J|A|C|S
COMMUNICATIONS

Published on Web 04/10/2007

Polymerase-Directed Synthesis of 2′-Deoxy-2′-fluoro-β-D-arabinonucleic Acids

Chang G. Peng and Masad J. Damha*

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC, Canada H3A 2K6

Received December 19, 2006; E-mail: masad.damha@mcgill.ca

Systematic evolution of ligands by exponential enrichment (SELEX)[1,2] is a powerful method for generating oligonucleotide aptamers with therapeutic utility, particularly when modified nucleoside 5′-triphosphates (NTPs) are used for the selection of aptamers with nuclease-resistant capability.[3] Two key requirements must be met for the effective utilization of modified NTPs in the SELEX process; that is, polymerases must recognize and incorporate the modified nucleoside 5′-triphosphates as building blocks with high fidelity, and the resulting products must be faithfully amplified into cDNA libraries. In addition to the most commonly used 2′-fluoro and 2′-amino rNTPs,[3] only a handful of other modified rNTPs[4−7] and even fewer dNTPs[8] are available for aptamer selection.

2-Fluoro-D-arabinose confers a DNA-like (south/east) conformation[9] to the oligonucleotide while rendering it more nuclease resistant.[10] In addition, 2-fluoro-D-arabinose raises the T_m of duplexes (ca. +0.5 to 1 °C/nt),[11] triplexes (ca. +0.8 °C/nt),[12] and C-rich (ca. +1 °C/nt, pH < 4.0),[13] and G-rich tetraplexes (ca. +2 °C/nt). (Peng, C.-G.; Damha, M. J., unpublished results.) Furthermore, DNA or RNA containing FANA units exhibit gene silencing efficacy (via mRNA targeting) in the low nanomolar range.[14] For all of these reasons, we hypothesized that FANA-modified strands could also have considerable potential as aptamers, and we sought to test this hypothesis by first examining the ability of 2′-deoxy-2′-fluoro-β-D-arabinonucleoside 5′-triphosphates (2′F-araNTPs) to serve as substrates for various DNA polymerases, thereby rendering these modified NTPs amenable to DNA aptamer selection. To our knowledge, there are no reports on the biosynthesis of FANA in vitro, though 2′F-araNTP derivatives have been studied as potential chain terminators for antiviral and anticancer applications[15] and positron emission tomography.[16]

We adapted primer extension assays[7,17] to first screen various DNA polymerases[18] for their ability to incorporate 2′F-araNTPs (N = A, G, T, and C, Figure 1). Family B polymerases (DV, 9N, Th, Ph) effectively incorporated all four 2′F-araNTPs to yield full-length FANA products (Figure S2, Supporting Information), whereas family A polymerases (*Bst*, *Taq*, KF) and MMLV generated premature products containing only two to four 2′F-araNTP residues (for names of enzymes, see note 19). The divergence in DNA polymerase classification and structure[18] and NTP discrimination has been observed with other modified nucleotides, for example, ddNTPs and acyclic NTPs,[20] suggesting that significant differences exist in the active site of these two families (A and B) of enzymes.

Performing SELEX using modified NTPs requires necessary fidelity in the polymerization reaction so that the selection of functional sequences can be faithfully maintained and enriched from one round to another. Thus, we next conducted "dropout assays"[21] to assess *apparent* fidelity of 2′F-araNTP incorporation by DV and 9N. In one of these assays, 2′F-araATP was removed from the pool, and the ensuing synthesis assessed in comparison with a control reaction containing all four 2′F-araNTPs. For comparison, dropout experiments containing dNTPs were run in parallel and the results obtained are shown in Figure 2. Full-length DNA and FANA products were obtained when all four dNTPs (groups 1 and 5) and

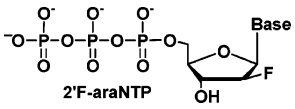

Figure 1. Chemical structure of 2′F-araNTPs (*N* = A, G, T, and C).

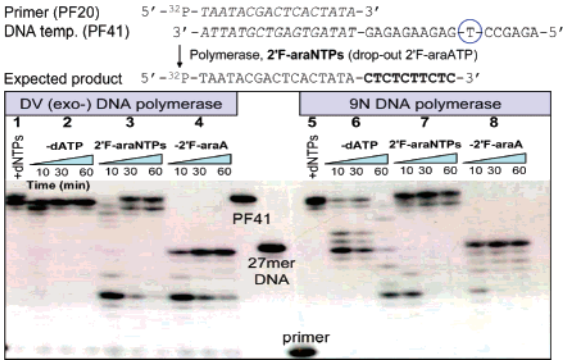

Figure 2. Fidelity study on 2′F-araATP incorporation by DV and 9N DNA polymerases on DNA template PF41. Experimental details about this dropout assay are provided in the Supporting Information. Reaction conditions: 0.8 unit DV and 9N, 25 μM triphosphates at 55 °C in 30 μL of reaction volume; incubation time, 30 min. Groups 1 and 5, dNTPs; groups 2 and 6, dropout of dATP; groups 3 and 7, 2′F-araNTPs; groups 4 and 8, dropout of 2′F-araATP; 27mer DNA is PF39 in Table S1, Supporting Information.

2′F-araNTPs (groups 3 and 7) were available, although some pausing was evident during synthesis of the arabinose modified oligomers. Compared with dATP, both DV and 9N exhibited excellent selectivity on 2′F-araATP, as demonstrated by efficient chain termination at the position where 2′F-araATP was required (groups 4 and 8). In contrast, dropping out dATP in these assays (groups 2 and 6) afforded full-length in addition to premature products (Table 1), suggesting that 2′F-araATP is more strictly selected than dATP under these conditions.

Next, we applied the same dropout experiments to the remaining three triphosphates, namely, 2′F-araGTP, 2′F-araCTP, and 2′F-araTTP (Table 1; Figures S3−S4, Supporting Information). Again, DV and 9N demonstrated higher fidelity with respect to 2′F-araGTP incorporation relative to the native dGTP. Selectivity toward the pyrimidine-based 2′F-araNTPs was significantly less, but the same was true for the corresponding pyrimidine-containing dNTPs. Factors governing fidelity of base substitutions by polymerases are complex and not completely understood.[22]

Preferential geometry,[23] sugar pucker,[24] and topology and size of the polymerase-active site[23] are all important considerations. In addition, it has also been suggested that dropout essays underestimate the fidelity of base substitution because there would be more opportunity for NTP misincorporation when the normal competition between the correct and incorrect NTP is lacking in these experiments.[21] Regardless of the mechanisms that operate, the selectivity for 2′F-araNTP incorporation by DV and 9N polymerases appears to be comparable, if not better, than that of dNTPs.

Next, we examined Phusion DNA polymerase (Ph), an enzyme with higher processing capacity and fidelity compared to other

10.1021/ja069100g CCC: $37.00

Table 1. Apparent Fidelity of FANA Synthesis in Dropout Assays

		% apparent fidelity[a]			
polymerase	2′F-araNTP		dNTP	template used	figure
Ph	A:	>99%	>99%	PF21	3
	G:	>99%	>99%		
	T:	>99%	>99%		
	C:	>99%	>99%		
DV	A:	>99%	1%	PF41	2
	G:	>99%	62%	PF43	S3
	T:	3%	19%	PF21	S4
	C:	50%	3%	PF23	S4
9N	A:	>99%	80%	PF41	2
	G:	98%	84%	PF43	S3
	T:	76%	32%	PF21	S4
	C:	43%	82%	PF23	S4

[a] Apparent fidelity is calculated according to the equation 1 − [(% full-length-2′F-araNTP)/ (% full-length+2′F-araNTP)] at 30 min reaction time (sample equation for dNTP values); >99% means that the full-length product is not detectable in our dropout assays.

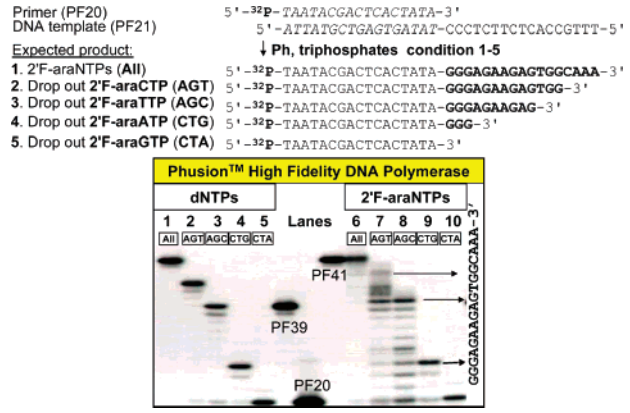

Figure 3. Fidelity study on 2′F-araNTP incorporation by Ph on DNA template PF21. Reaction conditions: 0.8 unit Ph, 25 μM triphosphate at 55 °C in 30 μL of reaction volume for 30 min. Lane 1, dNTPs; lane 2, drop out dCTP (i.e., dATP, dGTP, and dTTP, shorten as AGT; same coding for lanes 3−5), lane 6, 2′F-araNTPs; lane 7, drop out 2′F-araCTP (i.e., 2′F-araATP, 2′F-araGTP, 2′F-araTTP, shorten as AGT; same coding for lanes 8−10). Template PF41 and primer PF20 are shown; PF39 is a 27-nt DNA control.

thermophilic polymerases (e.g., the error rates for Ph[25] and DV[26] are 4.4×10^{-7} and 2.2×10^{-4}, respectively). All four dropout experiments were conducted with the same DNA template PF21 (Figure 3), or PF41 (Figure S5, Supporting Information). For the dNTPs, syntheses stopped at the expected termination sites, yielding products lacking the required dNTP at the 3′-terminus. A similar pattern was observed with 2′F-araNTPs, although in the latter, FANA synthesis proceeded with more difficulty (Figure 3; lanes 6−10). This effect, however, was observed only in the dropout assays, that is, the reaction containing all four 2′F-araNTPs produced excellent yields of the full-length FANA product with virtually no pausing observed. We speculate that in a dropout assay, where polymerization activity is suboptimal, the pausing bands observed may result, at least in part, by the shuttling (cycling) between the strong Ph 3′-5′ exonuclease and polymerase activities.[18,27] Similar results were observed with the DNA template PF41 (Figure S5). Of note, apparent fidelity among Ph, DV, and 9N is correlated to 3′-5′ exonuclease proofreading activity, with Ph having the strongest exonuclease activity and also 2′F-araNTP fidelity, and DV having no exonuclease activity and low 2′F-fidelity. We recently became aware of Wengel's work that Ph can incorporate a few LNA triphosphates in a growing DNA strand.[28] Thus, FANA and LNA NTPs appear to be the first examples of modified NTPs incorporated by Ph.

We are currently testing the limits of these procedures by exploring the synthesis of (a) DNA−FANA on a DNA−FANA template and (b) DNA on an all-FANA template. Preliminary results obtained indicate that DV, 9N, and Ph can synthesize FANA−DNA strands on FANA−DNA templates (Figure S6, Supporting Information), and that KF and *Bst* DNA can catalyze FANA template-directed DNA synthesis (Figure S7, Supporting Information). While we have not been able to synthesize an all-FANA strand on an all-FANA template, it is possible for DV, 9N, and Ph polymerase to drive the formation of multiple FANA−FANA base pairs within a DNA−FANA chimeric duplex (Figure S6; Supporting Information).

In summary, family B thermophilic DNA polymerases such as DV, 9N, and Ph can utilize 2′F-araNTPs, or a combination of 2′F-araNTPs and dNTPs, to generate FANA or chimeric FANA−DNA strands, respectively. In addition, these polymerases were shown to synthesize chimeric FANA−DNA strands on a chimeric FANA−DNA template. KF and *Bst* (family A) DNA polymerases were able to incorporate dNTPs on a template FANA strand. These results suggest that it should be possible to evolve FANA-modified aptamers via SELEX.

Acknowledgment. This work was supported by the Canadian Institutes of Health Research and Topigen Pharmaceuticals, Inc. Dedicated to Prof. Kelvin K. Ogilvie on the occasion of his 65th birthday.

Supporting Information Available: Experimental details and gel pictures (Figures S2−S7). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818−822.
(2) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505−510.
(3) Ruckman, J.; Green, L. S.; Beeson, J.; Waugh, S.; Gillette, W. L.; Henninger, D. D.; Claesson-Welsh, L.; Janjic, N. *J. Biol. Chem.* **1998**, *273*, 20556−20567.
(4) Lato, S. M.; Ozerova, N. D. S.; He, K.; Sergueeva, Z.; Shaw, B. R.; Burke, D. H. *Nucleic Acids Res.* **2002**, *30*, 1401−1407.
(5) Jhaveri, S.; Olwin, B.; Ellington, A. D. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2285−2290.
(6) Dineva, M. A.; Ivanov, I. G.; Petkov, D. D. *Nucleosides Nucleotides* **1997**, *16*, 1875−1882.
(7) Kato, Y.; Minakawa, N.; Komatsu, Y.; Kamiya, H.; Ogawa, N.; Harashima, H.; Matsuda, A. *Nucleic Acids Res.* **2005**, *33*, 2942−2951.
(8) Kuwahara, M.; Hanawa, K.; Ohsawa, K.; Kitagata, R.; Ozaki, H.; Sawai, H. *Bioorg. Med. Chem.* **2006**, *14*, 2518−2526.
(9) Trempe, J.-F.; Wilds, C. J.; Denisov, A. Y.; Pon, R. T.; Damha, M. J.; Gehring, K. *J. Am. Chem. Soc.* **2001**, *123*, 4896−4903.
(10) Kalota, A.; Karabon, L.; Swider, C. R.; Viazovkina, E.; Elzagheid, M.; Damha, M. J.; Gewirtz, A. M. *Nucleic Acids Res.* **2006**, *34*, 451−461.
(11) Wilds, C. J.; Damha, M. J. *Nucleic Acids Res.* **2000**, *28*, 3625−3635.
(12) Wilds, C. J.; Damha, M. J. *Bioconjug. Chem.* **1999**, *10*, 299−305.
(13) Wilds, C. J. Ph.D. Thesis, McGill University, Montreal, Canada, 2000.
(14) Dowler, T.; Bergeron, D.; Tedeschi, A. L.; Paquet, L.; Ferrari, N.; Damha, M. J. *Nucleic Acids Res.* **2006**, *34*, 1669−1675.
(15) Wright, G. E.; Brown, N. C. *Pharmacol. Ther.* **1990**, *47*, 447−97.
(16) Shields, A. F. *Mol. Imaging Biol.* **2006**, *8*, 141−150.
(17) Chaput, J. C.; Szostak, J. W. *J. Am. Chem. Soc.* **2003**, *125*, 9274−9275.
(18) Steitz, T. A. *J. Biol. Chem.* **1999**, *274*, 17395−17398.
(19) DNA polymerases abbreviations: deep vent (3′-5′ exo-), DV; $9^{o}N_{m}$, 9N; Therminator, Th; *Bst* large fragment, *Bst*; *Taq*, *Taq*; Phusion high-fidelity, Ph; Klenow fragment (3′-5′ exo-), KF; Moloney Murine leukemia virus reverse transcriptase, MMLV.
(20) Gardner, A. F.; Jack, W. E. *Nucleic Acids Res.* **2002**, *30*, 605−613.
(21) IIchida, J. K.; Zou, K.; Horhota, A.; Yu, B.; McLaughlin, L. W.; Szostak, J. W. *J. Am. Chem. Soc.* **2005**, *127*, 2802−2803.
(22) Kunkel, T. A. *J. Biol. Chem.* **2004**, *279*, 16895−16898.
(23) Kool, E. T. *Annu. Rev. Biochem.* **2002**, *71*, 191−219.
(24) Marquez, V. E.; Ezzitouni, A.; Russ, P.; Siddiqui, M. A.; Ford, H., Jr.; Feldman, R. J.; Mitsuya, H.; George, C.; Barchi, J. J., Jr. *J. Am. Chem. Soc.* **1998**, *120*, 2780−2789.
(25) NEB manufacturer's instruction.
(26) Huang, H. X.; Keohavong, P. *DNA Cell Biol.* **1996**, *15*, 589−594.
(27) Kunkel, T. A.; Bebenek, K. *Annu. Rev. Biochem.* **2000**, *69*, 497−529.
(28) Veedu, R. N.; Vester, B.; Wengel, J. *Conference Book*; Haenni, D. L., Ed.; International Roundtable on Nucleosides, Nucleotides and Nucleic Acids: Bern, Germany, 2006; p 292.

JA069100G