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Ligating DNA with DNA

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Abstract: Cloning DNA typically involves the joining of target DNAs with vector constructs by enzymatic ligation. A commonly used enzyme for this reaction is bacteriophage T4 DNA ligase, which requires ATP as the energy source to catalyze the otherwise unfavorable formation of a phosphodiester bond. Using in vitro selection, we have isolated a DNA sequence that catalyzes the ligation of DNA in the absence of protein enzymes. We have used the action of two catalytic DNAs, an ATP-dependent self-adenylating deoxyribozyme (AppDNA) and a self-ligating deoxyribozyme, to create a ligation system that covalently joins oligonucleotides via the formation of a 3′,5′-phosphodiester linkage. The two-step process is conducted in separate reaction vessels wherein the products of deoxyribozyme adenylation are purified before their use as substrates for deoxyribozyme ligation. The final ligation step of the deoxyribozyme-catalyzed sequence of reactions mimics the final step of the T4 DNA ligase reaction. The initial rate constant (k_{obs}) of the optimized deoxyribozyme ligase was found to be 1×10^{-4} min^{-1}. Under these conditions, the ligase deoxyribozyme promotes DNA ligation at least 10^5-fold faster than that generated by a simple DNA template. The self-ligating deoxyribozyme has also been reconfigured to generate a trans-acting construct that joins separate DNA oligonucleotides of defined sequence. However, the sequence requirements of the AppDNA and that of the 3′ terminus of the deoxyribozyme ligase limit the range of sequences that can be ligated.

Introduction

DNA can be used as a medium to create new catalysts that imitate the functions of enzymes made of protein or RNA.[1,2] By using various in vitro selection strategies,[3−5] enzyme engineers have created several distinct deoxyribozymes that specifically catalyze a variety of chemical transformations.[2,6−8] Several classes of deoxyribozymes catalyze important self-processing reactions such as DNA cleavage,[9−12] DNA coupling (via a more reactive phosphorimidazolide),[13] and cleavage of embedded RNA linkages.[14−20] Surprising efficacy in the control of gene expression in cells[21,22] and in whole organisms[23] is exhibited by an RNA-cleaving deoxyribozyme, thus highlighting the possibility that chemically reactive DNA constructs could find utility in these and other biotechnology or nanotechnology applications.[24,25]

In an effort to further define the catalytic potential of DNA, we initiated a program to create a series of self-processing DNAs that catalyze the reactions necessary to covalently join DNA. Previously, we isolated nearly 50 classes of deoxyribozymes that catalyze self-phosphorylation[26] and 12 classes of deoxyribozymes that catalyze self-adenylation,[27] thereby demonstrating that DNA catalysts can use ATP as an energy source. In the latter example, the deoxyribozymes catalyze the formation of a 5′,5′-pyrophosphate linkage in a reaction that expends a single ATP molecule (Figure 1A). This high-energy phosphoanhydride structure (AppDNA) is identical to that generated by the protein enzyme T4 DNA ligase,[28] which targets the linkage

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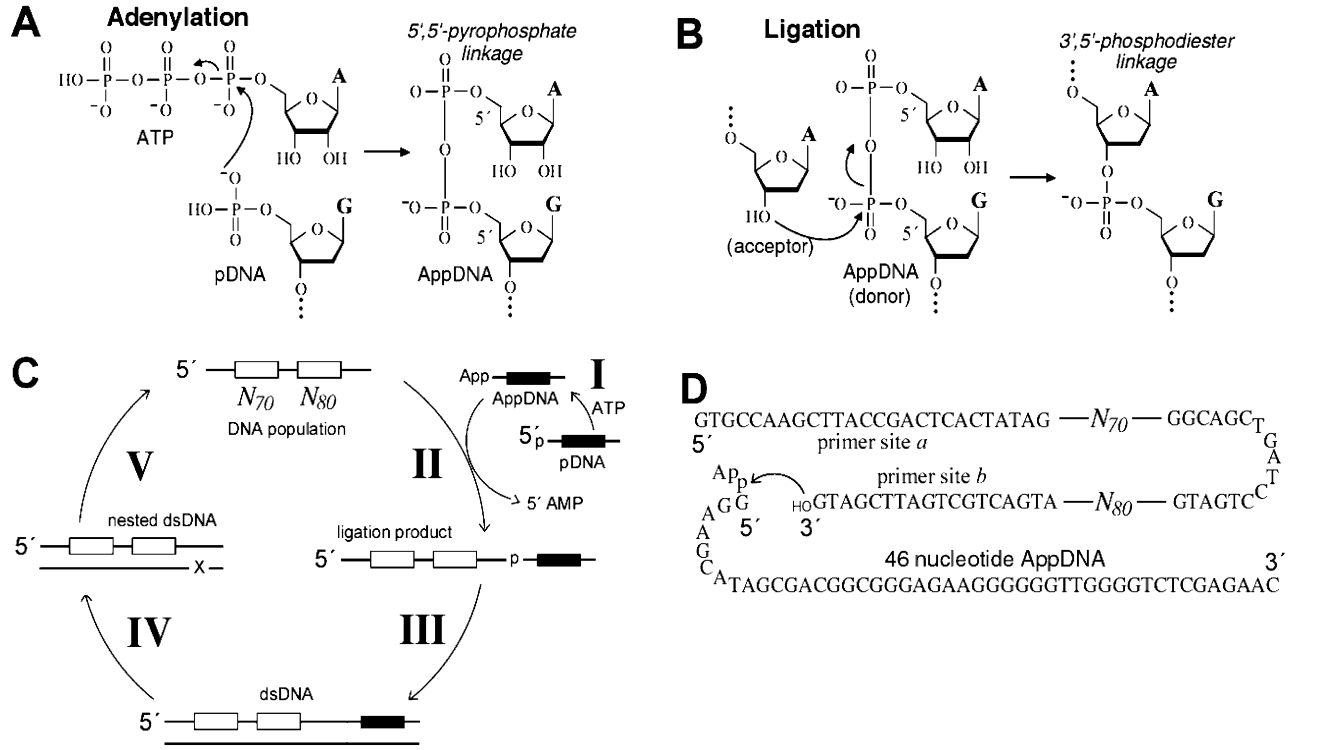

Figure 1. Chemical mechanism of deoxyribozyme-mediated ligation of DNA and the isolation of DNAs that ligate DNA. (A) Mechanism of formation of a 5′,5′-pyrophosphate linkage by a class I self-adenylating deoxyribozyme[27] to form the 46-nucleotide AppDNA substrate. Dotted line represents DNA. (B) Last step of 3′,5′-phosphodiester bond formation catalyzed by T4 DNA ligase[28] and by the self-ligating deoxyribozyme isolated in this study. (C) In vitro selection scheme used to isolate self-ligating deoxyribozymes.[29] Synthetic self-adenylating deoxyribozymes (pDNA) are (I) incubated in the presence of ATP to generate precharged substrate molecules (AppDNA) that carry a 5′,5′-pyrophosphate linkage.[27] A population of DNA constructs (N represents random sequence) is (II) incubated with substrate under selection conditions, and the ligation products are (III) isolated by denaturing (8 M urea) 6% polyacrylamide gel electrophoresis (PAGE) and amplified by the polymerase chain reaction (PCR; primers 3 and 4), (IV) reamplified by PCR (primers 3 and 5) to introduce a chemically labile RNA linkage in one strand (to permit destruction of the template strand), and the next generation of full-length DNAs are (V) isolated by treatment with alkali followed by denaturing PAGE. (D) DNA population and substrate constructs used for in vitro selection. DNA primers (not shown) are as follows: primer 3 is homologous to primer site a, primer 4 is complementary to substrate, and primer 5 is complementary to primer site b. Primer 5 carries a 3′-terminal ribonucleotide to facilitate recovery of full-length single-stranded deoxyribozymes.[29] Primer 6 corresponds to primer 5 but carries an additional 5′-uridyl moiety that encodes for a 3′-terminal adenosyl moiety in the ligase strand. Again, the ribo-U was required to facilitate recovery of a full-length single-stranded deoxyribozyme after NaOH treatment and gel purification.

in a subsequent reaction to form a 3′,5′-phosphodiester bond between acceptor and donor DNA molecules (Figure 1B). In this study, we extend the catalytic function of DNA to include the covalent coupling of DNA by a mechanism that is analogous to the last step of the joining reaction catalyzed by T4 DNA ligase.[28]

Results and Discussion

In Vitro Selection of Self-Ligating Deoxyribozymes. We speculated that a self-ligating deoxyribozyme could be created that uses the AppDNA construct as a substrate in a reaction that is analogous to the last step of DNA coupling promoted by T4 DNA ligase (Figure 1B). To achieve this, we employed an in vitro selection strategy (Figure 1C) that permits selective amplification of DNA molecules from a random-sequence population (Figure 1D) that catalyze the ligation of a DNA substrate to their 3′ terminus.[29] For this study, we employed an activated substrate that consists of a 46-nucleotide self-adenylating deoxyribozyme[27] that was precharged by reaction with ATP (Figure 1D).

The DNA population isolated after 12 rounds (G12) of in vitro selection exhibits significant DNA ligation activity, with ~14% of the precursor DNA converting to ligated product in 24 h (data not shown) under in vitro selection conditions (see Experimental Procedures). At this point, we assumed that the DNA joining reaction involved the formation of a 3′,5′-phosphodiester linkage because this is the only product that is expected to permit selective amplification of DNA by PCR. However, an explicit experimental verification of the ligation product has not been carried out.

Engineering a Minimized Self-Ligating Deoxyribozyme. Sequencing of clones from the G12 population revealed that the population is composed mainly of deoxyribozyme variants of a single structural class. We also observed that each clone includes the sequence of the substrate DNA following the 3′ terminus of the deoxyribozyme sequence. This result is expected if each variant was selectively amplified on the basis of its ability to catalyze DNA ligation. Surprisingly, each clone carries an additional adenosine nucleotide at the ligation junction that was not included in the design of the original DNA construct. This nonencoded nucleotide presumably is the result of the spurious (but essential) addition of an adenosine moiety prior to the DNA-catalyzed ligation event. The most likely source of this additional adenosine is the nontemplated nucleotide additions that are known to be efficiently incorporated by several DNA polymerase enzymes, including the Taq polymerase enzyme used for PCR.[30]

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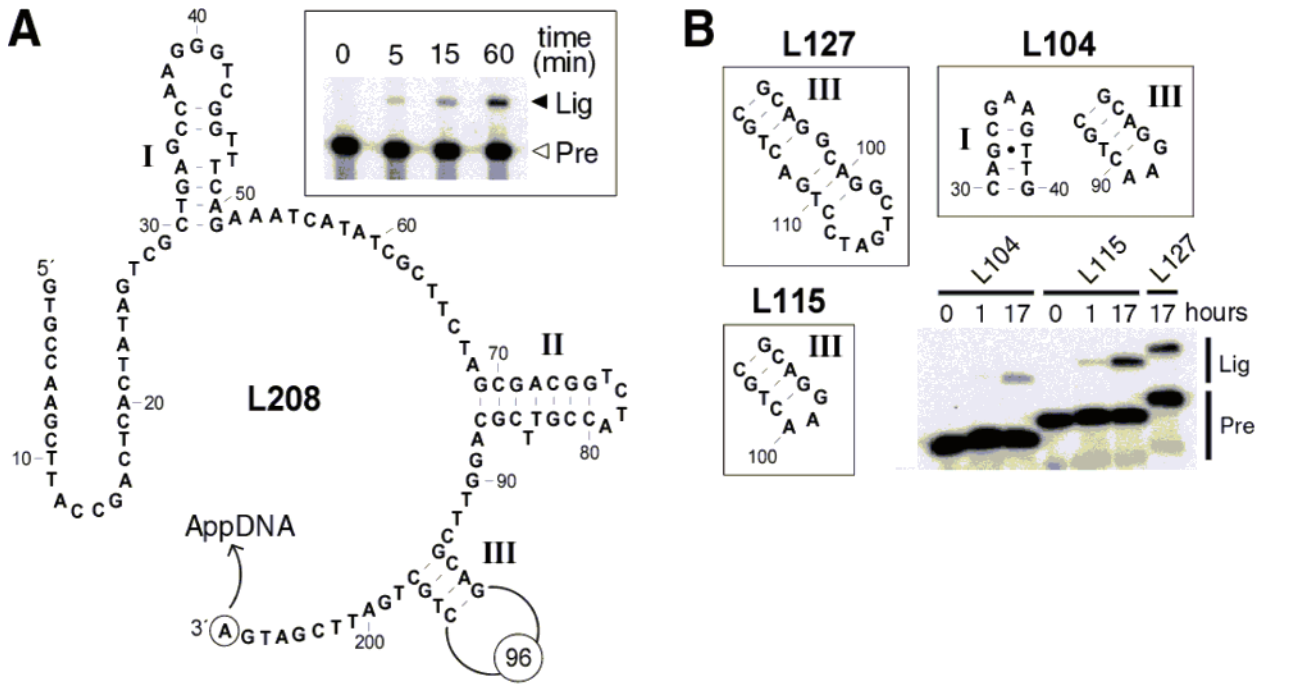

Figure 2. Sequence, structure, and activity of a representative self-ligating deoxyribozyme. (A) The L208 deoxyribozyme has three predicted stem structures (I−III). The encircled A (position 208) is the additional nucleotide whose 3′ hydroxyl serves as the nucleophile for DNA ligation to the 46-nucleotide AppDNA substrate (Figure 1D). Inset: time course for the DNA ligation reaction where Pre and Lig identify bands corresponding to the precursor L208 deoxyribozyme and ligated 254-nucleotide product, respectively. Internally ^{32}P-labeled L208 was incubated under assay conditions for the times indicated. Assay products were separated by denaturing PAGE and analyzed by phosphorimager. (B) Truncated deoxyribozymes are identical to L208 with the exception of the boxed structural elements as depicted for each construct. Assays were conducted as described for panel A with internally ^{32}P-labeled constructs that were generated by use of the appropriate primers during PCR amplification of the L208 or L115 deoxyribozymes as templates.

In our case, it appears that the acquisition of the nontemplated adenosine during each round of selection occurs to a significant portion of the PCR-amplified DNAs, as judged by the extent of deoxyribozyme ligation. For example, comparison of identical G12 populations of deoxyribozymes amplified with primer 5 (no encoded 3′-terminal A) or primer 6 (primer 5 with encoded 3′-terminal A) gave similar ligation yields of ∼14% and ∼18%, respectively, when incubated for 24 h under standard assay conditions (data not shown). These results indicate that at least 14% of the pool prepared with primer 5 has a nontemplated A residue, whereas intentional introduction of a templated A residue provides only a modest improvement in yield. Although we did not explore the numerous factors that might influence the extent of nontemplate A addition and ligation yield, we chose to include this extra 3′-terminal nucleotide in the deoxyribozyme constructs for all subsequent experiments.

An active clone from the G12 population was mutated via chemical synthesis and the resulting population of deoxyribozyme variants was subjected to six additional rounds of selective amplification for DNA ligase function. A representative clone from the reselected pool experienced a decrease in length from 211 nucleotides present in the original construct to 208 nucleotides, most likely due to the inherent mutagenic nature of the selective amplification process. This L208 enzyme has a DNA ligation activity of about 4.3% in 1 h (inset, Figure 2A) and about 20% in 17 h (data not shown). A secondary structure model for this L208 deoxyribozyme (Figure 2A; L208 represents a 208-nucleotide ligase deoxyribozyme) was generated with the aid of the DNA mfold algorithm (the DNA mfold server can be accessed on the Internet at http://bioinfo.math.rpi.edu/∼mfold/dna/form1.cgi). The L208 sequence has a surprisingly low propensity for secondary structure formation, and only three potential stem−loop elements (I, II, and III) were apparent in the original random-sequence region. Upon examining the artificial phylogeny of variants from reselection (18 clones), we find that random mutations occur with high frequency in the region encompassed by nucleotides 107−187, indicating that this region is not essential for deoxyribozyme function. In contrast, most mutations that occurred in regions encompassed by nucleotides 30−51 and 69−87 are compensatory and thus are consistent with the formation of stems I and II. As expected, deletion of the majority of the nonconserved nucleotides to form the L127 and L115 deoxyribozymes resulted in truncated constructs that retained DNA ligase activity (approximately 15% in 17 h for both enzymes; Figure 2B). In addition, we found that L104 exhibits $^1/_{10}$ ligase activity (1.4% in 17 h; Figure 2B) compared to the original construct.

Kinetic Characteristics of DNA Ligation. The L115 construct was used to establish the kinetic characteristics of deoxyribozyme-mediated DNA ligation. The maximum initial rate constant observed (k_{obs}) for DNA ligation under in vitro selection conditions is 1×10^{-4} min^{-1} (Figure 3A). These reaction conditions provide saturating concentrations of AppDNA substrate (Figure 3B) but subsaturating amounts (1 mM) of Mn^{2+} (Figure 3C), the latter of which serves as an essential metal ion cofactor. Under these conditions, the maximum k_{obs} measured for L115 is more than 100 000-fold greater than the rate constant for the spontaneous ligation of acceptor and donor DNAs that are aligned on a complementary DNA template (Figure 3D). The rate enhancement exhibited by L115 also compares favorably with a previously reported deoxyribozyme

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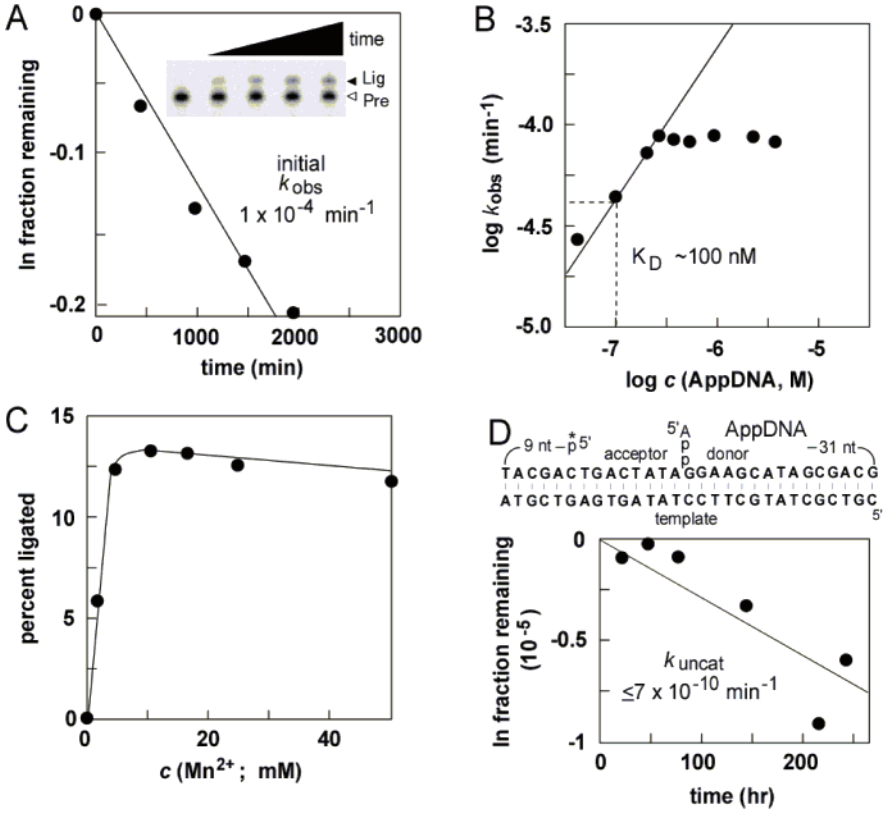

Figure 3. Kinetic characteristics of the L115 self-ligating DNA. (A) Rate constant for 5′ ^{32}P-labeled L115 deoxyribozyme (trace = <50 nM) under selection conditions. k_{obs} was established by determining the negative slope of the line resulting from a plot of the natural logarithm of the fraction of DNA that remains unligated versus time. (B) Log plot of k_{obs} versus substrate concentration (c) is consistent with a saturable binding site for the 46-nucleotide AppDNA with an apparent dissociation constant (K_D) of ~100 nM (where activity is half-maximal). The solid line represents a slope of 1, which is expected for the binding of a single AppDNA molecule per deoxyribozyme. (C) Plot of the percent of L115 ligated at various concentrations of Mn^{2+} after a 24 h incubation under selection conditions reveals that the enzyme is saturated with metal cofactor at concentrations above 5 mM. The assay is conducted as described for selection but in the absence of other types of divalent metal ions. (D) Template-catalyzed rate of DNA ligation with the acceptor and 5′-adenylated DNAs shown. Assay contained 0.5 μM 5′ ^{32}P-labeled acceptor (asterisk), 1 μM template, and 1.5 μM donor (46-nucleotide AppDNA) molecules that were incubated under assay conditions for the times indicated. Reaction products were separated by denaturing 10% PAGE and analyzed by phosphorimager. The rate constant was established as described for panel A.

that catalyzes DNA coupling via a more reactive phosphorimidazolide activation chemistry.[13] We have not examined the extent of reaction that is reached upon exhaustive incubation, and therefore we cannot discount the possibility that the rate constants reported are affected by misfolding of the ligase deoxyribozyme or its corresponding AppDNA substrate.

Although the L115 deoxyribozyme promotes DNA ligation by more than 5 orders of magnitude over that generated by a simple DNA template, the maximum k_{obs} measured for the deoxyribozyme is approximately 5 orders of magnitude lower than that of the corresponding step exhibited by the protein-based DNA ligase from *Escherichia coli*.[28,31] The protein-catalyzed reaction might be even faster because its rate constant is established under multiple turnover conditions, and therefore it could possibly be limited by slower conformational changes. Then again, the 5 orders of magnitude discrepancy in the rate of reaction between the deoxyribozyme and the protein enzyme in part may be explained by the misfolding of the deoxyribozyme. Thus it is possible that the rate constant for the chemical step of the deoxyribozyme might be considerably higher. Regardless, we expect that faster ligase deoxyribozymes could be isolated by continued selection efforts, thus narrowing the performance gap between engineered deoxyribozymes and natural protein enzymes. The reaction conditions required to permit self-adenylation by the substrate deoxyribozyme and to permit ligation by the L115 deoxyribozyme are sufficiently different as to prevent consecutive catalysis in a single mixture. Specifically, self-adenylation requires Cu^{2+} and is most active when low K^+ and high Na^+ are used.[27] In contrast, the ligase deoxyribozyme has been selected by use of a buffer that has high K^+, no Na^+ and no Cu^{2+}. Our attempts to catalyze both reactions in a single step were not successful because of these contrasting reaction conditions. This precluded us from determining the overall rate enhancements of the two reactions as they might occur in a single mixture. However, the rate enhancements generated even by these prototypic catalytic DNAs are sufficient to promote the two successive, albeit separate, steps of DNA ligation on a time scale of hours, which otherwise would occur far more slowly (years) than the rate of the competing reaction of spontaneous DNA cleavage.

Sequence-Specific Ligation of DNA by DNA. To further examine the structure and function of the ligase deoxyribozyme, we generated a tripartite assembly of DNAs that retains ligase activity. This complex comprises a 78-nucleotide deoxyribozyme core (L78), a separate 19-nucleotide DNA that encompasses the 3′ terminus of the deoxyribozyme (3′ frag), and an adenylated substrate represented by the 46-nucleotide

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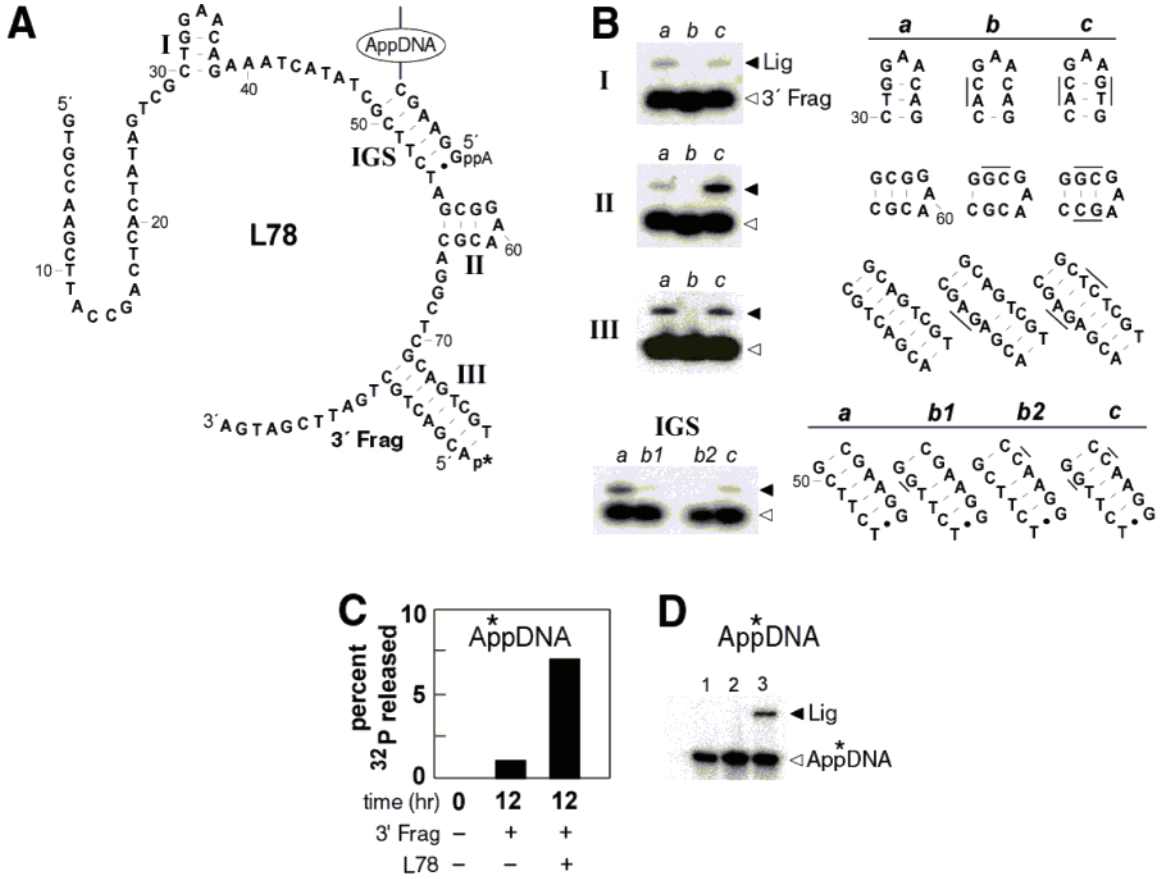

Figure 4. Trimolecular deoxyribozyme ligase system. (A) Sequence and secondary structure model for the complex formed between the L78 deoxyribozyme, the 46-nucleotide self-adenylating deoxyribozyme (AppDNA) substrate, and the 3′-terminal oligonucleotide substrate (3′ Frag). IGS represents the internal guide sequence for recognition of the adenylated DNA substrate. The asterisk designates a ^{32}P-labeled phosphate group. (B) Analysis of predicted base-pairing elements of the trimolecular L78 construct. The DNA ligase activity of an unmodified complex (*a*) is compared to the activities of variant complexes with disrupted (*b*) or restored (*c*) base-pairing elements. Reactions containing 0.5 μM 3′ Frag, 1 μM L78, and 1.5 μM AppDNA substrate were incubated under assay conditions for 24 h and analyzed by denaturing 10% PAGE and phosphorimager. The more active L78 variant depicted as mutant c for stem II was used in the assays for stem III and IGS. (C) Release of the α-phosphorus of AppDNA as a low molecular weight product upon L78-catalyzed DNA ligation. A preparation of AppDNA labeled with ^{32}P at the α-phosphate (relative to the adenyl group; asterisk) was incubated for 12 h with the trimolecular L78 complex and analyzed as described for panel B to establish the percentage of radioactivity released from AppDNA as a high-mobility band on PAGE (5′-adenosine monophosphate). (D) Incorporation of the β-phosphorus of AppDNA into the ligated (Lig) product created by the trimolecular L78 complex. A preparation of AppDNA substrate that was labeled with ^{32}P at the β-phosphate (relative to the adenyl group; asterisk) was used as a marker (lane 1), or incubated in the absence (lane 2) or presence (lane 3) of the L78 deoxyribozyme as described for panel C.

self-adenylating deoxyribozyme (Figure 4A). This tripartite arrangement permits the ligation of two substrate DNAs by use of a third catalytic DNA strand. Activity assays of variants that represent the systematic use of disruptive and compensatory mutations in each of the putative stem elements I, II, and III are consistent with the predicted secondary structure (Figure 4B). The results seen in Figure 4B for stem II c, wherein the stem−loop structure containing a GAA triloop with a CG closing base pair has >20% ligation activity compared to the 5% ligation for wild type, may in part be due to the stabilizing effects of this triloop.

In addition, we speculated that a highly conserved region of the L208 deoxyribozyme (nucleotides 49−54 of L78) might serve as an internal guide sequence (IGS) for binding the AppDNA substrate. Similar guide elements are observed in the natural group I and group II self-splicing ribozymes.[32,33] We hypothesize that the putative IGS sequence interacts with the 5′-most domain of the self-adenylating deoxyribozyme, which can make six base pairs between the two deoxyribozymes. To test for this possible interaction, we prepared two forms of AppDNA by self-adenylation of the 46-nucleotide deoxyribozyme depicted in Figure 1D and by self-adenylation of a variant deoxyribozyme wherein the G at the 5th nucleotide position has been changed to a C. We find that the matched combinations of IGS variants of L78 with their complementary AppDNA variants exhibit greater catalytic activity compared to mismatched variants (14% for *a* versus 0.5% for *b1*; 0.1% for *b2* versus 5% for *c*; Figure 4B, IGS), which is consistent with the presence of the proposed IGS−substrate interaction. Unfortunately, we are unable to exhaustively examine the putative IGS−substrate interaction because we must abide by the sequence requirements of two enzymes when testing mutations and compensatory mutations. We were fortunate to have evidence that the variant self-capping enzyme used herein can tolerate a mutation in this apparently critical 5′-most domain, such that we could prepare a self-adenylating mutant that actually self-adenylated.[27]

Although additional mutations in the self-adenylating deoxyribozyme might be used to more rigorously test the IGS model, we suspect that most would not self-adenylate and thus would

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not be useful in the ligase assay. However, we have several lines of evidence that are consistent with our IGS hypothesis: (A) the IGS element is conserved in the artificial phylogeny; (B) the IGS is complementary to the 5′-most domain of the self-adenylating deoxyribozyme; and (C) the single example of a self-adenylating deoxyribozyme variant that retains its activity also permits function by the ligase deoxyribozyme when the corresponding mutation is made in the IGS domain. These results indicate that substrate specificity by the ligase deoxyribozyme is most likely determined by base-pairing interactions and that these can be engineered to permit a limited number of sequence changes to be made.

Exploring the Mechanism of Ligation. The mechanism of the reaction was examined in greater detail by conducting assays with differently radiolabeled AppDNA substrates. A ^{32}P-labeled phosphate group was placed in either the α-phosphate (Figure 4C) or β-phosphate (Figure 4D) position relative to the adenyl group of AppDNA by reacting the 46-nucleotide self-adenylating deoxyribozyme with [α-^{32}P]ATP or by reacting a 5′ ^{32}P-labeled self-adenylating deoxyribozyme with unlabeled ATP, respectively. Upon deoxyribozyme action, the radiolabeled phosphate in the α position is released from DNA, while the radiolabeled phosphate in the β position is integrated with the ligated product. These results indicate that the ligase deoxyribozyme catalyzes the same chemical transformation as the last step of the ligation reaction promoted by T4 DNA ligase (Figure 1B).

The mechanism of the second step of this deoxyribozyme reaction series is similar to that catalyzed by certain RNA ligase ribozymes that have been created by in vitro selection (e.g., see ref 34 and citations therein). In each case, a 3′,5′-phosphodiester linkage is formed at the expense of a phosphoanhydride linkage (with the exception of several ribozymes that promote the formation of 2′,5′-phosphodiester linkages). We have not determined whether the terminal adenosyl moiety of the AppDNA substrate is required for ligation, but if necessary, its presence would most likely be required for molecular recognition purposes only. Several reports of engineered ribozymes that catalyze RNA ligation at the expense of a 5′-terminated triphosphate moiety provide convincing evidence that numerous RNA folds can promote such coupling reactions.[34] Given the recent reports of deoxyribozymes that ligate RNA[35] and deoxyribozymes that ligate DNA via other activated linkages,[13,36] we speculate that a great diversity of mechanisms could be used by engineered DNA enzymes.

Conclusions

T4 DNA ligase catalyzes DNA ligation by forming a 5′,5′-pyrophosphate linkage between the 5′-phosphate of the donor DNA and the α-phosphate of ATP via a covalent AMP-enzyme intermediate. This capped DNA donor is then used as an activated substrate that is attacked by the 3′ oxygen of the acceptor DNA to form the new 3′,5′-phosphodiester linkage. We have previously demonstrated that capped DNA can be formed by single-stranded DNA without the aid of protein enzymes.[27] In the present study, we have isolated and characterized deoxyribozymes that mimic the final step of ligation catalyzed by T4 DNA ligase. The isolation of a deoxyribozyme ligase that uses the product of a self-adenylating deoxyribozyme as a substrate is demonstrative of a family of more complex chemical transformations that DNA could be made to catalyze. Improved versions of such self-modifying DNAs would be of significant use in biotechnology and nanotechnology applications. In the present example, the self-adenylating deoxyribozyme serves as a substrate for DNA ligation. This ligase deoxyribozyme can be tailored to use variants of an AppDNA substrate by altering the sequence of stem III, and a variant construct can ligate separate DNA substrates of defined sequence. However, the range of sequences that can be ligated by this particular deoxyribozyme is limited by the requirements of the self-adenylating deoxyribozyme and by the sequence requirements of the 3′ frag domain. The recent demonstration that T4 DNA ligase is capable of accumulating adenylated DNA in an acceptor-free system independent of the DNA sequence[38] opens the avenue to evolve deoxyribozyme ligases for various DNA sequences. Future engineering efforts should give rise to even more versatile deoxyribozymes and to deoxyribozyme systems that harness the energy of ATP or other high-energy chemicals to carry out multistep processing reactions. The rate enhancements generated by these deoxyribozymes in the two-step process of DNA ligation support the hypothesis that DNA has sufficient catalytic power to serve as a useful agent of chemical change.

Experimental Procedures

Oligonucleotides. Synthetic DNAs were prepared by standard automated synthesis (Keck Biotechnology Resource Laboratory, Yale University) and were purified by preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE) prior to use. Single-stranded DNAs were either internally ^{32}P-labeled during synthesis by the polymerase chain reaction (PCR) with [α-^{32}P]dGTP or labeled at the 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase. AppDNA was prepared as described previously.[27] The mutagenized population of L208 deoxyribozymes was created by joining two synthetic DNAs with T4 DNA ligase and the appropriate DNA template. Nucleotides 27−193 of the final construct were prepared with a degeneracy of 0.12 per position.[37] In vitro selection was carried out as described.[29] Reselection was carried out as described in the caption to Figure 1C.

Selection Conditions. In vitro selection was carried out as described.[29] Selection reactions were conducted in 50 mM HEPES (pH 7.0 at 23 °C), 200 mM KCl, 15 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM $MnCl_2$, 0.3 μM precursor DNA (unless otherwise stated), and 0.6 μM substrate DNA. The first round of selection was initiated by combining 316 pmol ($\sim 10^{14}$ variants) of the 211-nucleotide DNA construct with 630 pmol of AppDNA substrate in selection buffer, followed by incubation at 23 °C for 48 h. Subsequent rounds were conducted with $\sim 1/10$ scale reactions.

Deoxyribozyme Assays. Assays were conducted in 50 mM HEPES (pH 7.0 at 23 °C), 200 mM KCl, 15 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM $MnCl_2$, trace ($\sim$25 nM) precursor, and 1 μM AppDNA substrate unless otherwise indicated. Reactions were incubated at 23 °C for specified periods. Assays were terminated by the addition of an equal volume of solution containing 95% formamide and 100 mM EDTA.

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