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A DNA enzyme that mimics the first step of RNA splicing

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We have discovered an artificial DNA enzyme that mimics the first step of RNA splicing. *In vitro* selection was used to identify DNA enzymes that ligate RNA. One of the new DNA enzymes carries out splicing-related catalysis by specifically recognizing an unpaired internal adenosine and facilitating attack of its 2′-hydroxyl onto a 5′-triphosphate. This reaction forms 2′,5′-branched RNA and is analogous to the first step of *in vivo* RNA splicing, in which a ribozyme cleaves itself with formation of a branched intermediate. Unlike a natural ribozyme, the new DNA enzyme has no 2′-hydroxyl groups to aid in the catalytic mechanism. Our finding has two important implications. First, branch-site adenosine reactivity seems to be mechanistically favored by nucleic acid enzymes. Second, hydroxyl groups are not obligatory components of nucleic acid enzymes that carry out biologically related catalysis.

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Catalytic RNA forms the essential core of both the ribosome[1] and likely the spliceosome[2]. In the latter RNA-protein machine, as well as in related protein-free self-splicing group II introns[3], RNA splicing is initiated by reaction of an internal 2′-hydroxyl group with the 5′ splice site, forming a 2′,5′-branched (lariat) RNA intermediate[4]. The reactive 2′-hydroxyl is almost invariably located on an unpaired, bulged adenosine nucleotide[5]. An understanding of splicing mechanisms is enhanced by exploring the ability of nucleic acid enzymes to catalyze reactions related to the first step of RNA splicing[6]. *In vitro* selection provides information about the catalytic properties of nucleic acids[7,8] and proteins[9,10]. Here we describe the use of *in vitro* selection to discover an artificial DNA enzyme (deoxyribozyme)[11–14] that mimics the first step of RNA splicing. Our findings have implications for the historical evolution of RNA splicing mechanisms and for the catalytic capabilities of prebiotic nucleic acid polymers.

RESULTS

Identification of the 7S11 DNA enzyme that ligates RNA

The new DNA enzyme (7S11) emerged as a consensus sequence from an *in vitro* selection experiment (**Fig. 1**)[15]. In the selection procedure, the left-hand (L) and right-hand (R) RNA substrates were bound to constant DNA regions surrounding a random 'enzyme region' of 37 nucleotides (nt). The R substrate bears a 5′-triphosphate group that typically reacts with 2′- or 3′-hydroxyls in RNA ligation reactions catalyzed by nucleic acid enzymes[16–19]. After seven rounds of selection, the key step of which is shown in **Figure 1a**, 7S11 was identified by aligning sequences of active clones and prepared independently by solid-phase synthesis (**Fig. 1b**). The closely related 7S10 deoxyribozyme identified in the same selection was also prepared.

The 7S11-mediated RNA ligation reaction proceeds rapidly in >90% yield, with k_{obs} ~0.5 min^{-1} at 37 °C, pH 9.0 and 40 mM Mg^{2+} (see below). Under the same incubation conditions, the background ligation rate for L + R substrates held together by an exactly complementary DNA splint is ~5×10^{-6} min^{-1} (data not shown). Therefore, the rate enhancement for 7S11 is at least 10^5-fold. The mass of the ligated product as determined by MALDI-TOF mass spectrometry is as expected from ligation of L + R with loss of pyrophosphate (observed m/z = 11,020 ± 11; expected m/z = 11,009). Consistent with this, if the R substrate is 5′-monophosphate or 5′-hydroxyl instead of 5′-triphosphate, then 7S11 activity is lost (<0.5% ligation; data not shown).

Branched structure of the ligated RNA product

Several previously identified *in vitro* selected ribozymes[16] provide either 3′–5′-linked or 2′–5′-linked linear RNA by reaction of the 3′-terminal nucleotide of L with the 5′-triphosphate of R. In contrast, the 7S11 DNA enzyme recognizes a specific adenosine within the L substrate and mediates reaction of its 2′-hydroxyl group with the 5′-triphosphate of R, thus forming a 2′,5′-branched RNA (**Fig. 2a**)[18]. Evidence for this branched structure was obtained in several ways. The product migrates anomalously on a denaturing polyacrylamide gel compared with a linear standard (**Fig. 2b**); this strongly suggests a branched RNA structure[4]. Partial alkaline hydrolysis (**Fig. 2c**) unambiguously maps the branch site to the assigned adenosine. Finally, the natural debranching enzyme Dbr[20] cleaves the product, confirming its 2′,5′-branched connectivity (**Fig. 2d**).

Unexpected interactions between the DNA enzyme and RNA

As shown in **Figure 3**, we found that 7S11 does not follow the reaction format initially programmed into the selection procedure. In particular, the L RNA substrate does not interact with its corresponding DNA binding arm, as intended by design (**Fig. 3a**). During selection, several mutations that destabilize Watson-Crick interactions accumulated within this binding arm (**Fig. 3b**), as permitted by the selection strat-

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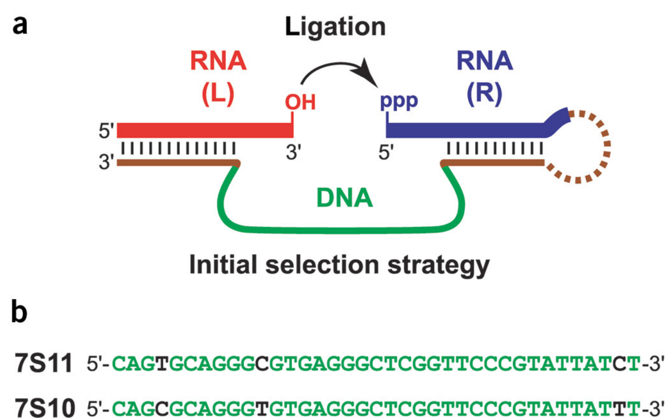

Figure 1 Selection and sequence of a DNA enzyme that ligates RNA. (**a**) The selection strategy. See **Supplementary Figure 1** online for details. In the final isolated DNA enzyme, the binding format has changed (see **Fig. 4**). The dashed loop on the right is present during selection but is not required for ligation activity. (**b**) Sequence of the 7S11 deoxyribozyme, along with the variant 7S10. Only the enzyme regions are shown, with the three nucleotide differences marked in black.

egy (see **Supplementary Fig. 1** online). Notably, different mutations were found in the binding arms of the 7S11 and 7S10 deoxyribozymes, although their ligation activities are similar. We hypothesized that the DNA binding arm may not interact at all with the L substrate, despite the initial design. Consistent with this hypothesis, the mutations identified within the 7S11 and 7S10 original left-hand DNA binding arms are scattered throughout the binding arm; this may maximize their destabilizing effects. Indeed, truncation of the deoxyribozyme by complete removal of the DNA binding arm does not reduce the RNA ligation rate or yield (**Fig. 3c**). Furthermore, restoring the DNA sequence of the binding arm to full complementarity with the L substrate completely abolishes ligation activity (**Fig. 3c**). This indicates that interaction between the binding arm and the L substrate is strongly detrimental to the ligation reaction.

In the 37-nt DNA 'enzyme region' of 7S11, two sequence blocks of lengths 5 and 7 nt (blocks A and B) are exact Watson-Crick matches for the RNA nucleotides that flank the branch-site adenosine in L (**Fig. 4a**). By making compensatory mutations within the DNA and RNA sequences of each of these two blocks, we showed that the DNA nucleotides indeed base-pair with those in the RNA substrate, leaving only the branch-site adenosine unpaired (**Fig. 4b**; see **Supplementary Fig. 2** online for full experimental details). In contrast to the L substrate, the R substrate interacts with its corresponding DNA binding arm via Watson-Crick base pairs (see **Supplementary Fig. 3** online). The branch-site nucleotide in L must be adenosine and have a 2′-hydroxyl for the ligation reaction to occur (**Fig. 4c**; a branch-site guanosine reacts with ~30-fold lower rate). The L substrate is shown in **Figure 4a** with extended 5′ and 3′ ends on the basis of an experiment in which extension at either end still permitted ligation activity (see **Supplementary Fig. 4** online).

Role of the leaving group during ligation

The 7S11-catalyzed branch-forming reaction (**Fig. 4a**) and natural RNA splicing reactions[3,5] differ in the details of the leaving group. In 7S11, the leaving group is the weakly basic pyrophosphate (PP_i), whereas in group II introns and the spliceosome, the leaving group is the more basic 3′-hydroxyl of an oligonucleotide (the 5′-exon). The 7S11 ligation product could not be cleaved (debranched) with PP_i in the presence of the DNA enzyme (data not shown), indicating that the ligation reaction is irreversible. To examine the role of the leaving group in more detail, we tested the ability of PP_i to inhibit the ligation reaction. Even when present in 10^3-fold excess relative to the DNA enzyme, PP_i did not detectably inhibit ligation (**Fig. 5a**). Inhibition would be expected if 7S11 were to make functionally critical contacts with the leaving group during branch formation. More conclusively, a 5′-adenylated R substrate (5′-AppRNA, **Fig. 5b**) used in place of the 5′-triphosphate R substrate supported nearly equivalent 7S11-mediated ligation activity (**Fig. 5c**). We conclude that the better (less basic) leaving group used by 7S11 merely allows the reaction to occur on a practical time scale, but the DNA enzyme does not interact strongly (if at all) with the leaving group.

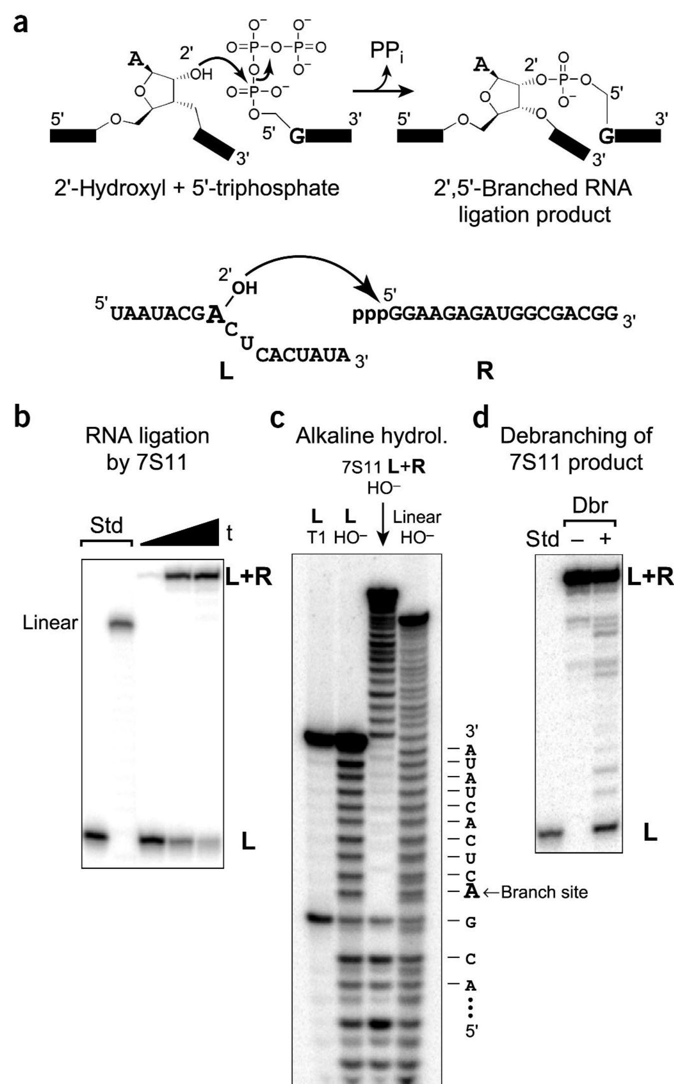

Figure 2 Analysis of the 2′,5′-branched RNA product formed by the 7S11 DNA enzyme. (**a**) The branch-forming reaction catalyzed by 7S11. (**b**) The ligation product (L + R) migrates anomalously compared with a linear 3′–5′-linked standard RNA (t = 20 s, 20 min and 1.5 h). (**c**) Partial alkaline hydrolysis assay reveals the site of branching as a specific adenosine. T1, RNase T1 digestion (cleavage to the 3′ side of G only); HO−, partial alkaline hydrolysis. (**d**) Debranching enzyme (Dbr)[20] successfully debranches the 7S11 ligation product.

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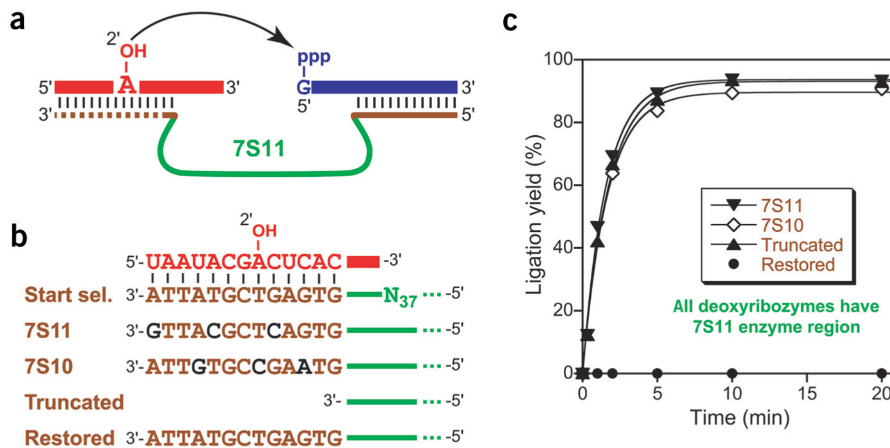

Figure 3 Analysis of the role of the left-hand DNA binding arm. (**a**) Sites of change in the DNA binding arm. The dashed brown line indicates the binding arm that was mutated or removed. (**b**) Sequences of the left-hand DNA binding arm as present at the start of selection, found in the 7S11 and 7S10 clones, removed entirely, or restored to the original sequence. Brown nucleotides are identical to those used at the start of selection; black nucleotides are mutations identified in either 7S11 or 7S10. (**c**) The left-hand DNA binding arm does not interact with the RNA substrate as depicted in **a**. Triple mutation or complete removal of the binding arm does not disrupt 7S11 ligation activity (k_{obs} ~0.6 min^{-1}). In contrast, restoring the binding arm sequence to that present at the start of selection abolishes ligation activity altogether.

DISCUSSION

7S11-mediated branch formation and *in vivo* RNA splicing

The overall reaction mediated by 7S11 is shown in **Figure 6a**. Remarkably, this chemical reactivity closely parallels the well-known natural RNA splicing reactions that are catalyzed by group II introns and the spliceosome (**Fig. 6b**)[3,5]. The detailed mechanistic relationship between the 7S11 DNA enzyme and *in vivo* splicing-related catalysis is unclear based on the available data. Nevertheless, it is notable that such robust DNA-catalyzed activity (>90% yield on a rapid timescale) emerges from a brief selection experiment that had no bias toward the use of an unpaired adenosine. Indeed, to achieve the splicing-related reactivity of **Figure 4a**, the binding interactions between the 7S11 DNA enzyme and left-hand RNA substrate were actively repositioned during selection (similar repositionings have been observed by others[21,22]). The initially programmed binding arm–substrate interaction (**Fig. 3a**) was discarded via accumulation of multiple mutations during selection (**Fig. 3b**), and restoring this interaction is detrimental to the ligation activity (**Fig. 3c**). Presumably this latter effect occurs by direct competition with formation of the active deoxyribozyme–RNA substrate complex, because the binding interactions of **Figures 3a** and **4a** clearly cannot occur simultaneously.

Two additional observations support the similarity between 7S11-mediated branch formation and *in vivo* RNA splicing. First, 7S11 strongly prefers a branch-site adenosine over the other nucleosides (**Fig. 4c**). Second, 7S11 can form lariat RNA in addition to noncircular 2′,5′-branched RNA (data not shown)[18,19], in a similar manner to the *in vivo* splicing reactions of **Figure 6b**. Considered together, these findings suggest that reactivity of an unpaired branch-site adenosine 2′-hydroxyl group, such as that shown in **Figure 6**, is mechanistically favored by nucleic acid enzymes. This hypothesis has clear implications

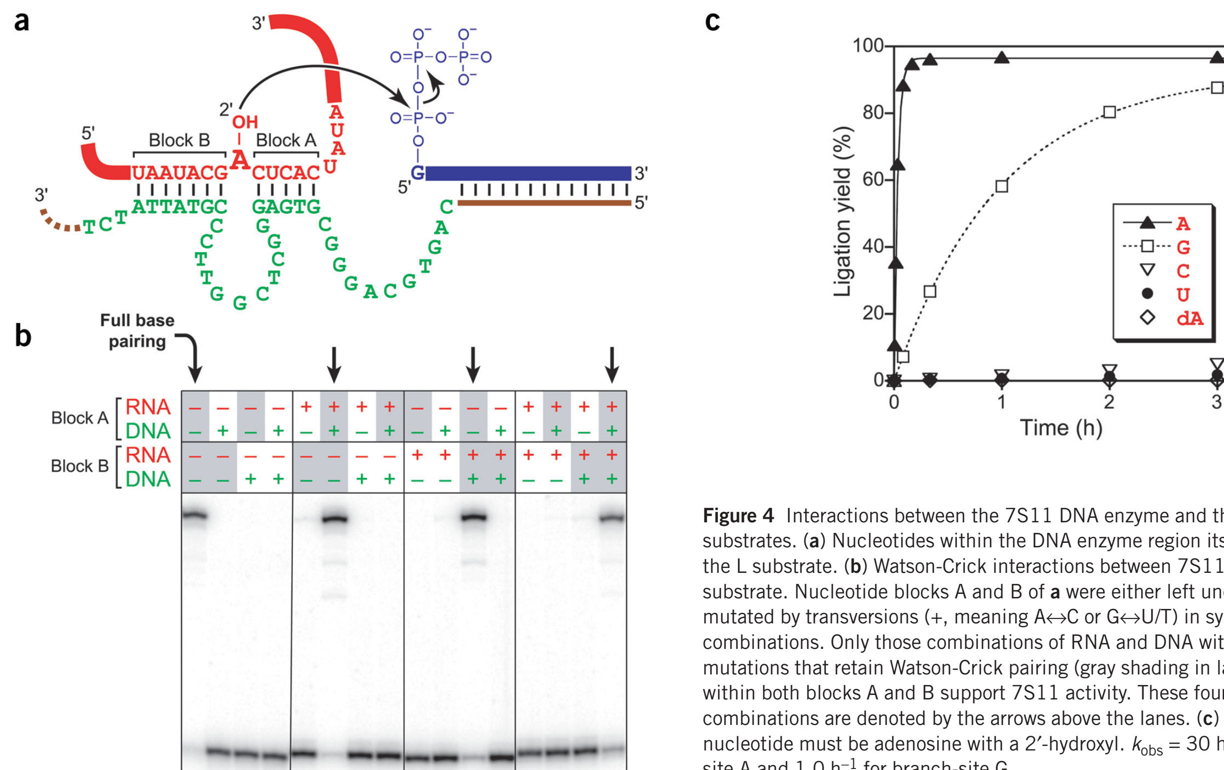

Figure 4 Interactions between the 7S11 DNA enzyme and the RNA substrates. (**a**) Nucleotides within the DNA enzyme region itself (green) bind the L substrate. (**b**) Watson-Crick interactions between 7S11 and the L substrate. Nucleotide blocks A and B of **a** were either left unchanged (–) or mutated by transversions (+, meaning A↔C or G↔U/T) in systematic combinations. Only those combinations of RNA and DNA with compensatory mutations that retain Watson-Crick pairing (gray shading in lane headers) within both blocks A and B support 7S11 activity. These four active combinations are denoted by the arrows above the lanes. (**c**) The branch-site nucleotide must be adenosine with a 2′-hydroxyl. k_{obs} = 30 h^{-1} for branch-site A and 1.0 h^{-1} for branch-site G.

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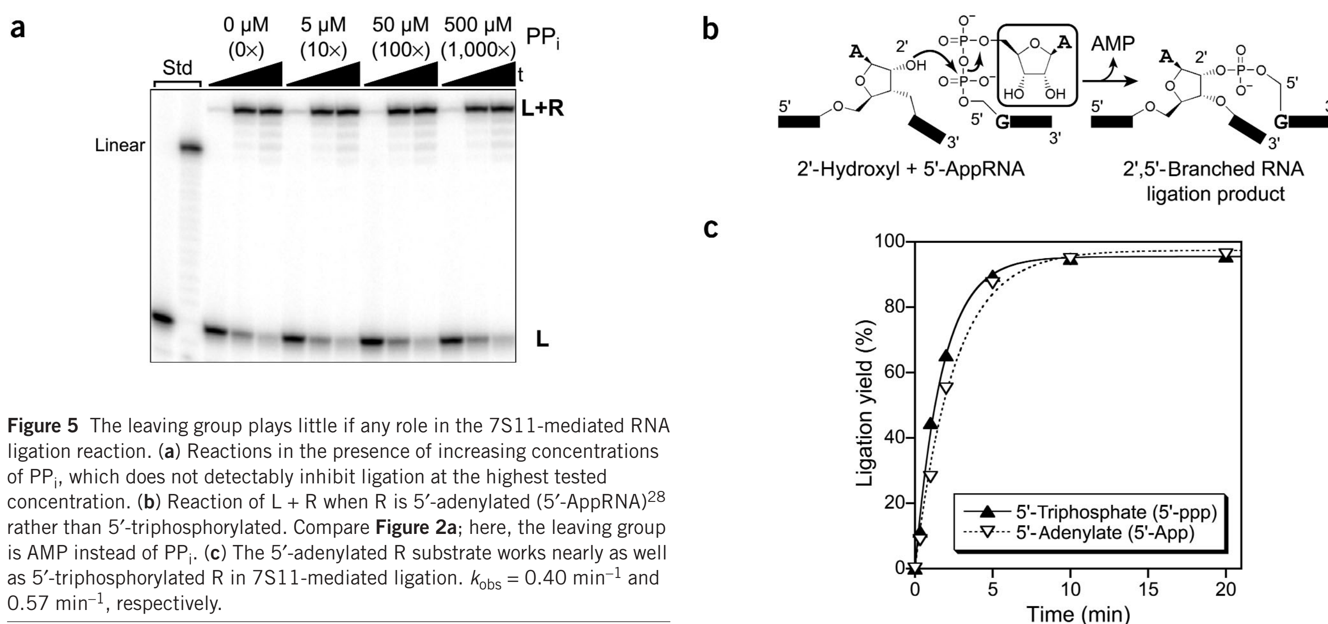

Figure 5 The leaving group plays little if any role in the 7S11-mediated RNA ligation reaction. (**a**) Reactions in the presence of increasing concentrations of PP_i, which does not detectably inhibit ligation at the highest tested concentration. (**b**) Reaction of L + R when R is 5′-adenylated (5′-AppRNA)[28] rather than 5′-triphosphorylated. Compare **Figure 2a**; here, the leaving group is AMP instead of PP_i. (**c**) The 5′-adenylated R substrate works nearly as well as 5′-triphosphorylated R in 7S11-mediated ligation. k_{obs} = 0.40 min^{-1} and 0.57 min^{-1}, respectively.

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for the historical emergence of natural branch-forming activity. Further study of 7S11 is warranted to understand its mechanism of branch formation and its relationship to *in vivo* splicing-related catalysis[6].

We have previously reported independently selected deoxyribozymes (such as 9F7) that form 2′,5′-branched RNA by reaction of an adenosine 2′-hydroxyl with a 5′-triphosphate[18,19]. Although the products from 7S11 have the same 2′,5′-branched connectivity as those from 9F7, the structural and mechanistic implications of these two sets of studies are quite different. In particular, 9F7 binds its RNA substrate in a similar manner to that depicted in **Figure 1**, where the reactive adenosine is located in the short 3′-overhang of the left-hand RNA substrate and is therefore flanked by a duplex region on only one side. Thus, 9F7 and natural splicing enzymes do not bind their RNA substrates in the same way, and no mechanistic implications should be drawn; this is in sharp contrast to the close structural relationship between 7S11 and natural splicing enzymes (**Fig. 6**). This structural relationship leads to the mechanistic implications discussed above.

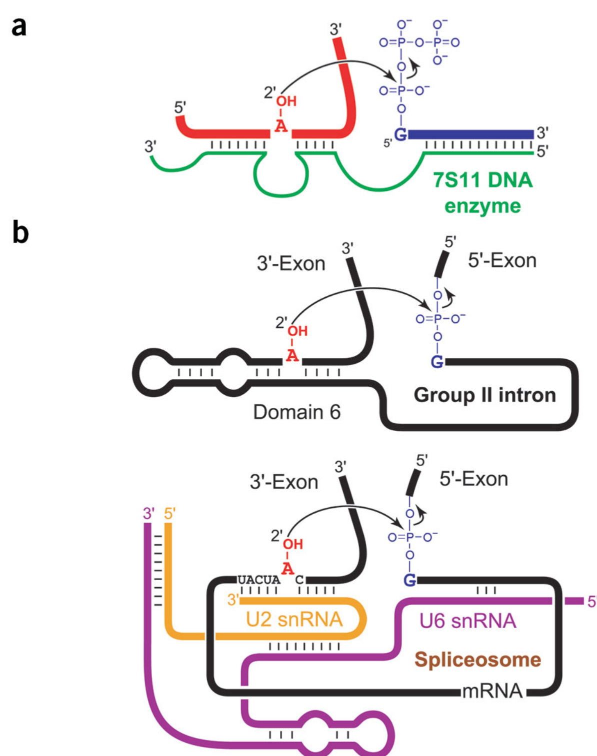

Figure 6 Close relationship between the branch-forming reaction catalyzed by 7S11 and the first step of *in vivo* RNA splicing. (**a**) The 7S11-catalyzed reaction. (**b**) The first step of RNA splicing in group II introns[3] and the spliceosome[5]. In all three cases, the unpaired branch-site adenosine (red) is flanked by Watson-Crick duplex regions.

Implications for prebiotic chemistry

Ribozymes have received attention because of their key role in the 'RNA world' hypothesis, which invokes a primordial epoch in which RNA served both catalytic and information-storage roles[23,24]. DNA is derived from RNA simply by removal of all 2′-hydroxyl groups. This renders DNA much more stable than RNA because intramolecular cleavage is suppressed[12]. However, this stability is often thought to reduce the catalytic potential of DNA, perhaps by reducing the available three-dimensional structures[14] or simply by removing all instances of a particular functional group (hydroxyl) relative to RNA. Here we have shown that the 7S11 DNA enzyme catalyzes a reaction closely related to the first step of *in vivo* RNA splicing (**Fig. 6**). In addition to the mechanistic implications discussed above, the conspicuous absence of 2′-hydroxyl groups in the 7S11 DNA enzyme leads us to conclude that the ribose 2′-hydroxyl is dispensable as a component of biologically relevant catalysis by nucleic acids, at least for the very important case of RNA splicing. One important corollary of this conclusion is that primordial biopolymer candidates lacking hydroxyl groups, such as threose nucleic acid (TNA)[25,26], should receive further experimental attention as potential contributors to prebiotic chemical reactions.

METHODS

RNA and DNA sequences, and selection and cloning procedures. During selection, the sequence of the L RNA substrate was 5′-UAAUACGACUCACUAUA-3′ with free 5′ and 3′ ends, and that of the R RNA substrate was

5′-pppGGAAGAGAUGGCGACGG-3′, where the 5′ end is a triphosphate. The L substrate and linear 3′–5′-linked L + R standard RNA were prepared by solid-phase synthesis (Dharmacon). The R substrate was synthesized by *in vitro* transcription with T7 RNA polymerase from a template prepared by annealing two DNA oligonucleotides[27]; such transcription automatically provides a 5′-triphosphate. The DNA was obtained from IDT and purified by denaturing PAGE. The 5′-AppRNA substrate of **Figure 5** was prepared from the 5′-monophosphorylated R substrate with ATP and T4 RNA ligase[28] (see **Supplementary Methods** and **Supplementary Fig. 1** online). The selection and cloning procedures were done essentially as described in our previous report[15] (see **Supplementary Fig. 1** online). The 7S11 DNA enzyme was prepared as an oligonucleotide by solid-phase synthesis (IDT) and purified by denaturing PAGE. The sequence of 7S11 (parent clone, which was identified as the eleventh clone from the seventh round of the selection denoted 'S' in our laboratory) was the 69-mer 5′-CCGTCGCCATCTC**CAGTGCAGGGCGTGAGGGCTCGGTTCCCGTATTATCTT**TAG<u>GTGACTCGCATTGTCC</u>-3′. The nucleotides in boldface compose the enzyme region, and the 16 underlined nucleotides were removed in the truncation experiment of **Figure 3**.

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Kinetic and product assays. All of the kinetic assays used a trimolecular format in which the L and R substrates were incubated together with the deoxyribozyme (the format of **Fig. 4a**). The L substrate was prepared by *in vitro* transcription with T7 RNA polymerase to permit synthesis of sequence variants, and it therefore includes the leading nucleotides 5′-GGA followed by those listed above. The dephosphorylated and 5′-^{32}P-radiolabeled left-hand RNA substrate L was the limiting reagent relative to the deoxyribozyme E and the right-hand substrate R. The ratio L/E/R was generally ~1:5:15, with the concentration of E equal to ~0.5 μM. Increasing the concentration of E or R (or both) did not substantially change the observed kinetics or yields (data not shown), indicating that the observed activities were not limited by the availability of E or R. Values of k_{obs} and final yield were obtained by fitting the yield-versus-time data directly to first-order kinetics: yield = $Y(1 - e^{-kt})$, where $k = k_{obs}$ and Y = final yield. See our earlier report for a detailed description of the methods of sample preparation and ligation analysis[15].

The partial alkaline hydrolysis and Dbr assays of **Figure 2** were done as described[18]. The debranching reaction of **Figure 2d** was carried to partial conversion to avoid excessive nonspecific nuclease degradation, some of which is visible in the lane labeled '+'. The ligation assays of **Figures 2–5** were done at 50 mM CHES, pH 9.0, 40 mM $MgCl_2$ and 37 °C. For the experiment in **Figure 5a**, the concentrations of L, 7S11 and R were ~0.5, 1.5 and 3 μM, respectively, with [PP_i] from 0 to 500 μM as indicated (0 to 1,000-fold excess PP_i relative to 7S11; L was 5′-^{32}P-radiolabeled).

For the experiment shown in **Figure 4b**, samples loaded on the gel were from timepoints taken at 20-min reaction. Lanes 1, 6, 11 and 16 of this experiment represent the four possible Watson-Crick matched combinations of RNA and DNA in both blocks A and B. Detailed kinetic data for these particular combinations are shown in **Supplementary Figure 2** online. The remaining combinations in the other lanes are all RNA-DNA mismatches in at least one of blocks A or B (or both). For the combinations in lanes 5, 10 and 15, a product band is detected, albeit at substantially reduced intensity (≤2% ligation in 20 min for the experiment in **Figure 4b**; ≤8% ligation in 2 h in a separate experiment). The combinations in the remaining lanes led to no detectable product in 2 h.

Preparative ligation to form branched RNA using the 7S11 DNA enzyme. The 7S11 deoxyribozyme was used to prepare branched RNA on a nanomole scale. A sample containing 2.0 nmol of left-hand substrate L, 2.2 nmol of 7S11 deoxyribozyme E (truncated version from **Fig. 3b**), and 2.4 nmol right-hand substrate R in 140 μl of 5 mM HEPES, pH 7.5, 15 mM NaCl, 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and then cooling on ice for 5 min. The volume was increased to 200 μl containing 50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl and 40 mM $MgCl_2$. The 200-μl solution was incubated at 37 °C for 1.5 h and then mixed with an equal volume of low-dye stop solution (80% (v/v) formamide, 1× TB, 50 mM EDTA, 0.0025% (w/v) each bromophenol blue and xylene cyanol). The sample was purified by denaturing PAGE as described[19], providing 1.0 nmol of branched RNA product and 1.1 nmol of recovered DNA enzyme (see **Supplementary Fig. 5** online for gel image). The MALDI-TOF mass spectrum of the branched product was obtained in the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign School of Chemical Sciences (Urbana, Illinois, USA).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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