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Conversion of a Ribozyme to a Deoxyribozyme through In Vitro Evolution

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Summary

An RNA ligase ribozyme was converted to a corresponding deoxyribozyme through in vitro evolution. The ribozyme was prepared as a DNA molecule of the same sequence, and had no detectable activity. A population of randomized variants of this DNA was constructed and evolved to perform RNA ligation at a rate similar to that of the starting ribozyme. When the deoxyribozyme was prepared as an RNA molecule of the same sequence, it had no detectable activity. Thus, the evolutionary transition from an RNA to a DNA enzyme represents a switch, rather than a broadening, of the chemical basis for catalytic function. This transfer of both information and function is relevant to the transition between two different genetic systems based on nucleic acid-like molecules, as postulated to have occurred during the early history of life on Earth.

Introduction

The discovery, more than 20 years ago, of RNA molecules with catalytic activity [1, 2] broadened the notion of an enzyme to include informational macromolecules composed of either amino acids or nucleotides. The first several reported examples of RNA enzymes (ribozymes) all are naturally occurring RNA molecules that catalyze the cleavage or formation of an RNA phosphoester. More recently, the large subunit ribosomal RNA was recognized to be a ribozyme that catalyzes the peptidyl transfer step of protein synthesis [3]. Beginning in the early 1990s, it became possible to develop artificial ribozymes through a process of in vitro evolution [4, 5]. This has led to numerous ribozymes that catalyze a broad range of chemical reactions (for reviews, see [6, 7]). Similar techniques have been used to obtain DNA enzymes (deoxyribozymes) with various functions (for reviews, see [8, 9]), although there is no known example of a deoxyribozyme in nature.

Structural and mechanistic studies of ribozymes often make use of the site-specific replacement of ribonucleotides by deoxyribonucleotides to assess the importance of particular 2′-hydroxyl groups within the RNA. Of course, there are other important differences between RNA and DNA, such as the tendency of RNA to adopt a C3′-*endo* sugar pucker compared to the preferred C2′-*endo* pucker of DNA. RNA lacks a methyl group at the C5-position of uridine, while this group is present at the corresponding position of thymidine in DNA. When in the double-stranded form, RNA tends to adopt an A-form helix, while DNA adopts a B-form helix. The structure of single-stranded regions within RNA and DNA differs in idiosyncratic ways even when the sequences are the same. In light of these differences, it is not surprising that there is no known example of a ribozyme that retains catalytic activity when prepared as the corresponding DNA molecule. There is a case of an 18 nucleotide DNA that forms a G-quartet structure and binds hemin, resulting in a 250-fold enhancement of hemin-dependent peroxidase activity [10]. When this DNA is prepared as the corresponding RNA, it binds hemin with 30-fold reduced affinity, but retains a similar catalytic rate enhancement in the hemin-dependent reaction [11].

It has been possible to make multiple deoxynucleotide substitutions within a ribozyme and retain at least a low level of activity. The best-studied case is the hammerhead ribozyme, which is a simple motif that catalyzes the site-specific cleavage of RNA. It contains a catalytic core of 13 highly conserved residues, supported by three flanking stems of variable sequence [12]. When all but 7 of the conserved residues are replaced by the corresponding deoxynucleotides the catalytic rate is reduced by about 20-fold, and when all but the 4 most critical residues are replaced by DNA, the catalytic rate is reduced by 300-fold [13, 14]. However, an all-DNA version of the hammerhead ribozyme has no detectable activity [15, 16]. Similarly, the hairpin ribozyme, which also catalyzes the site-specific cleavage of RNA, was shown to require four critical RNA residues in order to retain catalytic activity [17].

In vitro evolution may provide a means to convert a ribozyme to a corresponding deoxyribozyme. The potential for such a conversion has been demonstrated with the *Tetrahymena* ribozyme, which was evolved to cleave DNA as well as RNA [4, 18], and subsequently evolved to cleave DNA in preference to RNA [19]. This change of behavior required mutating less than 5% of the ribozyme's residues. Similarly, the RNase P ribozyme was evolved to cleave DNA as well as RNA, which also required mutating less than 5% of the ribozyme's residues [20]. It is expected to be more difficult to convert an enzyme, rather than its substrate, from RNA to DNA.

In the present study, the R3C RNA ligase ribozyme [21] was converted to a corresponding deoxyribozyme through in vitro evolution. This ribozyme has an unusual pedigree. Its ancestor was obtained by in vitro evolution, starting from a population of random-sequence RNAs that contained only adenosine, guanosine, and uridine, but lacked cytosine. This led to the development of the R3 (random 3-letter) ribozyme, which catalyzes the ligation of two oligonucleotides, one terminating in a 2′,3′-hydroxyl, and the other terminating in a 5′-triphosphate, forming a 3′,5′-phosphodiester linkage and releasing inorganic pyrophosphate. The R3 ribozyme subsequently was evolved to a cytosine-containing ribozyme, the R3C

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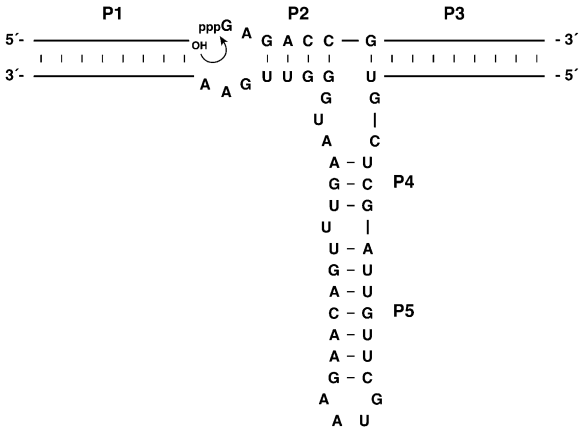

Figure 1. Sequence and Secondary Structure of the R3C RNA Ligase Ribozyme

The five paired regions (P1–P5) are indicated (see [21]). Nucleotides of variable sequence within the two substrates and complementary template portions of the ribozyme are indicated by horizontal lines. Curved arrow indicates attack of a 3′-hydroxyl on a 5′-triphosphate, resulting in RNA ligation.

ligase, which performs the same reaction, but with a 20-fold faster catalytic rate [21]. The R3 ligase also was evolved to a ribozyme that contains only diaminopurine and uracil residues and exhibits a 20-fold slower catalytic rate compared to the R3 ligase [22].

The R3C ligase ribozyme contains about 55 nucleotides, including two template regions of variable length that bind the two RNA substrates (Figure 1). The 2′,3′-hydroxyl-bearing substrate is bound by a single region of Watson-Crick pairing (P1 helix), while the 5′-triphosphate-bearing substrate is bound by two regions of Watson-Crick pairing (P2 and P3 helices) that are interrupted by a central stem-loop (P4 and P5 helices). The 5′-triphosphorylated substrate can be linked to the ribozyme via a hairpin loop, resulting in a bimolecular reaction that proceeds with a k_{cat} of 0.3 min^{-1} and K_m of 0.4 μM. Alternatively, this substrate can be provided separately in a trimolecular reaction that proceeds with a k_{cat} of 0.2 min^{-1} and K_m of 0.1 μM [21].

When the R3C ribozyme was converted to a DNA molecule of the same sequence (replacing U with T), it had no detectable activity. This DNA molecule was used as a starting point to generate a population of random variants, which were selected for the ability to catalyze the RNA ligation reaction. Ten rounds of in vitro evolution were carried out, resulting in a deoxyribozyme with RNA ligase activity. When the deoxyribozyme was prepared as the corresponding RNA molecule, it had no detectable activity. Thus, the evolutionary transition from an RNA to a DNA enzyme represents a switch, rather than a broadening, of the chemical basis for catalytic function.

Results

In Vitro Evolution

A starting population of ~5 × 10^{12} different molecules was constructed based on a DNA version of the R3C ligase sequence, with random mutations introduced at a frequency of 12% per nucleotide position at 51 positions that encompass the catalytic domain (Figure 2A). This population included all possible variants containing up to seven mutations relative to the wild-type sequence, as well as a sampling of the higher-error mutants. The population of DNA molecules was joined via a hairpin loop to a 5′-triphosphate-bearing RNA substrate (**S2**), which had the sequence 5′-pppGAGACC GUAAUGAGUAG-3′. The joined molecules were challenged to react with a separate 2′,3′-hydroxyl-bearing substrate (**S1**), which had the sequence 5′-d(GAACT GACGAACTGATGCTCAC)-r(UAUA)-3′. Molecules that performed the ligation reaction were selected based on their reduced mobility in a denaturing polyacrylamide gel. The gel-purified products were further selected by PCR amplification, employing one primer that was complementary to the 3′ end of the DNA, and a second primer that matched the sequence at the 5′ end of **S1**. The PCR products then were used to generate a progeny population of DNA molecules, which were enriched for the desired activity.

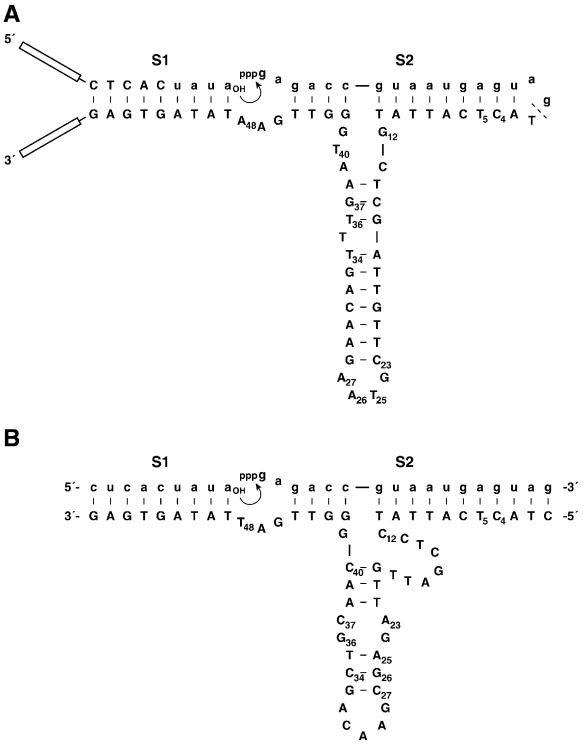

Figure 2. Composition of the Starting DNA and Evolved 10-18 Deoxyribozyme

(A) The starting pool was based on a DNA version of the R3C ribozyme that was joined to the **S2** substrate and allowed to react with a separate **S1** substrate. DNA residues are in uppercase; RNA residues are in lowercase. Dashed line indicates the site of joining between **S2** and the first deoxynucleotide that was randomized. Boxed regions at the 5′ end of **S1** and the 3′ end of the DNA correspond to primer binding sites used during selective amplification.
(B) The 10-18 deoxyribozyme contains 57 residues, including a 5′-terminal C that was added to allow pairing with the 3′-terminal G of **S2**. Mutated positions relative to the starting sequence are numbered. The C→A change at position 4 and T→C change at position 5 were reverted to restore complementarity with **S2**.

The DNA-catalyzed reaction was carried out in the presence of 25 mM $MgCl_2$ at pH 8.5 and 23°C, employing 5 μM **S1** and 0.1 μM **S2**-DNA conjugate. The time allotted

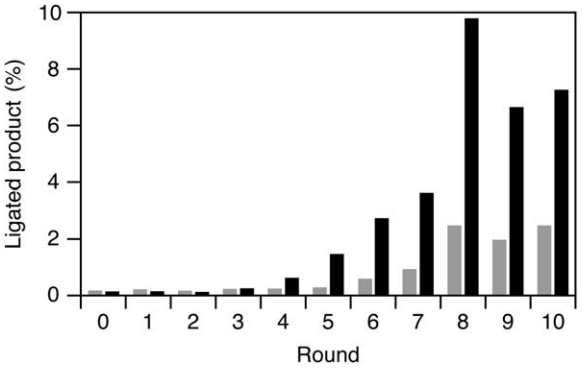

Figure 3. Activity of the Population of Molecules in the Starting Pool and after Each Round of In Vitro Evolution

The population was assayed in the bimolecular reaction format, employing 0.1 μM labeled **S2**-DNA and 5 μM **S1** that contained either one or four 3′-terminal ribonucleotides (gray and black bars, respectively). The fraction of **S2**-DNA that was converted to ligated products was determined after 30 min.

Table 1. Aligned Sequences of 22 Clones of the Evolved RNA Ligase Deoxyribozyme

```
          10        20        30        40        50
      5′ ...|....|....|....|....|....|....|....|....|.. 3′
wt    ---TACTCATTATGCTCGATTGTTCGTAAGAACAGTTTGAATGGGTTGAATATAGTGAG-
con   ............C.........A.A.C........G...C.......T..........
18    .....AC......C..........A.AGC......C.GC..C.......T..........
13    .....AC..C...C.AT......AA.AGC.G....C.GC..C.......T.......G.G
9     .....AC......C..........A.A.C........GC..C.......T.......G..
19    ..TA..C......CT....-....A.A.C......C.GC..C.......T..........
15    ....CGC......C..T..G....A.A.C........GC..C.......T..........
7     .....TG......C..T..C....A.ATC..G.....GC..C.......T..........
22    .....AC......C.CT.......A.A.C........GCG.C.......T........C.
6     ......G......C.CT.G.....A.A.C..G.....GC..C.......T........C.
21    ...A.AC......C........CAA.A.C..G.....G...C.......T.G.......G
3     .CA..T....C..C.....C..CAA.A.C........G...C.................
5     ....C........T.A...A..A.A.C.C...T...G....C.......T.......GCG
17    ....C........T..A..A..A.A.C.C...T...G....C.......T...G...GCG
4     ...AG........T.A...A..A.-.C.C...T...G....C.......T.......G..
12    .AACC........C.A.C.A..A.A.C.C...T...G...GC.......T..........
20    ......C......T.A...A..A.A.C.C...T...G....C.......T........C.
14    -AGG........A.........C...A.C....G...G.C.C.......T.......--.
8     ...GCAG...........CA..CC..A.C....G...G.C.C.......T.......G..
2     ---G...GG..T......T..C......G......A.GT..........-.......GCG
10    ...G...GGC.C......T..C....A.T......A.GT.G........-......C.C..
16    ...G...A..C........A................C..TGC....G.............
1     ACTG......AC...A..C....C.C....GT-....GCGCA..-.G..........G..
11    ......GGG.....G.T....A.A.....A.......AGG...A....TTGG.C...-..
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Mutations relative to the starting sequence (wt) are indicated for the consensus sequence (con) and for individual clones (numbered). Deleted nucleotides are indicated by a dash; unchanged nucleotides are indicated by a period. Scale at the top corresponds to nucleotide positions 2–57 of the deoxyribozyme (Figure 2B). Note that the C residue at position 1 is not present in the cloned DNA, but was added to allow pairing with the 3′-terminal G of the **S2** substrate.

for the reaction was reduced progressively over the course of ten rounds of in vitro evolution, beginning with 14 hr in the first round and ending with 15 s in the final two rounds. Random mutations were introduced at a frequency of 0.7% per nucleotide position following the fifth, sixth, and seventh rounds by a PCR mutagenesis procedure [23]. The population as a whole was assayed after each round, demonstrating a progressive increase in ligation activity (Figure 3). Following the tenth round, individuals were cloned from the population and sequenced.

Examination of 22 cloned sequences revealed that about half fell into a major sequence cluster, although no two sequences were identical (Table 1). The mean number of mutations per clone was 14.7. Individuals from the major sequence cluster were found to exhibit the highest level of catalytic activity. One of these, designated "10-18" (round 10, clone 18), was chosen for further study (Figure 2B). It contains 12 mutations relative to the wild-type sequence, including 7 mutations that are present in the consensus sequence of all cloned individuals. Two of the nonconsensus mutations within the 10-18 deoxyribozyme occur in a portion of the template region that binds the 3′ end of the **S2** substrate (P3 helix). These mutations were reverted to the wild-type, restoring perfect complementarity in this region without affecting catalytic activity. All of the other mutations had a more significant effect on activity, as will be discussed below.

Properties of the 10-18 Deoxyribozyme

The kinetic properties of the 10-18 deoxyribozyme first were examined in the bimolecular reaction format, analogous to that employed during in vitro evolution. A shortened form of the **S1** substrate was used that lacked the nucleotides at the 5′ end that had provided a primer site for PCR amplification. The shortened substrate had the sequence 5′-d(CTCAC)-r(UAUA)-3′, which is perfectly complementary to the corresponding template region of the deoxyribozyme. With trace amounts of [5′-^{32}P]-labeled **S1** and various concentrations of the **S2**-DNA conjugate, the reaction was found to occur with a k_{cat} of 0.030 min^{-1} and K_m of 2.5 μM, measured in the presence of 25 mM $MgCl_2$ at pH 8.5 and 23°C (Figure 4A). This is about 10-fold slower than the corresponding rate of reaction of the R3C ligase ribozyme. When a different version of **S1** was used that contained all-DNA residues except for a single 3′-terminal ribonucleotide, the reaction rate was about 4-fold slower, with a k_{cat} of 0.0073 min^{-1} and a K_m of 2.1 μM.

The regiospecificity of the reaction was determined with a [5′-^{32}P]-labeled **S1** substrate that contained either one or four 3′-terminal ribonucleotides. Following the reaction, the 5′-labeled products were purified by denaturing polyacrylamide gel electrophoresis, then digested with NaOH, RNase U2, or RNase φM. NaOH cleaves both 2′,5′- and 3′,5′-phosphodiesters, while the ribonucleases cleave only 3′,5′-phosphodiesters (RNase U2 cleaves after A and G residues; RNase φM cleaves after A and U residues). The phosphodiester at the ligation junction was cleaved by NaOH, but not by either RNase, indicating that this is a 2′,5′ linkage. In contrast, the phosphodiester formed by the R3C ribozyme is a 3′,5′ linkage that is readily cleaved by the two ribonucleases.

The 10-18 deoxyribozyme was next tested in the trimolecular reaction format, employing an all-RNA version of **S1**, having the sequence 5′-CUCACUAUA-3′, a detached version of **S2**, having the sequence 5′-pppGA GACCGUAAUGAGUAG-3′, and a 57-mer deoxyribozyme containing template regions that were complementary to the two substrates (Figure 2B). The reaction was carried out under two different kinetic regimes to assess the saturation behavior of each substrate. The first regime used a trace amount of [5′-^{32}P]-labeled **S1** and varying, but equal, concentrations of **S2** and deoxyribozyme, demonstrating a k_{cat} of 0.052 min^{-1} and K_m(**S1**) of 0.45 μM (Figure 4B). Under the same conditions the R3C ribozyme exhibited a k_{cat} of 0.14 min^{-1}

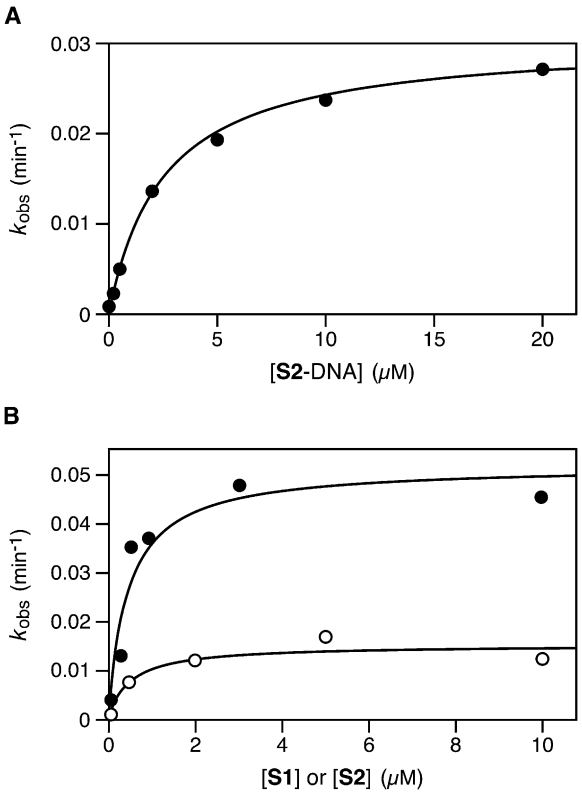

Figure 4. Catalytic Activity of the 10-18 Deoxyribozyme

(A) Bimolecular reaction, employing 5 nM **S1** and 0.05–20 μM **S2**-DNA.

(B) Trimolecular reaction, employing either 5 nM **S1** and 0.05–10 μM **S2** (solid circles), or 5 nM **S2** and 0.05–10 μM **S1** (open circles). The concentration of deoxyribozyme was always equal to that of the more concentrated substrate. Values for k_{obs} were obtained for each concentration of reactants and fit to a Michaelis-Menten saturation plot based on the equation: $k_{obs} = k_{cat}[S]/(k_m + [S])$.

and K_m(**S1**) of 0.11 μM. The second kinetic regime used trace amounts of [α-^{32}P]ATP-labeled **S2** and varying, but equal, concentrations of **S1** and deoxyribozyme, demonstrating a k_{cat} of 0.015 min^{-1} and K_m(**S2**) of 0.48 μM (Figure 4B). Neither the RNA version of the 10-18 deoxyribozyme nor the DNA version of the R3C ribozyme had any detectable activity.

It was possible to change the sequence of both substrates, together with the complementary template region of the deoxyribozyme, without disrupting catalytic activity. For example, an alternative version of **S1**, having the sequence 5′-GAAUACGUU-3′, was ligated by the corresponding deoxyribozyme with a k_{cat} of 0.035 min^{-1} and K_m(**S1**) of 0.75 μM. Not surprisingly, the deoxyribozyme was unable to ligate an all-DNA version of **S1**. The deoxyribozyme reacts with the terminal 2′-hydroxyl of **S1**, which is not present in DNA. A very low level of activity was seen with an all-RNA version of **S1** and an all-DNA version of **S2**. The observed rate in that case was $\sim 2 \times 10^{-6}$ min^{-1}, which is only about 10-fold faster than the uncatalyzed, template-dependent rate of reaction (see below).

Site-Directed Mutagenesis

Site-directed mutagenesis studies were carried out to assess the role of individual mutations acquired by the 10-18 deoxyribozyme and to investigate possible alteration of its secondary structure relative to that of the R3C ribozyme. The deoxyribozyme, together with 18 of 22 clones that were sequenced (Table 1), contains an A→T change at nucleotide position 48. This position lies immediately downstream from the ligation junction, opposite the 5′-terminal guanosine triphosphate of **S2** (Figure 2B). When the mutation was reverted to A, the catalytic rate decreased from 0.052 to 0.028 min^{-1}, and when it was changed to C, the rate was 0.012 min^{-1}. Thus, there does not appear to be a strict geometrical constraint at this position, even though it lies adjacent to the site of reaction. The ability to tolerate a C residue at position 48, which could form a C-G pair with the 5′-terminal nucleotide of **S2**, suggests that it may be possible to extend the P1 helix across the ligation junction. However, it was not possible to replace the entire bulge loop between the P1 and P2 stems with Watson-Crick pairs, either by substituting 5′-TC-3′ for 5′-GAT-3′ at positions 46–48 of the enzyme, or by substituting 5′-ATC-3′ for 5′-GA-3′ at the 5′ end of **S2**.

Eighteen of the 22 clones, including the 10-18 deoxyribozyme, contain a T→C mutation at nucleotide position 40. This position is located on the 3′ side of the central stem-loop, close to the three-way junction (Figure 2). Reverting this mutation to T reduced the catalytic rate by about 30-fold to 0.0018 min^{-1}. The 10-18 deoxyribozyme also contains a G→C change at position 12, located on the opposite side of the central stem-loop, also close to the three-way junction. This mutation is present in 11 of the 22 clones, and 4 other clones have a G→T change at this position. Reverting this mutation to G reduced the catalytic rate of the 10-18 deoxyribozyme to 0.0003 min^{-1}. When both of these mutations were reverted simultaneously, there was no detectable activity.

The hairpin loop at the end of the P5 stem of the R3C ribozyme (Figure 1) has no effect on catalytic activity. The sequence of this loop can be altered, and the loop can even be deleted, without affecting the catalytic rate of the ribozyme [21]. Thus, one might expect that the mutations at the corresponding positions of the 10-18 deoxyribozyme are neutral with regard to phenotype. This is not the case. Reverting the deoxyribozyme sequence at positions 23–27 from 5′-AGAGCG-3′ to 5′-CGTAAG-3′ eliminated catalytic activity. Similarly, replacing the former loop sequence by the especially stable DNA tri-loop 5′-GAA-3′ [24] resulted in no detectable activity. This suggests that either the deoxyribozyme requires a particular loop sequence, or it has an altered secondary structure compared to that of the R3C ribozyme. When two adjacent base pairs within the former P5 stem (G20-C31 and T21-A30) were exchanged, there was a complete loss of activity, further indicating that the secondary structure had changed.

Secondary structural prediction of the 10-18 deoxyribozyme with "mfold" [25] suggested an alternative folded structure for the central stem-loop region. This structure involves a shorter stem, a shifted position for the hairpin loop, and a larger internal bulge loop on the 5′ side adjacent to the three-way junction (compare Figures 2A and 2B). In order to test this structure, the predicted loop sequence 5′-GAACA-3′ was replaced by 5′-GTTTA-3′, which reduced the catalytic rate to 0.0001 min^{-1}. The predicted base pairs A25-T35 and

G26-C34 were mutated to C25-G35 and A26-T34, which resulted in a catalytic rate of 0.0015 min^{-1} and 0.010 min^{-1}, respectively. When both of these base pairs were mutated simultaneously, the catalytic rate was 0.0030 min^{-1}, which is only 17-fold slower than the rate of the 10-18 deoxyribozyme. Further support for this short stem comes from comparative sequence analysis of the cloned individuals. Thirteen of 22 clones, including all those in the major sequence cluster, had the putative A25-T35 pair, while 5 other clones had a G25-C35 pair (Table 1). Similarly, 2 clones (including 10-18) had the putative G26-C34 pair, while 16 others had an A26-T34 pair.

The alternative secondary structure has a predicted internal bulge loop at positions 12–19, whereas in the R3C ribozyme, all but two of these positions are involved in Watson-Crick pairing (Figure 2). When the 5′-CTC-3′ sequence at positions 13–15 of the deoxyribozyme was replaced by 5′-TTT-3′, the catalytic rate was reduced by about 10-fold to 0.0050 min^{-1}. When the entire bulge loop sequence was changed to 5′-TTTTTTTT-3′, the catalytic rate was 0.0002 min^{-1}. Among the 22 clones, there are 17 different sequences in the bulge loop region, and 3 or 4 different nucleotides occur at each of nucleotide positions 12–18.

The suggested alternative secondary structure of the central stem-loop region of the 10-18 deoxyribozyme should be regarded as conjectural. There is no sequence covariation to support the putative C27-G33 pair, although 17 clones contain an A→C change at position 27. Similarly, there is no covariation to support the G20-C40 pair, although 18 clones contain a T→C change at position 40. When the putative T21-A39 pair was mutated to A21-T39 there was no catalytic activity, and when T22-A38 was mutated to A22-T38 activity was only barely detectable. This is a small catalytic motif, which makes it difficult to discern nucleotides that only play a role in supporting the secondary structure, except for the well-defined regions involved in binding the two substrates.

Uncatalyzed Template-Directed Ligation

RNA molecules bearing a 5′-triphosphate normally are prepared by in vitro transcription. However, this approach is limited to sequences with a 5′-terminal purine residue, preferably beginning with pppGpG [26]. In order to prepare 5′-triphosphorylated RNAs of arbitrary sequence, as well as 5′-triphosphorylated DNAs, a synthetic approach is required. Based on literature precedent [27–29], chemical triphosphorylation of RNA or DNA was carried out on the solid support following automated synthesis, but prior to deprotection. The 5′-terminal hydroxyl was detritylated, reacted with salicyl phosphorochloridite to form the phosphite, then reacted with tributylammonium pyrophosphate to form the cyclic trimetaphosphite, which was oxidized to give the linear 5′-triphosphate. Following standard deprotection, the product was purified by denaturing polyacrylamide gel electrophoresis. Provided that the reagents were kept exquisitely dry, 5′-triphosphorylated RNA or DNA up to the 20-mer in length could be obtained in 80%–90% yield.

Both an RNA and DNA version of both the **S1** and **S2** substrates were prepared (see Experimental Procedures). In the absence of a template there was no detectable ligation of these substrates. The reaction then was carried out in the presence of a saturating concentration (15 μM) of either an RNA or a DNA template. The RNA-directed ligation of two RNA substrates occurred at a rate of $1.4 \pm 0.1 \times 10^{-7}$ min^{-1}, measured in the presence of 25 mM $MgCl_2$ at pH 8.5 and 23ºC. This is consistent with previous measurements of this reaction under somewhat different conditions [30]. Not surprisingly, the reaction occurred at a similar rate in the presence of a DNA template, with a k_{obs} of $1.2 \pm 0.3 \times 10^{-7}$ min^{-1}. Thus, the R3C ligase ribozyme exhibits a rate enhancement of 1×10^6-fold compared to the uncatalyzed reaction on an RNA template, and the 10-18 deoxyribozyme exhibits a rate enhancement of 4×10^5-fold compared to the uncatalyzed reaction on a DNA template.

A more intriguing question concerns the uncatalyzed rate of template-directed DNA ligation, which has not been measured previously. This reaction is thought to be more difficult than RNA ligation because of the reduced nucleophilicity of the DNA 3′-hydroxyl compared to the RNA 2′,3′-hydroxyl [31]. Surprisingly, the rate of DNA-DNA ligation on a DNA template was found to be $3.6 \pm 1.1 \times 10^{-8}$ min^{-1}, which is only 4-fold slower than the rate of RNA-RNA ligation on an RNA template. The reduced rate is attributable to the DNA version of the **S1** substrate, because ligation of **S1**-RNA and **S2**-DNA on a DNA template proceeds with a k_{obs} of $2.1 \pm 0.3 \times 10^{-7}$ min^{-1}, which is slightly faster than the rate of RNA-RNA ligation.

An initial attempt was made to further evolve the RNA ligase deoxyribozyme so that it could function as a DNA ligase. The diverse population that existed following round seven of the initial in vitro evolution procedure was used as the starting point for further evolution. Nine more rounds of evolution were carried out, selecting for DNA ligase activity. Random mutations were introduced by mutagenic PCR after each of the first three rounds. Individuals were cloned from the final selected population, sequenced, and tested for catalytic activity. A typical individual exhibited a catalytic rate of 2×10^{-5} min^{-1}, which corresponds to a 600-fold rate enhancement compared to the uncatalyzed template-directed reaction. This meager rate enhancement was not deemed sufficient to merit further investigation until a more robust DNA ligase deoxyribozyme can be obtained.

Discussion

Transfer of Macromolecular Information and Function

All known organisms operate according to the "central dogma of molecular biology," whereby the transfer of sequential genetic information can proceed from nucleic acid to nucleic acid, and from nucleic acid to protein, but not from protein to protein, or from protein to nucleic acid [32, 33]. The transfer of information from either DNA or RNA to either DNA or RNA is the most straightforward, relying on the one-to-one correspondence of Watson-Crick pairing. There are instances in biology of all four of these nucleic acid information transfer pathways. The transfer of information from RNA to protein is more complex, but retains the crucial feature of

"sequentialization" [32] based on the three-to-one correspondence of the genetic code. In contrast, the transfer of function from nucleic acid to nucleic acid, or from nucleic acid to protein, does not occur in contemporary biology, and is difficult to envision because function cannot be conveyed in a sequential manner. Instead, in the case of protein synthesis, function is encoded in nucleic acid sequences that are translated to corresponding protein sequences, and function is expressed at the level of the proteins.

During the early history of life on Earth, in an era commonly referred to as the "RNA world," both genetic information and catalytic function are thought to have resided in RNA [34, 35]. With the advent of protein synthesis, catalytic function became largely the province of proteins. It is not clear the extent to which RNA-based function was transferred to proteins, perhaps through ribonucleoprotein intermediates [36–38], but it is highly unlikely that sequential information in RNA that encoded an RNA-based function was translated to a corresponding protein sequence that encoded the same function.

A different situation may have existed with regard to the transfer of function between two different nucleic acid-like molecules. It has been suggested that the RNA world was preceded by a simpler "pre-RNA world," based on a nucleic acid-like molecule that would have occurred more readily on the abiotic Earth [39–41]. Among the candidate macromolecules for the pre-RNA world are peptide nucleic acid (PNA) [42], threose nucleic acid (TNA) [43], and glycol nucleic acid (GNA) [44], all of which form base-paired structures with themselves and with RNA. Cross-pairing would allow genetic information to be transferred from such a pre-RNA molecule to RNA. In addition, as the present study suggests, the catalytic function of a PNA, TNA, or GNA enzyme might be transferred to the corresponding RNA enzyme following the acquisition of a few critical mutations.

The transfer of function between different nucleic acid-like molecules is possible because, despite their chemical differences, these molecules all form secondary structural elements as a consequence of the specificity of base pairing. Often the precise sequence of a stem-loop element does not affect function, so long as the proper secondary structure is maintained. Similarly, alteration of the sugar-phosphate backbone may be tolerated at positions that support secondary structure but are not involved in unusual local structure, tertiary interactions, or catalysis. Most ribozymes, including the R3C ligase, contain a preponderance of residues that are amenable to substitution. For the relatively small number of residues that must be precisely specified, simple evolutionary pathways may exist to allow adaptation to differences in base identity and backbone composition.

Comparative Biochemistry of an RNA and DNA Enzyme

The R3C ribozyme and 10-18 deoxyribozyme provide a unique example of members of two different classes of macromolecules that have the same function and are related by evolutionary descent. Their function is not exactly the same because the ribozyme has a k_{cat} of 0.14 min^{-1} and K_m(**S1**) of 0.11 μM, while the deoxyribozyme has a k_{cat} of 0.052 min^{-1} and K_m(**S1**) of 0.45 μM (measured in the trimolecular reaction format). In addition, the ribozyme forms a 3′,5′-phosphodiester, while the deoxyribozyme forms a 2′,5′-phosphodiester between the two RNA substrates. The ~10-fold difference in catalytic efficiency (k_{cat}/K_m) might be overcome by a few additional rounds of evolution, but the difference in the regiospecificity of ligation is more substantive.

The R3C ribozyme is unusual among RNA and DNA enzymes with RNA ligase activity because it forms a 3′,5′-phoshodiester, even though it has imperfect Watson-Crick pairing surrounding the ligation junction. The uncatalyzed template-dependent ligation of RNA favors formation of a 3′,5′-phosphodiester [45], but unless efforts are made during in vitro evolution either to enforce Watson-Crick pairing at the ligation junction [46] or to provide selection pressure favoring 3′,5′ linkages [47, 48], there is a strong tendency to obtain ligases that have imperfect pairing at the ligation junction and form a 2′,5′-phosphodiester. The parent of the R3C ligase, the cytosine-free R3 ligase, was the only one of seven isolated clones that forms a 3′,5′-phosphodiester [21]. The regiospecificity of the R3 ligase was carried forward to the R3C ligase, despite the continued lack of Watson-Crick pairing at the ligation junction. The 10-18 deoxyribozyme, which was derived from a DNA version of the R3C ligase that had no catalytic activity, reverted to the usual tendency to form a 2′,5′ linkage once catalytic activity was re-established. It seems plausible that a 3′,5′-regiospecific deoxyribozyme could be obtained starting from DNA variants of the R3C ribozyme, provided that Watson-Crick pairing is enforced in the region between the P1 and P2 stems throughout the evolution procedure.

The above differences notwithstanding, there is a high degree of sequence and functional similarity between the R3C ribozyme and 10-18 deoxyribozyme. The deoxyribozyme contains 12 mutations out of a total of 57 residues (Figure 2B), but two of these (positions 4 and 5) have no effect and one (position 48) has only a modest effect on catalytic activity. Two other mutations (positions 12 and 40), which are located at the proximal end of the central stem-loop, can each be reverted to the sequence of the R3C ribozyme without eliminating activity. Thus, the seven mutations located more distally in the central stem-loop (positions 23, 25–27, 34, 36, and 37) appear to be responsible for the conversion of an RNA sequence to a DNA sequence, each providing RNA ligase activity. These seven mutations alter the secondary structure of the central stem-loop, but not that of the regions involved in Watson-Crick pairing with the two substrates.

The two endpoints of the evolutionary transition are mutually exclusive: when either the ribozyme is prepared as a corresponding DNA or the deoxyribozyme is prepared as a corresponding RNA there is no detectable activity. It would be interesting to construct a population of randomized variants of the RNA version of the 10-18 deoxyribozyme and employ in vitro evolution to restore activity in an RNA context. With repeated interconversion between RNA- and DNA-based catalytic function it may be possible to evolve a sequence that retains activity when prepared as either an RNA or a DNA molecule.

The Expanding Repertoire of Deoxyribozyme Ligases

The first reported example of a deoxyribozyme ligase is a Zn^{2+}/Cu^{2+}-dependent enzyme that catalyzes the ligation of two DNA substrates, one bearing a 5′-hydroxyl and the other a reactive 3′-phosphorimidazolide [49]. Another reported deoxyribozyme catalyzes the ligation of two DNAs, one with a 5′-terminal adenosine-5′,5′-pyrophosphate and the other with a 3′-hydroxyl [50]. Most efforts concerning deoxyribozyme ligases, however, have focused on the template-directed ligation of RNA molecules, either bearing a 5′-hydroxyl and 2′,3′-cyclic phosphate [51–53], or bearing a 2′,3′-hydroxyl and 5′-triphosphate [46–48, 54, 55]. As discussed above, unless special precautions are taken to favor the formation of a 3′,5′-phosphodiester, in vitro evolution almost invariably leads to catalysts that form a nonnative 2′,5′ linkage [56]. In some instances the nucleophile is an internal 2′-hydroxyl rather than the terminal 2′-hydroxyl, resulting in formation of a branched RNA, analogous to the lariat intermediate of RNA splicing [54]. The ability to form either 2′,5′-linked linear or branched RNAs has practical applications for preparing these materials for laboratory studies [51, 55].

Deoxyribozyme ligases that form a 3′,5′-phosphodiester may be useful in preparing long synthetic or semisynthetic RNAs, especially those that contain modified nucleotides that cannot be incorporated during in vitro transcription by an RNA polymerase. In most cases, one would employ either T4 RNA ligase in the absence of a template [57] or T4 DNA ligase in the presence of a complementary DNA template [58]. A potential advantage of deoxyribozyme ligases is that they can operate on RNA substrates bearing either a 2′,3′-cyclic phosphate or 5′-triphosphate terminus, as would result from ribonuclease cleavage or in vitro transcription, respectively. Another potential advantage is that the chemistry of ligation, especially for ligases that utilize a 5′-triphosphorylated substrate, can be applied to polymerization reactions. There are several examples of ribozymes that catalyze the template-directed ligation of an oligonucleotide 3′-hydroxyl and oligonucleotide 5′-triphosphate, and that can also extend an oligonucleotide primer through the addition of mononucleoside 5′-triphosphates [59–61]. In one case, an RNA ligase ribozyme was evolved to function as an accurate and general RNA polymerase [62]. A similar result might be achieved with an RNA ligase deoxyribozyme.

The repertoire of deoxyribozyme ligases might be further expanded by evolving variants of the 10-18 enzyme that catalyze the ligation of two DNA substrates, completing the transition from an all-RNA to an all-DNA reaction system. An initial attempt to develop this activity resulted in molecules with a catalytic rate enhancement of only 600-fold compared to the uncatalyzed template-directed reaction. The uncatalyzed rate of DNA ligation was found to be only 4-fold slower than the comparable reaction with RNA. This does not imply that it is only slightly more difficult to catalyze DNA ligation compared to RNA ligation, because a catalytic strategy that exploits the terminal 2′-hydroxyl to assist in deprotonation of the adjacent 3′-hydroxyl or to provide binding interactions close to the reaction site would not be available for DNA. However, with the ability to prepare synthetic 5′-triphosphorylated DNA and the advantageous starting point provided by the 10-18 deoxyribozyme, the evolutionary search for a DNA ligase deoxyribozyme may be worth exploring.

Significance

The transfer of sequence information between two different classes of nucleic acid-like molecules, for example, between RNA and DNA, is straightforward because it relies on the one-to-one correspondence of Watson-Crick pairing. The transfer of function is more difficult because function is an overall property of a macromolecule and cannot be conveyed in a sequential manner. The present study demonstrates the evolutionary conversion of a ribozyme to a deoxribozyme of the same function through the acquisition of a few critical mutations. Evolutionary pathways such as this may exist between other classes of nucleic acid-like molecules, for example, between the postulated predecessor of RNA and RNA during the early history of life on Earth.

Experimental Procedures

Materials

Histidine-tagged T7 RNA polymerase was purified from *Escherichia coli* strain BL21 containing plasmid pBH161 (kindly provided by William McAllister, SUNY Downstate Medical Center). *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA and purified as described previously [63]. RNase ϕM and RNase U2 were purchased from Industrial Research Ltd. through the RNA Society. SuperScript III reverse transcriptase was from Invitrogen, and T4 polynucleotide kinase and T4 DNA ligase were from New England Biolabs. Nucleoside 5′-triphosphates (NTPs) and deoxynucleoside 5′-triphosphates (dNTPs) were from Amersham Pharmacia Biotech. [α-^{32}P]ATP (3 μCi/pmol), [α-^{32}P]dATP (3 μCi/pmol), and [γ-^{32}P]ATP (7 μCi/pmol) were from ICN. Synthetic oligonucleotides were prepared with an Expedite nucleic acid synthesis system (Applied Biosystems), with phosphoramidites from either Glen Research or Applied Biosystems. All oligonucleotides were deprotected by the recommended protocol, purified by denaturing polyacrylamide gel electrophoresis, and desalted on a C18 SEP-Pak Cartridge (Waters).

Solid-Phase Synthesis of 5′-Triphosphorylated Oligonucleotides

Oligonucleotides were synthesized on the 1.0 μmol scale employing standard methods. The 5′-terminal dimethoxytrityl group was removed, but the other protecting groups were left intact, and the oligonucleotide was retained on the controlled-pore glass (CPG) solid support. The entire synthesis column was dried under vacuum (<200 mTorr) for 1 hr and then mounted between two plastic syringes with a three-way stopcock at the top and a plunger at the bottom. The stopcock permitted access to either an argon source or an injection port. The apparatus was flushed with argon, then the CPG-supported material was washed with two 300 μl volumes of 1:3 (v/v) pyridine:dioxane. While maintaining a positive flow of argon, the material was suspended in 200 μl 1:3 (v/v) pyridine:dioxane, to which was added 100 μl 0.3 M salicyl phosphorochloridite in dioxane [28, 29]. After 15 min at 23ºC, the reagent was removed. A total of 250 μl of a solution of 2.0 M tributylammonium pyrophosphate in 1:2 (v/v) tributylamine:dimethylformamide (DMF), which had been kept dry by storage over 4 Å molecular sieves under argon, was added, and the reaction was allowed to proceed at 23ºC for 20 min while agitating frequently with the bottom syringe. The reagent was removed and the CPG-supported material was washed with 500 μl DMF, followed by 500 μl acetonitrile. The α-phosphite was oxidized to the phosphate by reacting with 250 μl 3:2:20:75 (v/v) iodine:water:pyridine:tetrahydrofuran at 23ºC for 30 min, and

then the material was washed with 500 μl acetonitrile. The oligonucleotide was cleaved from the support and deprotected according to standard procedures for either DNA or RNA, and then evaporated to dryness. The product was purified by denaturing polyacrylamide gel electrophoresis, desalted on a C18 SEP-Pak Cartridge, and verified by MALDI-TOF mass spectrometry.

In Vitro Transcription

5′-Triphosphorylated RNAs were prepared by in vitro transcription, employing 400 nM DNA template, 2 mM each of the 4 NTPs, 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 7.5), and 25 U/μl T7 RNA polymerase, which were incubated at 37ºC for 2 hr. The transcription products were purified by denaturing polyacrylamide gel electrophoresis and desalted on a C18 SEP-Pak Cartridge.

In Vitro Evolution

The starting population was prepared by extending 1 μM RNA primer, having the sequence 5′-pppGAGACCGUAAUGAGUA-3′, in the presence of 2 μM DNA template, having the sequence 5′-TAGTT CATGTATCAGTCTCTCAC*TATATTCAACCCATTCAAACTGTTCTTA CGAACAATCGAGCATAATGAGTAC*TACTCATTACGGTCTCAAAAAA AAA-3′ (italicized letters indicate positions where each of the other 3 nucleotides were present at a frequency of 4%; the primer binding site is underlined). The RNA primer was annealed to the DNA template and extended in a reaction mixture containing 0.2 mM each of the 4 dNTPs, 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, 50 mM Tris-HCl (pH 8.3), and 10 U/μl of SuperScript III reverse transcriptase, which was incubated at 55ºC for 60 min. The full-length extension products were purified by denaturing polyacrylamide gel electrophoresis and desalted on a C18 SEP-Pak Cartridge.

The population of 5′-triphosphorylated RNA-DNA molecules were challenged to react with a substrate having the sequence 5′-GAACT GACGAACTGATGCTCAC-r(UAUA)-3′ (primer region is underlined). The population and substrate were preincubated separately in the presence of 25 mM $MgCl_2$ and 25 mM EPPS (pH 8.5) at 23ºC for 5 min, and then mixed to start the reaction. After the desired incubation time, the reaction was quenched by adding an equal volume of gel-loading buffer containing 25 mM Na_2EDTA and 18 M urea. The ligated products were purified by denaturing polyacrylamide gel electrophoresis, and then used to generate a complementary DNA strand by extending 1 μM DNA primer (primer 1), having the sequence 5′-TAGTTCATGTATCAGTCT-3′, under the conditions described above, except that it was carried out at 42ºC. The extension products were desalted with a NAP-5 Sephadex column (Amersham Biosciences), evaporated to dryness, and redissolved in 100 μl 1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA.

A portion (~50%) of the extension products were PCR amplified in 100 μl volume with primer 1 and a second primer (primer 2), having the sequence 5′-GAACTGACGAACTGATG-3′. A small portion (~1%) of the PCR products were amplified in a second, large-scale (3 ml) PCR, employing primer 1 and an internal primer, having the sequence 5′-CTCACTATAGAGACCGTAATGAGTA-rG-3′, which restored the 5′ end of the population of selected molecules. The second PCR amplification was performed under mutagenic conditions following rounds five, six, and seven of the in vitro evolution procedure [23]. The PCR products were ethanol precipitated, redissolved in 50 μl H_2O, and incubated in the presence of 0.2 N NaOH at 90ºC for 20 min to cleave the single ribonucleotide linkage (following rG) within one of the two strands [64]. After neutralizing with HCl, the uncleaved strand was isolated by denaturing polyacrylamide gel electrophoresis, eluted from the gel, and desalted with a C18 SEP-Pak Cartridge. The purified DNA then was used as the template for a primer extension reaction employing the RNA primer, as described above, in order to begin the next round of evolution.

Analysis of Individual Clones

Following PCR amplification with primers 1 and 2, individuals were cloned from the population with the TOPO-TA cloning kit (Invitrogen). Individual colonies were isolated and inoculated into 3 ml cultures. Plasmid DNA was isolated with the QIAPrep Spin miniprep kit (Qiagen), and the insert was sequenced by the Nucleic Acids Core Facility at The Scripps Research Institute. The sequences were aligned and clustered with Vector NTI Advance 9.0 software (Invitrogen). Secondary structure predictions were made with the DNA and RNA folding algorithms on the mfold web server [25].

Kinetic Analyses

Ligation assays were carried out in the presence of 25 mM $MgCl_2$, 40 μg/ml BSA, and 50 mM EPPS (pH 8.5) at 23ºC. The catalyst and substrate(s) were preincubated separately under these conditions for 5 min, and then mixed to start the reaction. Aliquots were removed at various times and quenched by adding an equal volume of gel-loading buffer containing 25 mM Na_2EDTA and 18 M urea. The reactants and products were separated by denaturing polyacrylamide gel electrophoresis and quantitated with a PhosphorImager (Molecular Dynamics). The fraction reacted at each time point was determined, and these data were fit to the equation: $y = a\,(1 - e^{-kt})$, where y is the fraction reacted at time t, a is the fraction reacted at $t = \infty$, and k is the observed rate constant. Values for k_{cat} and K_m were obtained from a Michaelis-Menten saturation plot of k_{obs} versus substrate concentration.

The uncatalyzed, template-dependent rate of ligation was measured under saturating conditions, employing either an RNA or a DNA version of the template, and either an RNA or a DNA version of each of the two complementary substrates. The RNA template was prepared by in vitro transcription, and had the sequence 5′-GGUACUCAUUACGGUCUCUAUAGUGAG-3′. The DNA template was prepared by solid-phase synthesis, and had the sequence 5′-TACTCATTACGGTCTCTATAGTGAG-3′. Both the RNA and DNA versions of **S1** were prepared by solid-phase synthesis. The RNA version of **S2** was prepared by in vitro transcription. The DNA version of **S2** was prepared by synthesizing two oligonucleotides, 5′-ppp GAGACCGT-3′ and 5′-pAATGAGTA-3′, which were ligated in the presence of a complementary DNA template by T4 DNA ligase, and then purified by denaturing polyacrylamide gel electrophoresis. The uncatalyzed reaction was carried out over a 6 day period under the same conditions as for the enzyme-catalyzed reaction, employing a trace amount of [5′-^{32}P]-labeled **S1**, 15 μM **S2**, and 15 μm template. The rate of ligation was determined from a linear fit of a plot of fraction reacted versus time.

Analysis of Regiospecificity

The products of ligation of [5′-^{32}P]-labeled **S1** and unlabeled **S2** were purified by denaturing polyacrylamide gel electrophoresis, and then digested by NaOH, RNase ϕM, or RNase U2. Alkaline digestion employed 0.2 N NaOH and incubation at 23ºC for 20 min. Enzymatic digestion employed 6 M urea, 24 mM sodium citrate (pH 5.0 for RNase ϕM, pH 3.5 for RNase U2), 1 mM Na_2EDTA, and 0.04 U/μl RNase, which were incubated at 50ºC for 60 min. The products were separated by denaturing polyacrylamide gel electrophoresis.

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References

1. Kruger, K., Grabowski, P.J., Zaug, A.J., Sands, J., Gottschling, D.E., and Cech, T.R. (1982). Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. Cell *31*, 147–157.
2. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell *35*, 849–857.

3. Nissen, P., Hansen, J., Ban, N., Moore, P.B., and Steitz, T.A. (2000). The structural basis of ribosome activity in peptide bond synthesis. Science *289*, 920–930.
4. Beaudry, A.A., and Joyce, G.F. (1992). Directed evolution of an RNA enzyme. Science *257*, 635–641.
5. Bartel, D.P., and Szostak, J.W. (1993). Isolation of new ribozymes from a large pool of random sequences. Science *261*, 1411–1418.
6. Wilson, D.S., and Szostak, J.W. (1999). In vitro selection of functional nucleic acids. Annu. Rev. Biochem. *68*, 611–647.
7. Joyce, G.F. (2004). Directed evolution of nucleic acid enzymes. Annu. Rev. Biochem. *73*, 791–836.
8. Emilsson, G.M., and Breaker, R.R. (2002). Deoxyribozymes: new activities and new applications. Cell. Mol. Life Sci. *59*, 596–607.
9. Peracchi, A. (2005). DNA catalysis: potential, limitations, and open questions. ChemBioChem *6*, 1316–1322.
10. Travascio, P., Li, Y., and Sen, D. (1998). DNA-enhanced peroxidase activity of a DNA aptamer-hemin complex. Chem. Biol. *5*, 505–517.
11. Travascio, P., Bennet, A.J., Wang, D.Y., and Sen, D. (1999). A ribozyme and a catalytic DNA with peroxidase activity: active sites versus cofactor-binding sites. Chem. Biol. *6*, 779–787.
12. Uhlenbeck, O.C. (1987). A small catalytic oligoribonucleotide. Nature *328*, 596–600.
13. Perreault, J., Wu, T., Cousineau, B., Ogilvie, K.K., and Cedergren, R. (1990). Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity. Nature *344*, 565–567.
14. Yang, J.H., Usman, N., Chartrand, P., and Cedergren, R. (1992). Minimum ribonucleotide requirement for catalysis by the RNA hammerhead domain. Biochemistry *31*, 5005–5009.
15. Jeffries, A.C., and Symons, R.H. (1989). A catalytic 13-mer ribozyme. Nucleic Acids Res. *17*, 1371–1377.
16. Chartrand, P., Harvey, S.C., Ferbeyre, G., Usman, N., and Cedergren, R. (1995). An oligodeoxyribonucleotide that supports catalytic activity in the hammerhead ribozyme domain. Nucleic Acids Res. *23*, 4092–4096.
17. Chowrira, B.M., Berzal-Herranz, A., Keller, C.F., and Burke, J.M. (1993). Four ribose 2′-hydroxyl groups essential for catalytic function of the hairpin ribozyme. J. Biol. Chem. *268*, 19458–19462.
18. Tsang, J., and Joyce, G.F. (1994). Evolutionary optimization of the catalytic properties of a DNA-cleaving ribozyme. Biochemistry *33*, 5966–5973.
19. Tsang, J., and Joyce, G.F. (1996). Specialization of the DNA-cleaving activity of a group I ribozyme through in vitro evolution. J. Mol. Biol. *262*, 31–42.
20. Cole, K.B., and Dorit, R.L. (1999). Acquisition of novel catalytic activity by the M1 RNA ribozyme: the cost of molecular adaptation. J. Mol. Biol. *292*, 931–944.
21. Rogers, J., and Joyce, G.F. (2001). The effect of cytidine on the structure and function of an RNA ligase ribozyme. RNA *7*, 395–404.
22. Reader, J.S., and Joyce, G.F. (2002). A ribozyme composed of only two different nucleotides. Nature *420*, 841–844.
23. Cadwell, R.C., and Joyce, G.F. (1992). Randomization of genes by PCR mutagenesis. PCR Methods Appl. *2*, 28–33.
24. Yoshizawa, S., Kawai, G., Watanabe, K., Miura, K., and Hirao, I. (1997). GNA trinucleotide loop sequences producing extraordinarily stable DNA minihairpins. Biochemistry *36*, 4761–4767.
25. Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. *31*, 3406–3415.
26. Milligan, J.F., Groebe, D.R., Witherell, G.W., and Uhlenbeck, O.C. (1987). Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. *15*, 8783–8798.
27. Ludwig, J. (1987). A simple one flask synthesis of nucleoside 5′-triphosphates from unprotected nucleosides via nucleoside 5′-cyclotriphosphates. In Biophosphates and Their Analogues—Synthesis, Structure, Metabolism and Activity, K.S. Bruzik and W.J. Stec, eds. (Amsterdam: Elsevier Science Publishers), pp. 201–204.
28. Gaur, R.K., Sproat, B.S., and Krupp, G. (1992). Novel solid phase synthesis of 2′-*O*-methylribonucleoside 5′-triphosphates and their α-thio analogues. Tetrahedron Lett. *33*, 3301–3304.
29. Lebedev, A.V., Koukhareva, I.I., Beck, T., and Vaghefi, M.M. (2001). Preparation of oligonucleotide 5′-triphosphates using solid support approach. Nucleosides Nucleotides Nucleic Acids *20*, 1403–1409.
30. Rhogati, R., Bartel, D.P., and Szostak, J.W. (1996). Kinetic and mechanistic analysis of nonenzymatic, template-directed oligoribonucleotide ligation. J. Am. Chem. Soc. *118*, 3332–3339.
31. Izatt, R.M., Hansen, L.D., Rytting, J.H., and Christensen, J.J. (1965). Proton ionization from adenosine. J. Am. Chem. Soc. *87*, 2760–2761.
32. Crick, F.H.C. (1958). On protein synthesis. Symp. Soc. Exp. Biol. *12*, 138–163.
33. Crick, F. (1970). Central dogma of molecular biology. Nature *227*, 561–563.
34. Gilbert, W. (1986). The RNA world. Nature *319*, 618.
35. Joyce, G.F. (2002). The antiquity of RNA-based evolution. Nature *418*, 214–221.
36. Di Giulio, M. (1997). On the RNA world: evidence in favor of an early ribonucleoprotein world. J. Mol. Evol. *45*, 571–578.
37. Poole, A.M., Jeffares, D.C., and Penny, D. (1997). The path from the RNA world. J. Mol. Evol. *46*, 1–17.
38. Noller, H.F. (2004). The driving force for molecular evolution of translation. RNA *10*, 1833–1837.
39. Shapiro, R. (1984). The improbability of prebiotic nucleic acid synthesis. Orig. Life Evol. Biosph. *14*, 565–570.
40. Joyce, G.F., Schwartz, A.W., Miller, S.L., and Orgel, L.E. (1987). The case for an ancestral genetic system involving simple analogues of the nucleotides. Proc. Natl. Acad. Sci. USA *84*, 4398–4402.
41. Joyce, G.F., and Orgel, L.E. (2006). Progress toward understanding the origin of the RNA world. In The RNA World, Third Edition, R.F. Gesteland, J.F. Atkins, and T.R. Cech, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 23–56.
42. Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B., and Nielson, P.E. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature *365*, 566–568.
43. Schöning, K.-U., Scholz, P., Guntha, S., Wu, X., Krishnamurthy, R., and Eschenmoser, A. (2000). Chemical etiology of nucleic acid structure: the α-threofuranosyl-(3′→2′) oligonucleotide system. Science *290*, 1347–1351.
44. Zhang, L., Peritz, A., and Meggers, E. (2005). A simple glycol nucleic acid. J. Am. Chem. Soc. *127*, 4174–4175.
45. Rhogati, R., Bartel, D.P., and Szostak, J.W. (1996). Nonenzymatic, template-directed ligation of oligoribonucleotides is highly regioselective for the formation of 3′-5′ phosphodiester bonds. J. Am. Chem. Soc. *118*, 3340–3344.
46. Coppins, R.L., and Silverman, S.K. (2004). Rational modification of a selection strategy leads to deoxyribozymes that create native 3′-5′ RNA linkages. J. Am. Chem. Soc. *126*, 16426–16432.
47. Wang, Y., and Silverman, S.K. (2005). Directing the outcome of deoxyribozyme selections to favor native 3′-5′ RNA ligation. Biochemistry *44*, 3017–3023.
48. Purtha, W.E., Coppins, R.L., Smalley, M.K., and Silverman, S.K. (2005). General deoxyribozyme-catalyzed synthesis of native 3′-5′ linkages. J. Am. Chem. Soc. *127*, 13124–13125.
49. Cuenoud, B., and Szostak, J.W. (1995). A DNA metalloenzyme with DNA ligase activity. Nature *375*, 611–614.
50. Sreedhara, A., Li, Y., and Breaker, R.R. (2004). Ligating DNA with DNA. J. Am. Chem. Soc. *126*, 3454–3460.
51. Flynn-Charlebois, A., Wang, Y., Prior, T.K., Rashid, I., Hoadley, K.A., Coppins, R.L., and Silverman, S.K. (2003). Deoxyribozymes with 2′-5′ RNA ligase activity. J. Am. Chem. Soc. *125*, 2444–2454.
52. Flynn-Charlebois, A., Prior, T.K., Hoadley, K.A., and Silverman, S.K. (2003). In vitro evolution of an RNA-cleaving DNA enzyme into an RNA ligase switches the selectivity from 3′-5′ to 2′-5′. J. Am. Chem. Soc. *125*, 5346–5350.
53. Prior, T.L., Semlow, D.R., Flynn-Charlebois, A., Rashid, I., and Silverman, S.K. (2004). Structure-function correlations derived from faster variants of a RNA ligase deoxyribozyme. Nucleic Acids Res. *32*, 1075–1082.

54. Wang, Y., and Silverman, S.K. (2003). Deoxyribozymes that synthesize branched and lariat RNA. J. Am. Chem. Soc. *125*, 6880–6881.
55. Coppins, R.L., and Silverman, S.K. (2004). A DNA enzyme that mimics the first step of splicing. Nat. Struct. Mol. Biol. *11*, 270–274.
56. Semlow, D.R., and Silverman, S.K. (2005). Parallel selections in vitro reveal a preference for 2′-5′ RNA ligation upon deoxyribozyme-mediated opening of a 2′,3′-cyclic phosphate. J. Mol. Evol. *61*, 207–215.
57. Wittenberg, W.L., and Uhlenbeck, O.C. (1985). Specific replacement of functional groups of uridine-33 in yeast phenylalanine transfer ribonucleic acids. Biochemistry *24*, 2705–2712.
58. Moore, M.J., and Sharp, P.A. (1992). Site-specific modification of pre-mRNA: the 2′-hydroxyl groups at the splice sites. Science *256*, 992–997.
59. Ekland, E.H., and Bartel, D.P. (1996). RNA-catalysed RNA polymerization using nucleoside triphosphates. Nature *382*, 373–376.
60. McGinness, K.E., and Joyce, G.F. (2002). RNA-catalyzed RNA ligation on an external RNA template. Chem. Biol. *9*, 297–307.
61. Ikawa, Y., Tsuda, K., Matsumura, S., and Inoue, T. (2004). De novo synthesis and development of an RNA enzyme. Proc. Natl. Acad. Sci. USA *101*, 13750–13755.
62. Johnston, W.K., Unrau, P.J., Lawrence, M.S., Glasner, M.E., and Bartel, D.P. (2001). RNA-catalyzed RNA polymerization: accurate and general RNA-templated primer extension. Science *292*, 1319–1325.
63. Pluthero, F.G. (1993). Rapid purification of high-activity *Taq* DNA polymerase. Nucleic Acids Res. *21*, 4850–4851.
64. Walder, R.Y., Hayes, J.R., and Walder, J.A. (1993). Use of PCR primers containing a 3′-terminal ribose residue to prevent cross-contamination of amplified sequences. Nucleic Acids Res. *21*, 4339–4343.