Bridging the Gap between Proteins and Nucleic Acids: A Metal-Independent RNAseA Mimic with Two Protein-Like Functionalities

David M. Perrin,*[,§] Thérèse Garestier,[†] and Claude Hélène[†]

Contribution from the Laboratoire de Biophysique, Museum National d'Histoire Naturelle, 43 rue Cuvier, 75005, Paris, France

Received September 6, 2000

Abstract: Two synthetically modified nucleoside triphosphate analogues (adenosine modified with an imidazole and uridine modified with a cationic amine) are enzymatically polymerized in tandem along a degenerate DNA library for the combinatorial selection of an RNAse A mimic. The selected activity is consistent with both electrostatic and general acid/base catalysis at physiological pH in the absence of divalent metal cations. The simultaneous use of two modified nucleotides to enrich the catalytic repertoire of DNA-based catalysts has never before been demonstrated and evidence of general acid/base catalysis at pH 7.4 for a DNAzyme has never been previously observed in the absence of a divalent metal cation or added cofactor. This work illustrates how the incorporation of protein-like functionalities in nucleic acids can bridge the gap between proteins and oligonucleotides underscoring the potential for using nucleic acid scaffolds in the development of new materials and improved catalysts for use in chemistry and medicine.

Introduction and Formulation of the Problem

In vitro selection has been used to sample up to 10^{15} different nucleic acid sequences for the identification of new ligands to medically important targets as well as novel catalytic activities with no biological precedent.[1,2] Nevertheless, k_{cat} values of in vitro selected activities often fall far short of those found in polypeptide (protein) enzymes. This may be due in large part to the lack of functional groups such as an imidazole (His) and a cationic amine (Lys) which are found almost ubiquitously at enzyme active sites where they play roles of electrostatic stabilization, general acid−general base catalysis, and metal coordination.[3] The dearth of such functionalities has been forwarded as an explanation for why nucleic acids catalysts are inferior to enzymes (and abzymes),[4] even when comparing a specific reaction catalyzed by both.[5−7]

The incorporation of imidazoles and cationic amines onto oligonucleotides and their phosphoramidite precursor monomers would seem to be an attractive means for expanding and enhancing the catalytic potential of DNA. Antecedent efforts to this end have focused on a "rational" or "directed" approach.[8−11] More recently, considerable effort has been devoted to demonstrating the potential of a *combinatorial* approach that uses modified monomer *triphosphates* that are substrates for polymerases.[12−18] In addition, at least two studies have defended the utility of a single modified nucleotide by demonstrating that post-selection resynthesis with the unmodified analogue resulted in loss of the selected activity.[19,20] Nevertheless, if at the outset of the selection, no modified nucleotide is used, the *same* genre of activity may be indepen-

* To whom correspondence should be addressed: Professor D. M. Perrin, Department of Chemistry, University of British Columbia−Vancouver, 2036 Main Mall, Vancouver, BC, V6T-1Z1 Canada. E-mail: dperrin@chem.ubc.ca. Telephone: (604) 822-0567. Fax: (604) 822-2847.

§ Prior Address: Laboratoire de Biophysique, Museum National d'Histoire Naturelle, 43 rue Cuvier, 75005, Paris, France. E-mail: biophy@mnhn.fr. Telephone: 1-40793708. Fax: 1-40793705.

† E-mail: biophy@mnhn.fr. Telephone: 1-40793708. Fax: 1-40793705.

(1) Jensen, K. B.; Atkinson, B. L.; Willis, M. C.; Koch, T. H.; Gold, L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12220−12224.

(2) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54−57.

(3) Sträter, N.; Lipscomb, W. N.; Klabunde, T.; Krebs, B. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2024−2055.

(4) Narlikar, G. J.; Herschlag, D. *Annu. Rev. Biochem.* **1997**, *66*, 19−59.

(5) Prudent, J. R.; Uno, T.; Schultz, P. G. *Science* **1994**, *264*, 1924−7.

(6) Uno, T.; Ku, J.; Prudent, J. R.; Huang, A.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 3811−3817.

(7) Compare: Hilvert, D.; Hill, K. W.; Nared, K. D.; Auditor, M.-T. M. *J. Am. Chem. Soc.* **1989**, *111*, 9261−9262, with Morris, K. N.; Tarasow, T. M.; Julin, C. M.; Simons, S. L.; Hilvert, D.; Gold, L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 13028−13032.

(8) Bashkin, J. K.; Gard, J. K.; Modak, A. S. *J. Org. Chem.* **1990**, *55*, 5125−5132.

(9) Bashkin, J. K.; McBeath, R. J.; Modak, A. S.; Sample, K. R.; Wise, W. B. *J. Org. Chem.* **1991**, *56*, 3168−3176.

(10) Bashkin, J. K.; Sondhi, S. M.; Modak, A. S.; Sampath, U. S.; d'Avignon, D. A. *New J. Chem.* **1994**, *18*, 305−316.

(11) Reynolds, M. A.; Beck, T. A.; Say, P. B.; Schwartz, D. A.; Dwyer, B. P.; Daily, W. J.; Vaghefi, M. M.; Metzler, M. D.; Klem, R. E.; Arnold, L. J. *Nucleic Acids Res.* **1996**, *24*, 760−765.

(12) Dewey, T. M.; Mundt, A. A.; Crouch, G. J.; Zyzniuewski, M. C.; Eaton, B. *J. Am. Chem. Soc.* **1995**, *117*, 8474−8475.

(13) Benner, S. A.; Battersby, T. R.; Eschgfaller, B.; Hutter, D.; Kodra, J. T.; Lutz, S.; Arslan, T.; Baschlin, D. K.; Blattler, M.; Egli, M.; Hammer, C.; Held, H. A.; Horlacher, J.; Huang, Z.; Hyrup, B.; Jenny, T. F.; Jurczyk, S. C.; Konig, M.; von Krosigk, U.; Lutz, M. J.; MacPherson, L. J.; Moroney, S. E.; Muller, E.; Nambiar, K. P.; Piccirilli, J. A.; Switzer, C. Y.; Vogel, J. J.; Richert, C.; Roughton, A. L.; Schmidt, J.; Schneider, K. C.; Stackhouse, J. *Pure Appl. Chem.* **1998**, *70*, 263−266.

(14) Sakthivel, K.; Barbas, C. F. *Angew. Chem., Int. Ed.* **1998**, *37*, 2872−2875.

(15) Perrin, D. M.; Garestier, T.; Helene, C. *Nucleosides Nucleotides* **1999**, *18*, 377−391.

(16) Lee, S.; Mignet, N.; Vyle, J.; Whitton, L.; Grasby, J.; Williams, D. M. *Collect. Symp. Ser.* **1999**, *2*, 35−38.

(17) Matulic-Adamic, J.; Daniher, A. T.; Karpeisky, A.; Haeberli, P.; Sweedler, D.; Beigelman, L. *Bioorg., Med. Chem. Lett.* **2000**, *10*, 1299−1302.

(18) Vaish, N. K.; Fraley, A. W.; Szostak, J. W.; McLaughlin, L. W. *Nucleic Acids Res.* **2000**, *28*, 3316−3322.

10.1021/ja003290s CCC: $20.00 © 2001 American Chemical Society
Published on Web 02/02/2001

dently selected with comparable, or even greater, rate constants.[21,22] For instance, most recently, Joyce and co-workers selected a zinc-dependent RNAse activity by incorporating an imidazole-modified deoxyuridine monomer.[23] Although micromolar (5−30 μM) divalent zinc (in the presence of 1 mM divalent magnesium) was required to give a k_{cat} of 4 min^{-1}, no significant catalytic boost was observed over antecedent, *unmodified* catalysts selected in 2 mM magnesium by the same investigators[24] or over *unmodified* catalysts that have recently been selected to cleave in 100 μM zinc absent of magnesium, as reported by Lu and co-workers.[25] This would raise doubts as to the advantage of using modified nucleotides since comparable rate constants may be obtained in the absence of *any* modification.[26]

Given these observations, one might ask: why use synthetically modified bases when a well-placed divalent magnesium (or zinc) ion can suffice? Moreover, one might also ask: why do modified catalysts, selected in the presence of metals, fail to exhibit significantly higher rate constants than those of independently selected, unmodified counterparts? The answers may be found by considering the practical methodologies used for catalyst selection. Answering these questions may, in turn, help define conditions whereby modifications will boost catalytic rates and indicate a means of evaluating their utility.

Although a diverse set of activities (including peptide synthase,[27] Diels−Alderase,[28] biphenyl-rotamase,[29] and ferrochelatase[30,31]) have been selected, reports of RNAse activities have populated the literature, largely because of the relative ease in selection techniques. As such, RNAse activity may be taken as a "standard" for evaluating k_{cat} values. Traditionally, nucleic acid catalysts (modified or unmodified) that cleave RNA have been selected in the presence of a divalent metal cation to hydrolyze a phosphodiester linkage at a single, internal ribose.[32] Catalytic rate constants generally do not exceed a value of 4 min^{-1}. This value seems to represent an upper-limit, which we contend is simply due to a rather obvious practical constraint: when selecting for an intramolecular catalytic reaction, the selective separation of active strands from inactive strands *in practice* requires roughly 2 min during which time catalysts with first-order (intramolecular) cleavage rates of 4 min^{-1} have cleaved 99.96%.[33] Thus, it is most likely practice (and not theory) that severely limits the extent to which selective pressure may be applied to favor rate constants any higher than 4 min^{-1}. Otherwise, we must entertain an assertion that nucleic acid catalysts are intrinsically and inherently poor catalysts and that a k_{cat} of 4 min^{-1} is the best that may be expected. Our results seem to contradict this latter assertion and suggest that, given the appropriate selection schemes, nucleic acids should be able to exhibit much higher rate constants.

If selection procedures are limited to discovering catalysts with k_{cat}'s in the range of 4 min^{-1}, the presence of divalent metal ions in a selection probably overrides any real advantage of incorporating synthetic modifications and consequently precludes a forthright evaluation regarding the advantages of using modified bases. Thus, it is only in the absence of metals, where the intrinsic rate of intramolecular 2′OH-assisted phosphodiester hydrolysis at ribose is appreciably reduced, that we may hope to effectively address the utility (if any) of selecting modified catalysts. This intrinsic rate was identified by Geyer and Sen who selected and then *re*selected DNAs that catalyzed intramolecular phosphodiester hydrolysis with a k_{cat} of 0.0015 min^{-1} in 0.2 M NaCl in the absence of *both* modified bases *and* divalent metal ions (strictly speaking, intramolecular cleavage was not truly catalytic since no turnover was observed).[34,35] Their work now provides a "benchmark minimum" for evaluating any catalytic boost that might be achieved by the addition of metals, cofactors, or modified bases.

With this in mind, we undertook a selection analogous to theirs using $dA^{im}TP$ (8-(2-(4-imidazolyl)ethylamino)-2′deoxyriboadenosine-5′-triphosphate) and $dU^{aa}TP$ (5-(3-aminoallyl)-2′deoxyribouracil-5′-triphosphate) in the absence of the unmodified substrate analogues dATP and dTTP (Figure 1). Previously, we had described the potential for increasing the chemical diversity of in vitro selected DNA by demonstrating that both $dA^{im}TP$ and $dU^{aa}TP$ satisfied the four conditions for use in combinatorial selections: (1) retention of base-pairing capacity during the iterative cycling of information transfer, (2) substrate activity for at least one DNA polymerase, (3) capability for replacing unmodified counterparts at *every* position specified by the sequence, and (4) capability of transferring genetic information to unmodified cDNA complements during each round of PCR amplification.[36]

In pursuing the selection, we excluded metals *not only* to gauge the utility of such modifications but also to characterize the consequent activity without having to entertain complicating factors such as (i) metal chelation by the modified bases, (ii)

(19) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54−57.

(20) Tarasow, T. M.; Tarasow, S. L.; Tu, C.; Kellogg, E.; Eaton, B. E. *J. Am. Chem. Soc*. **1999**, *121*, 3614−3617.

(21) Seelig, B.; Jaschke, A. *Chem. Biol.* **1999**, *6*, 167−176.

(22) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54−57.

(23) Santoro, S. W.; Joyce, G. F.; Sakthivel, K.; Gramatikova, S.; Barbas, C. F. *J. Am. Chem. Soc*. **2000**, *122*, 2433−2439.

(24) Santoro, S. W.; Joyce, G. F *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262−4266.

(25) Li, J.; Wenchao, Z.; Kwon, A. H.; Lu, Y. *Nucleic Acids Res*. **2000**, *28*, 481−488.

(26) It is difficult to make a direct, side-by-side comparison of the catalytic activity reported in ref 23 (modified) and ref 25 (unmodified). Under optimal conditions for each, k_{cats} are roughly equivalent: The modified catalyst exhibits a k_{cat} of 1.5 min^{-1} when measured in 10 μM Zn^{2+}, 1 mM Mg^{2+}, 150 mM NaCl, 50 mM EPPS pH 7.5, 37 °C (see Figure 4 of ref 23). The unmodified catalyst exhibited a k_{cat} of 1.8 min^{-1} when measured in 100 μM Zn^{2+}, (no Mg^{2+}) 50 mM HEPES pH 7.0, 37 °C (see Figure 2 of ref 25). However, under simulated physiological conditions i.e., 20 μM Zn^{2+}, 2 mM Mg^{2+}, 150 mM NaCl, 50 mM EPPS pH 7.5, 37 °C (differing from those used in the selection), the k_{cat} of the unmodified catalyst was found to be approximately 4−5-fold lower: 0.38 min^{-1}. By this measure, the modified catalyst is superior. Nevertheless, it was deliberately selected under physiological conditions (Mg^{2+} present), whereas the unmodified catalyst was not and deviation from the conditions used during the selection resulted in slightly reduced activity. It is important to note that the modified catalytic motif is four bases shorter than the unmodified one. If size is a criterion where smaller is superior, then a modified catalyst may be desirable.

(27) Wiegand, T. W.; Janssen, R. C.; Eaton, B. *Chem. Biol.* **1997**, *4*, 675−683.

(28) Seelig, B.; Jaschke, A. *Chem. Biol.* **1999**, *6*, 167−176.

(29) Prudent, J. R.; Uno, T.; Schultz, P. G. *Science* **1994**, *264*, 1924−1927.

(30) Conn, M. M.; Prudent, J. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 7012−7013.

(31) Li, Y.; Sen, D. *Nat. Struct. Biol*. **1996**, *3*, 743−747.

(32) Breaker, R.; Joyce, G. F. *Chem. Biol.* **1995**, *2*, 655−660.

(33) The separation method usually consists of an avidin−biotin couple that is employed to retain inactive catalysts on solid-phase avidin, while active ones are collected in the supernatant. Other methods include flow elution from an immobilized avidin on solid support and do not significantly decrease the time for differentiation between inactive (bound) and active (eluted). A catalyst with an intramolecular k_{cat} of 4 min^{-1} has a half-life of 10.35 s. During a 2 min wash, the reaction will have gone 99.96% to completion.

(34) Geyer, C. R.; Sen, D. *Chem. Biol.* **1997**, *4*, 579−593.

(35) On reselection, Geyer and Sen found an improved k_{cat} of 0.006 min^{-1} in 1 M salt (prior to reselection, the initial selection gave a k_{cat} = 0.004 min^{-1}). Linear extrapolation back to 0.2 M NaCl (Figure 2A in the above reference) gives an estimated value for k_{cat} of 0.0015 min^{-1}.

(36) Reference 15.

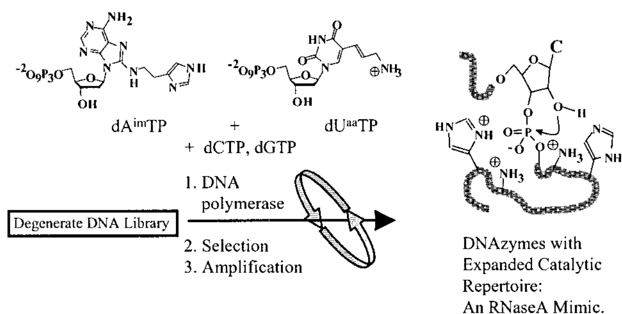


Figure 1. Two synthetic nucleoside triphosphates, one presenting an imidazole, the other a cationic amine, are enzymatically polymerized, in lieu of their unmodified homologues, along a degenerate DNA template comprising 10^{12} sequences. Selection resulted in a divalent metal-independent RNAse activity schematically represented at the right of the figure. The synthesis of dA^{im}TP is given in the Experimental Section.

metal-induced polarization of the phosphate, and (iii) metal activation of the 2′OH. This is the first report to (1) incorporate *two* modified monomers for improved catalytic function and (2) to rigorously evaluate the advantages of such modifications. By excluding metals, we provide evidence that both electrostatic complementarity and general acid/base catalysis may assist cleavage. Although we excluded divalent metals, our results indirectly suggest the potential for developing more robust metalloenzymes and hydrolytic "chemical" nucleases, as well as antigene agents that may be used in a fully physiological context for the control of gene expression (vide infra).

Experimental Section

Extensive details can be found in Supporting Information).

Suppliers. dU^{aa}TP were obtained from Sigma-Aldrich. All oligonucleotides were obtained from Eurogentec (Belgium) and repurified on denaturing 10−20% PAGE. Ultrapure dNTPs and lambda-exo nuclease were obtained from Pharmacia. Sequenase 2.0 and pyrophosphatase were obtained from Amersham. Avidin magnetic particles were obtained from Boerringer-Manheim/Roche. pCR-Script Amp SK(+) Cloning Kit was obtained from Stratagene.

Synthesis of 8-(2-(4-Imidazolyl)ethylamino)-2′deoxyriboadenosine-5′-triphosphate (dA^{im}TP). All syntheses were adapted from established protocols.[37,38] 8-Bromo-dAMP was converted to 8-(2-(4-imidazolyl)ethylamino)-2′deoxyriboadenosine-5′-monophosphate (dA^{im}MP) in the presence of histamine (free base).[39] 8-(2-(4-Imidazolyl)ethylamino)-2′deoxyriboadenosine-5′-monophosphate (dA^{im}MP) was converted to the triphosphate (dA^{im}TP) by standard methods.[40] Prior to the enzymatic incorporations used in the selection, 200 nmol portions of dA^{im}TP were further purified by normal-phase TLC and subsequently applied to an analytical HPLC Nucleosil-RPC4 column (R_f = 5.2 ± 0.1 min, flow: 1 mL/min, gradient 5% → 40% MeCN in 50 mM NH_4OAc pH 6.8). Using the ϵ-max for dA^{im}TP, the phosphate/purine ratio was determined to be 3 from a phosphomolybdate assay at 822 nm (within an error of 5%).[41] MALDI-TOF analysis was performed to ascertain purity: (M−H) calcd 599.0570 obsd 599.0821 (MH$^+$) calcd 601.0727 obsd 601.1339.

Oligonucleotides (Shown 5′3′). **1** biotin-GCGTGCC**rC**GTCTGTTGGGCCC TACCAACA, **2** GAGCTCGCGGGGCGTGCN$_{20}$CTGTTGGTAGGGCCCAACAGA CG, **3** phosphate-CGTCTGTTGGGCCCTACCA, **4** GAGCTCGCGGGGCGTGC, **5** phosphate-ACGACACAGAGCGTGCCCGTCTGTTGGGCCCTACCA, **6** T$_{20}$GA GCTCGCGGGGCGTGC, **7** phosphate-TAATACGACTCACTATAGGGAGCTCGC GGGGCGTGC, **8** T$_9$GAGC TCGCGGGGCGTGCCTTCACTACGGATGAGAACT GTTGGTAGGGCCCAACAGAGGGCACGCTCGTGTCGT, **9** biotin-GCGTGCC**C** GTCTGTTGGGCCCTACCAACA, **10** biotin-GCGTGCC**C$^{2'OMe}$**GTCTGTTGGGCC CTACCAACA. Bold face print at the eighth position indicates a ribose in oligo **1**, a deoxyribose in oligo **9**, and a 2′-*O*-methyl ribose in oligo **10**, respectively.

Buffers. **1** (cleavage): 50 mM sodium cacodylate pH 7.4, 0.2 M NaCl, 1 mM EDTA was then DEPC-treated and autoclaved. **2** (elution): 1% $LiClO_4$/7 mM triethylamine in water. **3** (metals): 50 mM sodium cacodylate pH 7.4, 0.2 M NaCl and 500 μM M^{2+} (M^{2+} = Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) or 5 mM Mg^{2+}. **4** (pH variance) 200 mM NaCl, 1 mM EDTA, and 50 mM A^-/HA where A^-/HA = cacodylate pH 6, HEPES pH 7, cacodylate pH 7.4, Tris pH 8, borate pH 9, glycine pH 9, and pyrophosphate pH 9. The pH of all buffers was adjusted in the presence of 200 mM NaCl. **5** (pre-wash/resuspension): 25 mM sodium cacodylate pH 7.4, 1 mM EDTA.

Preparation of Modified DNA for Selection. Oligo **1** (biotin-GCGTGCC**rC**GTCTGTTGGGCCCTACCAACA), was annealed to template DNA (T$_{20}$GAGCTCGCGGGGCGTGCN$_{20}$CTGTTGGTAGGGCCCAACAGAGGGCACGCTCGTGTCGT prepared by nested PCR from oligos **6** and **7**) and modified DNA was enzymatically polymerized at 37 °C with Sequenase 2.0 in the presence of both modified nucleotides as well as dCTP and dGTP and trace amounts of dGTPα^{32}P. Approximately 7−15 pmol modified DNA was synthesized as determined from back-calculating the specific radioactivity of dGTP and assuming an average 13.5 G's per strand.

Selection of Modified DNAs. Selection followed protocols of Geyer and Sen that had been pioneered by Breaker and Joyce using avidin magnetic particles.[42,43] Particles were resuspended in 250 μL of buffer **1** and incubated for decreasing amounts of time at 37 °C. Following magnetization, the supernatant was removed, precipitated, resuspended, and resolved by 7% 8 M urea denaturing PAGE. The species corresponding to phosphodiester cleavage at ribose was eluted, precipitated, and desalted for eventual PCR amplification.

PCR Synthesis of cDNA Template. Generally, a nested double amplification was used. For the selection, the gel-purified modified DNA was first amplified using oligos 3 and 4 and an internal label, and then purified by sequencing-D-PAGE. An aliquot was then reamplified with a second set of oligos 5 and 6 and treated with lambda exonuclease.[44] A 100−200 pmol single-stranded DNA template was purified by 8% urea mini-PAGE and identified by UV shadowing.

Cloning of cDNAs. The selected modified DNA from the ninth generation was PCR-amplified with oligos **3** and **7** and blunt-end ligated into the pCR-Script vector. Colonies were screened initially by blue/white selection on IPTG/Xgal−LB-Amp plates. Twenty-five white colonies yielding plasmids with only one insert (verified by *Pvu*II digestion) were grown up in 2 mL overnight inoculations, and plasmids were purified by the rapid TELT-procedure in *Current Protocols in Molecular Biology*. Individual plasmids were PCR-amplified with oligos **6** and **7** and cDNA templates prepared and isolated as during the selection. A 5 × 5 matrix of pooled, individually cloned cDNA templates was used to prepare modified DNA that was screened for metal-independent RNAse activity. A single clone (9_{25}-11: ninth generation, 11th clone, selected from 25 clones) was obtained from matrix deconvolution: the column and row that gave the highest activity narrowed the screen to a unique sequence that was fully investigated for activity reported herein. Eurogentec Inc. sequenced the one clone that was found to be most active. A synthetic template (oligo 8) of the same sequence was used to prepare the modified DNA used in the kinetic assays.

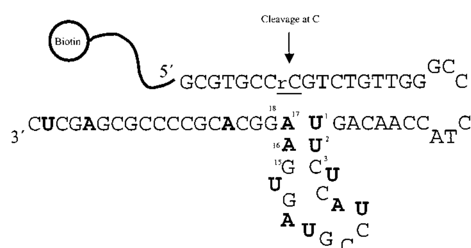
Kinetic Analysis of Intramolecular Cleavage. Following five short washes with 0.2 M NaOH/1 mM EDTA, the avidin-bound modified DNA strands were washed in 2 × 100 μL buffer 5 and then resuspended as a slurry in a volume corresponding to 5 μL/time point. Five microliters of the slurry was then added to polypropylene tubes

(37) Wlassoff, W. A.; Dobrikov, M. I.; Safronov, I. V.; Dudko, R. Y.; Bogachev, V. S.; Kandaurova, V. V.; Shichkin, G. V.; Dymshits, G. M.; Lavrik, O. I. *Bioconjugate Chem.* **1995**, *6*, 352−360.
(38) Trayer, I. P.; Trayer, H. R.; Small, D. P.; Bottomley, R. C. *Biochem. J.* **1974**, *139*, 609−623.
(39) Gillam, I. C.; Tener, G. M. *Nucleosides Nucleotides* **1989**, *8*, 1453−1462.
(40) Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785−1788.
(41) Ames, B. N.; Dubin, D. T. *J. Biol. Chem.* **1960**, *235*, 769−785.
(42) Geyer, C. R.; Sen, D. *Chem. Biol.* **1997**, *4*, 579−593.
(43) Breaker, R.; Joyce, G. F. *Chem. Biol.* **1995**, *2*, 655−660.
(44) Higuchi, R. G.; Ochman, H. *Nucleic Acids Res.* **1989**, *17*, 5865.

(previously coated with calf thymus DNA and SDS) containing 100 μL of buffer 1, 3, or 4. Samples were incubated at 37 °C for varying times; 400 μL 3% $LiClO_4$ in acetone was added to arrest the reaction, and samples were precipitated, washed twice with 400 μL of ethanol, air-dried, and finally resuspended in formamide (containing 20 mM EDTA and 1 mM biotin) and heated at 95 °C just prior to resolution by sequencing 7% urea PAGE.

Results

Both modified nucleoside triphosphates, in lieu of their unmodified analogues, were simultaneously polymerized along a degenerate template (T_{20}GAGCTCGCGGGGCGTG CN_{20}-CTGTTGGTAGGGCCCAACAGAGGGCACGCTCGTGTC-GT) initially containing 20 degenerate positions corresponding to a library of $\leq 10^{12}$ different sequences. Cleaved (active) strands were obtained in the supernatant of 50 mM cacodylate pH 7.4, 200 mM NaCl, and 1 mM EDTA and revealed by the appearance of a shorter product (see Figure 3). Over the course of nine generations, the incubation time, during which the catalyst was to intramolecularly cleave and liberate itself from the avidin magnetic particles, was shortened from 60 to 5 min in an attempt to select for catalysts that would display higher k_{cat} values. Despite the considerable degeneracy observed in the library,[45] the 10th generation DNA was cloned, and the most active clone among 25 screened was isolated and characterized. The sequence and hypothetical 2D map of 9_{25}-11 are shown in Figure 2.



Sequence and 2D structure of clone 9_{25}-11

Figure 2. Sequence and hypothetical 2D structure of clone 9_{25}-11. The construct is biotinylated at the 5′-terminus. The target sequence is derived from the HIV-LTR promoter and contains one target ribose that is underlined. The fixed sequences contain an internal guide sequence that is partially complementary to the target sequence. The first base contained within the degenerate region is denoted by number 1. The last base found in the degenerate region bears number 18. Bold-faced A's indicate the position of imidazoles whereas bold-faced U's indicate the position of cationic allyl-amines. Two modified A's and one modified U are necessarily incorporated within the fixed region as well.

During the selection, the number of bases in the degenerate region dwindled from 20 to 18 despite two PAGE-purification steps that were implemented to ensure the selection of a catalytic domain containing 20 bases. This loss may indicate that evolutionary pressures were operating to favor shorter sequences more in keeping with the 10−15 base motifs elucidated by Santoro and Joyce. Significantly shorter catalytic motifs might have appeared if no selective pressure had been applied by the PAGE purification step to ensure isolation of the correct product.[46]

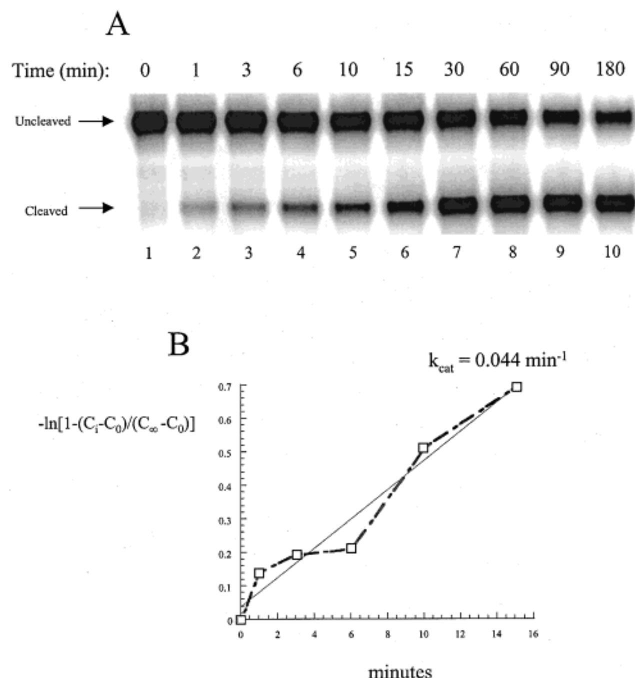


Figure 3. (A) Representative kinetics of intramolecular cleavage. Clone 9_{25}-11 containing both modifications is internally labeled with dGTPα^{32}P. Following isolation on the avidin column, it is resuspended in cleavage buffer (50 mM cacodylate pH 7.4, 200 mM NaCl, and 1 mM EDTA). Cleavage proceeded at 37 °C for noted times before samples are arrested with $LiClO_4$ in acetone, precipitated, denatured in formamide, and resolved by urea PAGE (B) Graphical analysis of the kinetics. The initial rate within the first 1 min approaches a value of approximately 0.1 min^{-1}. The linear fit of the data shown in the natural log/linear plot has a slope corresponding to a k_{cat} of 0.044 min^{-1}.

The first-order rate constant for intramolecular cleavage of the cloned 9_{25}-11 sequence containing four modified adenosines and six modified uridines was measured in 50 mM cacodylate pH 7.4, 200 mM NaCl, 1 mM EDTA, and representative cleavage is shown in Figure 3A. The initial rate constant (measured within the first minute of cleavage) approached a value of 0.1 min^{-1}; however, when measured over longer times, a lower value of 0.044 ± 0.01 min^{-1} was observed (Figure 3B) (three independent measurements on different days with different preparations of modified DNA). Cleavage reached a maximum of approximately 60%, and no further conversion was observed after 90 min. Incomplete cleavage has also been observed by at least two other teams.[47] Geyer and Sen suggested differential folding as a possible explanation for incomplete cleavage.[48] This possibility was discounted in the case of 9_{25}-11 by the following observation: after a 90 min incubation, samples were reheated either at 65 or 95 °C for 2 min, rapidly cooled at 20 °C for 5 min, and then allowed to cleave for an additional 90 min at 37 °C. If the catalyst were to adopt active and inactive conformations in a ratio of 3:2, then reheating would presumably denature the remaining 40% that would, upon renaturation, proportionate into active and inactive conformations resulting in 84% total cleavage after a second 90 min incubation. Although a small increase in cleavage (5−15%) was indeed observed, these experiments suggest that differential folding is probably not the full explanation for incomplete cleavage.

Another explanation may be nonspecific binding to the reaction tubes such that the rate of cleavage diminished over

(45) See Supporting Information.
(46) Santoro, S. W.; Joyce, G. F *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262−4266.
(47) See specifically page 1043 in Carmi, N.; Shultz, L. A.; Breaker, R. R. *Chem. Biol.* **1996**, *3*, 1039−1046.
(48) Geyer, C. R.; Sen, D. *Chem. Biol.* **1997**, *4*, 579−593.

time: following a 30 min incubation, when the 100 μL reaction was aspirated into a pipet, it was noted that up to 30% of the cpm remained on the side of the tube despite having precoated the tube with SDS and denatured calf thymus DNA. Aggregation on the beads also may have occurred. During workup in the kinetic assay, samples were simply precipitated and then resuspended in formamide, which effectively resolubilized nonspecifically bound species. Heating at 95 °C in 1 mM biotin served to dissociate the uncleaved species bound to avidin to quantify cleaved and uncleaved products by PAGE.

Yet, another explanation is that the incorporation of the modified bases may be less than optimal, particularly when using a DNA polymerase that lacks a proofreading activity. As such, any misincorporation event may result in a heterogeneous population containing active, partially active, and completely inactive species. The presence of partially active species that cleave within 3 h may explain why the initial rate constant seems to be greater than the overall rate constant. The presence of inactive species may account for incomplete cleavage[49] and may also explain why no increase in cleavage was seen after the ninth generation.

Such explanations would, if anything, lead to an *underestimation* of k_{cat}, and thus we are cautious when reporting a k_{cat} (intramolecular cleavage constant) value of 0.044 min^{-1}. In any event, the respectable rate constant of clone 9_{25}-11, begged further characterization of the activity with respect to ionic strength, the role of the modifications, divalent metal ions, pH, and the role of the 2′-OH at the labile ribose linkage.

The cleavage rate increased with increasing ionic strength: the extent of cleavage measured at 15 min was half-maximal at 100 mM NaCl and reached a maximum at 200 mM NaCl with no increase observed at 500 mM NaCl (data not shown—see Supporting Information). Subsequently, we wanted to substantiate that the observed catalysis was indeed due to the incorporation of the modified bases. Isosequential strands were polymerized under conditions where one or both modified bases were replaced with the unmodified analogue (i.e., dATP in place of $dA^{im}TP$, or dTTP in place of $dU^{aa}TP$, or simply with dATP and dTTP; dCTP and dGTP were invariably present). Following polymerization, strands were allowed to suffer intramolecular cleavage. Even after 10^4 minutes, no cleavage at ribose was observed. Exogenous addition of 10 units of RNAse A revealed the cleaved fragment within 15 min. This is demonstrated in Figure 4 and would suggest that both modified bases are necessary for catalytic competence.

The activity we report is not affected by 5 mM Mg^{2+}. This is in contrast to the unmodified, metal-independent RNAse activity reported by Geyer and Sen in which case the addition of Mg^{2+} completely inhibited activity. In this work, Ca^{2+}, as with Mg^{2+}, had no effect, whereas Co^{2+} gave mildly inhibitory effects. The inconsequence of the two former divalent metals on activity would suggest the possibility of using the 9_{25}-11 motif to target messenger RNA under physiological conditions. Strikingly however, divalent copper, nickel, and zinc, all of which are known to have high affinity for amines and imidazoles, completely abolished cleavage at 500 μM concentrations. These results are summarized in Figure 5.

Imidazole-bearing nucleosides should afford general acid/base catalysis, a role that cytosine ($pK_a \approx 4.2$) [50] appears to play in

(49) Since the selected strands were eluted after 5 min, any partially active strands cleaving thereafter would not have been amplified in the selection. Any misincorporation event i.e., mutation resulting in loss of activity would not have survived selection since inactive strands are retained on the avidin magnetic particles. Kinetics conducted over 3 h would reveal the presence of partially active (and totally inactive) species that would normally not have survived the stringency of the late-generation selection.

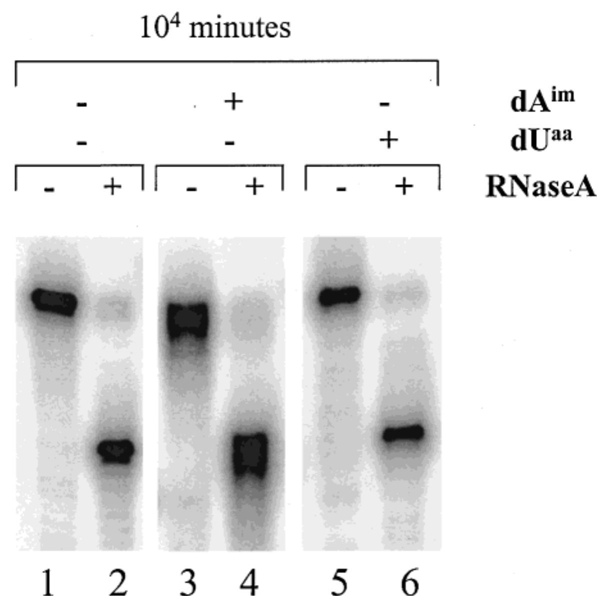


Figure 4. Demonstration of the necessity for both modifications. Lanes 1 and 2 represent clone 9_{25}-11 prepared in the absence of both modified bases, 3 and 4 represent clone 9_{25}-11 prepared in the absence of $dU^{aa}TP$, and 5 and 6 represent clone 9_{25}-11 prepared in the absence of $dA^{im}TP$. No cleavage is observed even after 1 week's time. Lanes 2, 4, and 6 were treated with RNAse A to reveal the cleavage product.

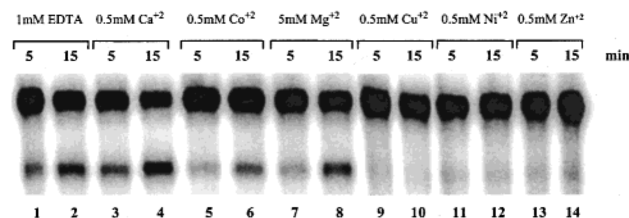


Figure 5. Investigation of the effects of various divalent metal cations. Lanes 1 and 2 are the control lane for 5 and 15 min in the presence of 1 mM EDTA. All other lanes indicate the effects of calcium, cobalt, magnesium, copper, nickel, and zinc. No EDTA is present in lanes 3−14, and all metals were present at 0.5 mM except for magnesium which is present at 5 mM. Cleavage occurs in 50 mM cacodylate pH 7.4, 200 mM NaCl.

divalent metal-independent RNAse activities selected at pH 5.2 (maximum k_{cat} observed at pH 4) as well as in naturally occurring ribozymes.[51−53] In addition, adenosine has been recently implicated in general acid catalysis of peptide synthesis within the ribosome.[54] Nevertheless, to date, no evidence for general acid/base catalysis has been presented either for the aforementioned unmodified and modified, Zn-dependent DNAzymes or for the metal-independent DNAzyme selected by Geyer and Sen. In contrast, 9_{25}-11 shows a pronounced catalytic optimum at pH 7.4 that is entirely consistent with an imidazole pair contributing to general acid/bases catalysis. These results are presented in Figure 6A. The sharp drop in activity from pH 8 to 9 was reexamined in three different buffers (borate, glycine, and pyrophosphate) to exclude the possibility of buffer-specific counterion inhibition (Figure 6B). Although these results strongly suggest a general acid/base catalytic couple with a pK_a

(50) Hartman, K. A.; Rich, A. *J. Am. Chem. Soc.* **1965**, *87*, 2033.
(51) Jayasena, V. K.; Gold, L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10612−10617.
(52) Nakano, S.-i.; Chjadalavada, D. M.; Bevilacqua, P. C. *Science* **2000**, *287*, 1493−1497.
(53) Perrotta, A. T.; Shih, I.-h.; Been, M. *Science* **1999**, *286*, 123−126.
(54) Muth, G. W.; Ortoleva-Donnelly, L.; Strobel, S. A. *Science* **2000**, *289*, 947−950.

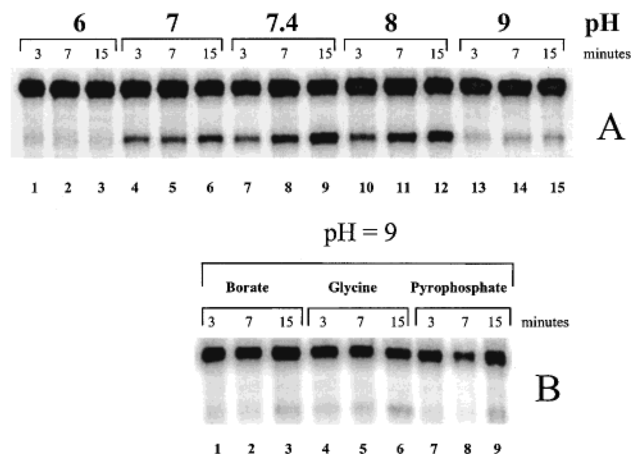


Figure 6. (A) The effects of pH are measured at five different pH values at three time points: 3, 7, and 15 min. Maximum cleavage efficiency is observed at pH 7.4. (B) The cleavage at pH 9 was further examined with three different buffer systems to rule out any buffer-specific counterion inhibition.

tuned to the selected pH of 7.4, the absence of cleavage at elevated pH may also be due to structural denaturation via deprotonation at G residues found in both the internal guide sequence and in the catalytic domain.

Finally, we wanted to implicate the 2′-OH as the nucleophile assisting cleavage. An all-DNA species and a 2′-*O*-methyl species were prepared using oligos 9 and 10, and both were found to be entirely resistant to cleavage (data not shown) indicating that cleavage most likely proceeds via intramolecular 2′OH attack, as in RNAse A.

Discussion and Conclusions

Nucleic acids represent the only polymer where activity is intrinsically encoded. This encoding, along with PCR, permits parallel sampling of exceedingly large (10^{12-17}) numbers of molecules. A "SELEX" approach would seem to represent the "ultimate" in parallel screening—both for ligand binding and for catalysis. Combinatorial selection of nucleic acid catalysts has met with phenomenal success in recent years, in part because direct selection for catalytic activity does not require a stable transition-state analogue. This is particularly advantageous for developing nucleic acid phosphodiesterases that stabilize a pentacoordinate phosphate for which no stable analogue exists; although vanadate has been implicated as a transition-state analogue as it inhibits phosphodiesterase activity, this has been recently questioned.[55] Moreover, even if vanadate esters were to mimic the transition state, they are not sufficiently stable to be used in immunizations or in vitro selections.

Transition-state stabilization is an important question in the development and discovery of catalysts—particularly when the catalyst is selected for affinity to a transition-state analogue. At present, the only example where a nucleic acid catalyst compares favorably with antibody and enzyme catalysts is in the case of a "ferrochelatase" activity where a cationic *N*-methylporphyrin had been employed as the transition-state analogue in an affinity selection for the catalyst.[56,57] In terms of discovering catalysts for one of the most elementary chemical reactions, ester saponification, antibodies have been selected for a high-affinity binding to transition-state analogues composed of phosphate, phosphonate, or phosphoramidate esters that mimic the anionic tetrahedral transition state. Interestingly, structural studies indicate that antibodies assemble lysines and histidines around these anionic transition-state analogues to provide for catalysis.[58,59] Considering that unmodified nucleic acids are entirely devoid of a cationic residue and replete with a negatively charged backbone, it is difficult to imagine how nucleic acids could ever be selected to recognize an anionic transition-state analogue with high affinity at physiological pH. Recent work would corroborate this assertion; an affinity selection for a nucleic acid that recognized a phosphodiester analogue was undertaken to discover an activity that hydrolyzes a carbonate ester only to reveal disappointingly low rate constants: $k_{cat} = 2.1 \times 10^{-5}$ min^{-1} and $k_{cat}/k_{uncat} = 111$.[60] Our work now demonstrates the possibility of delivering a more complete electrostatic complement to nucleic acid catalysts to aid in the recognition of anionic transition-state analogues.

Hijacking imidazoles and cationic amines onto nucleic acids would suggest a means of increasing the catalytic repertoire of nucleic acid catalysts by making them more "protein-like". A notable example of how two imidazoles and two cationic amines deliver excellent rate enhancements is that of RNAse A which exhibits a metal-independent activity with a k_{cat} of 8.4×10^4 min^{-1}.

Nevertheless, in general, modifications have not increased catalytic rates: in the presence of divalent metals, rate constants have been limited to values approaching 4 min^{-1}. Although this limit may have more to do with practical rather than chemical concerns, no evidence exists to show that rate constants of catalytic nucleic acids can indeed surpass what seems to be an upper limit. What is clear is that the presence of divalent metals has made it difficult to properly evaluate the advantage of using modified bases, thus raising questions as to their real utility.

Although a few selections have exploited one modified base, to date, no combinatorial selection (irrespective of divalent metal ion content or reaction type) has successfully used more than one modified nucleotide. To more clearly evaluate any catalytic advantage of incorporating modified bases, we sought to establish a rigorous logic for gauging the catalytic boost that synthetic functionality may deliver. An important premise in this logic is the exclusion of divalent metal cations to establish conditions under which nucleic acids are known to be quite poor catalysts. In this manner, we discovered a ribonuclease activity analogous to that of RNAse A. We further show that multiple incorporation of two modified bases, as intended, appears to deliver both electrostatic complementarity and general acid/base catalysis at pH 7.4. Although the catalytic boost we report is modest, we believe that k_{cat} values may still increase by an additional order of magnitude, or more, to approach the upper limit of 4 min^{-1} imposed by selection practices, provided that subtle changes are made to let strands to explore deletions or expansions.[61] An unmodified, metal-independent catalyst has already attained the practical limit at pH 4 where hemiprotonated cytosine can consequently play the role of general acid/base catalysis.

(55) Messmore, J. M.; Raines, R. T. *J. Am. Chem. Soc.* **2000**, *122*, 9911−9916.
(56) Conn, M. M.; Prudent, J. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 7012−7013.
(57) Li, Y.; Sen, D. *Nat. Struct. Biol.* **1996**, *3*, 743−747.
(58) Kristensen, O.; Vassylyev, D. G.; Tanaka, F.; Morikawa, K.; Fujii, I. *J. Mol. Biol.* **1998**, *281*, 501−511.
(59) Haynes, M. R.; Heine, A.; Wilson, I. A. *Isr. J. Chem.* **1996**, *36*, 133−142.
(60) Chun, S.-M.; Jeong, S.; Kim, J.-M.; Chong, B.-O.; Park, Y.-K.; Park, H.; Yu, J. *J. Am. Chem. Soc.* **1999**, *121*, 10844−10845.
(61) It is important to note that the very first selections of this nature exhibited a k_{cat} value of 0.0025 min^{-1} in 0.25 M NaCl and 1 mM $MgCl_2$ (see ref 32). Changing the selection conditions and increasing the number of generations resulted in much more efficient catalysts (see ref 46). We believe that the same improvement may emanate from the work presented herein.

Our initial findings demonstrate that modified nucleotides can increase k_{cat} values by approximately 25–100 fold over unmodified catalysts—at least where RNA cleavage is concerned. Superimposing this 25–100-fold increase onto the values obtained in metal-dependent selections, it should be possible to obtain k_{cat} values on the order of 250–1000 min^{-1} *provided* that new selection techniques are developed to allow discrimination for the truly fast catalysts ($k_{cat} > 4$ min^{-1}). These techniques might include the use of a stop-flow apparatus equipped with FRET-based fluorescence detection.

Although electrostatic complementarity is more difficult to address in the absence of structure, the absolute necessity of the dU^{aa} for activity suggests that electrostatic stabilization of the anionic transition state is also involved in catalysis. Although a previous selection had exploited a nucleoside presenting a cationic amine to improve phosphate recognition,[62] our work is the first to demonstrate the incorporation of a cationic residue for catalysis and should underscore the potential for discovering other catalysts that deliver electrostatic stabilization of anionic transition states.

We deliberately excluded metals to permit a discrete demonstration of general acid/base catalysis—a feature that has not to date been observed at pH 7–7.4 with combinatorially selected nucleic acids, be they modified or not. It is nevertheless difficult to definitively conclude that two imidazoles are acting in concert as we cannot exclude the possibility that the modified bases act indirectly to properly fold the catalytic domain and perturb the pK_a's of G and C via new H-bonding motifs, electrostatic interactions, and even hydrophobic effects.[63] If the observed activity is in fact due to *unmodified* bases contained within new folding motifs that are promoted by the modified bases, such structures should prove quite interesting as well.

Although we are cautious in our conclusions, it is nonetheless interesting to note that there are two allyl-amines (cations) and two imidazoles in the immediate vicinity of the target ribose (See Figure 2). Could these residues be playing the roles analogous to lysines 7 and 41 and histidines 12 and 119 found in RNAse A?[64] It is tempting to make such an argument; significant enrichment for dU^{aa} in positions 1, 2, and for dA^{im} in positions 16, and 17 was observed by the ninth generation despite a significant degeneracy elsewhere within the random region, suggesting that modifications in these regions are important to the catalytic motif (data not shown—see Supporting Information). It is also interesting to speculate on the putative GC base pair formed by residues 3 and 15? Could this serve to "staple" a minimal catalytic motif containing merely two imidazoles and two cationic amines? If so, selections that impose no constraint on length will most likely confirm this.

In addition, directed synthesis of deletion constructs will aid in establishing the minimum motif required for catalysis. The automated synthesis of the catalyst with duly modified phosphoramidite precursors will help identify which bases play the role of catalysis. Chemical rescue by exogenously added histamine or imidazole in the presence of unmodified adenosines might also provide information as to how many imidazoles contribute to catalytic competence and may help differentiate between catalytic versus structural (folding) motifs.[65] Nevertheless chemical rescue may not definitively separate "catalysis" from "structure" because folding will likely depend on the C1′–N glycosidic bond angle which in turn will depend on the modification; 8-substituted purines often exhibit a marked preference for the *syn* rather than *anti* conformation, which is more readily adopted by their unmodified counterparts.[66] Ongoing characterization will not only involve the aforementioned chemical rescue experiment but will likely require tests with other ethylamino analogues of $dA^{im}TP$ that present subtle modifications such as pyrrole, oxazole, pyrazole, isoxazole, isothiazole, and thiazole cycles.[67]

It is equally significant that neither Mg^{2+} nor Ca^{2+} inhibited cleavage, indicating that 9_{25}-11 might have immediate utility in a physiological context to target the extant HIV proviral sequence GCGTGCCCGTCTGT (see Figure 2), provided that the activity can be directed to cleave *in trans* an all-RNA sequence (only one target ribose was incorporated on an otherwise all-DNA target sequence that cleaved intramolecularly). In terms of increasing the potential for exploiting metals in catalysis, the fact that other metals, notably copper, nickel, and zinc,completely abolished activity at 500 μM suggests that the modified bases, particularly the dA^{im} with its imidazole and guanidine-like functionalities, already provide high-affinity binding sites for such metals. Reselection in the presence of various transition metals will likely result in new classes of highly efficient (possibly heterodinuclear) metalloenzymes.

Given these very promising results, chemical, enzymatic, structural, and pharmacological studies on 9_{25}-11 are underway as is the development of other novel catalysts, gene-delivery reagents, and ligands that exploit this enriched repertoire of functionalities.

Also under consideration is derivatization of both dG and dC to further equip nucleic acid catalysts with nucleophiles and electrophiles. Expanding the catalytic repertoire to include other functionalities must be accompanied by a more thorough synthetic investigation of modified monomer triphosphates that will have no sequence constraints on their incorporation so that sequence space remains maximal.[68]

Modified bases may be used in a combinatorial selection to find irregular motifs that extend triplex recognition beyond the polypurine strand. Either unmodified or modified bases may also be used to select a catalytic activity that selectively deaminates cytosine to uracil or adenine to inosine on targeted RNA (or DNA). This might lead to a "gene modification" strategy (as contrasted with an inactivating antisense/antigene strategy) whereby one might "correct" protein expression, restore wild-type, and introduce transdominant protein function.

Expanding the repertoire of catalytic activities that relate to synthesis/biosynthesis is also a paramount goal. DNA should be recognized as a coding scaffold for combinatorial chemistry and as a material for catalyzing synthetically useful reactions. Unmodified nucleic acids should effectively catalyze polyene condensations/cyclizations—a remarkable class of reactions that proceed via cationic transition states at physiological pH. On the other hand, modified bases should enhance the catalysis of Aldol and Claisen condensations where the cationic amine should be able to form a Schiff's base with the donor, while imidazoles will chelate metals to afford appropriate polarization

(62) Battersby, T. R.; Ang, D. N.; Burgstaller, P.; Jurczyk, S. C.; Bowser, M. T.; Buchanan, D. D.; Kennedy, R. T.; Benner, S. B. *J. Am. Chem. Soc.* **1999**, *121*, 9781–9789.

(63) Kirby, A. J.; Marriott, R. E. *J. Am. Chem. Soc.* **1995**, *117*, 833–834.

(64) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Company: New York, 1977; pp 426–32.

(65) Roth, A.; Breaker, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6027–6031.

(66) Birnbaum, G. I.; Shugar, D. *Biochim. Biophys. Acta* **1978**, *517*, 500–510.

(67) Tarasow, T. M.; Tarasow, S. L.; Tu, C.; Kellogg, E.; Eaton, B. E. *J. Am. Chem. Soc.* **1999**, *121*, 3614–3617.

(68) On discrete template strands presenting isosequential T's, we found that polymerases would incorporate at least four and up to seven contiguous $dA^{im}TP$'s. After the incorporation of the fourth base, yields dropped off precipitously (unpublished observations).

of the acceptor. Selection of nucleic acid catalysts capable of aryl-ether and acetylene coupling, for which transition-state analogues are difficult to design, is an added possibility.

Acknowledgment. We thank Dr. Alain Blond for NMR characterization and Dr. Jean-Pierre Le Caer for mass spectroscopic characterization of $dA^{im}TP$. D.M.P. thanks Dr. Natalie Mignet and Professors Steven Benner and Michael Jung for encouragement. D.M.P. was supported as a postdoctoral fellow by The National Science Foundation of the United States of America, The North Atlantic Treaty Organization (NATO), The French Government's "Chateaubriand" Fellowship Program, and by a postdoctoral grant from the French AIDS Foundation: A.N.R.S - l'Association Nationale pour la Recherche Contre le SIDA.

Note Added in Proof. The reader's attention is directed to two highly germane references that have appearaed in print since the submission of this work: (i) In Vitro Selection of a Ligase Ribozyme Carrying Alkylamino Groups in the Side Chains. Teramoto, N.; Imanishi, Y.; Ito, Y. *Bioconjugate Chem.* **2000**, *11*(6), 744−48; (ii) In Vitro Evolution of the Hammerhead Ribozyme to a Purine-specific Ribozyme using Mutagenic PCR with Two Nucleotide Analogues. Kore, A. R.; Vaish, N. K.; Morris, J. A.; Eckstein, F. *J. Mol. Biol.* **2000**, *301*(5), 1113−1121.

Note Added after ASAP Posting

This article was released ASAP on 2/2/2001 with an error in the contribution line. The correct version was posted on 2/21/2001.

Supporting Information Available: Data regarding the dependence of cleavage on ionic strength and sequencing of the degenerate library as well as a detailed synthetic procedure for the synthesis of $dA^{im}TP$ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA003290S