

Convergent and General One-Step DNA-Catalyzed Synthesis of Multiply Branched DNA

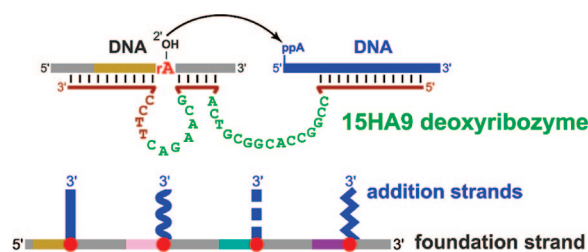
Timothy P. Mui and Scott K. Silverman*

Department of Chemistry, University of Illinois at Urbana–Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

scott@scs.uiuc.edu

Received July 10, 2008

ABSTRACT



We report a deoxyribozyme (DNA enzyme) that catalyzes the convergent and general synthesis of branched DNA. The 15HA9 deoxyribozyme mediates nucleophilic attack of the 2'-hydroxyl group of a ribonucleotide embedded within one DNA substrate into a 5'-adenylate of the second DNA substrate. This approach can be used to synthesize multiply branched DNA with a wide range of DNA sequences.

Covalently branched DNA molecules have a single nucleotide that is connected to three or more different DNA oligonucleotide strands.¹ The attachment point for the third strand may be the 2'-OH of an embedded ribonucleotide as shown in Figure 1A,² although in practice many other connection strategies have been used.^{3,4} Solid-phase synthesis approaches have typically been employed to prepare branched DNA.⁵ However, such methods are nonconvergent and relatively tedious, and they are inherently limited regarding the size of the final branched product. Qualitatively new synthetic approaches to branched DNA are desirable.⁶ In

(1) Covalently branched DNA is distinct from the branched DNA of DNA-based nanotechnology, which uses only linear DNA strands that Watson–Crick hybridize with strand exchange to form multihelix junctions (Seeman, N. C. *Mol. Biotechnol.* **2007**, *37*, 246–257). In the present report, “branched DNA” refers solely to covalently branched DNA.

(2) (a) Grötl, M.; Eritja, R.; Sproat, B. *Tetrahedron* **1997**, *53*, 11317–11346. (b) Damha, M. J.; Braich, R. S. *Tetrahedron Lett.* **1998**, *39*, 3907–3910. (c) Carriero, S.; Damha, M. J. *Org. Lett.* **2003**, *5*, 273–276. Carriero, S.; Damha, M. J. *J. Org. Chem.* **2003**, *68*, 8328–8338. (e) Mendel-Hartvig, M.; Kumar, A.; Landegren, U. *Nucleic Acids Res.* **2004**, *32*, e2.

(3) (a) Horn, T.; Urdea, M. S. *Nucleic Acids Res.* **1989**, *17*, 6959–6967. (b) Horn, T.; Chang, C. A.; Urdea, M. S. *Nucleic Acids Res.* **1997**, *25*, 4835–4841. (c) Horn, T.; Chang, C. A.; Urdea, M. S. *Nucleic Acids Res.* **1997**, *25*, 4842–4849.

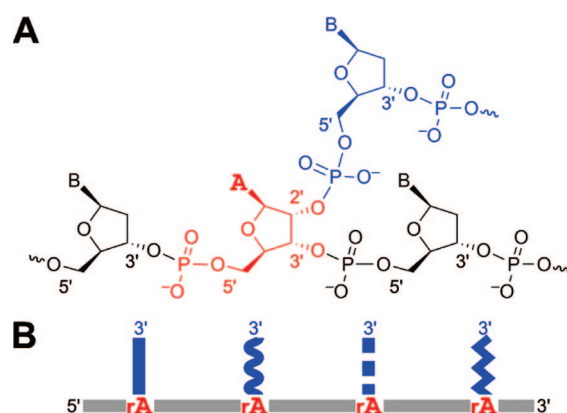


Figure 1. Covalently branched DNA. (A) Branch-site adenosine ribonucleotide (rA, red) embedded within a DNA strand (B = base). (B) Generic structure of multiply branched DNA. The vertical “addition strands” (blue) represent different DNA sequences appended to the common “foundation strand” (gray).

particular, multiply branched DNA (Figure 1B) is a valuable synthetic target because the increased surface density of

immobilized DNA strands should foster improved analytical properties.^{3,7}

Deoxyribozymes (also called DNA enzymes or DNAzymes) are DNA molecules with specific catalytic activities⁸ that are identified by in vitro selection.⁹ Our laboratory has reported several deoxyribozymes that synthesize 2',5'-branched RNA,¹⁰ which is the linkage formed during natural pre-mRNA splicing.¹¹ As exemplified by a particular DNA enzyme named 7S11 (Figure 2A),^{10c,d} the ligation reaction proceeds via attack of an internal 2'-OH group of one RNA substrate into the 5'-triphosphate of the second RNA substrate. The two oligonucleotide substrates are bound by Watson–Crick base pairs to the deoxyribozyme, which catalyzes the ligation reaction via a detailed mechanism that has not yet been elucidated. Neither 7S11 nor related deoxyribozymes can accept a 2'-OH donor substrate that comprises DNA rather than RNA. This failure may be due simply to the requirements of the individual deoxyribozymes that were tested, or it may suggest a greater inherent difficulty when the substrate is DNA. In either case, prior to the current efforts branched DNA could not be synthesized by a deoxyribozyme.

In this study, we performed in vitro selection to identify new deoxyribozymes that synthesize branched DNA. The iterated selection procedure was essentially the same as we have previously reported,^{10,12} with two changes to the key selection step in which branch formation takes place. (1) The “left-hand” (L) substrate was DNA rather than RNA, with a

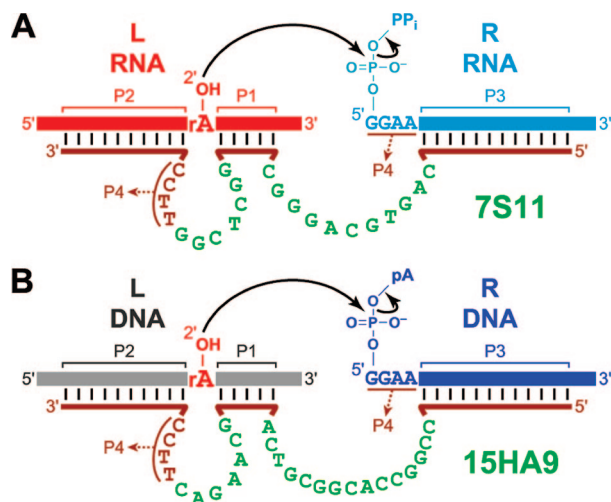


Figure 2. Deoxyribozymes for synthesis of branched nucleic acids. (A) The previously reported 7S11 deoxyribozyme, which synthesizes 2',5'-branched RNA.^{10c,d} (B) The new 15HA9 deoxyribozyme that synthesizes branched DNA.

single embedded ribonucleotide. (2) The “right-hand” (R) substrate was 5'-adenylated DNA rather than 5'-triphosphorylated RNA. The L substrate was prepared by solid-phase synthesis with a strategically located riboadenosine (rA) nucleotide that provides the 2'-OH nucleophile for branch formation. The R substrate was prepared by solid-phase synthesis as a 5'-phosphorylated oligonucleotide that was subsequently 5'-adenylated using ATP and T4 DNA ligase via our reported procedure.¹³ The nucleotide sequences of the L and R substrates were equivalent to those we have used previously.^{10,12} During the key step of each selection round, in which L and R become ligated, the incubation conditions were 50 mM CHES, pH 9.0, 40 mM MgCl₂, 150 mM NaCl, and 2 mM KCl at 37 °C for 2 h.

Ligation activity of the deoxyribozyme pool was detectable (0.9%) by round 9 and reached 17% by round 15.¹⁴ Individual deoxyribozymes were cloned and assayed for catalytic activity. One particular deoxyribozyme, named 15HA9 according to our laboratory's systematic nomenclature, showed promising activity and was prepared by solid-phase synthesis (Figure 2B). Although the [Mg²⁺] was 40 mM during selection, the *K*_{d,app} for Mg²⁺ was ca. 160 mM (data not shown), and 120 mM Mg²⁺ was used in all subsequent experiments.

The 7S11 and related deoxyribozymes have four Watson–Crick paired regions denoted P1–P4 between the deoxyribozyme and its RNA substrates (Figure 2A).^{10c,d} The selection strategy used to identify 15HA9 was designed to include the analogous paired regions at the outset of selection, and these regions are found within 15HA9 (Figure 2B). Detailed tests were performed to determine the extent to which 15HA9 tolerates sequence changes in its DNA

(4) (a) Shchepinov, M. S.; Udalova, I. A.; Bridgman, A. J.; Southern, E. M. *Nucleic Acids Res.* **1997**, *25*, 4447–4454. (b) Scheffler, M.; Dorenbeck, A.; Jordan, S.; Wustefeld, M.; von Kiedrowski, G. *Angew. Chem., Int. Ed.* **1999**, *38*, 3311–3315. (c) Eckardt, L. H.; Naumann, K.; Pankau, W. M.; Rein, M.; Schweitzer, M.; Windhab, N.; von Kiedrowski, G. *Nature* **2002**, *420*, 286. (d) Endo, M.; Majima, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 5744–5747. (e) Endo, M.; Majima, T. *J. Am. Chem. Soc.* **2003**, *125*, 13654–13655. (f) Stewart, K. M.; McLaughlin, L. W. *J. Am. Chem. Soc.* **2004**, *126*, 2050–2057. (g) Ogasawara, S.; Fujimoto, K. *ChemBioChem* **2005**, *6*, 1756–1760. (h) Chandra, M.; Keller, S.; Gloeckner, C.; Bornemann, B.; Marx, A. *Chem.—Eur. J.* **2007**, *13*, 3558–3564.

(5) Damha, M. J.; Zabarylo, S. *Tetrahedron Lett.* **1989**, *30*, 6295–6298. (6) Watson, K. J.; Park, S. J.; Im, J. H.; Nguyen, S. T.; Mirkin, C. A. *J. Am. Chem. Soc.* **2001**, *123*, 5592–5593.

(7) (a) Collins, M. L.; Irvine, B.; Tyner, D.; Fine, E.; Zayati, C.; Chang, C.; Horn, T.; Ahle, D.; Detmer, J.; Shen, L. P.; Kolberg, J.; Bushnell, S.; Urdea, M. S.; Ho, D. *Nucleic Acids Res.* **1997**, *25*, 2979–2984. (b) Flagella, M.; Bui, S.; Zheng, Z.; Nguyen, C. T.; Zhang, A.; Pastor, L.; Ma, Y.; Yang, W.; Crawford, K. L.; McMaster, G. K.; Witney, F.; Luo, Y. *Anal. Biochem.* **2006**, *352*, 50–60. (c) Gibbs, J. M.; Park, S. J.; Anderson, D. R.; Watson, K. J.; Mirkin, C. A.; Nguyen, S. T. *J. Am. Chem. Soc.* **2005**, *127*, 1170–1178. (d) Gibbs-Davis, J. M.; Schatz, G. C.; Nguyen, S. T. *J. Am. Chem. Soc.* **2007**, *129*, 15535–15540. (e) Stepp, B. R.; Gibbs-Davis, J. M.; Koh, D. L.; Nguyen, S. T. *J. Am. Chem. Soc.* **2008**, *130*, 9628–9629.

(8) (a) Peracchi, A. *ChemBioChem* **2005**, *6*, 1316–1322. (b) Baum, D. A.; Silverman, S. K. *Cell. Mol. Life Sci.* **2008**, *65*, 2156–2174. (c) Silverman, S. K. *Chem. Commun.* **2008**, 3467–3485.

(9) Joyce, G. F. *Annu. Rev. Biochem.* **2004**, *73*, 791–836.

(10) (a) Wang, Y.; Silverman, S. K. *J. Am. Chem. Soc.* **2003**, *125*, 6880–6881. (b) Wang, Y.; Silverman, S. K. *Biochemistry* **2003**, *42*, 15252–15263. (c) Coppins, R. L.; Silverman, S. K. *Nat. Struct. Mol. Biol.* **2004**, *11*, 270–274. (d) Coppins, R. L.; Silverman, S. K. *J. Am. Chem. Soc.* **2005**, *127*, 2900–2907. (e) Pratico, E. D.; Wang, Y.; Silverman, S. K. *Nucleic Acids Res.* **2005**, *33*, 3503–3512. (f) Wang, Y.; Silverman, S. K. *Angew. Chem., Int. Ed.* **2005**, *44*, 5863–5866. (g) Zelin, E.; Wang, Y.; Silverman, S. K. *Biochemistry* **2006**, *45*, 2767–2771.

(11) Peebles, C. L.; Perlman, P. S.; Mecklenburg, K. L.; Petrillo, M. L.; Tabor, J. H.; Jarrell, K. A.; Cheng, H. L. *Cell* **1986**, *44*, 213–223.

(12) Flynn-Charlebois, A.; Wang, Y.; Prior, T. K.; Rashid, I.; Hoadley, K. A.; Coppins, R. L.; Wolf, A. C.; Silverman, S. K. *J. Am. Chem. Soc.* **2003**, *125*, 2444–2454.

(13) Patel, M. P.; Baum, D. A.; Silverman, S. K. *Bioorg. Chem.* **2008**, *36*, 46–56.

(14) See the Supporting Information for experimental details.

substrates; a sufficient degree of such tolerance is necessary for the general utility of the deoxyribozyme. We systematically examined nucleotide changes to the P1–P4 regions, making suitable alterations in the 15HA9 binding arms (brown in Figure 2B) to retain Watson–Crick complementarity to the altered substrates. Via this approach, we determined the generality of 15HA9 with regard to its substrate sequences, including its practical utility for synthesizing branched DNA of significantly varying sequence.¹⁴

The 20-mer L substrate has the P1 and P2 regions (Figure 2B). The P2 portion of L was altered systematically from the parent (Par) sequence via either transitions (Tsn; A↔G, C↔T), transversions-1 (Tv1; A↔C, G↔T), or transversions-2 (Tv2; A↔T, G↔C), and the corresponding Watson–Crick changes were made in the appropriate 15HA9 binding arm. In all cases, substantial ligation activity was retained; indeed, the rate constant was as much as 3-fold higher with the variant L substrates.¹⁴ Therefore, 15HA9 is likely to be general for branch formation with any sequence in the P2 region. In contrast, the P1 region did not tolerate sequence changes. Systematic variations were also made in the 17-mer R substrate to determine the P3 and P4 generality (in all cases, the 5'-G of R was retained). The P3 region could have any sequence after its first two nucleotides while maintaining useful ligation rate and yield. All three nucleotides of P4 other than the 5'-G could also be varied without reduction in yield, although there was a modest (6-fold) variation in k_{obs} . Finally, the branch-site nucleotide itself could be any of rA, rG, rU, or rC with appreciable ligation activity; the k_{obs} changed in the order rU > rA ≫ rG > rC. Branch-site rA was retained in all further experiments. Combining the changes permitted in P2 with the changes allowed in most of P3, any of the four orthogonal R substrates could be attached site-selectively to a desired L

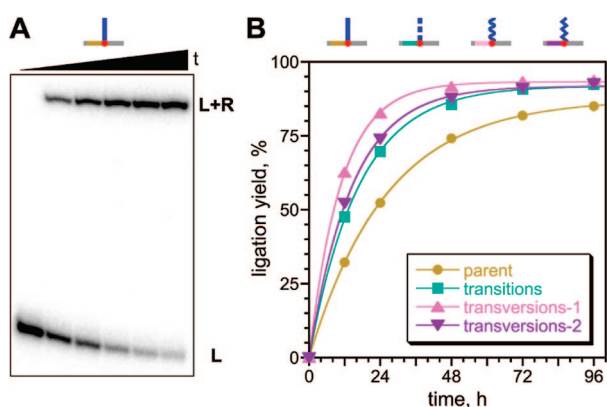


Figure 3. Demonstrating generality of the 15HA9 deoxyribozyme for synthesizing singly branched DNA. (A) 20% PAGE image for branch formation with the parent 20-mer 5'-³²P-radiolabeled L substrate and parent R substrate. Conditions: 50 mM CHES, pH 9.0, 120 mM MgCl₂, 150 mM NaCl, 2 mM KCl, 37 °C, 0–96 h. (B) Kinetic plots for branch formation using L and R substrates with systematic sequence changes in the P2 (L) and P3 (R) regions. k_{obs} values (top to bottom for data plots): 0.092, 0.069, 0.060, 0.038 h⁻¹.

substrate by appropriate choice of 15HA9 binding arm sequences (Figure 3; also see Abstract graphic for depiction of site-selectivity).

These data suggested that 15HA9 can likely be used for general and convergent synthesis of multiply branched DNA, including branched DNA with orthogonal sequences attached at different branch sites. This goal was explored using a longer 40-mer L substrate prepared by solid-phase synthesis with two embedded rA nucleotides at two remote branch sites. This L substrate is termed a “foundation strand”, to which 15HA9 attaches the two “addition strands” (i.e., R substrates). Two versions of the two-site foundation strand were synthesized. The first version has two concatenated copies of the parent P2-rA-P1 sequence (Par-Par); the second version has P2-rA-P1 followed by P2'-rA-P1, where P2 ≠ P2' but the same P1 sequence is present twice (Par-Tv1; see diagrams above gel image in Figure 4). Using these two

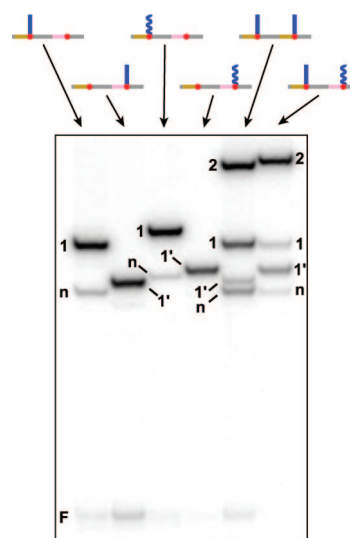


Figure 4. Synthesis of doubly branched DNA by the 15HA9 deoxyribozyme. Time points at 96 h are shown on 20% PAGE for six different combinations of foundation strand and addition strands, as depicted above the gel image. The 40-mer 5'-³²P-radiolabeled foundation strand (horizontal, gray) has two rA potential branch sites (red). The colors within the foundation strand denote alternative P2 sequence components that are used to direct branch formation with the two addition strands (vertical) by appropriate design of the deoxyribozyme sequence. In the gel image, the labels 1 and 2 mark singly and doubly branched products. For the singly branched intermediates, 1 indicates a single branch at the left side of the foundation strand and 1' indicates a single branch at the right side. The label F denotes the unmodified foundation strand. The label n marks a band from nonspecific cleavage at a nonbranched rA site; some additional nonspecific cleavage bands (fully assignable) that appear below the unmodified foundation strand are not shown. See the Supporting Information for full gel image and all DNA sequences.

foundation strands, doubly branched DNA was synthesized using the appropriate 15HA9 deoxyribozyme(s) and orthogonal 5'-adenylated addition strands as substrates (Figure 4). In all cases, only the expected branched products were observed, along with trace amounts of products from

nonspecific cleavage at unreacted rA sites. Importantly for synthetic applications, all of the observed bands were readily assigned, and no evidence was found for any undesired cross-reactivity (i.e., product from attachment of the incorrect addition strand at a branch site along the foundation strand). Furthermore, either addition strand can be attached to either site on the foundation strand in any desired combination, simply by proper choice of deoxyribozyme binding arm sequences.¹⁴

Singly and doubly branched DNAs were also synthesized on the preparative (nanomole) scale.¹⁴ Starting with 1 nmol of the one-site foundation strand, 720 pmol of the singly branched product was obtained after PAGE purification. Similarly, starting with 1 nmol of either the Par-Par or Par-Tv1 two-site foundation strand, 540 or 380 pmol of the doubly branched product was obtained after PAGE purification. MALDI-MS data were consistent with the assigned products.¹⁴

Finally, we examined the reactivity of an 80-mer four-site foundation strand, comprising either a repeated (4×) P2-rA-P1 sequence or a sequence in which the four P2 regions are Watson–Crick orthogonal. In the first case, one addition-strand sequence was attached four times; in the second case, four orthogonal addition strands were attached. On a preparative scale in both cases, 15HA9 successfully synthesized the quadruply branched DNA (Figure 5A). Starting with 1 nmol of foundation strand, 280 and 240 pmol of the branched DNAs were obtained after PAGE purification. MALDI-MS data were again consistent with the assigned products (Figure 5B).¹⁴

Synthesis of branched DNA by the 15HA9 deoxyribozyme requires 72–96 h to reach maximal yield (Figure 3B). Nonetheless, the 15HA9-catalyzed reaction is reproducible, general, and useful for convergent synthesis of multiply branched DNA (Figures 4 and 5). In additional experiments, we have applied 15HA9 in one step to synthesize a quadruply branched 5'-biotinylated DNA that has a total of 228 nucleotides (data not shown), which is larger than routinely achievable by direct solid-phase synthesis. Faster deoxyribozymes that synthesize branched DNA in <1 h have been identified but not yet characterized (T.P.M., C. S. Lee, and S.K.S., unpublished data).

In summary, we have identified the 15HA9 deoxyribozyme that is a useful catalyst for convergent and general synthesis of branched DNA. This includes one-step synthesis of multiply branched DNA with strands that have either identical or Watson–Crick orthogonal sequences. Straight-forward access to multiply branched DNA is likely to improve analytical assays that require increased surface

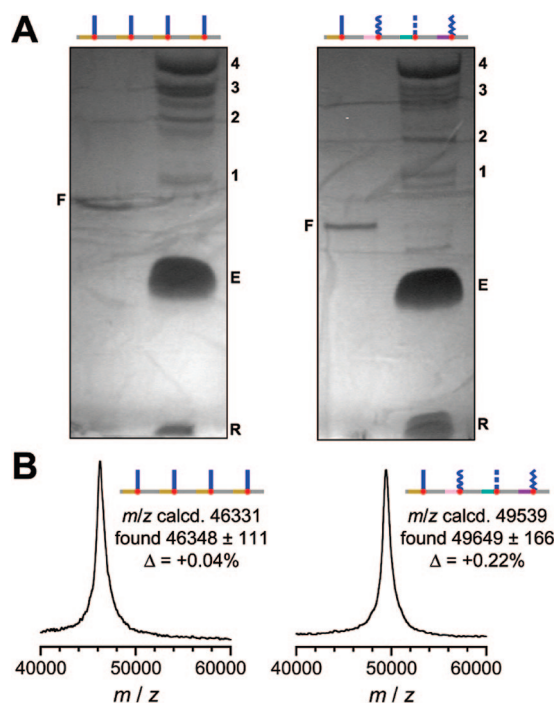


Figure 5. Preparative-scale syntheses of quadruply branched DNA by the 15HA9 deoxyribozyme. (A) 6% PAGE images demonstrating syntheses of the quadruply branched products (visualized by UV shadowing). The 80-mer foundation strand (horizontal, gray) has four rA potential branch sites (red). The colors within the foundation strand denote alternative P2 sequence components that are used to direct branch formation with the four addition strands (vertical) by appropriate design of the deoxyribozyme sequences. The labels 1–4 denote singly through quadruply branched products (note that four, twelve, and four different single, double, and triple addition products, respectively, can be formed). Also marked are the deoxyribozymes (E), unreacted R substrates (R), and foundation strands as standards (F). See the Supporting Information for DNA sequences and experimental procedures. (B) MALDI-MS spectra of the quadruply branched DNA products.

densities of immobilized DNA,^{3,7} as well as other applications.

Acknowledgment. This research was supported by the National Institutes of Health (GM-65966 to S.K.S.). S.K.S. is a Fellow of the David and Lucile Packard Foundation.

Supporting Information Available: Details of selection procedure and ligation assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

OL801568Q