Replication of an RNA ligase ribozyme under alternating temperature condition

Ki-Sun Kim\textsuperscript{a}, Woo-Hyung Choi\textsuperscript{a}, Bo-Ra Choi\textsuperscript{b}, Sangtaek Oh\textsuperscript{c}, Sung Su Yea\textsuperscript{d}, Moon-Young Yoon\textsuperscript{e}, Dong-Eun Kim\textsuperscript{b,}*  
\textsuperscript{a} Department of Biomaterial Control, Dong-Eui University, Busan 614-714, Republic of Korea  
\textsuperscript{b} Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, Republic of Korea  
\textsuperscript{c} PharmacoGenomic Research Center, Inje University College of Medicine, Busan 633-165, Republic of Korea  
\textsuperscript{d} Department of Biochemistry, Inje University College of Medicine, Busan 633-165, Republic of Korea  
\textsuperscript{e} Department of Chemistry, Hanyang University, Seoul 133-791, Republic of Korea  

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Abstract Self-replication process of the RNA ligase ribozyme molecules was investigated by using the modified RNA ligase ribozyme under alternating temperature condition that enhances turnover rate of the RNA ligation reaction. In our experiment, the RNA ligase ribozyme system mainly undergoes a cross-catalytic replication process, in which two ribozymes catalyze each other’s synthesis from a total of four RNA substrates under alternating temperature condition, resulting in time-dependent accumulation of additional copies of the starting ribozymes in a reaction mixture. The present study demonstrates that cross-catalytic replication in nucleic acids system can be efficiently devised under the alternating temperature condition.  
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1. Introduction

Self-replication is a pivotal process in living systems, by which genetic information is transferred from template nucleic acid molecules to newly synthesized, complementary products. The genetic molecule itself is not self-replicating in a cell, but with a help of special replication machinery forms a self-replicating system. This enables the genetic material to be perpetuated, living systems utilizes a process of semi-conservative replication, in which the coding strand of the genetic material directs the synthesis of a complementary macromolecule that copy the non-coding strand to produce a coding strand [1].

Chemistry-based non-biological systems of the form \( A + B \rightarrow T \) has been proposed, where \( A \) and \( B \) are substrates that bind to a complementary template \( T \) and become joined to form a new copy of \( T \) [2]. These reactions proceed in an autocatalytic manner if the new copies of \( T \) can direct the joining of \( A \) and \( B \). The autocatalytic replication of the template molecule has been demonstrated for activated oligonucleotides [2–4], peptides [5,6], and small organic compounds [7–9]. Among these, nucleic acids is an attractive system for self-replication because they can encode genetic information by recognizing their complementary partners and catalyze nucleic acid joining reactions [10–12]. Due to these properties, catalytic nucleic acids with a power to catalyze a broad set of chemical reactions greatly simplified the procedures needed to perform Darwinian evolution [13]. Recent progress with catalytic nucleic acids has been made by observing that an RNA poly- 

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temperature condition to enhance turnover rate of the RNA ligase ribozyme reaction. Under this condition, the RNA ligase ribozyme system mainly undergoes a cross-catalytic replication process by generating additional copies of the starting RNA ligase molecules.

2. Materials and methods

2.1. Enzymes, nucleotides, and oligonucleotides

T7 RNA polymerase was purified from *Escherichia coli* strain BL21 harboring plasmid pAR1219 (kindly provided by Dr. Smita S. Patel, Robert Wood Johnson Medical School, New Jersey, USA). T4 polynucleotide kinase and calf intestine phosphatase were purchased from New England Biolabs. Oligodeoxynucleotides were synthesized by Bioneer (Daejeon, Korea) and purified by denaturing polyacrylamide gel electrophoresis (PAGE).

2.2. Preparation of ribozyme and RNA substrates

The ribozymes T and T’ and the RNA substrates A, A’, B, and B’ were prepared by in vitro transcription. The transcription mixture contained 15 mM MgCl₂, 2 mM spermidine, 5 mM dithiothreitol, 50 mM Tris (pH 7.5), 0.4 μM DNA template, 0.8 μM synthetic oligodeoxynucleotide of the sequence 5’-GGACTAATCGACTACTATA-3’ (T7 promoter sequence underlined), 2 mM each of the four NTPs, and 30 U/μl T7 RNA polymerase. The mixture was incubated at 37 °C for 2 h, and then quenched with an equal volume of gel-loading buffer containing 15 mM EDTA and 18 M urea. The transcription products were purified by denaturing PAGE, eluted from the gel, and desalted using a C18 SEP-Pak cartridge (Waters). The substrates A and A’ were 5’-labeled using T4 polynucleotide kinase and [γ-³²P]ATP, following removal of the 5’ triphosphate with calf intestine phosphatase, then gel-purified and desalted.

2.3. RNA-catalyzed reaction

RNA ligation reactions were performed in a reaction mixture containing 2 μM ligation substrates (5’-labeled A and/or A’, unlabeled B and/or B’), 1 μM RNA ligase (unlabeled T and/or T’, 25 mM MgCl₂), and 50 mM N-[2-hydroxyethyl]-piperazine-N’-2-ethanesulfonic acid (EPPS, pH 8.5), which were incubated after interval (23 °C) or alternating temperature (periodical heat pulse, 55 °C, 2 min) condition. The reactions were initiated by mixing equal volumes of two solutions, one containing the substrates, and the other containing the ribozymes. Aliquots were taken at various times, and quenched by adding an equal volume of gel-loading buffer. The reaction products were separated by denaturing PAGE and quantitated using a Cyclone storage phosphor system (Packard Instrument Co., Meriden, CT, USA).

2.4. Kinetic analysis

The fraction reacted at each time point was determined, and the time-dependent accumulation of the ligation product was fit to an exponential function fraction reacted = a(1 - e⁻ᵏᵗ), where a and k are the amplitude and rate of exponential increase of the products, respectively. The initial rate of reaction was obtained by multiplying a and k. The data for cross-catalytic replication were fit to a logistic equation for a sigmoid growth: fraction reacted = al/(1 + be⁻ᵏᵗ), where l is the maximum extent and k is the rate of growth, respectively, and b is a floating parameter for fitting. All of the kinetic results represent the means of at least triplicate experiments and are presented as means ± S.E.

3. Results and discussion

3.1. Design of cross-replicating ligase ribozymes and their substrates

A pair of cross-replicating ribozymes was constructed based on the previously-described self-replicating ribozyme such that the 2-fold symmetry of the A · B · T complex was broken to attain cross-replication [16,18]. Two templates were redesigned (T and T’), each containing the catalytic core of the R3C ligase ribozyme [17]. Each template ligase ribozyme could bind two substrates (A’ and B’, A and B, respectively), resulting in RNA-catalyzed ligation to form the opposing template (Fig. 1). The specificity for substrate recognition derives from base-pairing interactions within the P1, P2, and P3 domains of the template ligase and substrate complex. P1, P2, and part of P3 domain are crucial for RNA ligation activity; hence the nucleotide sequences of the catalytic core are fixed (Fig. 2). However, terminal part of the P3 domain is changeable to sequence variation, and therefore was chosen as a site for discrimination between the two pairs of substrates. The three proximal base pairs of the P3 stem (the closest to the catalytic core) were kept constant and chosen to provide maximum ligation efficiency [16,18]. The proximal 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 A’ to T, and therefore for binding of B to T’.

The P3 stems of template A and its complementary substrates were subject to sequence change to find optimal enzyme-substrate complex construct that reacts efficiently in a highly discriminating manner; template T only catalyzes the ligation of A and B’, and template T’ only catalyzes the reaction of A’ and B. Under alternating temperature condition with a heat pulse the ligated product could be easily dissociated, not limiting the rate of entire catalytic cycle. Thus, stability of the P3 stem was kept higher than that of the previously designed ligase substrates pair [18] to allow tight binding of the cognate substrates. This was attained by simply inserting one G–C pair into the P3 stems (bases in bold character in Fig. 2). Other than the P3 stems, it was not required to change the sequence of the previously designed cross-catalytic ligase ribozyme [18] in order to obtain more efficient cross-replicating ribozymes. However, different lengths of the P5 stem (bases in green color in Fig. 2) were used to provide a means for distinguishing between two different templates. In fact, altering the length or sequence of the P5 stem had little effect on the catalytic efficiency of the ribozyme. Finally, the optimized design of the templates and corresponding substrates is shown in Fig. 2.

3.2. RNA ligation reactions with template ligase ribozymes

Each member of the pair of cross-replicating ribozymes first was tested individually, employing the two substrates with either the corresponding template or the mismatched template at the constant temperature of 23 °C (Fig. 3A). In the previous study, RNA substrates ligation was investigated with a varying concentration of the RNA ligase [16]. Initial rate of the ligation reaction was linearly dependent on the starting concentration of RNA ligase, implicating binding of substrates to the template RNA ligase follows a first-order reaction kinetics. Based on the previous RNA ligation reaction conditions [16,18], we have chosen 2 μM of RNA substrates and 1 μM of RNA template ligase as a standard reaction condition. The substrates A and B were ligated efficiently in the presence of the template T’, but not in the presence of the mismatched template T. Similarly, A’ and B’ only were ligated efficiently in the presence of T. Time course of both template-dependent
reactions exhibited exponential kinetics (Fig. 3B). Fitting of
the data provided amplitudes of 0.19 ± 0.008 and 0.30 ±
0.011, and an exponential rate of 0.017 ± 0.0019 min⁻¹ and
0.022 ± 0.0023 min⁻¹ for reactions catalyzed by T' and T,
respectively. The initial observed rate (kobs) of reaction by
template ligase T' and T was 3.2 × 10⁻³ min⁻¹ and 6.6 ×
10⁻³ min⁻¹, respectively. The rate of reaction was not affected
by changing the order in which the three RNA components
were added to the reaction mixture. In the presence of 2 μM
RNA substrates (A and B, or A' and B'), a linear relationship
was observed between the starting concentration of template
ligase and initial reaction rate (data not shown). The increase
in the initial reaction rate was mainly attributed by the increase
of amplitude of the exponential kinetics.

Since the ligated product (T · T') could be readily dissociated
at a high temperature, one of the ligation reaction mixtures (A
and B ligation by T') was subjected to an alternating temper-
ature condition. At every 30 min reaction vessel incubated at
23 °C was transferred to the other bath adjusted to a higher
temperature (55 °C) for 2 min and returned to the 23 °C bath,
which is similar to a conventional PCR temperature setting.
Condition for this periodical “heat-pulse” (55 °C for 2 min)
was chosen to assure that RNAs in the reaction was not
degraded with high temperature during the reaction and the
ligated product complex was fully dissociated. The heat-pulse
process was repeated throughout the entire time-course.
Ligated product (T) was progressively accumulated without
saturation and exceeded the amount of product that was
obtained from the reaction performed at the constant temper-
ature (Fig. 3B). In order to address whether heat pulses may
provide a condition for unlocking of misfolded ribozyme–sub-
strate complexes instead of efficient product dissociation, the
ligase reaction mixture was initially heated at 55 °C for 2 min
and was transferred to a vessel incubated at 23 °C. This prior
heat treatment did not enhance yield of ligation reaction prod-
ucts, providing similar kinetics compared to the one without

Fig. 1. Reaction cycle of cross-catalytic replication in the ligase ribozyme system. The ribozyme T catalyzes ligation of the substrates A' and B' to
form the ribozyme T. The ribozyme T' in turn catalyzes ligation of the substrates A and B to form the ribozyme T. Next cycle of cross-catalytic
replication is initiated by new copies of each ribozyme, which are released from the T · T' product. RNAs were drawn in red and blue to distinguish
two sets of substrates and their ligated product (template ligase).


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the heat treatment (data not shown). Thus, periodical heat treatment mainly contributes to dissociate the RNA ligation product complex, not affecting initial RNA folding state that may influence competent ligation reaction. However, we do not know why the average amplitudes of ligation reaction reach only up to 20–30% of total substrates present in the reaction mixture (isothermal reactions in Fig. 3B), which reflects the fraction of competent RNA ligase–substrates complex in a reaction. At present we are investigating a way to enhance amplitude of the ligation reaction by increasing initial population of the competent ligase–substrates complex for ligation. Since binding affinity of the P3 arms in the construct was increased, the entire ligation reaction cycle is likely limited by the product dissociation step not by the substrate binding. Heat-pulsed reaction thus yielded more products by taking an advantage of enforced product dissociation at higher temperature. Therefore, the alternating temperature condition is an appropriate tool to enhance a turnover rate of RNA ligation by the template ligase ribozyme in a reaction.

At a constant temperature of 23 °C, the template-directed RNA ligation reaction was also performed using all four substrates; [5'-32P]-labeled A and A', and unlabeled B and B'; each
Fig. 3. Time course of the RNA ligation reaction catalyzed by the individual ligase ribozyme. (A) The ligase ribozyme (1 μM) that is either matched (T) or mismatched (T) was allowed to react with an unlabeled 14 mer substrate (B, 2 μM) and labeled 52 mer substrate (A, 2 μM) at 23 °C (see Section 2). Matched ligase (T) formed a unique 66 mer ligation product (A-B), which is equal to the other template, T. The reaction was sampled at 8, 16, 32, 64, 92, and 200 min. Same reaction was repeated with the other template pair of RNA substrates, unlabeled 18 mer substrate (B) and labeled 60 mer substrate (A) in the presence of either T or T. The products were separated by electrophoresis in a denaturing polyacrylamide gel (12%), an autoradiogram of which is shown. (B) Time course of template formation in reaction mixtures performed in (A). Kinetics of ligation of A' and B' catalyzed by T (□) and ligation of A and B catalyzed by T (○) was fitted to the one-exponential function. The ligation reaction mixture (A and B ligated product was monitored (●) for formation of the promiscuous reaction products as well as the directed ligation products in the presence of either ribozyme (T or T). The template-directed A ligase reaction was performed using all four substrates; labeled A and A', and unlabeled B and B'. each present at 2 μM concentration in the absence or presence of a ligase ribozyme (either T or T templates, 1 μM) in a reaction mixture at 23 °C. Ligation products at increasing time points were detected in a denaturing PAGE (8%). (D) Time course of the promiscuous ligation product formation in reaction mixtures performed in (C) with monitored. Open symbols represent ligation reaction catalyzed by the previous ligase ribozymes [18]; ligation of A and B in the presence of T (□) and ligation of A' and B' in the presence of T (○). Closed symbols represents ligation reaction catalyzed by the new ligase ribozyme constructed in this study (Figs. 2 and 3C); ligation of A and B' in the presence of T (●) and ligation of A' and B in the presence of T (●). Each lane shows a ligase ribozyme complexed with two RNA substrates. Perfect matched portion was indicated with a shaded box, and the P3 stems at the end of the ligation contain mismatched sequences.

present of 2 μM substrate in the absence or presence of a ligase ribozyme (either T or T' templates, 1 μM) in a 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. In the absence of ribozyme, ligated product was not detected, whereas addition of each ligase (T or T') resulted in greatly increased formation of directed ligation product, T' or T, respectively (Fig. 3C). This result shows that a directed ligation reaction is predominant over the other possible ligation reactions at a constant temperature. However, in addition to the directed ligation reaction, there exists a possibility of a promiscuous reaction, in which A and B become ligated to form the chimeric product A-B. These chimeric products, once formed, might act as templates to direct the formation of additional copies of themselves through RNA-catalyzed self-replication (autocatalytic replication). Two of the four possible promiscuous reactions were found to occur (Fig. 3C). A' and B are ligated in the presence of T', while A' and B' are ligated in the presence of T. The template T' does not catalyze ligation of A and B', and the template 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 5'-hydroxyl-bearing substrate (A or A'), but not within the P3 stem that binds the 5'-triphosphate-bearing substrate (B or B') (inset in Fig. 3D). The former binding interaction also benefits from the P1 and P2 pairings, while the latter involves P2 but not P1 pairing.
Compared to the previously constructed ribozymes [18], the new pair of template ribozymes yielded less amounts of promiscuous ligated products (Fig. 3D). This result indicates that the increased binding affinity in the P3 stem provided more specificity to the template ligases. Dissociation rate of mismatched substrates is expected to be much faster than that of matched substrates, resulting in a decrease in the amount of competent ligation reaction complex with mismatched substrates. Thus, the yield of ligation products of the two promiscuous reactions is much lower than those of the directed ligation reactions with perfectly matched substrates. Stabilization of the directed ligation reaction complex over the promiscuous reaction is likely attributed to the enhanced specificity and product yield of the newly designed template ligases.

3.3. Cross-catalytic replication of RNA templates under alternating temperature condition

The cross-catalytic replication pathway (shown in Fig. 1) was initially investigated by employing both T and T' to all four substrates in a common reaction mixture. At a constant temperature of 23 °C the template-directed RNA ligation reaction was performed by employing unlabeled T and T' templates (1 μM each) to the substrates; [5'-32P]-labeled A and A', and unlabeled B and B', each present at 2 μM concentration in a reaction mixture. Ligations of each pair of substrates were observed and showed exponential kinetics (isothermal reaction in Fig. 4). The yield of both ligation reactions were about 5- to 10-fold lower when carried out in the presence of all four substrates compared to when carried out in the presence of only two matched substrates. This is likely due to the non-productive RNA complex formation and the promiscuous reaction that occur in the presence of all four substrates (Fig. 3C), which divert materials from the cross-catalytic replication pathway. Since there present four RNA substrates that might pair with each cognate partner in a reaction mixture, significant amount of RNA substrates could form non-productive dimeric complex, such as A·B' and A'·B in a ternary complex (A·B'·A'·B). Existence of these complexes due to perfect base pairing between substrates was observed in a gel-shift assay in the previous study [16]. Furthermore, a stable T·T' complex could be formed before the reaction due to a presence of T and T' in the same reaction mixture, leading to a decrease of initially available ligases. Thus, these non-productive complexes form during the beginning of the reaction decreases amount of proper reaction complexes (A·B'·T' or A'·B·T) that are eligible for ligation reaction.

The same ligation reaction was also repeated under the alternating temperature condition (heat-pulsed reaction in Fig. 4B). The ligation products were detected in the formation of [5'-32P]-labeled T and T' that were resulted from A and B.
and A' and B' ligation, respectively. The formation of the ligation products increased linearly over 6 h under the alternating temperature condition (Fig. 4B). Heat-pulses provided a condition for efficient products (T·T') dissociation, which enables newly formed RNA template ligases to participate a next cycle of ligation reaction, leading to multiple-turnover reactions. In contrast, multiple-turnovers of template-directed ligation was not observed in the same reaction performed at the constant temperature, in which accumulation of the ligation products was not continued after 2 h of reaction (Fig. 4B). This result suggests that the alternating temperature with a high temperature facilitates dissociation of the ligated product from the template ligase, which in turn serves to direct formation of the replication product. Due to increased binding affinity of the P3 arms in the ligase–product complex, product dissociation likely limits rates of subsequent steps. Alternating temperature condition likely limits rates of subsequent steps. Alternating temperature thus provides a favorable reaction condition not only to enhance the dissociation rate at the high temperature, but also to allow efficient binding of the cognate substrates by the template ligase at the ambient temperature. Benefit of the alternating temperature condition provided a favorable reaction condition not only to enhance the dissociation rate at the high temperature, but also to allow efficient binding of the cognate substrates by the template ligase at the ambient temperature. Benefit of the alternating temperature also contributed to amplification of the hybrid template ligase at the ambient temperature. Benefit of the alternating temperature condition formation of ligation products increased linearly over 6 h under the alternating temperature condition (Fig. 4B). Heat-pulses provided a condition for efficient products (T·T') dissociation, which enables newly formed RNA template ligases to participate next cycle of ligation reaction, leading to multiple-turnover reactions. In contrast, multiple-turnovers of template-directed ligation was not observed in the same reaction performed at the constant temperature, in which accumulation of the ligation products was not continued after 2 h of reaction (Fig. 4B). This result suggests that the alternating temperature with a high temperature facilitates dissociation of the ligated product from the template ligase, which in turn serves to direct formation of the replication product. Due to increased binding affinity of the P3 arms in the ligase–product complex, product dissociation likely limits rates of subsequent steps. Alternating temperature thus provides a favorable reaction condition not only to enhance the dissociation rate at the high temperature, but also to allow efficient binding of the cognate substrates by the template ligase at the ambient temperature. Benefit of the alternating temperature condition provided a favorable reaction condition not only to enhance the dissociation rate at the high temperature, but also to allow efficient binding of the cognate substrates by the template ligase at the ambient temperature. Benefit of the alternating temperature also contributed to amplification of the hybrid template ligase at the ambient temperature. Benefit of the alternating temperature also contributed to amplification of the hybrid template ligase at the ambient temperature.

To distinguish the cross-catalytic product from the preferential ligation product resulted from the complement template, one template ligase (T') instead of two template ligases was added to all four substrates in a common reaction mixture. The four substrates, each present at 2 μM concentration, were incubated at 23 °C in either the presence or absence of template ligase T. In the absence of template, labeled products were rarely detected (Fig. 3C). Addition of 1 μM T' resulted in significantly increased formation of T, with a yield of 5% after 6 h (isothermal reaction in Fig. 5). Under alternating temperature, condition formation of T (heat-pulsed reaction in Fig. 4B) was significantly increased compared to the amount of T formed at the constant temperature. Most importantly, under the alternating temperature condition reaction employing unlabeled A and B with all four substrates (5'-32P-labeled A and A' and unlabeled B and B') led to formation of labeled T, which was catalyzed by the newly generated ligation product (template ligase T) that was not present under either isothermal or alternating temperature condition in order to address whether the cross-catalytic product indeed leads to formation of additional copies of the starting template. To distinguish the cross-catalytic product from the preferential ligation product resulted from the complement template, one template ligase (T') instead of two template ligases was added to all four substrates in a common reaction mixture. The four substrates, each present at 2 μM concentration, were incubated at 23 °C in either the presence or absence of template ligase T. In the absence of template, labeled products were rarely detected (Fig. 3C). Addition of 1 μM T' resulted in significantly increased formation of T, with a yield of 5% after 6 h (isothermal reaction in Fig. 5). Under alternating temperature, condition formation of T (heat-pulsed reaction in Fig. 4B) was significantly increased compared to the amount of T formed at the constant temperature. Most importantly, under the alternating temperature condition reaction employing unlabeled A and B with all four substrates (5'-32P-labeled A and A' and unlabeled B and B') led to formation of labeled T, which was catalyzed by the newly generated ligation product (template ligase T) that was not present.

Fig. 5. Time course of RNA ligation reaction containing all four substrates, demonstrating formation of additional copies of the starting ribozyme. The mixture contained 2 μM each of the four RNA substrates and 1 μM of template ribozyme (T). (A) The ligation reactions were allowed to react with unlabeled RNA substrates (B and B') and labeled substrates (A and A') either at 23 °C (isothermal reaction) or under the alternating temperature condition (heat-pulsed reaction). The ligation products were sampled at various time points and separated in a denaturing polyacrylamide gel, an autoradiogram of which is shown. (B) Shown is kinetics of template ribozyme formation in reaction mixtures performed in (A). Time courses of ligation of A and B (to form T) catalyzed by T' either under isothermal condition (23 °C, □) or under the alternating temperature condition (heat-pulse indicated as spikes in the graph, ◇). Ligation of A' and B' (to form T') in the presence of T (●), but no starting amount of T under the alternating temperature condition were fitted to a logistic equation for a sigmoid growth (dashed line). The hybrid template (Tb) resulted from A' and B ligation (△) was also detected under isothermal reaction condition.
at first (Fig. 5A). This result indicates that the cross-catalytic product in turn led to the formation of additional copies of the starting ribozyme, thus completing reaction cycle (Fig. 1). The rate of formation of the replication product increased progressively over the course of the reaction with a sigmoidal kinetics, reflecting accumulation of the cross-catalytic product, which then served as the template for formation of the replication product (Fig. 5B). Because T preferentially catalyzes the synthesis of T', and T' preferentially catalyzes the synthesis of T, a reaction mixture containing a starting amount of T' but no T gives rise to additional copies of T' through a cycle of cross-catalytic replication. This cross-catalytic replication was substantiated by observing the sigmoidal accumulation of the starting ribozyme (closed circles in Fig. 5B); the rate of formation of additional copies of the starting ribozyme increases with time due to an increasing concentration of the cross-catalytic product [18]. However, this behavior of cross-catalytic replication was not observed under the isothermal reaction condition. This suggests that under isothermal reaction condition the ligation product (T) was not dissociated from the template ligase (T') due to a high affinity in the P3 stem, which leads to a failure of newly formed template ligase to complete the cross-catalytic reaction cycle. Instead, under the isothermal reaction condition, product of A' and B ligation that is equal to the hybrid template (74 nts) was slightly accumulated in the presence of template ligase T', as observed in the promiscuous reaction shown in Fig. 3C. This promiscuous reaction occurs under isothermal condition, but not observed under the alternating temperature condition. Under the alternating temperature condition A' and B ligation with template ligase T' seems to be less efficient than the cross-catalytic ligation reaction, because the template ligase has to compete in common substrates with a much slower rate in the B ligation due to partial mismatch within the P3 stem at the end of the ribozyme.

The present study demonstrates cross-catalytic template amplification in a reaction system involving two ribozymes that catalyze each other’s synthesis from a total of four RNA substrates under alternating temperature condition. We could devise more complex catalytic replication systems that involve various template ligases ensemble, which operate in a single reaction mixture. Given that the rate of the chemistry step that is phosphodiester bond formation between two RNA substrates is same among the template ligase ensemble, efficiency and specificity of the two component reactions are mainly dictated with two reaction steps: substrate binding and product dissociation. These steps are largely governed by the binding affinity in the P3 stem of the unit ribozyme constructs. Increase of binding affinity in the P3 stem enhances stability of ribozyme–substrate but decreases rate of product dissociation. Thus, the optimal binding affinity that balances these two opposite effects is required for survival of the fittest in the ligase ribozyme replication system. Diverting this prerequisite, the heat-pulse provides an artificial condition for monitoring the cross-catalytic replication reaction in a reasonable time scale. Using a perturbing parameter like an alternating temperature, it will be intriguing to simulate Darwinian evolution with more complex systems involving lager numbers of replicating components in a single reaction pot. Temperature-fluctuating convection system could be hypothesized in a primordial environment near hot springs and/or hydrothermal vents occurring within volcanic areas in sea-bed [19,20]. Thus, one may speculate that the RNA world thrives in those ecosystems, which appears to be the first event toward chemical evolution.

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