Chemistry & Biology, Vol. 11, 1505–1512, November, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.08.021

Cross-Catalytic Replication of an RNA Ligase Ribozyme

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Summary

A self-replicating RNA ligase ribozyme was converted to a cross-catalytic format whereby two ribozymes direct each other's synthesis from a total of four component substrates. Each ribozyme binds two RNA substrates and catalyzes their ligation to form the opposing ribozyme. The two ribozymes are not perfectly complementary, as is the case for replicating nucleic acid genomes in biology. Rather, the ribozymes contain both template elements, which are complementary, and catalytic elements, which are identical. The specificity of the template interactions allows the cross-catalytic pathway to dominate over all other reaction pathways. As the concentration of the two ribozymes increases, the rate of formation of additional ribozyme molecules increases, consistent with the overall autocatalytic behavior of the reaction system.

Introduction

The process of self-replication has special significance in biology. It provides the basis for heritability of genetic information at the level of molecules and heritability of form at the level of cells and whole organisms. Self-replication implies more than the production of additional copies of the "self;" it also refers to the ability of the copies to behave in a similar manner, thereby exhibiting autocatalytic behavior with the potential for exponential growth.

The means by which biological systems undergo self-replication is well understood. The genetic molecule, DNA or RNA, encodes and is replicated by a cellular machinery. The genetic molecule itself is not self-replicating, but together with the replicative machinery forms a self-replicating system. The coding strand of the genetic material directs the synthesis of functional macromolecules that copy the coding strand to produce a noncoding strand and copy the noncoding strand to produce a coding strand. Through this process of semiconservative replication, the genetic "self" becomes perpetuated [1].

Chemists have sought to capture self-replicative behavior in nonbiological systems. Most commonly, this has involved reactions of the form **A** + **B** → **T**, where **A** and **B** are two substrates that bind to a template **T** and become joined to form a new copy of **T**. The reaction proceeds autocatalytically if the new copies of **T** also can direct the joining of **A** and **B**. Chemical self-replication has been demonstrated for activated oligonucleotides [2, 3], peptides [4, 5], and even small organic compounds [6, 7]. In one case, a self-replicating oligonucleotide was formed by joining three substrates: **A** + **B** + **C** → **T**, with the rate of formation of both the intermediate **A-B** and the final product **A-B-C** (= **T**) being enhanced by **T** [8].

More complicated chemical self-replication systems have been devised that involve two templates that direct each other's synthesis: a template **T** directs the joining of **A**′ and **B**′ to form **T**′, while a template **T**′ directs the joining of **A** and **B** to form **T** [9, 10]. Such systems more closely resemble biological self-replication, which involves the synthesis of cross-complementary (rather than self-complementary) nucleic acid templates. Unlike biological systems, however, these chemical systems do not entail a replicative machinery. Once the substrates are bound at adjacent positions on the template, they become joined through a favorable reaction between reactive groups at their opposed ends. There also is an example of a cross-catalytic amplification system involving two deoxyribozymes, each of which catalyzes a cleavage reaction, rather than a joining reaction [11]. Although not self-replicating, that system demonstrates exponential amplification based on molecules that have both template and catalytic properties.

It is difficult to design a self-replicating system that involves a separate replicative machinery because the machinery must also be copied and provided to each of the "progeny." One approach toward this goal has been to devise self-replicating molecules that function as both template and machinery. Recently, for example, a self-replicating ribozyme was developed that binds two RNA substrates through Watson-Crick pairing and catalyzes their joining to form another copy of the ribozyme [12]. The copies behave in a similar manner, resulting in autocatalytic behavior.

The self-replicating ribozyme was derived from the R3C ligase, which catalyzes attack of the 3′-hydroxyl of one RNA substrate on the 5′-triphosphate of another RNA substrate, forming a 3′,5′-phosphodiester linkage and releasing inorganic pyrophosphate [13]. The ligase ribozyme has a catalytic rate of 0.32 min^{-1}, corresponding to a rate enhancement of about 10^6-fold compared to the uncatalyzed, template-dependent rate of reaction [14]. The secondary structure of the ribozyme consists of a central three-way junction, with one arm containing the ligation junction surrounded by the P1 and P2 stems, a second arm containing the P3 stem, and a third arm containing the P4 and P5 stems and most of the unpaired residues that are essential for catalytic activity (Figure 1A).

In the self-replicative format, the ribozyme (**T**) was made to ligate two substrates (**A** and **B**) that correspond to the 5′ and 3′ portions of the ribozyme itself (Figure 1B). The resulting enzyme-product complex must then dissociate to make available two ribozyme molecules

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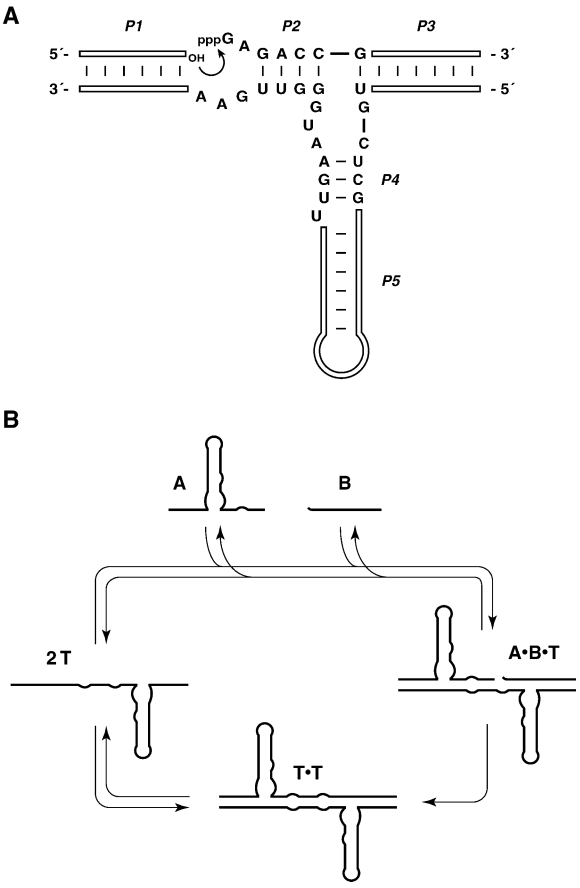

Figure 1. The R3C Ligase Ribozyme, which Has Been Made to Undergo Self-Replication

(A) Secondary structure of the ribozyme, consisting of five paired regions (P1–P5) and a total of 11 unpaired nucleotides [13]. The sequences of the P1, P3, and P5 stems can vary so long as base pairing is maintained. Curved arrow indicates attack of a 3′-hydroxyl on a 5′-triphosphate, resulting in RNA ligation.

(B) Replication cycle of the R3C ligase ribozyme [12].

that can enter the next cycle of replication. Dissociation of this complex was found not to be rate limiting, allowing the initial rate of formation of new ribozyme molecules to increase linearly with increasing starting concentration of ribozyme [12]. The self-replicating ribozyme does not exhibit sustained exponential growth, however, because the two component substrates can form a nonproductive **A•B** complex whose dissociation becomes rate limiting once the freely available reactants have been consumed. The 5′-terminal portion of **A** and the 3′-terminal portion of **B**, both of which are bound by **T**, are complementary to each other. This is necessary to maintain sequence identity between the ligated product **A-B** and the ribozyme **T**. However, it means that **A** and **B** can bind to each other in an intermolecular fashion, and the corresponding portions of **T** can bind to each other in an intramolecular fashion, both events potentially limiting the rate of self-replication. The requirement for self-complementarity at the termini also is a significant design constraint that may limit the discovery of more efficient replicators.

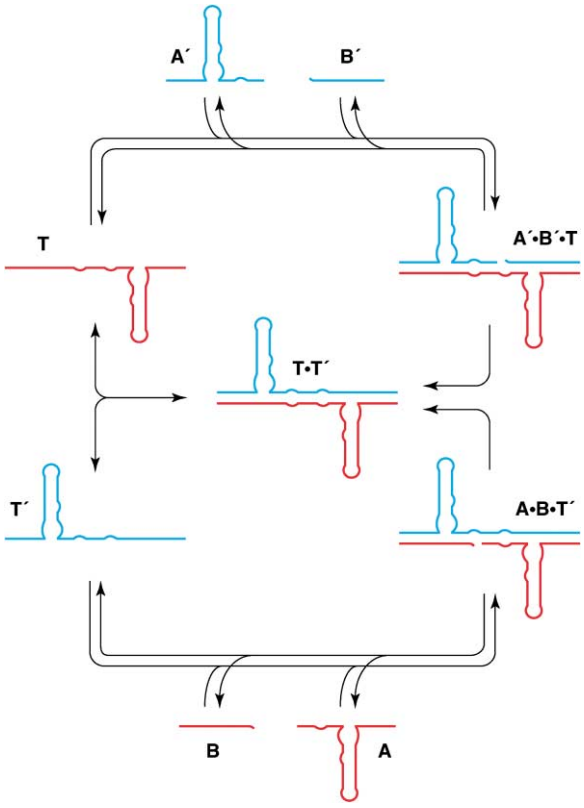

Figure 2. Cross-Catalytic Replication of the R3C Ligase Ribozyme

The ribozyme **T** (red) catalyzes ligation of the substrates **A′** and **B′** (blue) to form the ribozyme **T′**; the ribozyme **T′** (blue) catalyzes ligation of the substrates **A** and **B** (red) to form the ribozyme **T**. Dissociation of the **T•T′** product provides new copies of each ribozyme that can enter the next cycle of cross-catalytic replication.

The present study sought to break the symmetry of the self-replicating ribozyme by devising a cross-catalytic system involving two ribozymes that catalyze each other's synthesis from a total of four component substrates. This would replace the self-complementary relationship between **A** and **B** with cross-complementary relationships between **A** and **B′** and between **A′** and **B**. The ribozyme **T** would catalyze the ligation of **A′** and **B′** to form **T′**, and the ribozyme **T′** would catalyze the ligation of **A** and **B** to form **T** (Figure 2). The ribozymes **T** and **T′** would no longer be self-complementary at their termini, and there would be greater freedom to explore sequences that might be capable of more efficient replication.

Results

Design of Cross-Replicating Ribozymes

A pair of cross-replicating ribozymes was constructed based on the self-replicating R3C ligase ribozyme [12], but breaking the 2-fold symmetry of the **A•B•T** complex. Two ribozymes were designed (**T** and **T′**), each containing the catalytic core of the R3C ligase [13]. Each ribozyme could bind two substrates (**A′** and **B′**, **A** and **B**, respectively), resulting in RNA-catalyzed ligation to form the opposing ribozyme (Figure 2). The specificity

for substrate recognition derives from base-pairing interactions within the P1, P2, and P3 stems of the enzyme-substrate complex. The P3 stem is most amenable to sequence variation and therefore was chosen as the site for discrimination between the two pairs of substrates. The three proximal base pairs of the P3 stem (located closest to the catalytic core) were kept constant and chosen to provide maximum ligation efficiency. The distal portion of the P3 stem was varied to provide two distinct modes of substrate recognition: one for binding of **A′** to **T**, and therefore for binding of **B** to **T′**; the other for binding of **B′** to **T**, and therefore for binding of **A** to **T′**.

Initial studies were performed employing P3 stems of varying length and sequence. The goal was to find enzyme-substrate complexes that not only react efficiently but also in a highly discriminating manner so that **T** only catalyzes the ligation of **A′** and **B′**, and **T′** only catalyzes the ligation of **A** and **B**. In addition, it was important to minimize complementarity of the two P3 stems within both **T** and **T′** so that these molecules would not form intramolecular duplex structures. This also would minimize formation of nonproductive **A•B** or **A′•B′** complexes, which were found to limit the extent of exponential growth of the self-replicating ligase ribozyme [12]. Finally, the stability of the P3 stem was kept low to allow dissociation of the ligated product at a rate that would not limit catalytic turnover. The optimized design of the ribozymes and corresponding substrates is shown in Figure 3. Other than the P3 stems, it was not necessary to change the sequence of the self-replicating ligase to obtain cross-replicating ribozymes that met all of the above criteria. However, different lengths of the P5 stem were used to provide a convenient means for distinguishing the various ligated products. Altering the length or sequence of the P5 stem generally had little effect on the catalytic activity of the ribozyme. In addition, unpaired nucleotides were added to the 5′ and 3′ ends to facilitate preparation of the ribozymes by in vitro transcription.

Each member of the pair of cross-replicating ribozymes first was tested individually, employing the two substrates with or without the corresponding ribozyme (Figure 4A). The substrates **A′** and **B′** were ligated efficiently in the presence of the ribozyme **T** but not in the absence of ribozyme or in the presence of the mismatched ribozyme **T′**. Similarly, **A** and **B** only were ligated efficiently in the presence of **T′**. The time course of both ribozyme-dependent reactions exhibited biphasic kinetics, with an initial burst phase followed by a slower second phase. For the reaction catalyzed by **T**, the burst phase had a rate of $0.034 \pm 0.0036\ min^{-1}$ and an amplitude of 0.20 ± 0.014, followed by a linear phase with a rate of $5.0 \times 10^{-4}\ min^{-1}$. For the reaction catalyzed by **T′**, the burst phase had a rate of $0.026 \pm 0.0035\ min^{-1}$ and an amplitude of 0.11 ± 0.012, followed by a linear phase with a rate of $4.0 \times 10^{-4}\ min^{-1}$. The initial observed rate of reaction was $6.8 \times 10^{-3}\ min^{-1}$ and $2.9 \times 10^{-3}\ min^{-1}$ for the reaction catalyzed by **T** and **T′**, respectively. The rate of reaction was not affected by changing the order in which the three RNA components were added to the reaction mixture.

There also is the possibility of a "promiscuous" reaction, in which **A′** and **B** become ligated to form the chimeric product **A′-B** in a reaction catalyzed by either **T** or **T′**. Similarly, **A** and **B′** could become ligated to form **A-B′**. These chimeric products, once formed, might act as ribozymes to direct the formation of additional copies of themselves through RNA-catalyzed self-replication. Two of the four possible promiscuous reactions were found to occur (Figure 4B). **A′** and **B** are ligated in the presence of **T′** with an observed rate of $2.2 \times 10^{-4}\ min^{-1}$, and **A** and **B′** are ligated in the presence of **T** with an observed rate of $1.7 \times 10^{-4}\ min^{-1}$. The ribozyme **T′** does not catalyze ligation of **A** and **B′**, and the ribozyme **T** does not catalyze ligation of **A′** and **B**. Thus, the reaction appears to be tolerant of mismatches within the P3 stem that binds the 3′-hydroxyl-bearing substrate (**A** or **A′**) but not within the P3 stem that binds the 5′-triphosphate-bearing substrate (**B** or **B′**). The former binding interaction also benefits from the P1 and P2 pairings, while the latter involves the P2 but not the P1 pairing. The rates of the two promiscuous reactions are substantially slower than those of the two cross-catalytic reactions. In addition, dissociation of a mismatched substrate is expected to be much faster than dissociation of a matched substrate, further favoring the cross-catalytic reactions compared to the promiscuous reactions.

The observation of burst kinetics in the cross-catalytic reaction indicates that there is a rate-limiting step other than the ligation event, possibly the release of the ligated product. Pulse-chase experiments were carried out to investigate this possibility. First, radiolabeled **T** was incubated with an excess of unlabeled **T′** and allowed to bind to equilibrium. Then, a large excess of unlabeled **T** was added, and the mixture was sampled after various times. The amount of radiolabeled **T** that remained bound to **T′** was determined by a gel-shift assay employing nondenaturing polyacrylamide gel electrophoresis. This led to an estimate of the rate of dissociation of the **T•T′** complex, which was determined at various temperatures (Figure 5). The rate of dissociation was about $0.1\ min^{-1}$ at 23°C, increasing slightly between 23 and 43°C, then rising to $0.4\ min^{-1}$ at 48°C. This is faster than both the initial rate of ligation and the rate of the slower second phase of the reaction. Thus, the biphasic reaction kinetics appear to be due to a rate-limiting step other than product release.

Cross-Catalytic Replication

The cross-catalytic replication pathway first was investigated employing one of the two ribozymes (**T** or **T′**) and all four substrates (**A**, **A′**, **B**, and **B′**) in a common reaction mixture. All four potential ligated products could be distinguished by gel electrophoresis based on their size: **A-B** (equivalent to **T**) contains 66 nucleotides, **A′-B′** (equivalent to **T′**) contains 78 nucleotides, **A′-B** contains 74 nucleotides, and **A-B′** contains 70 nucleotides. The four substrates, each present at 2 μM concentration, were incubated at 30°C in either the presence or absence of a ribozyme. In the absence of a ribozyme, all four ligated products were detected, with a predominance of **A′-B** (Figure 6A). After 24 hr, the total yield of products was less than 4%. Addition of 1 μM **T** resulted in greatly increased formation of **T′**, with a yield of 23% after 24 hr (Figure 6B). The other three ligated products

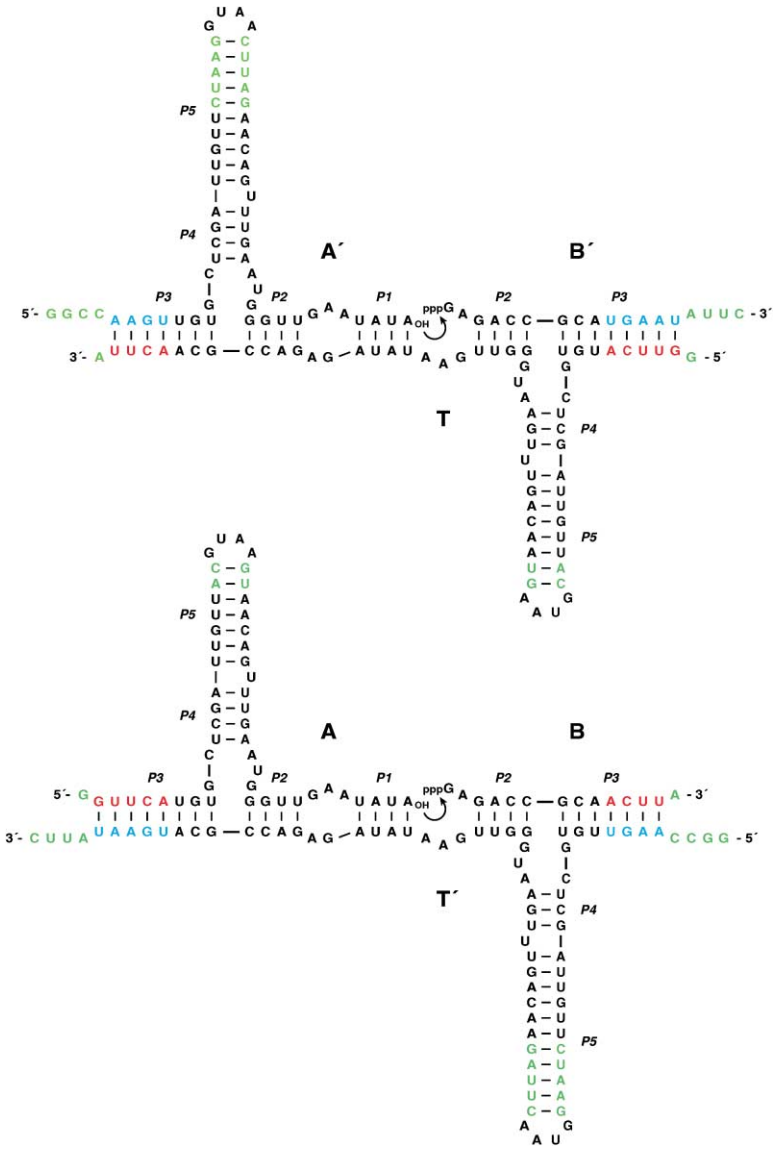

Figure 3. Sequence and Secondary Structure of the Ribozymes and Substrates Used to Carry Out Cross-Catalytic Replication

The ribozymes **T** and **T′** have largely identical sequences but with critical differences in the P3 stems (shown in red and blue, respectively) that allow them to bind selectively to their corresponding substrates. Noncritical differences also are present within the P5 stems and at the 5′ and 3′ ends (shown in green), which provide size discrimination and facilitate preparation of the ribozymes by in vitro transcription. Curved arrow indicates attack of a 3′-hydroxyl on a 5′-triphosphate, resulting in RNA ligation.

were produced at a much slower rate. Similarly, addition of 1 μM **T′** resulted in greatly increased formation of **T**, with a yield of 16% after 24 hr (Figure 6C). In that case, however, there was substantial production of **A′-B**, which was formed at about half the rate as the rate of formation of **T**.

Closer examination of the reactions carried out in the presence of either **T** or **T′** revealed that the cross-catalytic product in turn led to the formation of additional copies of the starting ribozyme, thus completing the replication cycle. Reactions employing unlabeled **T**, [5′-^{32}P]-labeled **A** and **A′**, and unlabeled **B** and **B′** led to the formation of labeled **T** (Figure 7A). Similarly, reactions employing unlabeled **T′**, [5′-^{32}P]-labeled **A** and **A′**, and unlabeled **B** and **B′** led to the formation of labeled **T′** (Figure 7B). In both cases, the rate of formation of the replication product increased progressively over the course of the reaction, reflecting accumulation of the cross-catalytic product, which then served to direct formation of the replication product. This behavior was not observed for the formation of the promiscuous products **A′-B** and **A-B′**. This suggests that the promiscuous products did not undergo self-replication via the autocatalytic pathway. When the starting ribozyme was omitted from the reaction mixture or replaced by an equivalent concentration of the component substrates, the yield of the replication product was greatly diminished, and there was no observed increase in the rate of product formation over time (Figure 7).

The formation of **T′** catalyzed by **T** and the formation of **T** catalyzed by **T′** both exhibited biphasic kinetics. For the reaction catalyzed by **T**, the burst phase had a rate of 0.0062 min^{-1} and an amplitude of 0.15, followed by a linear phase with a rate of 5.4×10^{-5} min^{-1} (Figure 6B). For the reaction catalyzed by **T′**, the burst phase had a rate of 0.0062 min^{-1} and an amplitude of 0.075, followed by a linear phase with a rate of 5.1×10^{-5} min^{-1} (Figure 6C). The rates of both reactions were about 5- to 10-fold slower when carried out in the presence of all four substrates compared to when carried out in the

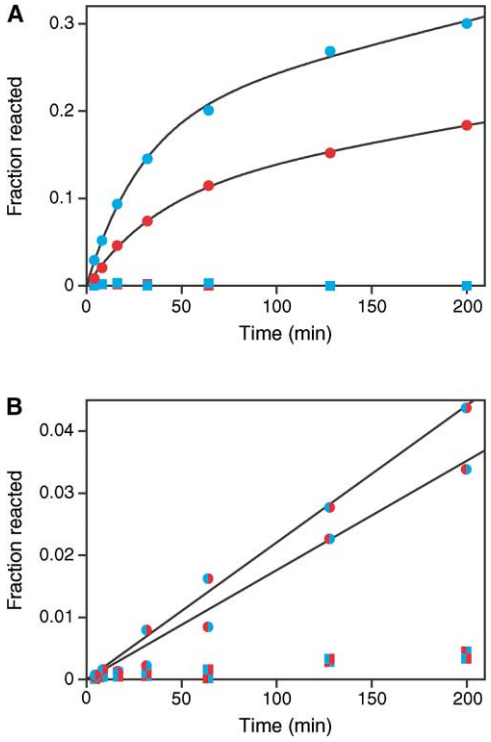

Figure 4. Time Course of the Individual RNA-Catalyzed Reactions

(A) Formation of the two cross-catalytic products in the presence of either a matched or mismatched ribozyme. Blue circles, ligation of **A′** and **B′** catalyzed by **T**; red circles, ligation of **A** and **B** catalyzed by **T′**; blue squares, ligation of **A′** and **B′** in the presence of **T′**; red squares, ligation of **A** and **B** in the presence of **T**.

(B) Formation of the promiscuous products in the presence of either ribozyme. Blue/red circle, ligation of **A′** and **B** in the presence of **T′**; red/blue circles, ligation of **A** and **B′** in the presence of **T**; blue/red squares, ligation of **A′** and **B** in the presence of **T**; red/blue squares, ligation of **A** and **B′** in the presence of **T′**. All reaction mixtures contained 1 μM ribozyme and 2 μM each of the two substrates.

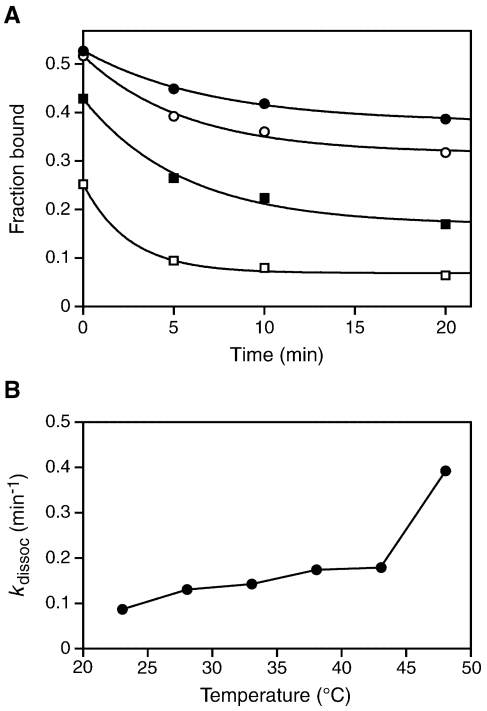

Figure 5. Dissociation of the **T•T′** Complex Investigated by Pulse-Chase Experiments

(A) Time course of **T•T′** dissociation at various temperatures, starting with an equilibrium mixture of 0.5 μM labeled **T** and 1.0 μM unlabeled **T′**, then chasing with a large excess of unlabeled **T**. The fraction of labeled **T** that remained bound to **T′** following the chase was determined by gel-shift analysis. The dissociation rate, k_{dissoc}, was calculated from a best fit of the data to an exponential decay equation. Filled circles, 28°C; open circles, 38°C; filled squares, 43°C; open squares, 48°C.

(B) Dissociation rate at various temperatures.

presence of only the two matched substrates. This is likely due to the two promiscuous reactions that occur in the presence of all four substrates, which divert materials from the cross-catalytic replication pathway. The combined rates of the two cross-catalytic reactions (Figures 6B and 6C) correspond roughly to the observed rate of formation of the replication products (Figure 7). Detailed analysis is complicated, however, by the competing promiscuous reactions and degradation of the ribozymes and substrates over long incubation times.

Discussion

By breaking the symmetry of the self-replicating RNA ligase ribozyme, it was possible to design a cross-catalytic system involving two ligase ribozymes that catalyze each other's synthesis from a total of four component substrates. Each ribozyme acts as both a template and catalyst and is specific for the substrates that lead to the formation of the desired cross-catalytic product. The two ribozymes, **T** and **T′**, are complementary to each other only within the P1, P2, and P3 stems, which are involved in substrate recognition (Figure 3). Discrimination between the substrates **A** and **A′** is determined by the sequence of the P3 stem at the 5′ end of the ribozyme, while discrimination between the substrates **B** and **B′** is determined by the sequence of the P3 stem at the 3′ end of the ribozyme. The central P1 stem and the two P2 stems together are "palindromic," containing paired strands that are both identical and complementary. The remainder of the nucleotides within the two ribozymes are identical, except for differences outside the catalytic core that provide size discrimination and facilitate in vitro transcription.

The ribozyme **T** directs the synthesis of **T′** but not **T**, and the ribozyme **T′** directs the synthesis of **T** but not **T′** (Figure 4A). At a much lower rate, **T** also directs the ligation of **A** and **B′**, and **T′** also directs the ligation of **A′** and **B** (Figure 4B). The two promiscuous reactions are less efficient because they involve partial mismatches within the P3 stem at the 5′ end of the ribozyme. They are potentially troublesome, however, because the products (**A-B′** and **A′-B**) have the potential to self-replicate in a reaction that involves perfect pairing within the P3 stem. Indeed, in the presence of all four substrates and no starting amount of ribozyme, **A′-B** arises and grows to dominate the reaction mixture (Figure 6A). When a starting amount of either **T** or **T′** is present,

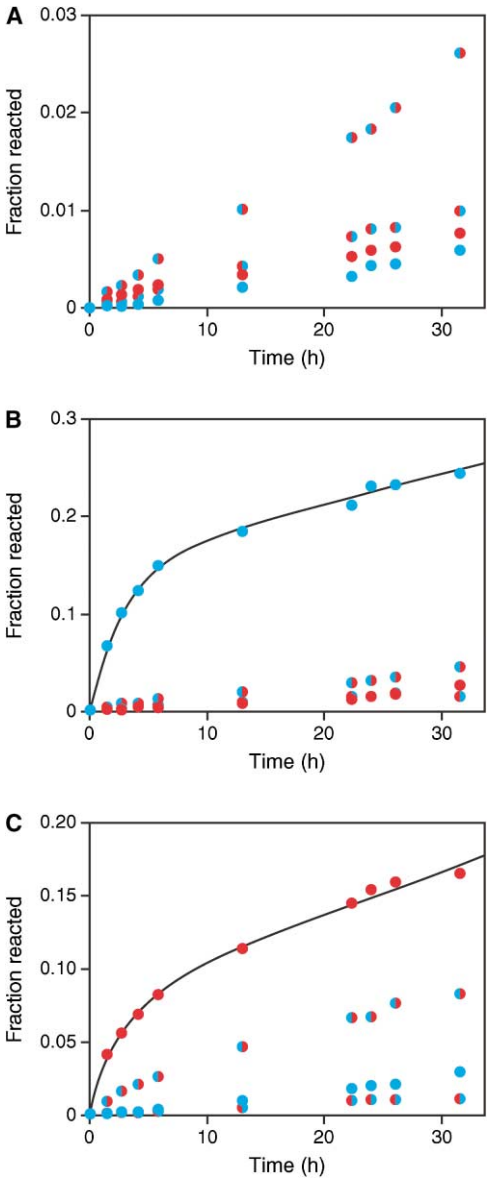

Figure 6. Time Course of RNA-Catalyzed Ligation in Reaction Mixtures Containing All Four Substrates

The mixtures contained 2 μM each of the four substrates and either (A) no ribozyme, (B) 1 μM **T**, or (C) 1 μM **T′**. Blue circles, ligation of **A′** and **B′**; red circles, ligation of **A** and **B**; blue/red circles, ligation of **A′** and **B**; red/blue circles, ligation of **A** and **B′**.

however, the desired cross-catalytic product remains dominant (Figures 6B and 6C).

Because **T** preferentially catalyzes the synthesis of **T′**, and **T′** preferentially catalyzes the synthesis of **T**, a reaction mixture that contains a starting amount of **T** but no **T′** gives rise to additional copies of **T** through a cycle of cross-catalytic replication (Figure 7A). Similarly, a reaction mixture that contains a starting amount of **T′** but no **T** gives rise to additional copies of **T′** (Figure 7B). In both cases, the rate of formation of additional copies of the starting ribozyme increases with time due to an increasing concentration of the cross-catalytic product.

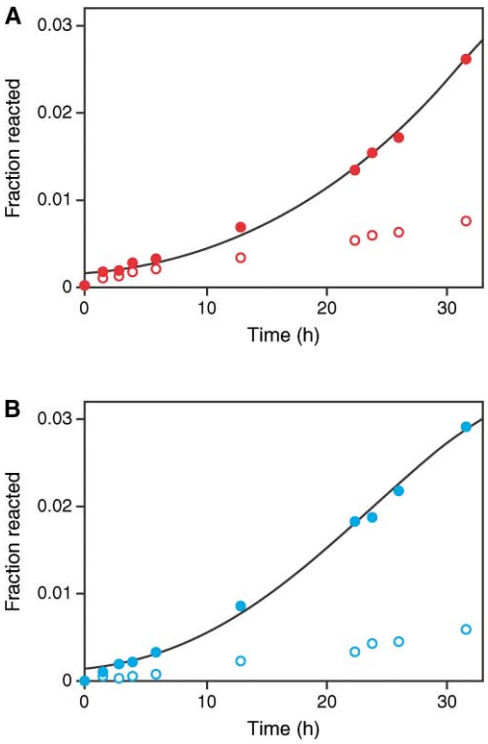

Figure 7. Time Course of RNA-Catalyzed Ligation in Reaction Mixtures Containing All Four Substrates, Demonstrating Formation of Additional Copies of the Starting Ribozyme

(A) Ligation of **A** and **B** (to form **T**) in either the presence (filled red circles) or absence (open red circles) of 1 μM **T** but no starting amount of **T′**.

(B) Ligation of **A′** and **B′** (to form **T′**) in either the presence (filled blue circles) or absence (open blue circles) of 1 μM **T′** but no starting amount of **T**. All reaction mixtures contained 2 μM each of the four substrates.

This is consistent with the overall autocatalytic behavior of the reaction system.

In principle, one could devise more complex catalytic cycles that involve three or more ribozymes. In a three-member cycle, for example, the ribozyme **T** would direct the ligation of **A′** and **B′** to form **T′**, the ribozyme **T′** would direct the ligation of **A″** and **B″** to form **T″**, and the ribozyme **T″** would direct the ligation of **A** and **B** to form **T**. One could envision a variety of catalytic cycles operating in a common reaction mixture, differing in their number of members and degree of promiscuity, with some cycles enforcing and others repressing each other's operation. It has been suggested that complex autocatalytic networks of this type offer a plausible route to the origin of living systems [15, 16]. Such networks, however, would involve molecules that differ in their template properties but not in their fundamental catalytic properties. It is difficult to see how one would devise autocatalytic networks that allow optimization of a replicative machinery that is distinct from the templating properties of the molecule.

The rate of cross-catalytic replication demonstrated in the present study is modest, especially considering that it involves only two joining reactions. In the presence of 2 μM each of the two corresponding substrates,

the initial rate of synthesis of **T′** catalyzed by **T** is 6.8×10^{-3} min^{-1}, and the initial rate of synthesis of **T** catalyzed by **T′** is 2.9×10^{-3} min^{-1}. These rates are reduced by 7- and 6-fold, respectively, when all four substrates are present. The rate of each half-reaction in the replication cycle is more than 100-fold slower than that of the R3C ligase ribozyme when it catalyzes RNA ligation in the presence of 2 μM (saturating) substrate [13]. When the R3C ligase was converted to a self-replicating ribozyme, it was necessary to reduce substantially the length of the P1 stem in order to reduce the rate of reaction in the absence of a starting amount of ribozyme, even though this reduced the catalytic rate of the ribozyme [12]. The rate of cross-catalytic replication might be increased by lengthening the P1 stem, but this would have the undesired consequence of increasing the rate of product-independent ligation as well as the product-dependent promiscuous reactions that involve partial mismatches within the P3 stem.

Converting the self-replicating ribozyme to a pair of cross-replicating ribozymes has several advantages. First, it breaks the symmetry of the enzyme-substrate complex, enabling the exploration of a broader range of sequences. Second, it allows the two P3 stems within each ribozyme to be noncomplementary, preventing them from forming an intramolecular duplex that would compete with binding of the two substrates. Third, it similarly prevents the two substrates from binding to each other, which was found to limit the performance of the self-replicating ribozyme [12]. In the case of the self-replicating ribozyme, this was remedied by first mixing **T** and **B**, then adding **A**; while for the cross-replicating ribozymes, all of the reaction components could be added simultaneously. Finally, the cross-replicative format should make it easier to design more complex replicating systems involving more than two joining reactions or employing multiple replicators in a common reaction mixture.

Significance

The process of self-replication is fundamental to biology and of considerable interest to chemists seeking to develop artificial systems that exhibit complex behaviors. Several examples of chemical self-replication systems have been reported previously, including one involving a ribozyme that catalyzes the joining of two RNA molecules to produce additional copies of itself [12]. The present study demonstrates cross-catalytic replication in a reaction system involving two ribozymes that catalyze each other's synthesis from a total of four RNA substrates. This system more closely resembles biological replication because it relies on a catalytic machinery to bring about the synthesis of two cross-complementary molecules. The efficiency and specificity of the two component reactions are sufficient to ensure that the cross-catalytic pathway dominates over all other reaction pathways. While the present system of cross-replicating ribozymes is not capable of undergoing Darwinian evolution, it is likely to be extensible to more complex systems employing larger numbers of replicating components.

Experimental Procedures

Materials

Synthetic oligodeoxynucleotides were prepared using an Expedite automated DNA/RNA synthesizer (Applied Biosystems) with phosphoramidites obtained from Glen Research (Sterling, VA). Synthetic oligoribonucleotides were prepared by Dharmacon Research (Boulder, CO). All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and desalted using a C18 SEP-Pak cartridge (Waters). Histidine-tagged T7 RNA polymerase was purified from *E. coli* strain BL21 containing plasmid pBH161 (kindly provided by William McAllister, State University of New York, Brooklyn). T4 polynucleotide kinase and calf intestine phosphatase were purchased from New England Biolabs. Nucleoside 5′-triphosphates and [γ-^{32}P]ATP (7 μCi/pmol) were purchased from Amersham Pharmacia.

Preparation of Ribozymes and Substrates

The ribozymes **T** and **T′** and the substrates **A** and **A′** were prepared by in vitro transcription. The transcription mixture contained 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 50 mM Tris (pH 7.5), 0.4 μM DNA template, 0.8 μM synthetic oligodeoxynucleotide having the sequence 5′-GGACTAATACGACTCACTATA-3′ (T7 promoter sequence underlined), 2 mM each of the four NTPs, and 25 units/μl T7 RNA polymerase. The mixture was incubated at 37°C for 2 hr, then quenched by adding an equal volume of gel-loading buffer containing 15 mM Na_2EDTA and 18 M urea. The transcription products were purified by denaturing PAGE, eluted from the gel, and desalted using a C18 cartridge. The substrates **A** and **A′** could not be obtained in good yield by in vitro transcription and instead were prepared synthetically. The synthetic RNAs were 5′ labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, then purified by denaturing PAGE and desalted using a C18 cartridge. The ribozyme **T** was 5′ labeled similarly, following removal of the 5′ triphosphate using calf intestine phosphatase.

Gel-Shift Analysis of Enzyme-Product Dissociation

A mixture containing 0.5 μM [5′-^{32}P]-labeled **T**, 1.0 μM **T′**, 25 mM $MgCl_2$, and 50 mM *N*-[2-hydroxyethyl]-piperazine-*N′*-[3-propanesulfonic acid] (EPPS, pH 8.5) was equilibrated at 23°C for 10 min, then incubated at the desired temperature for 10 min. A chase solution containing a large excess of unlabeled **T** (final concentration 15 μM), 25 mM $MgCl_2$, and 50 mM EPPS was added, maintaining the desired temperature. Aliquots were withdrawn at various times and analyzed by electrophoresis in a 10% nondenaturing polyacrylamide gel containing 12 mM $MgCl_2$, 2 mM Na_2EDTA, and 90 mM Tris-borate buffer (pH 8.0). The gel was run at a constant temperature of 4°C and analyzed using a PhosphorImager (Molecular Dynamics).

Kinetic Analysis

All RNA-catalyzed reactions were performed in a mixture containing [5′-^{32}P]-labeled **A** and/or **A′**, unlabeled **B** and/or **B′**, unlabeled **T** and/or **T′**, 25 mM $MgCl_2$, and 50 mM EPPS (pH 8.5), which was incubated at 23°C. The reactions were initiated by mixing equal volumes of two solutions, one containing the substrates, the other containing the ribozymes, and both containing 25 mM $MgCl_2$ and 50 mM EPPS (pH 8.5). Aliquots were withdrawn at various times and quenched by adding an equal volume of gel-loading buffer containing 25 mM Na_2EDTA and 18 M urea. The products were separated by denaturing PAGE and quantitated using a PhosporImager. The data for cross-catalytic ligation were fit to a burst kinetic equation: fraction reacted $= a(1 - e^{-kt}) + bt$, where a and k are the amplitude and rate of the burst phase, respectively, and b is the rate of the subsequent linear phase. The k_{obs} value for the initial rate of reaction was obtained by multiplying a and k. The data for cross-catalytic replication were fit to a logistic equation: fraction reacted $= a/(1 + be^{-kt})$, where a is the maximum extent and k is the maximum rate of growth.

Acknowledgments

This work was supported by research grant NNG04GA28G from the National Aeronautics and Space Administration.

Received: July 2, 2004
Revised: August 13, 2004
Accepted: August 19, 2004
Published: November 29, 2004

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