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

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Oligonucleotides

DNA oligonucleotides, including those incorporating one or more branch-site ribonucleotides, were prepared by solid-phase synthesis at IDT (Coralville, IA). The 5′-phosphate for each R substrate was either included during solid-phase synthesis or added after synthesis using ATP and T4 polynucleotide kinase. All oligonucleotides were purified by denaturing PAGE with running buffer 1× TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3) as described previously.[1]

All R substrates were activated by 5′-adenylation using our previously described procedure.[2] For R substrate of sequence 5′-GGAAGAGATGGCGACGG-3′, the template oligonucleotide was 5′-CCGTCGCCATCTC-TTCCTATAGTGAGTCGTATTAGAATTC-3′ (underlined sequence complementary to R), and the acceptor oligonucleotide was 5′-GAATTCTAATACGACTCACTATC-3′ (underlined C intentionally mismatched to T in the template sequence). In cases where the substrate was approximately the same length as the acceptor, the acceptor was shortened by several nucleotides at its 5′-end to avoid overlap on PAGE with the adenylated R substrate. To ensure clarity, we provide here the adenylation procedure for the parent R substrate (the procedure works equivalently whether the final three nucleotides are RNA or DNA). A sample containing 2.0 nmol of 5′-phosphorylated R, 2.2 nmol of template oligonucleotide, and 2.4 nmol of acceptor oligonucleotide in 260 μL of 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and cooling at room temperature for 20 min. The solution was brought to 400 μL total volume containing 1× T4 DNA ligase buffer (40 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT), 20 mM ATP, and 20 μL His_6-T4 DNA ligase (4 μg/μL). Final concentrations were 5.0 μM R substrate, 5.5 μM template, 6.0 μM acceptor and ~300 ng/μL His_6-T4 DNA ligase. The sample was incubated at 25 °C for 2 h, precipitated by addition of 40 μL of 3 M NaCl and 1200 μL of ethanol, and purified by 20% PAGE. A typical yield of 5′-adenylated R was 1.0–1.1 nmol.

The parent L and R sequences as used during selection are L, 5′-GGATAATACGaCTCACTATA-3′ (a = riboadenosine) and R, 5′-GGAAGAGATGGCGACgga-3′ (R is 5′-adenylated and has three 3′-terminal ribonucleotides to enable ligation using T4 RNA ligase during each round of selection). All R substrates used after the conclusion of the selection process had only deoxyribonucleotides and also lacked the 3′-terminal adenosine. When testing substrate sequence generality and subsequently when preparing multiply branched DNA using the 15HA9 deoxyribozyme, systematic variations in the L and R sequences were made. Relative to the parent (Par) sequences, these variations were named as transitions (Tsn = A↔G, C↔T), transversions-1 (Tv1 = A↔C, G↔T), and transversions-2 (Tv2 = A↔T, G↔C). Using the same colors as in the diagrams of Figures 4 and 5, the minimal L and R substrate sequences (i.e., foundation and addition strands) are shown in Figure S1. The longer foundation strands were designed by appropriately concatenating the illustrated 20 nt L sequences. For example, the 80 nt four-site foundation strand of Figure 5 was designed by concatenating one each of the four variant L sequences shown in Figure S1.

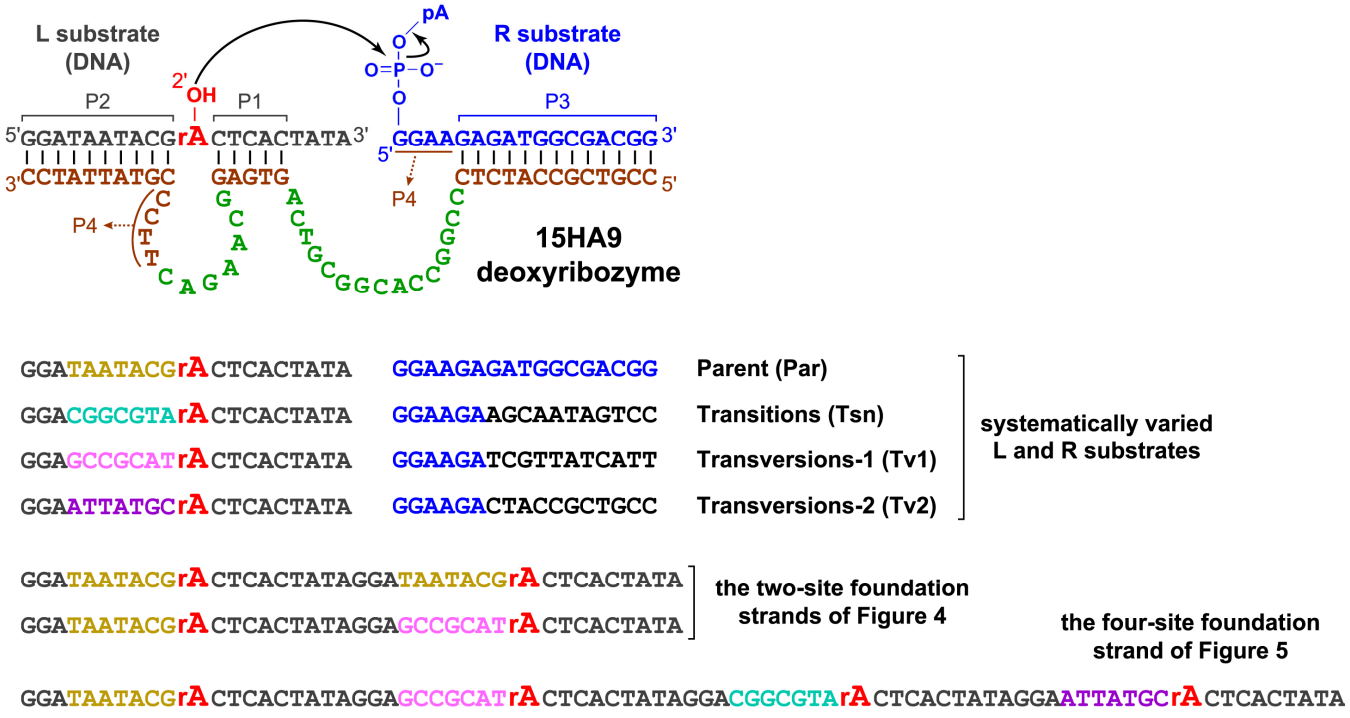

Figure S1. The 15HA9 deoxyribozyme and the systematically varied L and R substrate sequences (i.e., foundation and addition strands). The color scheme for the substrate variants is the same as in Figures 4 and 5. In some experiments as noted, each variant R substrate has two (Tv1), four (Tsn), or six (Tv2) extra nucleotides of sequence $(AC)_n$ at its 3′-terminus; this allows attachment of the different strands to be distinguishable by both PAGE and MS. On the basis of comprehensive generality data showing that P2 and most of P3 can be changed without suppressing branch formation (Figures S4–S6), the illustrated L and R variants were used in practice for synthesis of multiply branched DNA by 15HA9.

In vitro selection

The selection experiments were performed essentially as described previously.[3] The initial deoxyribozyme pool was 200 pmol (ca. 10^{14} molecules). In the key selection step (Figure S2A), the DNA pool—previously attached to the R substrate using T4 RNA ligase—was heated to 95 °C for 3 min in 5 mM Tris, pH 7.5, 15 mM NaCl and 0.1 mM EDTA, and then placed on ice for 5 min. The sample was adjusted to 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$, then incubated for 2 h at 37 °C. After incubation with 40 mM Mg^{2+}, the samples were separated by 8% denaturing PAGE. After round 15, where the pool ligation activity was 17% (Figure S2B), individual deoxyribozymes were cloned and surveyed for ligation activity. Cloning and initial screening of individual deoxyribozyme clones was performed using DNA strands prepared by PCR from miniprep DNA derived from individual *E. coli* colonies. The miniprep DNA samples were first checked by digestion with EcoRI to confirm the presence of the expected insert. The concentration of each PAGE-purified deoxyribozyme strand was estimated from the intensity (UV shadowing) relative to suitable standards. Each screening assay used ca. 0.5 pmol of 5′-^{32}P-radiolabeled L substrate, 3 pmol of the deoxyribozyme strand, and 15 pmol of R substrate. Samples were incubated at 37 °C with timepoints at 0, 1, and 2 h using the procedure described below for analytical-scale assays. The 15HA9 deoxyribozyme was chosen for further investigation on the basis of its relatively high ligation yield.

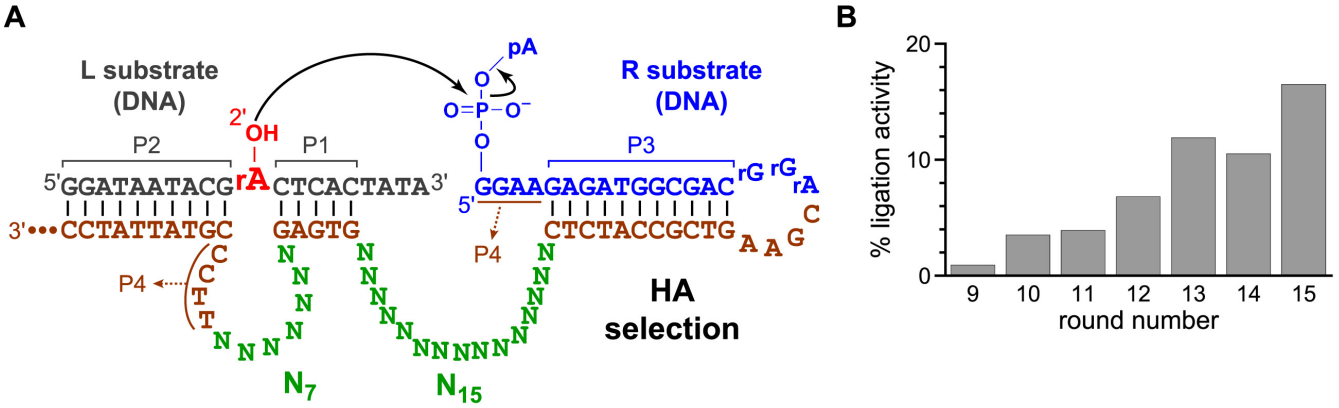

Figure S2. Selection design and activity progression. (A) Selection design. The 22 N nucleotides (*green*) were random at the outset of selection. Fifteen nucleotides (5′-AGCTGATCCTGATGG-3′) at the 3′-end of the deoxyribozyme strand (*brown*; part of a primer binding site) are not shown. Illustrated is the key selection step of each round. (B) Activity progression. Deoxyribozymes were cloned from round 15.

Representative analytical-scale branch formation assay procedure

The general approach has been described previously.[3] The 5′-^{32}P-radiolabeled L substrate (foundation strand) was the limiting reagent relative to the deoxyribozyme (E) and the R substrate (addition strand). A 7 μL sample containing 0.5 pmol of L, 3 pmol of E, and 15 pmol of R was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. When in some assays more than one E or R strand was included, the indicated amount of each strand was added. The sample was adjusted to final concentrations of 50 mM CHES, pH 9.0, 150 mM NaCl and 2 mM KCl by addition of 2 μL of an appropriate stock solution. The ligation reaction was initiated by addition of 1 μL of 1200 mM $MgCl_2$ (final total volume of 10 μL; final Mg^{2+} concentration of 120 mM), and the sample was incubated at 37 °C. Aliquots of 1–2 μL were removed at desired timepoints, quenched onto 8 μL of stop solution (80% formamide, 1× TBE, 50 mM EDTA,

0.025% bromophenol blue, 0.025% xylene cyanol), and analyzed by 20% PAGE. For synthesis of singly branched DNA, the yield versus time data were fit directly to first-order kinetics; i.e., yield = $Y\bullet(1 - e^{-kt})$, where $k = k_{obs}$ and Y = final yield. All yields were computed by taking into account the band due to nonspecific cleavage at any unreacted, non-branched rA site.

Assaying branch-site nucleotide tolerance of the 15HA9 deoxyribozyme

Using the assay procedure described above and the parent R substrate, the dependence of 15HA9 activity on the identity of the branch-site nucleotide was determined. Four variants of the L substrate with each of the four ribonucleotides at the branch site (rA, rG, rU, rC) were tested (Figure S3). All four branch sites led to substantial ligation yield, with k_{obs} values rU > rA >> rG > rC. Branch formation was clearly more effective with branch-site rU or rA rather than rG or rC.

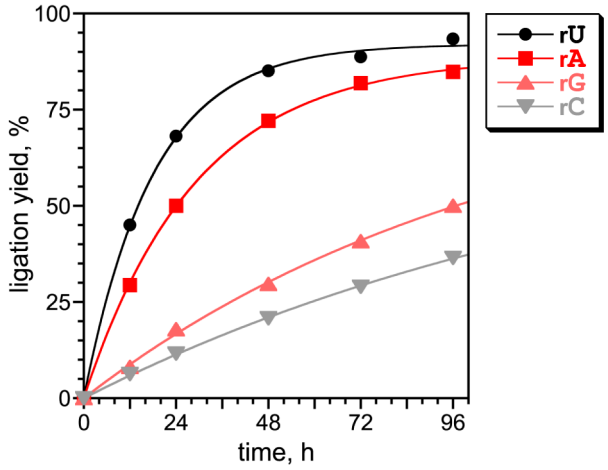

Figure S3. Branch-site nucleotide tolerance of the 15HA9 deoxyribozyme. k_{obs} values: rU 0.056, rA 0.034, rG 0.009, and rC 0.007 h^{-1}.

Assaying substrate sequence generality of 15HA9 in the P1–P4 regions

Systematic experiments were performed to determine the substrate sequence generality of the 15HA9 deoxyribozyme in each of the P1–P4 regions. These experiments used new L and R substrates that have the systematic variations (Par, Tsn, Tv1, Tv2) at specific nucleotides, as shown above in Figure S1. In all cases, any changed nucleotide of L or R was accompanied by a corresponding change in the appropriate deoxyribozyme binding arm nucleotide to retain Watson-Crick complementarity.

We initially investigated the L substrate by making the same number of Tv1 nucleotide changes on both sides of the branch-site rA nucleotide; i.e., in the P1 and P2 regions. When progressively fewer nucleotides on either side of the rA were retained unchanged, the ligation yield decreased substantially (Figure S4), implying a sequence requirement in either P1 or P2 (or possibly both). However, when all seven P2 nucleotides adjacent to the rA were changed without altering any P1 nucleotides, the ligation yield remained high, indicating that the sequence requirement is solely in P1. We concluded that all five P1 nucleotides adjacent to the rA must be retained for 15HA9 to function optimally. We also found that the four single-stranded nucleotides at the 3′-terminus of L could be changed while retaining significant ligation activity (data not shown).

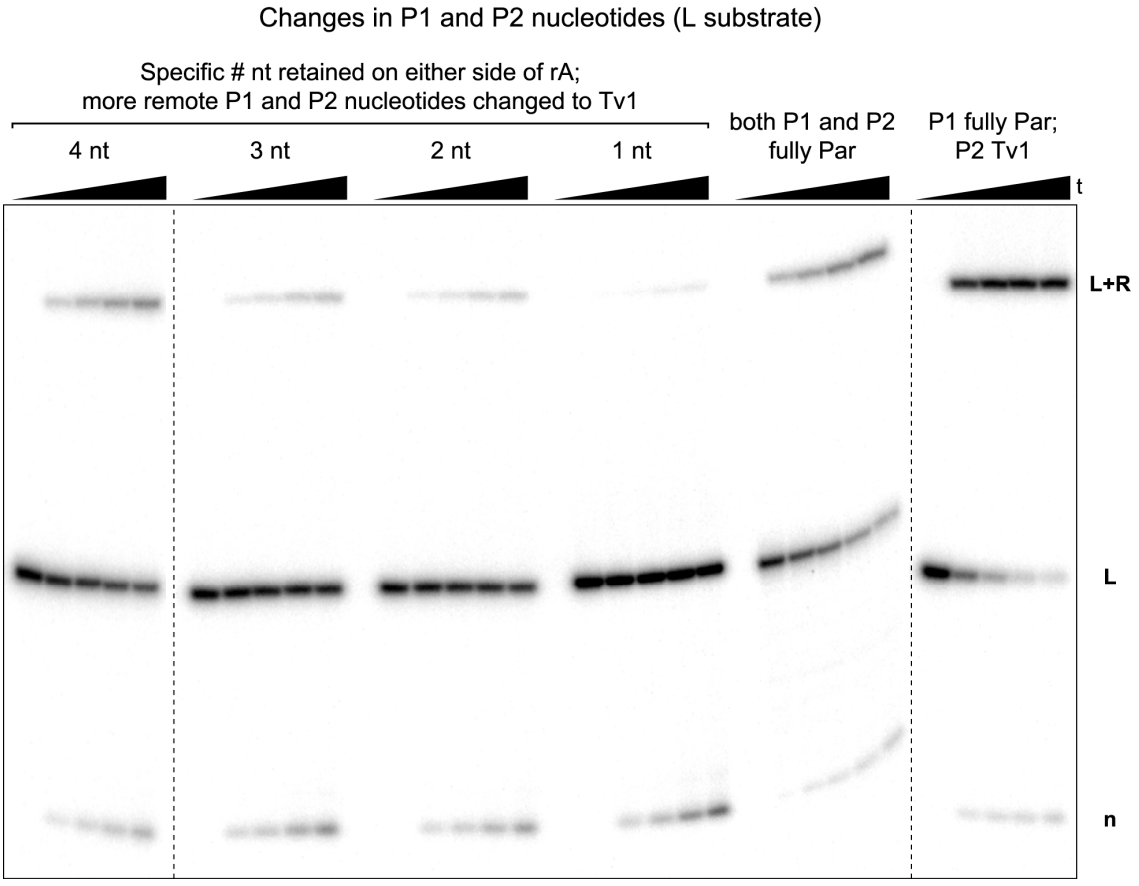

Figure S4. Variation of P1 and P2 nucleotides of the L substrate (see text for explanation). On the 20% PAGE image, the label n denotes nonspecific cleavage of the unreacted L substrate at the rA linkage. Timepoints are shown at 0, 12, 24, 48, and 72 h.

To validate the P2 generality in more comprehensive fashion, we used four different L substrates in which all seven P2 nucleotides adjacent to the rA were varied systematically (Par, Tsn, Tv1, and Tv2; Figure S1). The 5′-GGA was retained in all four L variants because changing seven nucleotides was considered sufficient to afford Watson-Crick orthogonality. When the corresponding deoxyribozymes were used with these four L substrates in branch formation assays, in all cases high ligation rates and yields were observed (Figure S5), confirming the P2 generality.

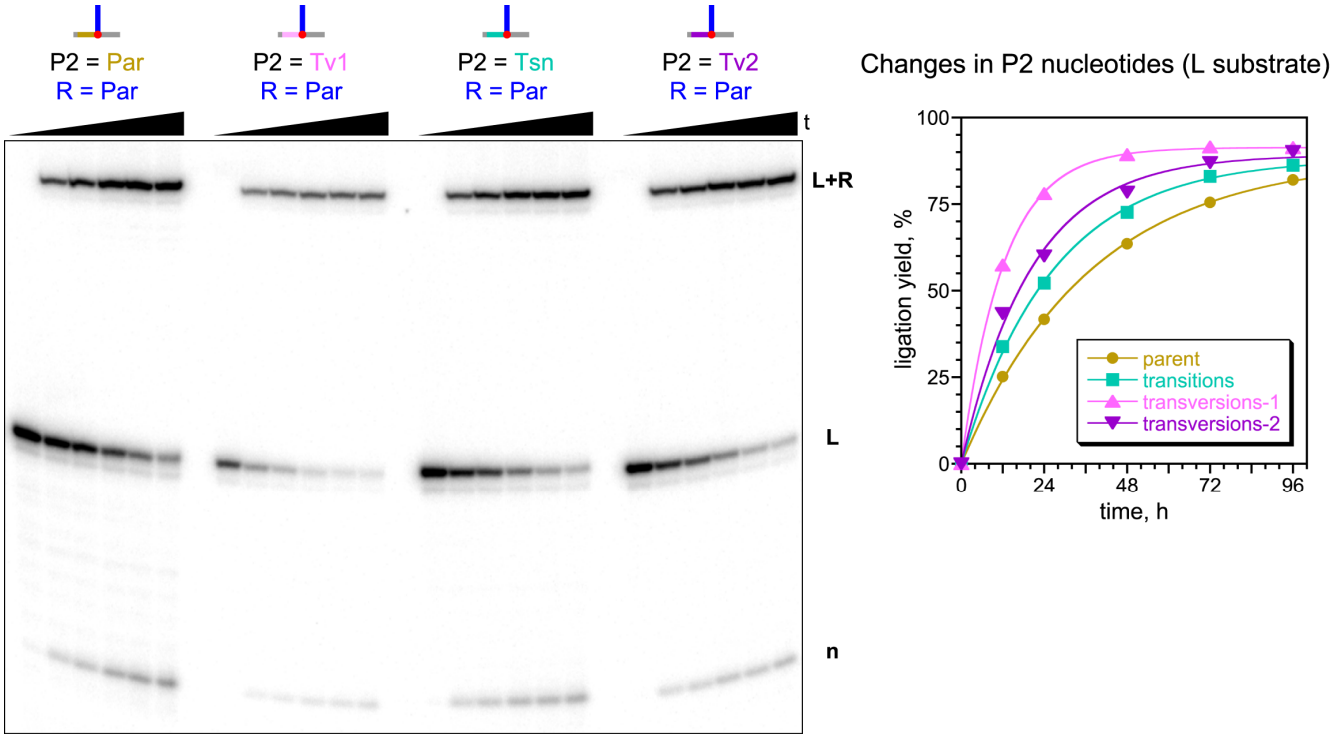

Figure S5. Variation of P2 nucleotides of the L substrate. All sequence combinations are tolerated well. On the 20% PAGE image, the label n denotes nonspecific cleavage of the unreacted L substrate at the rA linkage. k_{obs} values (top to bottom for data plots): 0.082, 0.049, 0.038, 0.027 h^{-1}.

We then investigated the R substrate. Preliminary experiments revealed that changing all nucleotides of the P3 region was not tolerated (data not shown). We synthesized a series of R substrates in which progressively more of the 5′-portion of the P3 region was retained as the parent (Par) sequence, and the remainder through the 3′-terminus was changed to Tv1. In all cases, the 15HA9 binding arm sequence was changed to maintain Watson-Crick complementarity. When even just two nucleotides at the 5′-end of the P3 region were retained, high ligation activity was observed (Figure S6). Therefore, the P3 sequence requirement is quite modest, extending over at most two nucleotides of R. We did not investigate whether retaining only one nucleotide of P3 is sufficient, because keeping two nucleotides nevertheless allows substantial sequence variation in the remaining 11 nucleotides of P3.

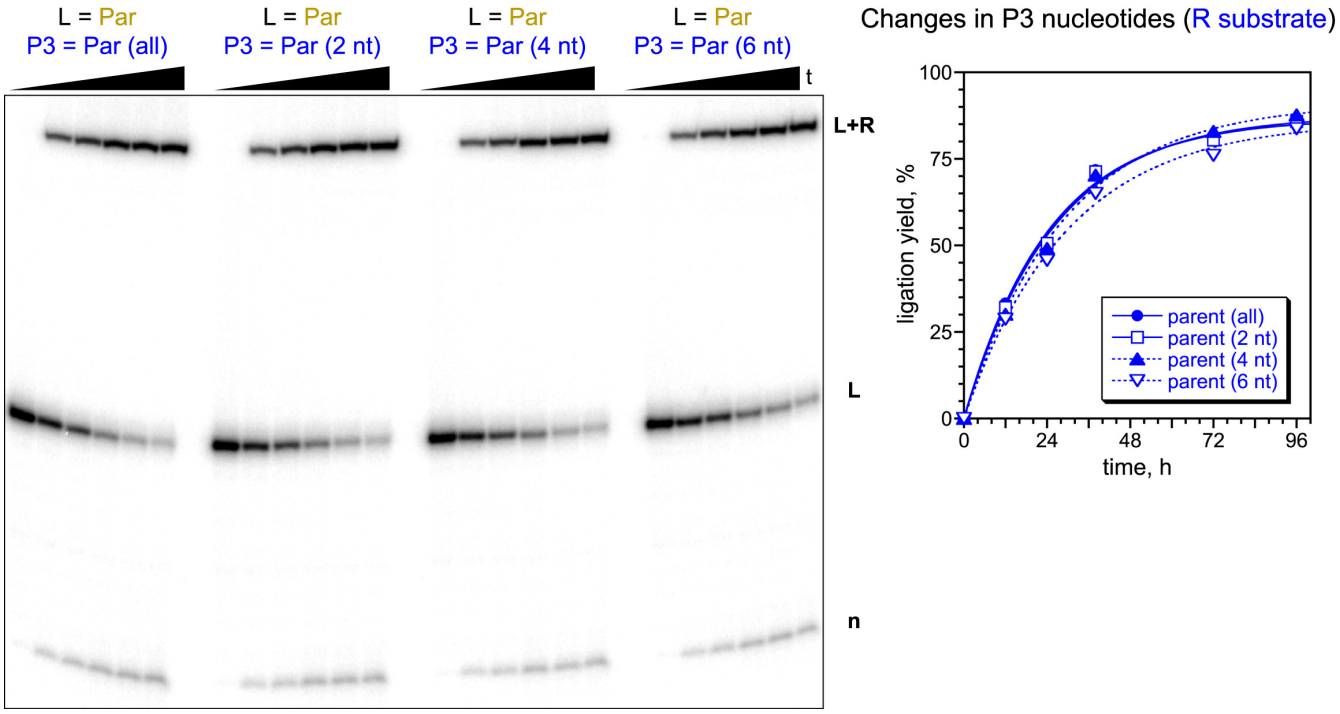

Figure S6. Variation of P3 nucleotides of the R substrate. In the first data set, the P3 region was all parent (Par) sequence. In the remaining three data sets, the P3 region was 2–6 nt of Par sequence followed by the remaining nucleotides as Tv1. All sequence combinations are tolerated well. On the 20% PAGE image, the label n denotes nonspecific cleavage of the unreacted L substrate at the rA linkage. k_{obs} values (top to bottom for legend entries): 0.040, 0.039, 0.034, 0.034 h^{-1}.

Finally, we examined nucleotides in the P4 region. The 5′-terminal G nucleotide of R was retained to allow consistency in the 5′-adenylation process, but the other three nucleotides of P4 were varied systematically. In all cases, substantial ligation activity was observed (Figure S7), indicating that P4 tolerates many nucleotide sequences. However, the six-fold variation in k_{obs} was larger than for changes in P2 or P3.

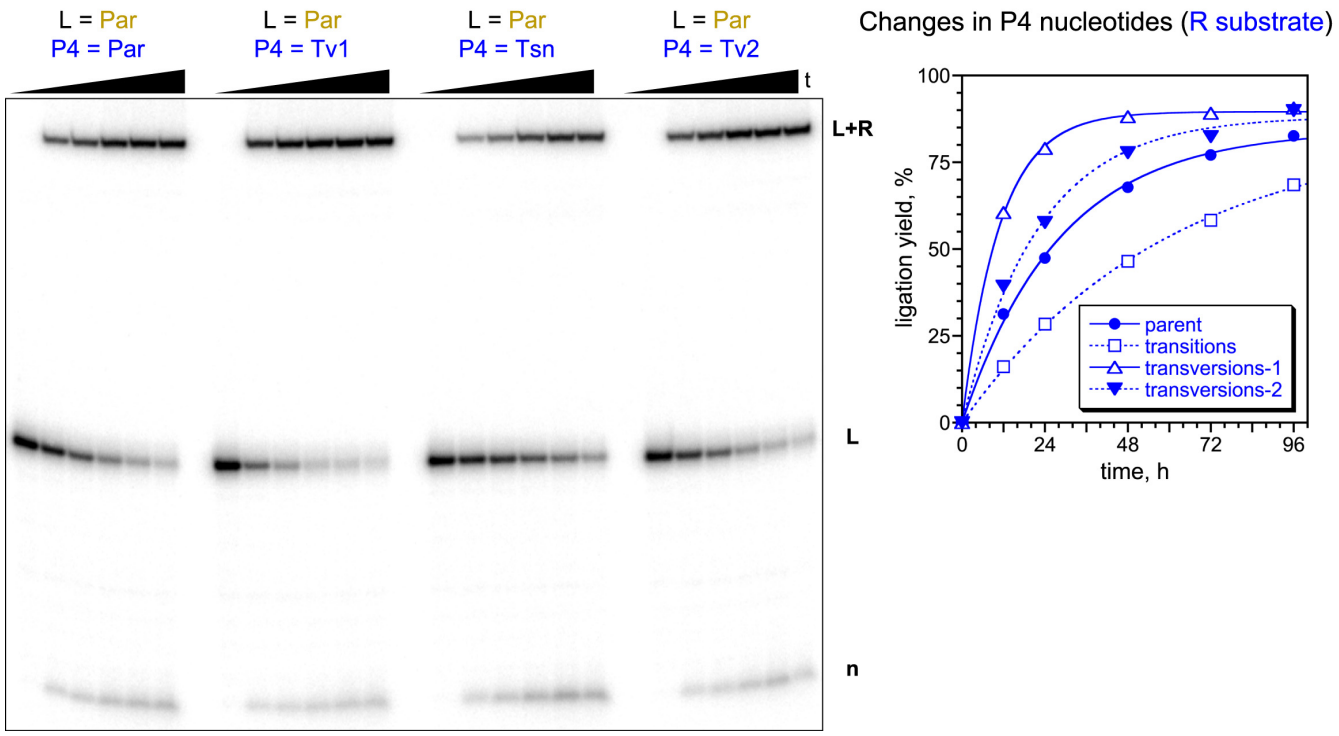

Figure S7. Variation of P4 nucleotides of the R substrate. All sequence combinations are tolerated, albeit with a range of reaction rates depending on sequence. On the 20% PAGE image, the label n denotes nonspecific cleavage of the unreacted L substrate at the rA linkage. k_{obs} values (top to bottom for data plots): 0.092, 0.046, 0.035, 0.017 h^{-1}.

Considering the combined results from Figures S4–S7, we concluded that highly site-selective branch formation can likely be achieved by carefully designing the sequences of the P2 region (in the L substrate) and the P3 region (in the R substrate). The deoxyribozyme sequences would then be dictated by Watson-Crick complementarity as required to synthesize the final desired branch. Although the P4 region also tolerates sequence variation as long as complementarity is maintained (Figure S7), we suspected that varying both P3 and P4 would not be required to achieve high selectivity (P3 should be sufficient). Furthermore, leaving the nucleotides of P4 unchanged would simplify the experiments and reduce the risk that the six-fold variation in k_{obs} with sequence could impact the overall ligation yield.

We designed four new R substrates in which the 5′-GGAAGA sequence was fixed and the remaining 11 nucleotides were varied systematically; this keeps the P4 region (5′-GGAA) and the first two nucleotides of the P3 region (...GA) unchanged relative to the parent sequence. We assayed appropriate 15HA9 deoxyribozymes using these R substrates along with L substrates having the same type of nucleotide changes (Par, Tsn, Tv1, or Tv2) in the P2 region (Figure S8). This experiment is analogous to that shown above in Figure S5, except that changes were made simultaneously in L and R instead of only in L. In all cases, high ligation rate and yield was observed, confirming that varying P2 and P3 at the same time is tolerated well by 15HA9. In these experiments, the Tv1, Tsn, and Tv2 R substrates had extensions of ...AC-3′, ...ACAC-3′, and ...ACACAC-3′, respectively relative to the parent R substrate. This was designed primarily for the ensuing experiments in which multiply branched DNA is formed, where having each R sequence variant with a different nucleotide length would be valuable.

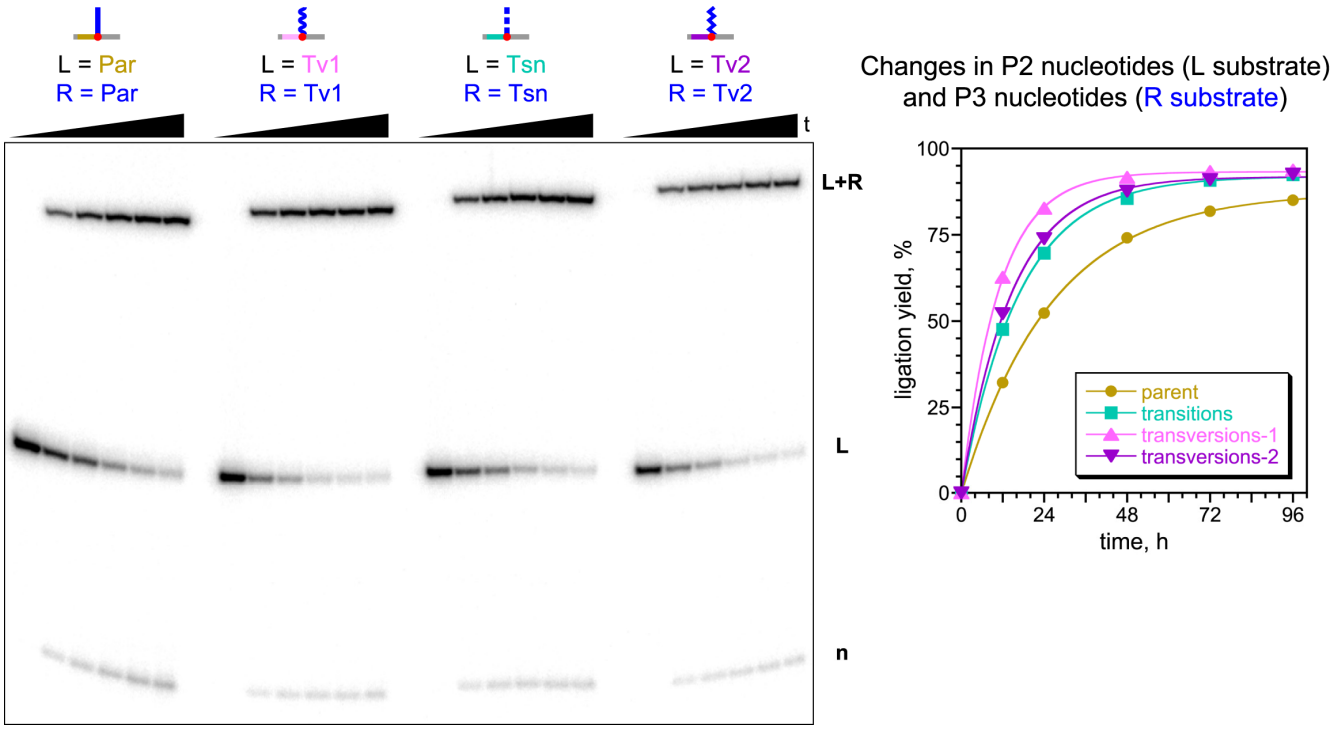

Figure S8. Comprehensive variation of P2 region nucleotides of the L substrate simultaneously with P3 region nucleotides of the R substrate. The P1 region of L and the P4 region of R were both fixed as the parent sequences, along with the first two nucleotides of the P3 region of R. All sequence combinations are tolerated well. In this experiment, the four different addition strands have zero, two, four, or six extra nucleotides of sequence $(AC)_n$ at their 3′-termini, as described in Figure S1, leading to the observed "staircase" in the ligation products. On the 20% PAGE image, the label n denotes nonspecific cleavage of the unreacted L substrate at the rA linkage. k_{obs} values (top to bottom for data plots): 0.092, 0.069, 0.060, 0.038 h^{-1}. A portion of this data is shown in Figure 3 in the manuscript.

Two additional assays were performed using one-site L substrates; i.e., 20 nt L substrates each with a single rA branch site. These assays were intended to demonstrate that the additional presence of orthogonal deoxyribozyme or addition strands does not interfere with the desired branch-formation reaction. In one experiment, including a mixture of all four R substrates did not suppress branch formation for any of the four L substrates tested individually (Figure S9), with the exception of a modest reduction in activity for the Par L substrate. The failure to observe incorrect products is strong evidence for the high fidelity of branch formation.

In a second experiment, including all four deoxyribozymes as well as all four R substrates did not suppress branch formation for any of the four L substrates tested individually (Figure S10), again with the exception of a modest reduction in activity for the Par L substrate. These results further suggested that formation of multiply branched DNA in a single step would be feasible, even when multiple deoxyribozymes and addition strands are required simultaneously. These results also indicated that caution is required when including "unmatched" R substrates; i.e., R substrates that will not become attached to an L substrate. The modest reduction in activity when unmatched Tsn, Tv1, and Tv2 R substrates are used along with the Par L substrate is likely due to binding of one of the unmatched R strands to the L substrate, although this is not readily predictable (e.g., by mfold) and has not been investigated further. It should be noted that this phenomenon was clearly not problematic when all included R substrates were "matched", as in Figures S12–S13 below.

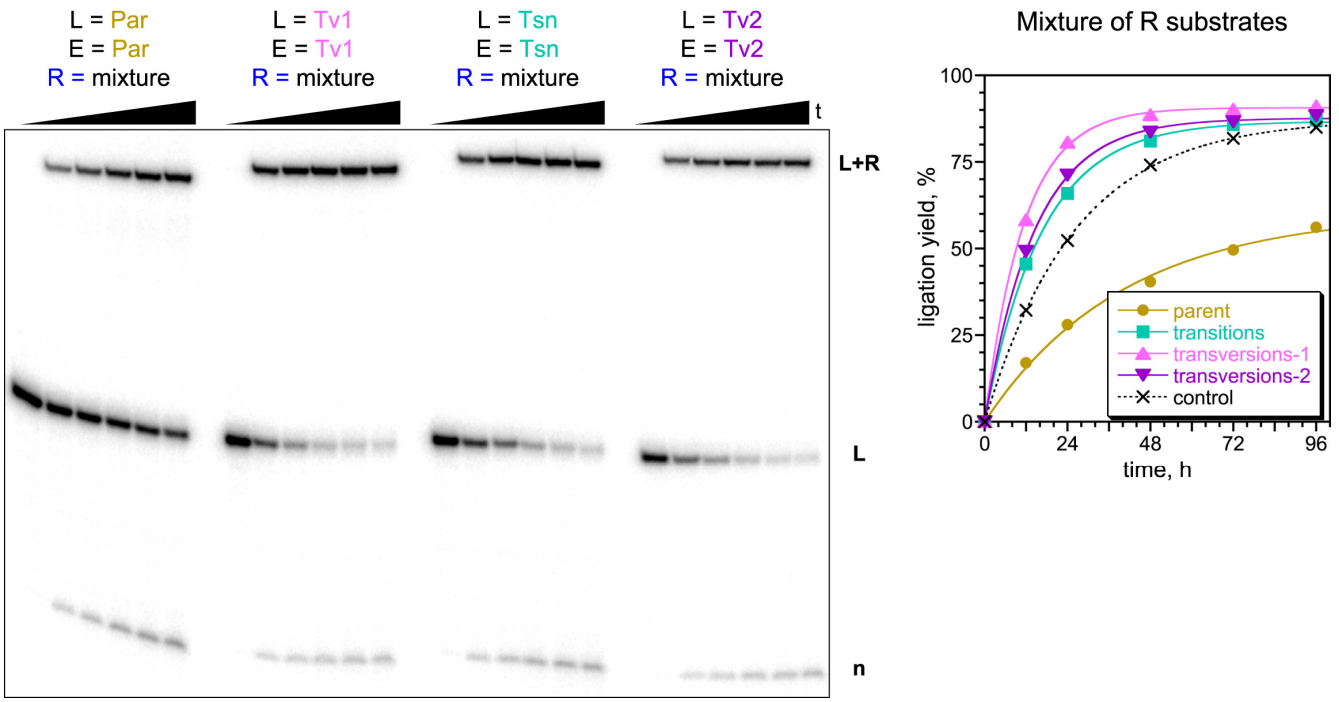

Figure S9. Assays with one L substrate, one deoxyribozyme (E), and a mixture of the four orthogonal R substrates. In the kinetic plot, "control" refers to the Par/Par combination from Figure S8. k_{obs} values (top to bottom for data plots, excluding "control"): 0.088, 0.069, 0.060, 0.025 h^{-1}.

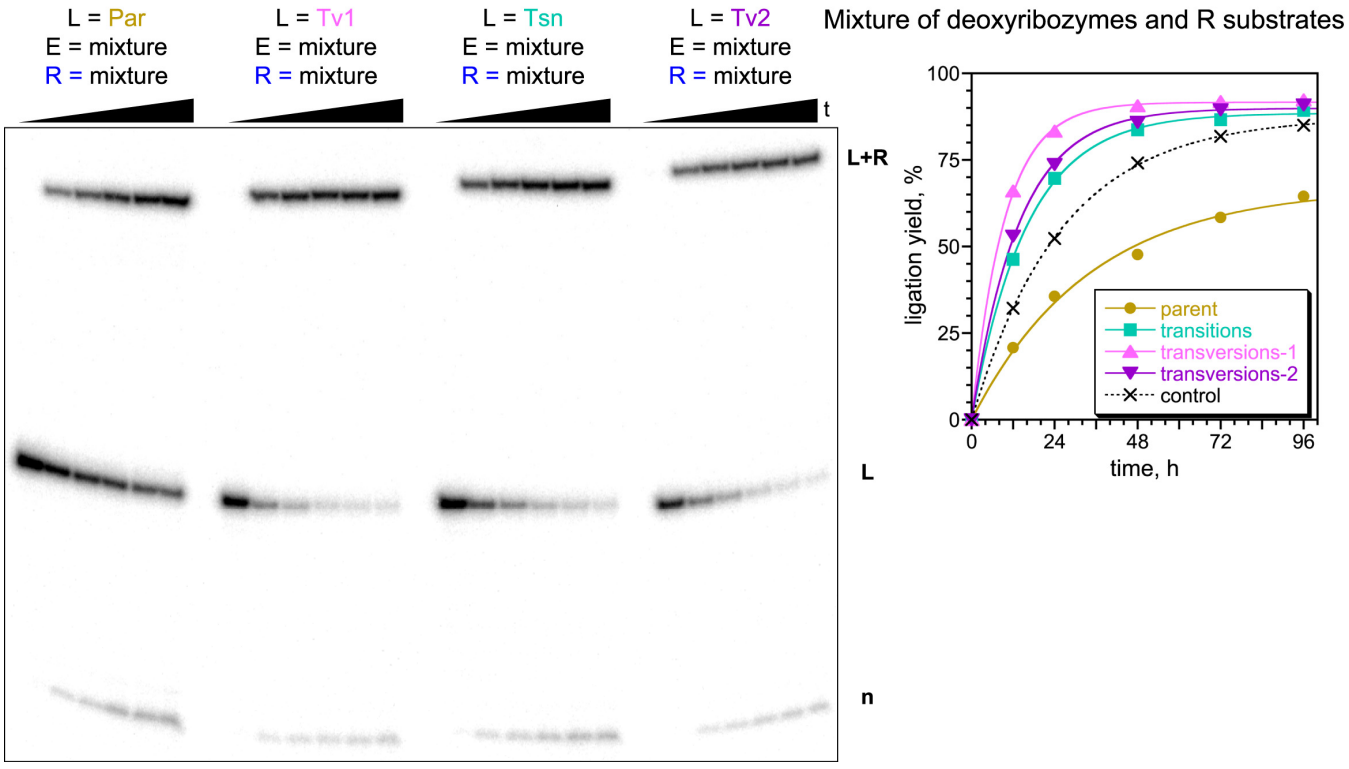

Figure S10. Assays with one L substrate, a mixture of the four orthogonal deoxyribozymes (E), and a mixture of the four orthogonal R substrates. In the kinetic plot, "control" refers to the Par/Par L/R combination from Figure S8. k_{obs} values (top to bottom for data plots, excluding "control"): 0.104, 0.073, 0.063, 0.029 h^{-1}.

Analytical-scale synthesis of doubly branched DNA by the 15HA9 deoxyribozyme

Experiments with two-site foundation strands are shown in Figures S11–S13. Collectively, these data clearly demonstrate that the addition strands can be attached at the desired branch site(s) of the foundation strand with no detectable side products, simply by choosing appropriate P2 and P3 region sequences for the foundation strand, addition strands, and deoxyribozymes.

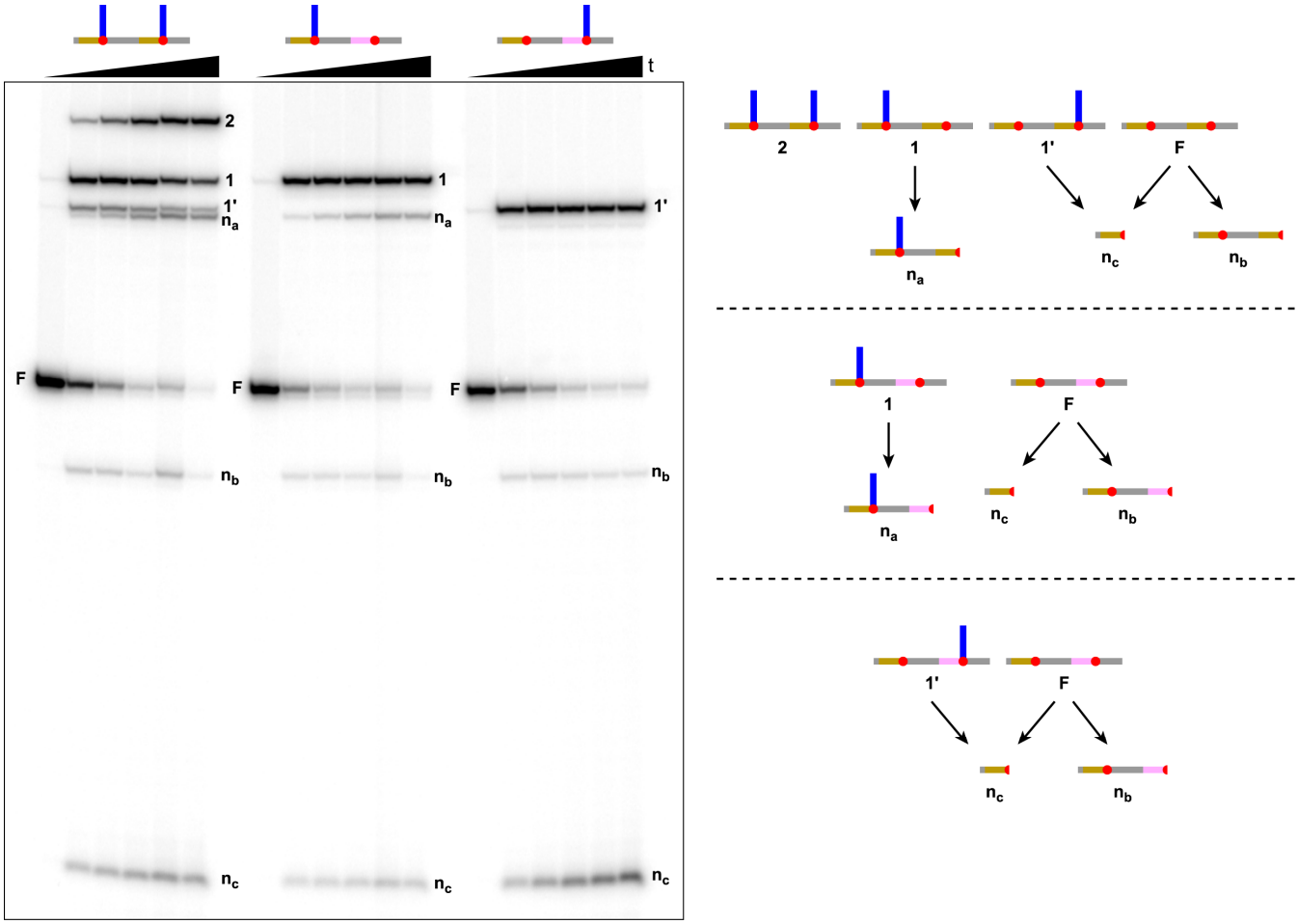

Figure S11. Synthesis of doubly branched DNA by the 15HA9 deoxyribozyme, demonstrating that a particular addition strand (vertical line, *blue*) can be attached with very high selectivity to either branch site (*red*) of the foundation strand (*gray*) as dictated by the P2 sequences (*gold* and *pink*). The diagrams on the right depict the identities of all observed bands, as labeled on the 20% PAGE image; arrows show the reactions that can directly form the nonspecific cleavage products (n_a–n_c). To reduce clutter in the diagrams, reactions arrows are not shown from n_b to n_c, from F to 1 and 1′, and from 1 and 1′ to 2.

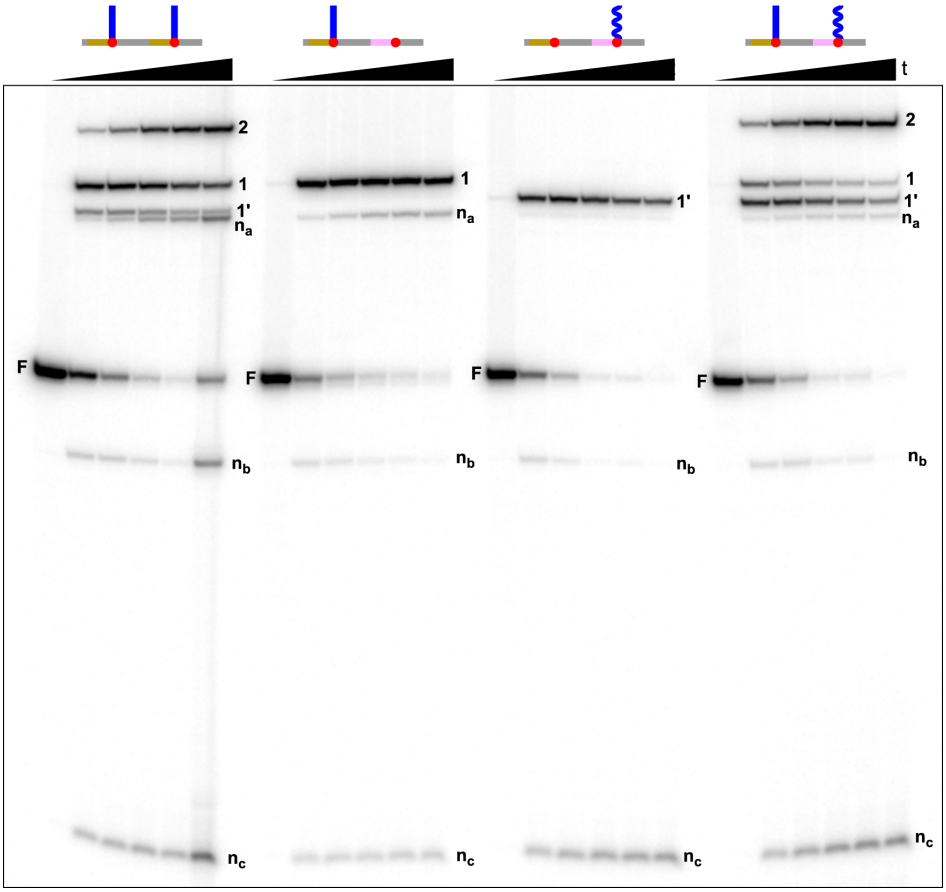

Figure S12. Synthesis of doubly branched DNA by the 15HA9 deoxyribozyme, demonstrating that two different addition strands (vertical lines, *blue*) can be attached with very high selectivity to two branch sites (*red*) of the foundation strand (*gray*) as dictated by the P2 sequences (*gold* and *pink*) and the P3 sequences (straight and wavy vertical lines, *blue*). In this experiment, the alternative addition strand (vertical squiggly line, *blue*) has two extra nucleotides of sequence AC at its 3′-terminus, as described in Figure S1.

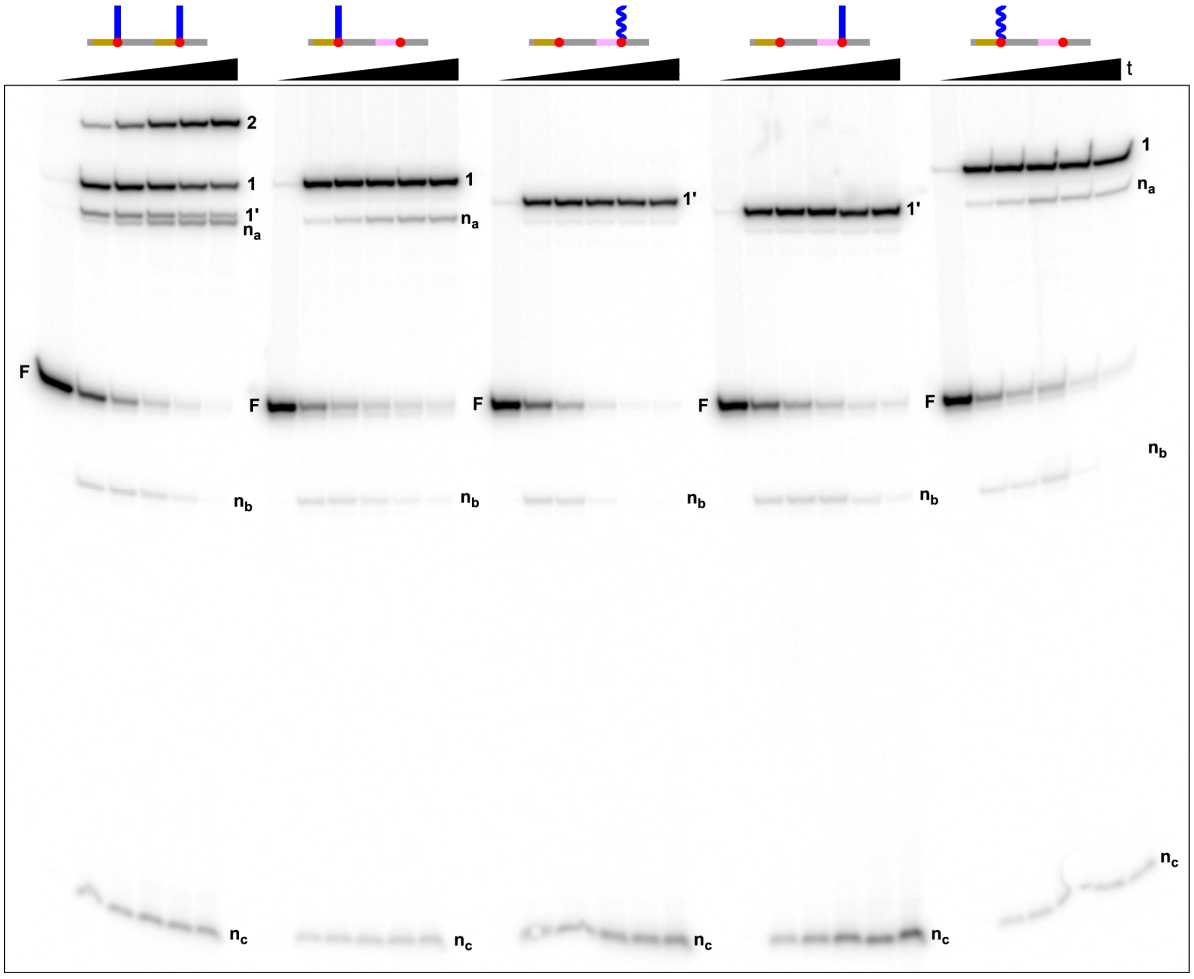

Figure S13. Synthesis of doubly branched DNA by the 15HA9 deoxyribozyme, demonstrating that two different addition strands (vertical lines, *blue*) can be attached with very high selectivity to either of two branch sites (*red*) of the foundation strand (*gray*) as dictated by the P2 sequences (*gold* and *pink*) and the P3 sequences (straight and wavy vertical lines, *blue*). In this experiment, the alternative addition strand (vertical squiggly line, *blue*) has two extra nucleotides of sequence AC at its 3′-terminus, as described in Figure S1.

Preparative-scale syntheses of branched DNA

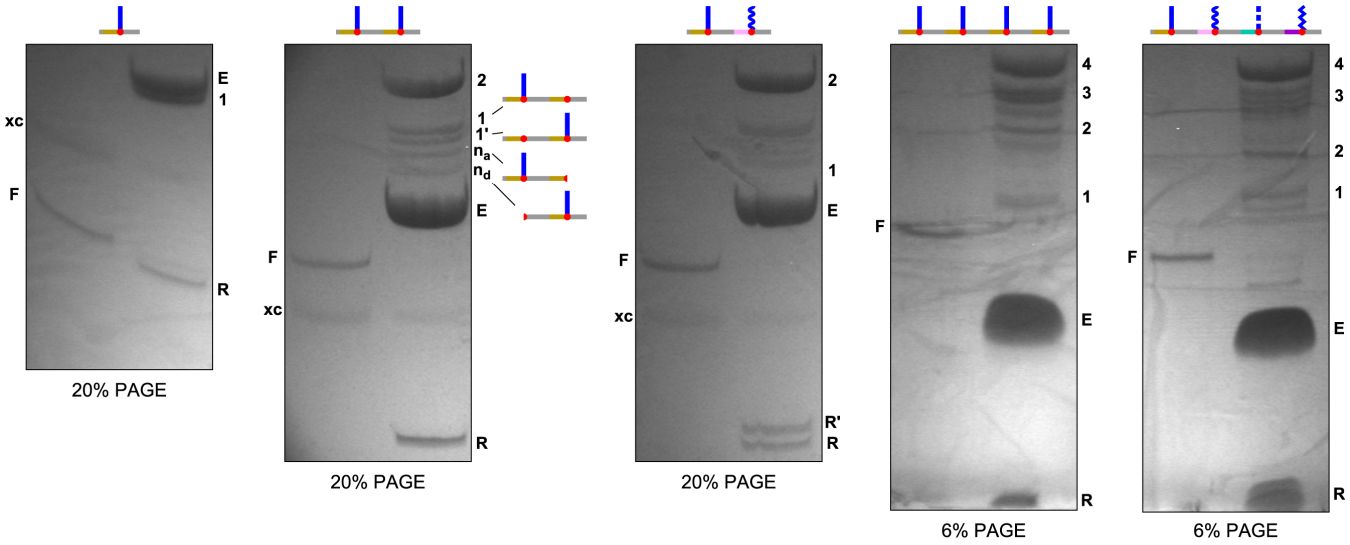

Figure S14. Preparative-scale syntheses of branched DNA. The images were acquired using a handheld UV viewer over a fluorescent TLC plate (xc = xylene cyanol). The two images on the far right are the same as in Figure 5. On the far left image, the very close proximity of the singly branched product (37 nt) and the deoxyribozyme (54 nt) reflects the expected anomalously slow PAGE migration of a branched nucleic acid. In cases where the branched product and the deoxyribozyme overlap exactly, the percentage of acrylamide in the gel may be changed, or additional nucleotides may be added to the deoxyribozyme sequence to shift its mobility above that of the branched product.

Procedure for preparative-scale synthesis of singly branched DNA. The L substrate was the limiting reagent relative to the deoxyribozyme (E) and R substrate. A 140 µL sample containing 1.0 nmol of L, 1.1 nmol of E, and 1.25 nmol of R was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling at room temperature for 20 min. The sample was adjusted to final concentrations of 50 mM CHES, pH 9.0, 150 mM NaCl and 2 mM KCl by addition of 48 µL of an appropriate stock solution. The ligation reaction was initiated by addition of 12 µL of 2 M $MgCl_2$ (final total volume of 200 µL; final Mg^{2+} concentration of 120 mM). The sample was incubated at 37 °C for 96 h and precipitated by addition of 20 µL of 3 M NaCl and 600 µL of ethanol. The products were separated by 20% PAGE as shown in Figure S14. The isolated yield of singly branched DNA was 720 pmol.

Procedure for preparative-scale synthesis of doubly branched DNA. The L substrate (foundation strand) was the limiting reagent relative to each deoxyribozyme (E) and each R substrate (addition strand). A 140 µL sample containing 1.0 nmol of L, 1.1 nmol of each E, and 1.25 nmol of each R was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling at room temperature for 20 min. (When the two addition strands were identical, the amounts were 1.0 nmol of L, 2.2 nmol of E, and 2.5 nmol of R.) The sample was adjusted to final concentrations of 50 mM CHES, pH 9.0, 150 mM NaCl and 2 mM KCl by addition of 48 µL of an appropriate stock solution. The ligation reaction was initiated by addition of 12 µL of 2 M $MgCl_2$ (final total volume of 200 µL; final Mg^{2+} concentration of 120 mM). The sample was incubated at 37 °C for 96 h and precipitated by addition of 20 µL of 3 M NaCl and 600 µL of ethanol. The products were separated by 20% PAGE as shown in Figure S14. The isolated yield of doubly branched DNA was 380 pmol (two different addition strands) or 540 pmol (two identical addition strands).

Procedure for preparative-scale synthesis of quadruply branched DNA. The L substrate (foundation strand) was the limiting reagent relative to each deoxyribozyme (E) and each R substrate (addition strand). A 220 μL sample containing 1.0 nmol of L, 1.1 nmol of each E, and 1.25 nmol of each R was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling at room temperature for 20 min. (When all four addition strands were identical, the amounts were 1.0 nmol of L, 4.4 nmol of E, and 5.0 nmol of R.) The sample was adjusted to final concentrations of 50 mM CHES, pH 9.0, 150 mM NaCl and 2 mM KCl by addition of 62 μL of an appropriate stock solution. The ligation reaction was initiated by addition of 18 μL of 2 M $MgCl_2$ (final total volume of 300 μL; final Mg^{2+} concentration of 120 mM). The sample was incubated at 37 °C for 96 h and precipitated by addition of 30 μL of 3 M NaCl and 900 μL of ethanol. The products were separated by 6% PAGE as shown in Figure S14. The isolated yield of quadruply branched DNA was 240 pmol (four different addition strands) or 280 pmol (four identical addition strands).

MALDI-MS analyses of branched DNA

MALDI-TOF mass spectrometry data were obtained for the branched DNA products synthesized on the preparative scale (Figure S15). In each case, the observed mass was within experimental error of the calculated value for the assigned product. Note that the $(AC)_n$ sequence at the 3′-terminus of each addition strand (n = 0 to 3 for each different strand) means that the doubly and quadruply branched DNAs with two or four different addition strands have unique masses that are substantially different from the masses expected if one or more of the same addition strands were added at multiple positions. The observation of the correct masses is corroborating evidence that the DNA-catalyzed ligation reactions are highly selective.

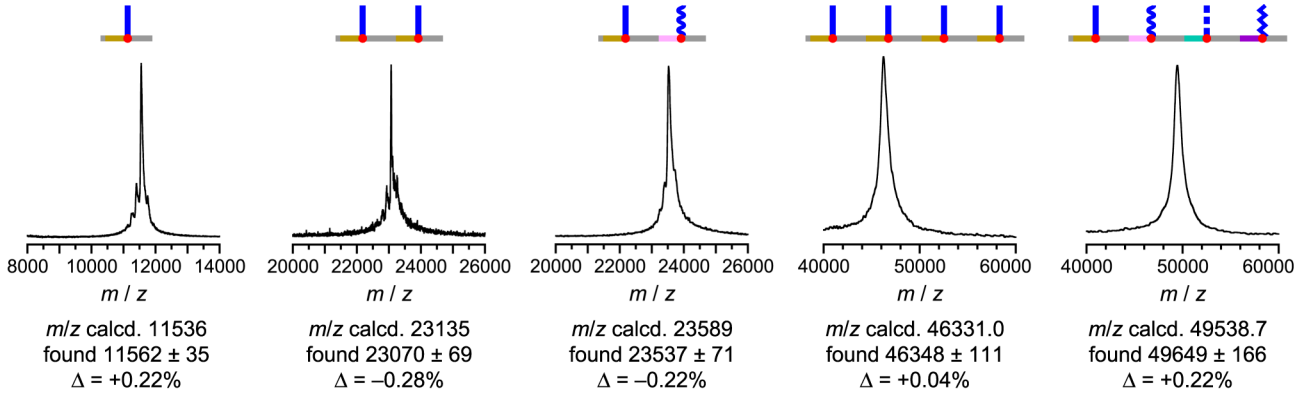

Figure S15. MALDI-MS analyses of branched DNA products. MALDI-TOF mass spectrometry data were obtained on an Applied Biosystems Voyager instrument in the Mass Spectrometry Laboratory of the University of Illinois School of Chemical Sciences.

The MALDI-MS spectra were calibrated as appropriate using standard DNA oligonucleotides (50 and 100 nt) of molecular weight 15358.3 and 30664.7 or in vitro transcribed P4-P6 RNA of molecular weight 51949.2.[4,5] Errors for the singly and doubly branched DNA products are reported as 0.3% of the calculated mass value. Errors for the quadruply branched DNA products were computed as the difference between the average mass value determined from five independently acquired spectra and the mass value determined for P4-P6 as an external standard. The mass values and errors for the quadruply branched DNA products were similar when P4-P6 was included in each sample as an internal standard (data not shown).

References for Supporting Information

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