k_{cat}, it is nevertheless difficult to compare the k_{cat} of 9_{25}-11, which is capable of catalytic turnover, with the "k_{obs}" of other DNA species that are not.