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RNA-Catalyzed RNA Ligation on an External RNA Template

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Summary

Variants of the hc ligase ribozyme, which catalyzes ligation of the 3′ end of an RNA substrate to the 5′ end of the ribozyme, were utilized to evolve a ribozyme that catalyzes ligation reactions on an external RNA template. The evolved ribozyme catalyzes the joining of an oligonucleotide 3′-hydroxyl to the 5′-triphosphate of an RNA hairpin molecule. The ribozyme can also utilize various substrate sequences, demonstrating a largely sequence-independent mechanism for substrate recognition. The ribozyme also carries out the ligation of two oligonucleotides that are bound at adjacent positions on a complementary template. Finally, it catalyzes addition of mononucleoside 5′-triphosphates onto the 3′ end of an oligonucleotide primer in a template-dependent manner. The development of ribozymes that catalyze polymerase-type reactions contributes to the notion that an RNA world could have existed during the early history of life on Earth.

Introduction

The RNA world hypothesis suggests that there was a time in early evolutionary history when RNA molecules were responsible for both genotype and phenotype and had roles akin to those of DNA and proteins, respectively, in modern biology [1]. In contemporary systems, RNA is capable of serving as a genetic molecule, in the form of certain viral genomes, and as a phenotypic molecule, in the form of ribozymes. Mechanisms exist for transferring RNA information into either DNA or protein, illustrating plausible evolutionary links between an RNA-based genetic system and current biological systems. In order for any sort of genetic system to be sustained, accurate and efficient information transfer is required. In an RNA world, information transfer probably would have involved an RNA molecule that was capable of copying RNA molecules through a polymerase-type activity [2].

Most laboratory investigations into the problem of information transfer in an RNA world have focused on the template-directed polymerization of activated mononucleotides to form oligomeric products that are joined by natural 3′,5′ phosphodiester linkages. Some of the earliest experiments along these lines involved the nonenzymatic template-directed polymerization of mononucleotides that were activated at the 5′ phosphate by either carbodiimide or imidazole [3]. In the presence of Zn^{2+}, poly(C) RNA templates were shown to direct the oligomerization of guanosine 5′-phosphorimidazolides to form 3′,5′-linked products [4]. 3′,5′ linkages also were formed when guanosine 5′-phosphoro-2-methylimidazolide was oligomerized on poly(C) templates [5]. All four activated mononucleotides could be oligomerized on poly(C,N) templates [6], but those reactions required a substantial excess of cytidine in the template [7, 8]. Accurate information transfer was demonstrated through the use of defined-sequence templates [9]. However, the number of different sequences that could be copied was limited by the requirement for an excess of cytidine in the template, the need to avoid intra- and intermolecular template structure, and the varied efficiency of oligomerization of the four nucleoside 5′-phosphoro-2-methylimidazolides [3]. Thus, it appears that nonenzymatic template-directed synthesis is not sufficiently robust to have provided the basis for generalized information transfer in an RNA world.

With the discovery of catalytic RNA [10, 11] and the ability to carry out in vitro evolution of nucleic acids [12–14], it has become possible to investigate the plausibility of RNA molecules that catalyze reactions related to RNA polymerization. Modern protein polymerases typically bind to a primer-template complex and utilize nucleoside 5′-triphosphates (NTPs) as substrates for template-directed extension at the 3′ end of the primer. This activity has served as the basis for developing RNA molecules that catalyze attack of the 2′ or 3′ hydroxyl group of a template bound primer on the α-phosphate of an adjacent template bound NTP or oligonucleotide 5′-triphosphate, with concomitant loss of inorganic pyrophosphate [15–22]. Of special interest are ribozymes that catalyze the formation of a 3′,5′-phosphodiester linkage [16, 17, 20–22], which is the connectivity that occurs in biological RNAs.

The class I ligase ribozyme, selected from a large pool of random-sequence RNAs, utilizes an internal template to recognize a substrate oligonucleotide through Watson-Crick pairing [16]. It catalyzes ligation of the 3′ hydroxyl of this substrate onto the 5′ end of the ribozyme. In a slightly modified format, the ribozyme also has been shown to catalyze the polymerization of several NTPs in a template-directed manner [23]. More recently, the class I ligase ribozyme has been utilized as a core catalytic domain, appended with a domain of random nucleotides at its 3′ end, to evolve a ribozyme that catalyzes polymerization on an external RNA template [22]. This new polymerase ribozyme exhibits sequence generality with respect to the primer-template complex and is able to extend an RNA primer by up to 14 nucleotides with high fidelity.

Starting from pools of random-sequence RNA molecules, several other ribozymes with 3′,5′ ligase activity have been evolved. These include motifs that are smaller than the class I ligase and exhibit some interesting catalytic properties. The L1 ligase can be activated by an

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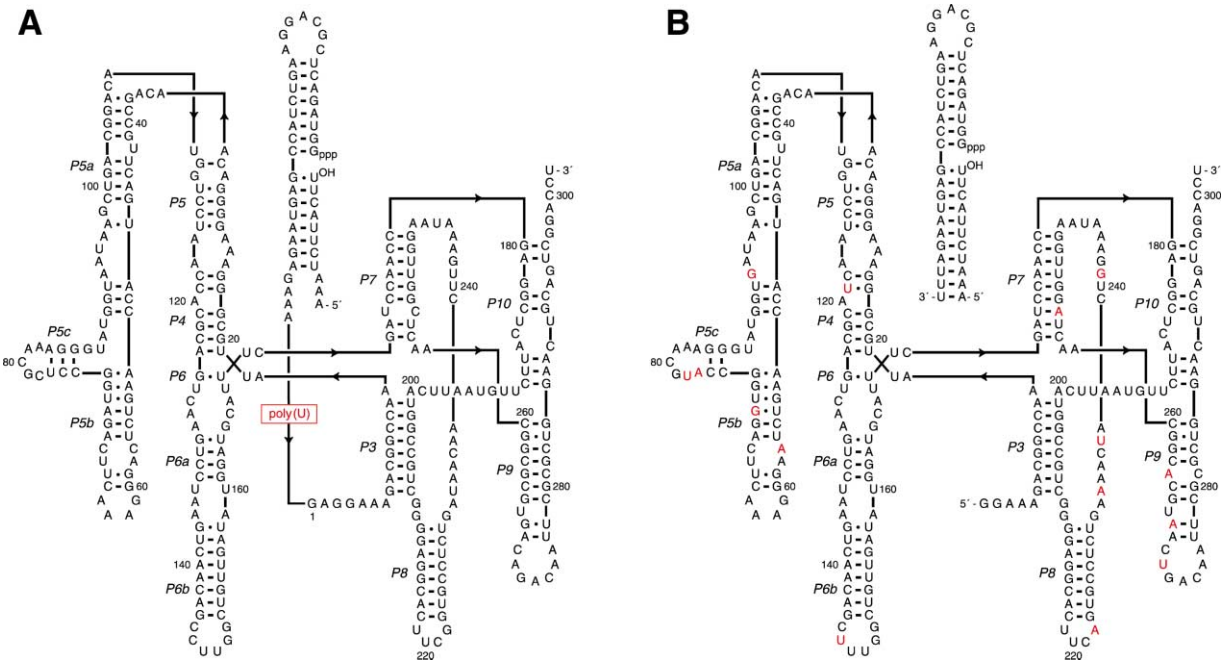

Figure 1. Starting Ribozyme and Mutations that Arose over the Course of Evolution

(A) Wild-type hc ligase ribozyme that was used to generate the starting population of ribozymes, with insertion of a poly(U) linker between the hairpin substrate and the 5′ end of the ribozyme.

(B) Evolved 18-2 ligase ribozyme, with mutations relative to the wild-type sequence shown in red.

oligonucleotide, small molecule, or protein effector to act as an allosteric ribozyme [17, 24, 25], suggesting a possible mechanism for primitive phenotypic regulation. The R3/R3C ligase adopts a simple three-way junction structural motif [21], strikingly similar to that of the L1 ligase. The R3 variant catalyzes RNA ligation within a well-defined nucleic acid structure that contains only three of the four nucleotides (it lacks cytidylate). The closely related R3C variant contains all four nucleotides and operates with a substantially faster catalytic rate compared to that of the R3 variant.

A somewhat different strategy was undertaken to evolve the hc ligase (Figure 1A) [20]. The initial pool of RNA molecules was based on a portion of the *Tetrahymena* group I ribozyme, with added regions of random nucleotides. The independently folding P4–P6 domain [26, 27] of the *Tetrahymena* ribozyme together with slightly modified versions of the P3 and P8 stems was used as a scaffold on which to build the new ribozyme. The substrate for the ligation reaction was presented to the ribozyme in a helical context, held by base-pairing interactions within a stem-loop region located at the 5′ end of the ribozyme. The resulting hc ligase took advantage of the P4–P6 domain, which was shown to fold into a structure similar to that seen in the *Tetrahymena* ribozyme. The evolved ligase joined the 3′ end of the substrate to its own 5′ end within the context of an extended region of Watson-Crick pairing. Ligation occurred even when the sequence of the stem loop structure was altered, so long as base pairing was maintained, indicating that the ribozyme may employ a sequence-independent mechanism for recognition of an RNA duplex that contains the site of ligation.

Portions of the P4–P6 domain of the *Tetrahymena* ribozyme have been implicated in the ability of that ribozyme to dock the substrate-containing duplex (P1 helix) into the active site through specific tertiary contacts. Critical interactions occur between two sheared A-A base pairs in the J4/5 region of the ribozyme and the G•U wobble pair that defines the site of reaction within the P1 helix [28–30]. Based on nucleotide analog interference mapping and substitution of various nucleotide analogs within the P1 helix, the minor groove surface of J4/5 was shown to interact with the P1 helix [30]. Specifically, the 2′-OH of A114 is thought to participate in a hydrogen-bonding interaction with the 2′-OH of the guanosine residue that forms the G•U wobble pair. In addition, the 2′-OH of A207 is thought to interact with the N2 amine of the wobble-paired G. The U residue that forms the G•U wobble pair is thought to participate in orienting the G for this interaction, as replacing this U by C (forming a Watson-Crick pair) impairs docking of the P1 helix [31, 32]. Certain 2′-OH groups on both strands of the P1 helix also have been implicated in docking interactions with the ribozyme [29, 33, 34], for example with A302 in the J8/7 region [35]. It was hypothesized that similar interactions might be realized in the hc ligase ribozyme by taking advantage of the unique geometry of the G•U wobble pair at the ligation junction to assist in binding and correctly orienting a substrate duplex that would be presented in an intermolecular reaction format.

It is inherently difficult to employ in vitro evolution methods to obtain catalysts that perform intermolecular reactions because the functional molecules must be altered as a consequence of the reaction in order to be

distinguished for selective amplification. Some strategies have been reported that overcome this challenge [36–39]. For example, one of the substrates may be covalently attached to the pool of potential catalysts through a flexible polyethylene glycol linker, thus creating a pseudo-intermolecular reaction format [37, 38]. In the current study, a pseudo-intermolecular reaction format was achieved by the introduction of a flexible poly(U) linker between the 5′ end of the ribozyme and the 3′ end of the stem loop region. The stem loop provides a helical context for ligation of the substrate oligonucleotide (Figure 1A). Ribozymes were evolved to catalyze pseudo-intermolecular ligation, then analyzed for their ability to carry out a true intermolecular ligation reaction involving a detached hairpin template and corresponding substrate oligonucleotide. The resulting catalysts also were analyzed with regard to their substrate generality and ability to catalyze mononucleotide addition reactions on an external RNA template.

Results

Evolution of Intermolecular Ligase Ribozymes

Evolution was initiated with a pool of randomized hc ligase variants that were generated by in vitro transcription of DNA templates that had been subjected to mutagenic PCR [40]. This procedure introduced mutations at a frequency of ~0.7% per nucleotide position and resulted in an average of two mutations per template molecule. A pool of 10^{13} different DNA templates was extended by *Taq* DNA polymerase to add the coding sequence for a hairpin template and 28 U residues, located upstream from the first nucleotide of the ribozyme (Figure 1A). The extended templates were transcribed to generate RNA molecules that were used as the input for the first round of in vitro selection. A total of eighteen rounds of selective amplification were performed in which the RNA molecules were required to bind an RNA substrate through Watson-Crick pairing and catalyze a ligation reaction that resulted in attachment of the 3′ end of the substrate to the 5′ end of the hairpin. The ribozyme concentration was held constant at 1 μM, and the substrate concentration was reduced from 5 μM during rounds 1–7 to 2 μM during rounds 8–18. During rounds 1, 2, 4, and 5, a short biotin-containing substrate molecule was used. Subsequent chromatography on a neutravidin column isolated RNA molecules that had ligated the substrate to their own 5′ end. During all other rounds, a longer substrate was used, and the reacted molecules were isolated based on their slower mobility in a denaturing polyacrylamide gel as compared to that of unreacted RNAs. The selected RNA molecules were reverse transcribed, PCR amplified, and forward transcribed to generate "progeny" RNAs that were used to initiate the next round of in vitro selection. The time of the reaction was reduced from 30 min to 10 min over rounds 1–7. Mutations were introduced into the population prior to round 4 by error-prone PCR [40]. After round 7, individuals were cloned, sequenced, and assayed for their ability to catalyze a true intermolecular ligation reaction involving a detached hairpin template and oligonucleotide substrate. The most active of these individuals was clone 7-20, which contained four mutations relative to the wild-type hc ligase. This sequence was mutagenized [40] to generate variants that were subjected to additional rounds of selective amplification.

Eleven more rounds of in vitro selection were performed, each with a reaction time of 30 s. Mutations were introduced by error-prone PCR prior to round 13 [40]. Following the 18th round, individuals again were cloned from the population and sequenced. A total of twenty different individuals were assayed for their ability to perform the intermolecular ligation reaction. A variety of active ligase ribozymes were identified, the most active of which was clone 18-2 (Figure 1B). This ribozyme contains 15 mutations relative to clone 7-20, almost twice as many as would have been expected for the average molecule based on the mutagenesis procedures that were performed. Thus, the selected molecule probably was rare or not represented in the initial population. The 15 mutations are distributed throughout the ribozyme. Interestingly, one occurs near the J4/5 region and several others are in the J7/8 region; both regions are involved in helix docking interactions in the context of the *Tetrahymena* ribozyme [28–35].

Biochemical Characterization of Intermolecular Ligation

A hairpin domain that was extended by three nucleotides to engage all of the substrate nucleotides in Watson-Crick base pairing was employed to analyze the kinetic properties of the 18-2 ribozyme. Gel-shift experiments were performed to ensure that the hairpin-substrate complex was fully formed under the conditions used for kinetic analyses. More than 80% of the hairpin-substrate complex was shown to be in a conformation that was competent for the RNA-catalyzed ligation reaction (our unpublished data).

Initial experiments were carried out to determine the optimal conditions for the intermolecular ligation reaction. Under multiple-turnover conditions, in which the concentration of hairpin-substrate complex was at least 5-fold greater than that of the ribozyme, increasing the KCl concentration above 50 mM resulted in a decrease in ligation efficiency, whereas eliminating KCl had no effect on the reaction rate (our unpublished data). Increasing the $MgCl_2$ concentration to as high as 700 mM resulted in a progressive increase in ligation efficiency, with no indication of $MgCl_2$ saturation (our unpublished data). Under multiple-turnover conditions involving an excess of substrate, an increase in the concentration of the ribozyme resulted in a less-than-proportional increase in the observed reaction rate, suggesting that the ribozyme engages in nonproductive ribozyme-ribozyme interactions at higher concentrations. This issue will be addressed in more detail below in the context of single-turnover reactions. Based on these results, kinetic analyses were performed under multiple-turnover conditions with 0.1 μM ribozyme in the presence of 100 mM $MgCl_2$ and no KCl at pH 7.5 and 37°C.

Kinetic parameters were determined for both the wild-type and evolved ligase ribozymes for intermolecular ligation within the hairpin-substrate complex (Figure 2). Both ribozymes exhibited Michaelis-Menten-type be-

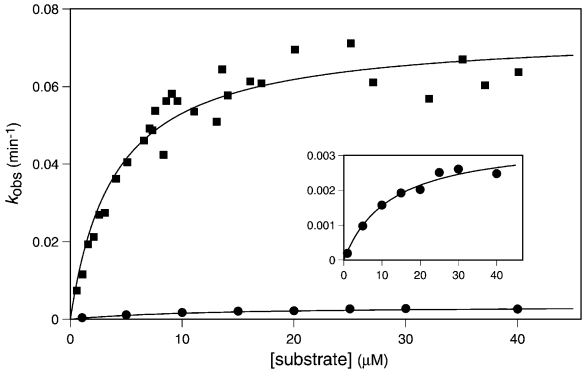

Figure 2. Catalytic Activity for RNA-Catalyzed Intermolecular Ligation with a Hairpin Substrate

Values for k_{obs} for the wild-type (circles) and 18-2 (squares) ribozymes were determined in the presence of 0.1 μM ribozyme and various concentrations of hairpin-substrate complex under standard reaction conditions (see Experimental Procedures). Data were fit to a curve based on the Michaelis-Menten equation: $k_{obs} = k_{cat}$[substrate]/(K_M + [substrate]).

havior. The wild-type hc ribozyme had a k_{cat} of 0.0035 min^{-1} and a K_M of 12 μM, corresponding to a catalytic efficiency, k_{cat}/K_M, of 290 M^{-1}·min^{-1}. The evolved ribozyme had a k_{cat} of 0.074 min^{-1} and a K_M of 3.9 μM, corresponding to a catalytic efficiency of 1.9×10^4 M^{-1}·min^{-1}, which is 65-fold improved compared to that of the wild-type ribozyme. The evolved ribozyme exhibited a linear increase in product formation over multiple turnovers under saturating conditions, indicating that product release is not the rate-limiting step of the reaction. The reaction has been followed for more than 30 turnovers.

The intermolecular ligation reaction also was studied under single-turnover conditions, with varying concentrations of ribozyme in excess of a subsaturating concentration of substrate. A plot of k_{obs} versus ribozyme concentration deviated from that which would be predicted based on the k_{cat} and K_M values obtained under multiple-turnover conditions (Figure 3A). In the single-turnover reactions, for ribozyme concentrations well below the K_M, the plot of k_{obs} versus ribozyme concentration was linear with a slope of 1.8×10^4 M^{-1}·min^{-1} (Figure 3B). This value, which should correspond to k_{cat}/K_M, is in fact almost identical to the value of 1.9×10^4 M^{-1}·min^{-1} that was calculated based on the k_{cat} and K_M values determined under multiple-turnover conditions. For ribozyme concentrations above 0.2 μM, however, the observed reaction rates were slower than predicted, presumably because of ribozyme-ribozyme interactions that lead to the formation of nonproductive complexes.

Estimates of the intramolecular ligation rates were obtained for both the wild-type and evolved ribozymes, with the hairpin either attached directly to the 5′ end of the ribozyme or joined to the ribozyme through a long poly(U) linker (Figure 1A). Catalytic rates were measured in the presence of saturating concentrations of substrate. The rate of intramolecular ligation for all four ribozyme constructs was approximately 0.01 min^{-1}, significantly slower than that of the evolved ribozyme in the intermolecular ligation format.

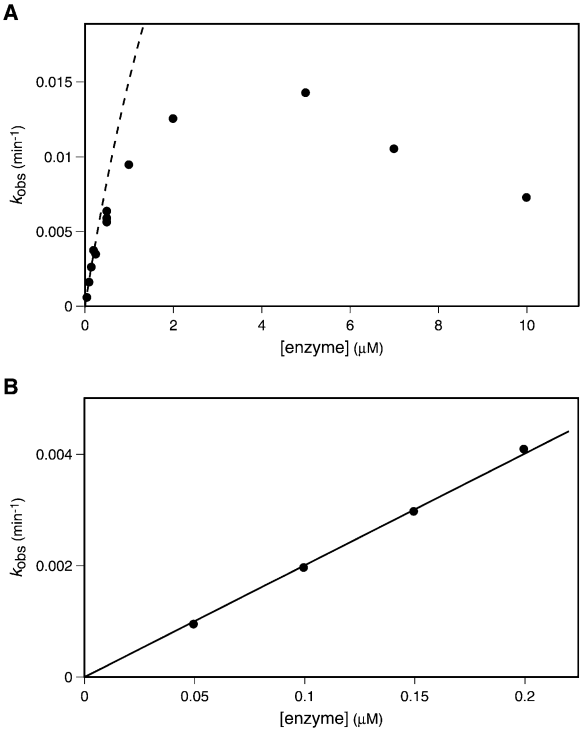

Figure 3. Catalytic Activity of the 18-2 Ribozyme under Single-Turnover Conditions

(A) Values for k_{obs} were determined in the presence of various concentrations of ribozyme, always in at least 5-fold excess over the hairpin-substrate complex. The predicted saturation curve (dashed line) was based on the Michaelis-Menten equation, $k_{obs} = k_{cat}$[enzyme]/(K_M + [enzyme]), with values for k_{cat} and K_M obtained from the multiple-turnover experiments.

(B) Single-turnover data for concentrations of ribozyme that were at least 10-fold below the K_M were fit to a curve based on the Michaelis-Menten equation: $k_{obs} = k_{cat}$[enzyme]/K_M.

The regiospecifity of RNA-catalyzed intermolecular ligation was examined with a [5′-^{32}P]-labeled 11mer substrate that contained a single ribonucleotide at its 3′ end, the remainder being composed of DNA. The rate of ligation with this substrate was approximately 10-fold slower than that observed with the all-RNA substrate under the same reaction conditions. The ligated product was isolated in a denaturing polyacrylamide gel, then treated with RNase A, RNase T2, or NaOH. The two ribonucleases specifically cleave 3′,5′-phosphodiester linkages of RNA, whereas NaOH cleaves both 2′,5′ and 3′,5′ linkages. Thus, the ribonucleases would be expected to generate either a labeled 11mer, if a 3′,5′ linkage had formed, or a labeled 12mer, if a 2′,5′ linkage had formed, whereas NaOH would generate a labeled 11mer in either case. Analysis of the degradation products indicated that the ribozyme catalyzed the formation of a 3′,5′ linkage, as is the case for intramolecular ligation catalyzed by the wild-type hc ligase.

Substrate Generality of Intermolecular Ligation

The sequence requirements for recognition of the hairpin-substrate complex by the ribozyme were examined by performing the reaction with various hairpins and

Figure 4. Substrate Sequence Requirements for RNA-Catalyzed Intermolecular Ligation

Observed ligation rates relative to that with the original hairpin-substrate complex were determined in the presence of 0.1 μM ribozyme, 10 μM 3′-hydroxyl-containing substrate, and an excess of the other oligonucleotide reactants. Sequence differences relative to the original hairpin-substrate complex are shown in red.

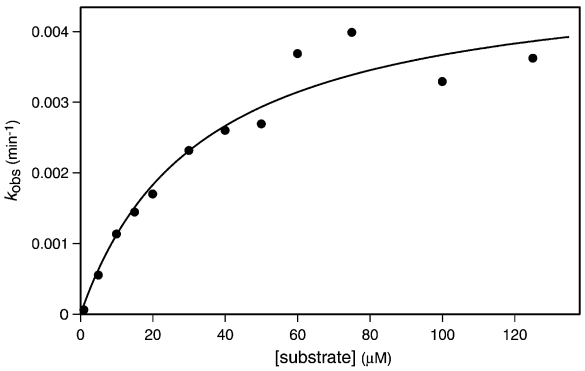

Figure 5. Catalytic Activity for RNA-Catalyzed Intermolecular Ligation with a Trimolecular Substrate Complex

Values for k_{obs} were determined in the presence of 0.1 μM ribozyme and various concentrations of the trimolecular substrate complex under standard reaction conditions (see Experimental Procedures). Data were fit to a curve based on the Michaelis-Menten equation: $k_{obs} = k_{cat}[\text{substrate}]/(K_M + [\text{substrate}])$.

complementary oligonucleotide substrates (Figure 4). The observed reaction rates were measured in the presence of 10 μM substrate, 11 μM hairpin, and 0.1 μM ribozyme and compared to those obtained with the original hairpin-substrate complex. These conditions ensured that the substrate was fully bound by the hairpin and that the resulting hairpin-substrate complex was in substantial excess over the ribozyme. However, the concentration of the substrate-hairpin complex was subsaturating. Thus, differences in the observed rates should be viewed as general trends that may reflect changes in either k_{cat} or K_M.

When the G•U wobble pair at the ligation junction was changed to a G-C Watson-Crick pair, the observed ligation rate decreased by about 7-fold, suggesting that the wobble pair participates in orienting the 3′ end of the substrate for efficient ligation. The sequence of the stem regions either upstream or downstream of the ligation site could be changed almost completely with only a small effect on the rate of ligation. When six of the eight base pairs in the downstream stem were altered, the observed ligation rate was 37% compared to that observed with the original substrate-hairpin complex. When nine of the eleven base pairs in the upstream stem were changed and a twelfth base pair was added, the observed ligation rate was 82% compared to that with the original complex. These results indicate a largely sequence-independent mechanism for substrate recognition by the ribozyme.

When the loop sequence of the hairpin was changed or displaced by 2 bp away from the ligation junction, there was a dramatic reduction in ligation rate. Replacing the 7 nucleotide loop by four guanylates reduced the ligation rate by 36-fold, whereas replacing it by four cytidylates resulted in no observable ligation under the conditions that were tested. Inserting two G-C base pairs adjacent to the loop reduced the ligation rate by about 20-fold. These results indicate that the ribozyme specifically recognizes the loop sequence and may rely on interaction with one or more loop nucleotides to correctly orient the substrate-hairpin complex within the active site.

The ribozyme is able to catalyze an intermolecular ligation reaction in which the hairpin loop is removed altogether and a trimolecular substrate complex is formed. With the original stem sequences, which are prone to forming competing self-structures, the ligation rate was 0.8% compared to that observed with the original hairpin-substrate complex (Figure 4). With a different trimolecular substrate complex, in which self-structure was avoided, the relative rate of ligation was 2.3%. These substrates provide for the ligation reaction a more polymerase-like context, in which the three components can be viewed as template, primer, and 5′-triphosphate-containing substrate for a template-directed extension reaction. The catalytic parameters for RNA-catalyzed ligation were determined for the more reactive of the two trimolecular substrate complexes (Figure 5). Under multiple-turnover conditions, the reaction exhibited Michaelis-Menten-type kinetics with an apparent k_{cat} of 0.0049 min^{-1} and an apparent K_M of 34 μM. Compared to the results of the reaction with the original hairpin-substrate complex, this corresponds to a 15-fold decrease in k_{cat} and a 9-fold increase in K_M, presumably reflecting the contribution made by the hairpin loop to

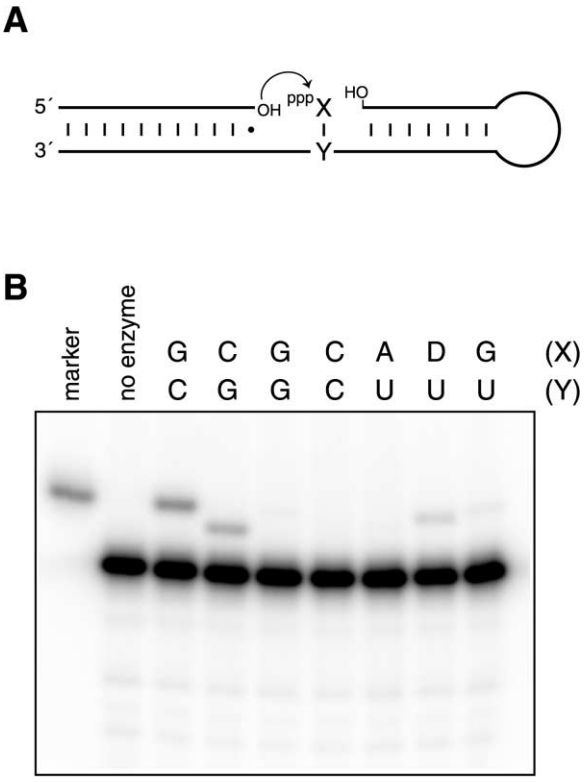

Figure 6. Addition of Mononucleoside 5′-Triphosphates on an External RNA Template

(A) Schematic representation of the mononucleotide addition reaction, which used a hairpin that lacks the 5′-terminal guanosine residue, that residue being supplied by an NTP substrate (X). Various templating residues (Y) were tested opposite the site of NTP addition.

(B) Extension of a [5′-^{32}P]-labeled primer by various NTPs. Reactions were carried out in the presence of 5 μM ribozyme, 5 μM primer, 5.5 μM hairpin, and 4 mM NTP under standard conditions (see Experimental Procedures) at 37°C for 24 hr. The products were separated by electrophoresis in a denaturing 20% polyacrylamide gel, a Phosphorimager scan of which is shown. The marker is a synthetic oligonucleotide of the same sequence as the primer except that it has an additional guanosine residue at its 3′ end. D indicates diaminopurine. Note that the product resulting from CTP addition has slightly faster gel electrophoretic mobility compared to that resulting from GTP addition.

substrate binding and positioning within the active site of the ribozyme.

Template-Directed Mononucleotide Addition

The evolved ligase ribozyme was tested for its ability to catalyze the template-directed addition of NTPs on an external RNA template. These experiments employed synthetic RNA hairpins that lacked the 5′-terminal guanosine residue and had a free hydroxyl group at their 5′ end. The missing residue was supplied by a separate NTP substrate, bound opposite various templating nucleotides (Figure 6A). The remainder of the sequence was identical to that of the wild-type hairpin molecule shown in Figure 1B. The reaction mixtures contained 5 μM ribozyme, 5.5 μM hairpin oligonucleotide, 5 μM oligonucleotide primer, and 4 mM NTP and were incubated at 37°C for 24 hr. The ribozyme was able to extend the substrate by one nucleotide, with varying efficiency depending on the templating nucleotide and the NTP that was supplied (Figure 6B).

In the absence of the ribozyme, no mononucleotide addition was observed. When the templating nucleotide was a C, GTP was added onto the 3′ end of 7% of the primer molecules. When the template nucleotide was changed to a G, it directed the addition of CTP onto 4% of the primer molecules. With G in the template, GTP was added onto 0.3% of the primer molecules, whereas with C in the template there was no detectable addition of CTP (<0.1%). When the templating nucleotide was a U, there was no detectable addition of ATP. However, a U-containing template directed the addition of diaminopurine nucleoside 5′-triphosphate (DAPTP) onto 0.2% of the primer molecules, presumably because DAPTP interacts more strongly with U than does ATP. Addition of GTP also was observed when the templating nucleotide was a U, albeit at an extent of only ~0.2%.

All of the other combinations of templating nucleotide and NTP were tested. When the templating nucleotide was a C, the ribozyme was able to extend the primer with GTP, but not with any of the other three standard NTPs. When the templating nucleotide was a G, addition of each of the four nucleotides was observed, with CTP being the most efficient (4.1%), UTP being less efficient (2.5%), and ATP and GTP addition occurring just above the limit of detection. No NTP addition was observed when A was the templating nucleotide, even when UTP was employed, presumably because of the weak binding of the monomer to the template. Finally, a synthetic RNA hairpin was provided that lacked two guanosine residues at the 5′ end and thus provided two GTP binding sites located opposite two templating C residues. In this case, the ribozyme was able to direct the addition of two or three G residues onto the 3′ end of the oligonucleotide primer. Thus, the ribozyme is capable of catalyzing the addition of multiple mononucleotides and provides a glimpse of the properties of a true polymerase enzyme.

The 18-2 ribozyme was tested for its ability to catalyze the template-directed addition of NTPs onto the 5′ end of a 5′-triphosphorylated hairpin oligonucleotide in what would represent addition in a 3′→5′ direction. These reactions employed a full-length hairpin molecule and a substrate that lacked the 3′-terminal residue and ended in a deoxynucleotide, forcing NTP addition to occur onto the 5′ end of the hairpin. The reactions were carried out similarly to those described above, but [α-^{32}P]UTP was employed as the radiolabeled material. Under these circumstances, the 18-2 ribozyme catalyzed NTP addition in a 3′→5′ direction but at a level that was just above the limits of detection.

Discussion

Ligation on an External Template

A pseudo-intermolecular reaction format was utilized to evolve ribozymes that are capable of catalyzing a true intermolecular RNA ligation reaction. These ribozymes, derived from the hc ligase [20], catalyze the joining of

two oligonucleotides on an external template and result in the formation a 3′,5′-phosphodiester linkage. The reaction takes place in the context of either an RNA hairpin that acts as both template and 5′-triphosphate-bearing substrate or a linear template that is complementary to two separate oligonucleotide substrates. The evolved ribozyme that was chosen for analysis contained 15 mutations relative to the wild-type hc ligase and exhibited a 65-fold improvement in catalytic efficiency in the intermolecular ligation reaction (Figure 1). The roles of the individual mutations are difficult to discern, but together they are responsible for the substantial differences in the biochemical properties of the starting and evolved ribozymes.

The wild-type hc ligase operates on an internal template with a catalytic rate of approximately 0.01 min^{-1} and on an external template with a catalytic rate of 0.0035 min^{-1}. In comparison, the evolved 18-2 ribozyme operates on an internal template with a catalytic rate of approximately 0.01 min^{-1} and on an external template with a catalytic rate of 0.074 min^{-1}. The fact that the intramolecular reaction catalyzed by both the wild-type and evolved ribozymes is slower than the intermolecular reaction catalyzed by the evolved ribozyme suggests that conformational constraints make it more difficult to orient an attached hairpin-substrate complex within the active site. Substrate orientation is less constrained in the intermolecular reaction format, although tertiary contacts are required to position the separate hairpin-substrate complex for efficient ligation. Normally, in an in vitro evolution experiment one expects to obtain catalytic properties that preferentially reflect the imposed selection constraints, in this case involving ligation on an internal rather than external template. Perhaps because of its heritage, deriving from the group I ribozyme, the 18-2 ribozyme was preadapted to evolve tertiary contacts that assist in binding a separate hairpin-substrate complex. In contrast, the class I-derived polymerase does not bind a separate template-substrate complex with high affinity [22], reflecting the heritage of the class I ligase, which operates on an internal template without the benefit of significant tertiary contacts [16].

The 18-2 ribozyme exhibits a high level of sequence generality when it operates on an external hairpin-substrate complex (Figure 4). The stem sequences both upstream and downstream of the ligation junction can be altered without significantly perturbing ligation activity, implying a largely sequence-independent mechanism for duplex recognition. Changes in the hairpin loop, however, have a dramatic effect on ligation activity, which is not the case for the wild-type ribozyme when it operates in the intramolecular reaction format [20]. Varying activity with different loop sequences may reflect the positive effects of specific tertiary contacts or the negative effects of unfavorable interactions. The possibility of unfavorable interactions was evident when the 7 nucleotide hairpin loop was replaced by four cytidylates, resulting in no observable ligation activity even though ligation proceeded when the loop was removed entirely.

Ligation reactions with hairpin-substrate complexes having different distances between the ligation junction and the loop provide additional information regarding the nature of ribozyme interactions with the loop nucleotides. The catalytic rate of the 18-2 ribozyme in the intermolecular reaction was reduced by 20-fold when the loop was displaced by 2 bp away from the ligation junction. A similar decrease in activity was seen with the wild-type ribozyme operating in the intramolecular reaction format when the loop was moved 6 bp closer to the ligation junction [20]. Thus, it appears that one or more loop nucleotides engage in specific interactions with the ribozyme to assist in correct orientation of the ligation junction within the active site.

One of the remarkable properties of the 18-2 ribozyme is its ability to bind the stem portion of the hairpin-substrate complex in a largely sequence-independent manner. Mutations in the evolved ribozyme compared to the wild-type ribozyme enhanced this binding ability, as reflected by a 3-fold decrease in K_M. The sequence generality of the reaction implies that binding occurs, at least in part, through contacts between the ribozyme and generic features of the hairpin-substrate complex. The hc ligase motif was derived from a substantial portion of the *Tetrahymena* ribozyme. The parent ribozyme makes generic contacts with an RNA duplex involving backbone interactions with 2′-OH groups on both strands [28–30, 35]. The evolved form of the hc ligase may employ a similar strategy. The *Tetrahymena* ribozyme also makes specific contacts with the G•U wobble pair that defines the 5′ splice site for the RNA-catalyzed self-splicing reaction [30–32]. The evolved hc ligase was trained on a wobble-containing substrate and retains a preference for a G•U wobble on the 5′ side of the ligation junction (Figure 4). Thus, the two ribozymes may employ a similar substrate binding strategy that involves specific recognition of nucleotides at the site of reaction and general recognition of the base-paired region upstream of the reaction site.

Polymerase-Like Reactions on an External Template

One of the goals of this study was to evolve ribozymes with polymerase-like properties, specifically the ability to bind a primer-template complex and catalyze template-directed primer extension reactions. The RNA-catalyzed, template-directed joining of an oligonucleotide 3′-hydroxyl and oligonucleotide 5′-triphosphate mimics this process, especially when carried out on an external RNA template. The ligation reaction is general with respect to the sequence both upstream and downstream of the ligation junction, although optimal activity requires the presence of a particular nucleotide sequence within the downstream hairpin loop (Figure 4). Recognition of duplex substrates, irrespective of sequence, would be required for polymerase activity in an RNA world in which a ribozyme is responsible for copying a large number of possible RNA sequences. A general mechanism for the binding of a duplex substrate and the template-directed polymerization of NTPs has been demonstrated recently for an in vitro-evolved ribozyme derived from the class I ligase [22]. The 18-2 ribozyme provides a second example of a ribozyme that may be capable of supporting general RNA polymerase reactions. These two ribozymes have no discernible sequence similarity

and have entirely independent evolutionary origins. This indicates that there may be many possible solutions to the problem of RNA-catalyzed information transfer in an RNA world.

The template-directed addition of NTPs onto the 3′ end of an oligonucleotide primer more directly addresses the issue of information transfer in an RNA world, if one assumes that 5′-activated mononucleotides were available for copying reactions. The 18-2 ribozyme catalyzes the template-directed addition of an NTP in the context of a hairpin molecule with a single-nucleotide gap (Figure 6). The efficiency and specificity of this reaction vary depending on the templating nucleotide and the NTP that is provided. For sustainable information transfer in an RNA world, both efficiency and specificity are important for copying a template RNA prior to its degradation.

The 18-2 ribozyme exhibits high efficiency and specificity for the addition of GTP on a templating C nucleotide, with a fidelity of more than 96%. The efficiency of NTP addition on a templating G nucleotide is approximately the same, but the fidelity of CTP addition is only 60%, primarily because of the addition of UTP as a G•U wobble pair. Reduced ribozyme fidelity due to wobble pairing also has been seen with the class I ligase and the class I-derived polymerase ribozyme, albeit to a lesser extent [22, 23]. The 18-2 ribozyme does not exhibit any detectable addition of UTP on a templating A or addition of ATP on a templating U, perhaps because of reduced template occupancy due to the relatively lower stability of A-U compared to G-C pairs. This does not fully explain the situation, however, because GTP addition occurs at a low level on a templating U despite the weakness of G•U pairing. Both the class I ligase [22] and the class I-derived polymerase [23] catalyze template-directed primer extension that employs all four NTPs, with an average fidelity of 85% and 97%, respectively. Similar to the 18-2 ribozyme, the class I ribozymes operate with highest fidelity when catalyzing the addition of GTP on a templating C residue. However, the class I ribozymes operate with greater generality and thus with a fidelity that reflects all 16 combinations of NTP and template.

The observation that multiple guanosine mononucleotides can be added onto the 3′ end of a primer opposite two templating C residues indicates that the 18-2 ribozyme has the potential to be evolved into a true polymerase ribozyme. In this study, the ribozyme was evolved to catalyze an intermolecular ligation reaction. However, it already exhibits many of the desirable properties of an RNA polymerase, most importantly the ability to bind a primer-template complex in a sequence-independent manner and to carry out the template-dependent addition of activated mononucleotides. Evolved forms of the 18-2 ribozyme might carry out mononucleotide addition reactions with enhanced efficiency and specificity and exhibit behaviors comparable to those required for sustainable information transfer in an RNA world.

Significance

The RNA world hypothesis proposes that there was a time in evolutionary history when both genotype and phenotype were the responsibility of RNA molecules. Information transfer in an RNA world probably would have relied on an RNA catalyst with RNA polymerase activity. In this study, the hc ligase ribozyme, which catalyzes the joining of the 3′ end of an oligonucleotide substrate to its own 5′-triphosphate within a helical context, was used as a starting point to evolve ribozymes that catalyze RNA ligation on an external RNA template. The evolved ribozyme recognizes duplex RNAs in a largely sequence-independent manner and catalyzes the template-directed ligation of the 3′ end of one oligonucleotide substrate with the 5′-triphosphate of another. This reaction occurs in the context of a hairpin molecule that acts as both template and triphosphate-containing oligonucleotide and in a format in which the 3′-hydroxyl- and 5′-triphosphate-bearing substrates are bound to a separate RNA template. The evolved ribozyme also can be made to catalyze the addition of NTPs onto the 3′ end of an oligonucleotide primer in the context of a hairpin molecule that contains a complimentary templating nucleotide. The efficiency and specificity of NTP addition varies for the different Watson-Crick pairs. Nonetheless, the ribozyme exhibits many of the properties that would have been required for an RNA polymerase in an RNA world and appears to provide a good starting point for the evolution of ribozymes with true polymerase activity.

Experimental Procedures

Materials

Synthetic oligonucleotides were prepared with a PerSeptive Expedite automated DNA/RNA synthesizer. Expedite DNA amidites and solid supports were purchased from Applied Biosystems, and TOM RNA amidites and solid supports were purchased from Glen Research. All oligonucleotides were deprotected according to the manufacturers' protocols and purified by denaturing polyacrylamide gel electrophoresis.

Histidine-tagged T7 RNA polymerase (T7 RNAP) was purified from *E. coli* strain BL21 containing plasmid pBH161 (kindly provided by William McAllister) by the use of His-Bind resin (Novagen) according to the manufacturer's protocol. *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA and purified as previously described [43]. Superscript II RNase H^- reverse transcriptase was obtained from Gibco-BRL, T4 polynucleotide kinase was from New England Biolabs, and Sequenase 2.0 modified T7 DNA polymerase was from U.S. Biochemical. Nucleoside 5′-triphosphates (NTPs) and deoxynucleoside 5′-triphosphates (dNTPs) were purchased from Pharmacia. Diaminopurine nucleoside 5′-triphosphate (DAPTP) was obtained from Trilink Biotechnologies, and dideoxynucleoside 5′-triphosphates (ddNTPs) were purchased from U.S. Biochemical. [γ-^{32}P]ATP, [α-^{32}P]ATP, and [α-^{35}S]dATP were obtained from ICN Radiochemicals. The TA cloning kit was purchased from Invitrogen, PCR purification kits were from Qiagen, and UltraLink immobilized neutravidin plus columns were from Pierce. RNase-free DNase I was purchased from Boehringer Mannheim. All in vitro-transcribed RNAs were treated with DNase I, purified by denaturing polyacrylamide gel electrophoresis, and subsequently precipitated with ethanol. Unless otherwise noted, all RNAs were quantitated by UV spectroscopy of the monomers resulting from complete NaOH hydrolysis [44].

Construction of the Initial Pool

Plasmid DNA encoding the wild-type hc ligase was used as a template for PCR mutagenesis [40], resulting in random mutations at a frequency of ~0.7% per nucleotide over positions 16–287 of the ribozyme. The reaction products were purified with a Qiagen PCR purification kit and used as input for a second PCR with the upstream

primer 5′-GGACTAATACGACTCACTATAGGTAGACTCGCAGGAAG TCTACCGAGTAAGAGAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTG AGGAAAGACGGCCA-3′ (T7 RNAP promoter sequence underlined), which introduced a poly(U) linker and hairpin region onto the 5′ end of the ribozyme. The downstream primer had the sequence 5′-AGGTCCGACTGCAGTTC-3′, corresponding to the last 17 nucleotides at the 3′ end of the ribozyme. The resulting DNA was extracted with phenol and chloroform and precipitated with ethanol. The DNA then was transcribed in vitro [45] in the presence of 40 μCi [α-^{32}P]ATP. The resulting RNA was purified by electrophoresis in a denaturing 5% polyacrylamide gel and quantitated by UV spectroscopy (extinction coefficient 3638.9 $mM^{-1} \cdot cm^{-1}$).

In Vitro Selection

Reaction mixtures contained 30 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[3-propane-sulfonic acid] (EPPS; pH 7.5), 25 mM $MgCl_2$, 50 mM KCl, 1 μM pool RNA, and either 5 μM (rounds 1–7) or 2 μM (rounds 8–18) substrate RNA, which were incubated at 37°C for 0.5–30 min. For rounds 1, 2, 4, and 5, a 5′-biotinylated substrate having the sequence 5′-biotin-ACUGAUGCCACUGAAA**UCUUACUU**-3′ (nucleotides that bind to the hairpin in bold) was employed, and the reacted molecules were isolated on a neutravidin column either prior to (rounds 2 and 5) or after (rounds 1 and 4) reverse transcription. For column isolation, 20 μl of neutravidin beads were washed three times with 200 μl of wash buffer (50 mM Tris [pH 7.5], 500 mM NaCl, 1 mM disodium ethylenediaminetetraacetate [Na_2EDTA], 0.1% sodium dodecyl sulfate [SDS], 0.25% Nonidet P-40 [NP40]), incubated with the reacted RNA in 100 μl of wash buffer at 23°C for 10 min, washed ten times with 200 μl of wash buffer, and then washed five times with 200 μl H_2O. RNAs were reverse transcribed either before or after binding to the column, eluted by the addition of 150 μl of a solution containing 0.1 M NaOH and 150 mM NaCl, neutralized by the addition of 150 μl 0.1 M HCl, and precipitated with ethanol. For rounds 3 and 6–18, a longer, nonbiotinylated substrate having the sequence 5′-GUACCGAUUCAGCACCGUUACUCGAAGCAAG CUAUCGCCAAA**UCUUACUU**-3′ (nucleotides that bind to the hairpin in bold) was employed, and reacted molecules were isolated by electrophoresis in a denaturing 5% polyacrylamide gel, followed by ethanol precipitation.

Reacted molecules were reverse transcribed with Superscript II RNase H^- reverse transcriptase and the primer 5′-AGGTCCGACTG CAGTTC-3′ and then precipitated with ethanol. The resulting cDNAs were PCR amplified with the upstream primer 5′-ACTGATGCCACT GAAATC-3′ (rounds 1, 2, 4, and 5) or 5′-GTACCGATTCAGCACCG-3′ (rounds 3 and 6–18), both of which bind specifically to the reacted materials, and downstream primer 5′-AGGTCCGACTGCAGTTC-3′. The PCR products were purified with a Qiagen PCR purification kit and then PCR amplified with upstream primer 5′-GGACTAATAC GACTCACTATAGGTAGACTCGCAGGAAG-3′ (rounds 1–7) or 5′-GGA CTAATACGACTCACTATAGGTAGACTCGCAGGAAGTCTACC GAGTAAGAGAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGGAAA GACGGCCA-3′ (rounds 8–18) to introduce the T7 RNAP promoter sequence (underlined) and downstream primer 5′-AGGTCCGACTG CAGTTC-3′. The products were purified with a Qiagen PCR purification kit, then in vitro transcribed in the presence of 20 μCi [α-^{32}P]ATP. The first of the two PCR amplifications was carried out in an error-prone manner [40] prior to rounds 4, 8, and 13. After rounds 7 and 18, individuals were cloned from the population with the TA cloning kit and sequenced by the dideoxynucleotide method, in which [α-^{35}S]dATP and Sequenase 2.0 modified T7 DNA polymerase were employed.

Kinetic Analysis of Ligation

Plasmid DNA encoding either the wild-type or evolved ribozyme was PCR amplified with primers 5′-GGACTAATACGACTCACTA TAGGAAAGACGGCC-3′ (T7 RNAP promoter sequence underlined) and 5′-AGGTCCGACTGCAGTTC-3′, then transcribed to generate RNA molecules lacking the hairpin at their 5′ end. The hairpin molecule was transcribed from a synthetic DNA template having the sequence 5′-AAATCTTACTCGGTAGACTCCTGCGAGTCTACCTAT AGTGAGTCGTATTAGTCC-3′, which was made double-stranded by primer extension in a PCR reaction employing 5′-GGACTAATAC GACTCACTATA-3′. The synthetic RNA substrate had the sequence 5′-AAAUCUUACUU-3′ and was [5′-^{32}P]-labeled with a specific activity of ~1.7 μCi/pmol by the use of T4 polynucleotide kinase.

For kinetic analyses, the hairpin RNA was preannealed prior to its addition to the reaction mixture by heating at 95°C for 1 min in 1 mM Tris (pH 7.5) and 0.1 mM EDTA. Incubation at 23°C for 5 min followed. Ligation experiments were performed under multiple-turnover conditions in the presence of 0.1 μM ribozyme, 0.5–40 μM labeled substrate, and hairpin RNA always in 1 μM excess over substrate RNA. The reaction mixtures also contained 100 mM $MgCl_2$ and 30 mM EPPS (pH 7.5). Reactions were allowed to proceed at 37°C for up to 6 hr, with aliquots taken at various times and quenched with an equal volume of a solution containing 9 M urea, 20% sucrose, 90 mM Tris-borate (pH 8.3), 0.05% bromophenol blue, 0.05% xylene cyanol, 0.1% SDS, and 100 mM Na_2EDTA. Reaction products were separated by electrophoresis in a denaturing 10% polyacrylamide gel and quantitated with a Molecular Dynamics Phosphorimager. The data were fit to a linear equation, and the resulting slopes were fit to a standard Michaelis-Menten saturation plot to determine k_{cat} and K_M.

Single-turnover experiments were carried out in a similar manner in the presence of 0.05–10 μM ribozyme, with substrate concentrations at least 5-fold below that of the enzyme and hairpin concentrations at least 0.1 μM above that of the substrate. The data were fit to a linear regression plot of $\ln[1 - F_{reacted}]$ versus time to determine k_{obs} for each enzyme concentration.

Kinetic parameters for the ligation reaction with the trimolecular substrate complex were determined in a similar manner. The 3′-hydroxyl-containing substrate had the sequence 5′-CACUCAGG CAUU-3′, the 5′-triphosphate-containing substrate was transcribed from a double-stranded DNA template and had the sequence 5′-GGUAGACU-3′, and the template RNA had the sequence 5′-AGU CUACCGAUGCCUGAGUG-3′. Ligation experiments were performed under multiple-turnover conditions in the presence of 0.1 μM ribozyme, 1–125 μM labeled 3′-hydroxyl-containing substrate, with the 5′-triphosphate-containing substrate always in 1 μM excess over the 3′-hydroxyl-containing substrate and the template RNA always in 2 μM excess over the 3′-hydroxyl-containing substrate.

Ribozymes for the intramolecular ligation reactions were transcribed from DNA templates that were generated by PCR amplification of plasmid DNA encoding either the wild-type or evolved ribozyme with upstream primer 5′-GGACTAATACGACTCACTATAGGT AGACTCGCAGGAAGTCTACCGAGTAAGATTTGAGGAAAGACGG CCA-3′ or 5′-GGACTAATACGACTCACTATAGGTAGACTCGCAGGA AGTCTACCGAGTAAGATTTTTTTTTTTTTTTTTTTTTTTTTTTTT GAGGAAAGACGGCCA-3′ (T7 RNAP promoter sequence underlined) and downstream primer 5′-AGGTCCGACTGCAGTTC-3′. Kinetic experiments were carried out as described above with 0.01 μM ribozyme, 0.1 μM [5′-^{32}P]-labeled substrate having the sequence 5′-AAAUCUUACUU-3′, 100 mM $MgCl_2$, 40 μg/ml BSA, and 30 mM EPPS (pH 7.5). No increase in rate was observed when the substrate concentration was increased by either 5- or 10-fold, indicating that the substrate was saturating at 0.1 μM concentration.

Analysis of Regiospecificity

The ligation reaction was performed under multiple-turnover conditions, as described above, in the presence of 10 μM [5′-^{32}P]-labeled substrate having the sequence 5′-CACTCAGGCA**TU**-3′ (single ribonucleotide in bold). The ligated products were purified by electrophoresis in a denaturing 10% polyacrylamide gel, precipitated with ethanol, and subsequently treated with (1) 0.6 M NaOH at 37°C for 1 hr, followed by neutralization with HCl and addition of an equal volume of gel loading buffer (9 M urea, 20% sucrose, 90 mM Tris-borate [pH 8.3], 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.1% SDS); (2) RNase T2 (5 U/μl) in 50 mM NaOAc (pH 5.2) and 2 mM Na_2EDTA at 50°C for 30 min, followed by addition of an equal volume of gel loading buffer; and (3) RNase A (0.1 U/μl) in 25 mM sodium citrate (pH 5.0), 5.9 M urea, 1 mM Na_2EDTA, 0.03% bromophenol blue, and 0.03% xylene cyanol at 50°C for 30 min. The products of either alkaline or RNase digestion were separated by electrophoresis in a denaturing 20% polyacrylamide gel.

Substrates for Analysis of Sequence Generality

The 5′-triphosphate-containing molecules, either hairpin or linear, were transcribed from double-stranded DNA templates encoding

the sequences shown in Figure 4. Sequences of the 3′-hydroxyl-containing substrates and linear templates also are shown in Figure 4. Reactions were carried out under the conditions described above with 10 μM substrate, 11 μM hairpin RNA, and 0.1 μM ribozyme for the hairpin-substrate reactions and 10 μM 3′-hydroxyl-containing substrate, 11 μM 5′-triphosphate-containing molecule, 12 μM template, and 0.1 μM ribozyme for the trimolecular reactions.

Mononucleotide Addition Reactions

Reaction mixtures contained 5 μM ribozyme, 5 μM [5′-^{32}P]-labeled oligonucleotide primer, 5.5 μM hairpin, 4 mM NTP, 100 mM $MgCl_2$, and 30 mM EPPS (pH 7.5), which were incubated at 37°C for 24 hr. The hairpins had the sequence 5′-GUAGACUCGCAGGAAGUCUA C**Y**GAGUAAGAUUU-3′ (**Y** = A, C, G, or U) or 5′-GUAGACUCGCAG GAAGUCUAC**CC**GAGUAAGAUUU-3′ (templating nucleotide(s) in bold). The oligonucleotide primer had the sequence 5′-AAAUCUUA CUU-3′. Reaction products were separated by electrophoresis in a denaturing 20% polyacrylamide gel and quantitated on a Molecular Dynamics Phosphorimager.

Reactions involving NTP addition in a 3′→5′ direction employed 5 μM ribozyme, 5.5 μM primer having the sequence 5′-AAAUCUU AC**T**-3′ (single deoxynucleotide in bold), 5 μM hairpin having the sequence shown in Figure 1B, 4 mM [α-^{32}P]UTP with a specific activity of 0.5 μCi/nmol, 100 mM $MgCl_2$, and 30 mM EPPS (pH 7.5), which were incubated at 37°C for 23 hr. Reactions were ethanol precipitated twice in the presence of 2 μg carrier tRNA and separated by electrophoresis in a denaturing 20% polyacrylamide gel.

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