Supporting Online Material for

Self-Sustained Replication of an RNA Enzyme

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Materials and Methods

Materials. Oligonucleotides were either purchased from Integrated DNA Technologies (San Diego, CA) or synthesized on an Expedite automated DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) using nucleoside phosphoramidites purchased from Glen Research (Sterling, VA). All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and desalted using a C18 SEP-Pak cartridge (Waters, Milford, MA). 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). *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA and purified as previously described (SI). M1 RNA, the catalytic subunit of RNase P, was obtained from *E. coli* genomic DNA (Sigma-Aldrich, St. Louis, MO) by PCR amplification using primers 5´-GGAC TAATACGACTCATACTATAGAAGGCTGACCAGACAGTGC-3´ and 5´-AGGTGAAACTGACCGATAAGC-3´ (T7 RNA polymerase promoter sequence underlined), followed by *in vitro* transcription. The PCR products were cloned into *E. coli* and their sequence was verified, as described below. Calf intestine phosphatase, *E. coli* poly(A) polymerase, and T4 polynucleotide kinase were purchased from New England Biolabs (Ipswich, MA), Superscript II RNase H+ reverse transcriptase was from Invitrogen (Carlsbad, CA), and calf thymus terminal transferase was from Roche Applied Science (Indianapolis, IN). Nucleoside and deoxynucleoside 5´-triphosphates were purchased from Sigma-Aldrich and [γ-32P]ATP (7 µCi/pmol) was from Perkin Elmer (Waltham, MA).

Preparation of RNA enzymes and substrates. All RNA enzymes and substrates were prepared by *in vitro* transcription. The transcription mixture contained 0.4 µM DNA template, 0.8 µM synthetic oligodeoxynucleotide having the sequence 5´-GGACTAATACGACTCATACTATA-3´ (promoter sequence underlined), 2 mM each of the four NTPs, 25 U/µL T7 RNA polymerase, 15 mM MgCl₂, 2 mM spermidine, 5 mM dithiothreitol, and 50 mM Tris-HCl (pH 7.5). The mixture was incubated at 37 °C for 2 h, then quenched by adding an equal volume of gel loading buffer containing 15 mM Na₂EDTA and 18 M urea. The transcription products were purified by PAGE, eluted from the gel, and desalted.

The A substrates could not be obtained reliably by *in vitro* transcription due to heterogeneity at the 3´ end of the transcripts. Instead, extended length RNAs were prepared that contained additional nucleotides, having the sequence 5´-GAGACCGCAACUUG-3´, located downstream from the A substrate sequence. The added nucleotides were removed using *E. coli* M1 RNA to generate a precise 3´ terminus. The cleavage reaction employed 20 µM RNA transcript, 20 µM external guide sequence RNA having the sequence 5´-GGUAAGUUGCGGUGCUCACCA-3´, 5 µM M1 RNA, 100 mM MgCl₂, 100 mM NH₄Cl, and 50 mM Tris-HCl (pH 7.5). Note that the guide RNA is complementary to the extended portion of the
transcript, with a 5’-terminal GG and 3’-terminal ACCA also present in the guide RNA (S2). The reaction mixture was incubated at 30 °C for 8 h, quenched, and the cleaved products were purified by PAGE, as described above. During the in vitro evolution procedure, the A’ substrates were prepared directly by in vitro transcription, but in all other instances these substrates were prepared using the M1 RNA cleavage procedure. For the A’ substrates, the added 3’-terminal nucleotides had the sequence 5´-GAGACCGCAU-GAAU-3’ and the external guide sequence RNA had the sequence 5´-GGAUUCAUGCGGUCUCACCA-3’.

**In vitro evolution.** DNA templates used to transcribe the starting pools of B-E’ and B’-E molecules were generated by a 10-cycle PCR employing two overlapping synthetic oligodeoxynucleotides, as listed below (promoter sequence underlined; nucleotides randomized at 12% degeneracy in italics). The resulting PCR products, each consisting of ~10¹⁴ molecules, were transcribed as described above, except that it was unnecessary to provide a synthetic oligodeoxynucleotide containing the second strand of the promoter.

**For B-E’**

5´-GGACTAATACGACTCACTATAGAGACCGCAACTTAG-3´ and 5´-GACAGATCAGTATTCAGTGCTCTCTTAAATTCAACCCATTCAAACGTGT-CTAAGTTTACCCTAGAACAATCGAGCACAACCTTACTAAGTTGCGGTTC-3’;

**For B’-E**

5´-GGACTAATACGACTCACTATAGAGACCGCATGAATAG-3´ and 5´-CTTCTGGATGGTCAAGTTGCGGTCTCTTTATTCAACCCATTCAAACGTGT-ACCTACGTAACAATCGACACATGAACACTATTTACTGCGGTTC-3’.

DNA templates used to transcribe the starting pools of A and A’ molecules were prepared directly as synthetic oligodeoxynucleotides (promoter sequence underlined; nucleotides randomized at 12% degeneracy in italics). The second strand of the promoter was supplied as a synthetic oligodeoxynucleotide. The transcribed A molecules were cleaved by M1 RNA, as described above.

**For A**

5´-CAAGTTGCGGTCTCTTTATTCACCCATTCAACGAGCTGAATCCGACTGTTTCTCTATTAATCGACACAC-3’;

**For A’**

5´-TAAATTCACAATTTTCATTCAACGAGCTGAATCCGACTGTTTCTCTATTAATCGACACAC-3’.

During each round of in vitro evolution, RNA-catalyzed RNA ligation was carried out in a reaction mixture containing 1 µM B-E´ (or B’-E), 5 µM A (or A’), 25 mM MgCl₂, and 50 mM EPPS (pH 8.5), which was incubated at 30 °C for various times. The ligated RNAs were gel purified, then reverse
transcribed in a reaction mixture containing ~0.4 µM RNA, 1 µM cDNA primer, 0.5 mM each of the four dNTPs, 3 mM MgCl₂, 75 mM KCl, 10 mM dithiothreitol, and 50 mM Tris-HCl (pH 8.3), which was incubated at 37 °C for 1 h. The resulting cDNAs were PCR amplified employing the same cDNA primer and a second primer, as listed below (promoter sequence underlined).

For A-B-E´
5´-GACAGATCAGTATTCATGC-3´ and
5´-GGACTAATACGACTCCTATAGCTAAACAGAGTTCA-3´;

For A´-B-E
5´-CTTCTGGATGTCAGTTGC-3´ and
5´-GGACTAATACGACTCCTATAGCCTGCTGCTGAATGTTCA-3´.

The PCR products were used to initiate nested PCR amplifications to generate templates for the transcription of progeny RNAs. For the B-E´ molecules, the products of this second PCR were transcribed directly. For the A molecules, it was necessary to perform three successive PCRs, rather than progressing directly from A-B-E´ to A, due to mispriming caused by sequence similarity near the 3´ ends of A and E´. The second PCR eliminated the 3´-terminal region of E´, allowing subsequent amplification of A. The products of the second PCR were incubated in the presence of 0.2 N NaOH for 20 min at 92 °C to bring about hydrolysis at the single ribonucleotide position, followed by neutralization with 0.2 N HCl. The shorter cleaved products were purified by PAGE and used as input for the third PCR. The products of the third PCR were transcribed to generate RNA, which was gel purified and cleaved by M1 RNA, as described above. The primers used for the various nested PCRs derived from A-B-E´ are listed below (T7 promoter underlined; ribonucleotide in bold).

For B-E´ (second PCR)
5´-GACAGATCAGTATTCATGC-3´ and
5´-GGACTAATACGACTCCTATAGCCAGCAGTTCA-3´;

For A (second PCR)
5´-GACAGATCAGTATTCATGC(rG)-3´ and
5´-GGACTAATACGACTCCTATAGGCTAAACAGAGTTCA-3´;

For A (third PCR)
5´-CTAAGTTGCGTGCTC-3´ and
5´-GGACTAATACGACTCCTATAGGCTAAACAGAGTTCA-3´.

For the B´-E molecules, the products of the second PCR were transcribed directly. For the A´ molecules, the products of the second PCR were subjected to alkaline hydrolysis as described above, then the cleaved products were purified by PAGE and used as input for a third PCR. The products of the third PCR also
were subjected to alkaline hydrolysis, the cleaved products were purified by PAGE, then used to transcribe the desired A’ molecules. The primers used for the various nested PCRs derived from A’-B’-E are listed below (T7 promoter underlined; ribonucleotide in bold).

**For B’-E (second PCR)**

5’-CTTCTGGATGGTCAAGTTGC-3’ and 
5’-GGACTAATACGACTCCTAGAGACCGCATGAATAG-3’;

**For A’ (second PCR)**

5’-CTTCTGGATGGTCAAGTTGC(rG)-3’
5’-GGACTAATACGACTCCTAGAGACCGCATGAATAG-3’;

**For A’ (third PCR)**

5’-CTATTCATGCGGTCT(rC)-3’ and 
5’-GGACTAATACGACTCCTAGAGACCGCATGAATAG-3’.

Six successive rounds of *in vitro* evolution were carried out as described above, with progressively shorter times for the RNA-catalyzed reaction:

<table>
<thead>
<tr>
<th>Round</th>
<th>A-B-E’</th>
<th>A’-B’-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 h</td>
<td>2 h</td>
</tr>
<tr>
<td>2</td>
<td>1 min</td>
<td>5 min</td>
</tr>
<tr>
<td>3</td>
<td>15 s</td>
<td>30 s</td>
</tr>
<tr>
<td>4</td>
<td>15 s</td>
<td>15 s</td>
</tr>
<tr>
<td>5</td>
<td>0.1 s</td>
<td>0.1 s</td>
</tr>
<tr>
<td>6</td>
<td>0.01 s</td>
<td>0.01 s</td>
</tr>
</tbody>
</table>

The last two rounds were conducted using a KinTek (Austin, TX) model RQF-3 quench-flow apparatus to achieve very short reaction times. Hypermutagenic PCR (S3) was performed following round 3 to increase diversity among the population of B-E’, B’-E, and A molecules. Standard mutagenic PCR (S4) was performed following round 3 for the A’ molecules.

Following round 6, the ligated molecules were gel purified, reverse transcribed, PCR amplified, and cloned into *E. coli* using the Invitrogen TOPO TA Cloning Kit. The bacteria were grown on LB agar plates containing 50 µg/ml carbenicillin. Samples were taken from individual colonies and evaluated by PCR to confirm they contained plasmid DNA with an insert of the appropriate length. Validated colonies were picked from the plate and cultured overnight in 2 mL LB medium containing 50 µg/ml carbenicillin. The plasmid DNA was isolated from the cells using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), then sequenced by Genewiz Inc. (La Jolla, CA).
Conversion of selected enzymes to corresponding substrates. A modified version of the nested PCR amplification procedure described above can be used to produce A and B molecules from corresponding E molecules, and to produce A´ and B´ molecules from corresponding E´ molecules. In this case, B and B´ are produced as separate molecules, rather than joined to E´ and E, respectively. This requires installing a primer binding site at the 3´ end of B and B´, which also encodes a recognition sequence for the “10-23” RNA-cleaving DNA enzyme (S5). Cleavage by the DNA enzyme is used to generate transcription products with a precise 3´ terminus (S6). A and A´ are produced as above, except that they are derived from PCR-amplified E and E´, rather than A-B-E´ and A´-B´-E, respectively. In addition, the primer binding site at the 5´ end of A and A´ is shifted upstream so as not to encroach on the genotype region of these molecules.

The ligated products E and E´ are purified by PAGE, reverse transcribed, and PCR amplified, as above. A second PCR is carried out to generate templates that are used to transcribe precursor substrates that contain additional nucleotides at their 3´ terminus. The added nucleotides are removed from A and A´ using M1 RNA, as described above. The added nucleotides are removed from B and B´ using a DNA enzyme. The downstream sequences for the various substrates and corresponding external guide sequence RNA or corresponding DNA enzyme are listed below (dot indicates the site for DNA-catalyzed RNA cleavage; substrate-binding domains within the DNA enzyme are underlined).

For A:
additional nucleotides 5´-GAGACCAGCAAGACCCCCCAG-3´,
guide RNA 5´-GGUCUUGCGGUCACCA-3´;

For A´:
additional nucleotides 5´-GAGACCAGCAUCUGAGACGAUGU-3´,
guide RNA 5´-GCGAGAUGCGGUCACCA-3´;

For B:
additional nucleotides 5´-AGACCCCCCAG•UACACACACC-3´,
DNA enzyme 5´-GGTGTGTGTAGGCTAGCTACAACGATGGGGGTCT-3´;

For B´:
additional nucleotides 5´-UCUGAGACGAUG•UUGAAAAGAGAG-3´,
DNA enzyme 5´-CTCTCTTTTCAAGGCTAGCTACAACGAATCGTCTCAGT-3´.

DNA-catalyzed cleavage is carried out in a reaction mixture containing 10 μM RNA, 30 μM DNA enzyme, 25 mM CaCl₂, and 30 mM EPPS (pH 7.5), which is heated to 70 °C for 2 min, then incubated at 37 °C for 45 min. Following RNA- or DNA-catalyzed cleavage, the desired products are purified by PAGE.
Serial transfer experiments. Reaction mixtures for exponential amplification of cross-replicating RNAs contained 5 µM each of the A, A’, B, and B’ substrates, 15 or 25 mM MgCl₂, and 50 mM EPPS (pH 8.5), which were incubated at 42 °C. The first reaction mixture in a serial transfer experiment contained 0.1 µM each of E and E’, but all subsequent mixtures contained only the E and E’ molecules that were carried over in the transfer. When multiple cross-replicating RNAs were employed, each was present at 0.1 µM concentration in the first reaction mixture, and 5 µM each of the component substrates were present in all of the reaction mixtures. The experiment involving E1 and E1’ alone (Fig. 2B) was carried out in the presence of 25 mM MgCl₂, while the experiments involving multiple pairs of cross-replicating enzymes (fig. S2A and Fig. 3A) were carried out in the presence of 15 mM MgCl₂.

The experiment involving 12 pairs of cross-replicating enzymes was pre-initiated by amplifying each cross-replicator in isolation for 10 h, determining the concentrations of E and E’ that had been produced, and employing an aliquot from these mixtures containing a total of 0.2 µM enzymes to initiate the first reaction of the serial transfer procedure. The enzymes E11 and E11’ amplified so poorly that in their case 0.1 µM of each enzyme was employed directly. The pre-initiation procedure was carried out so that the first reaction of the serial transfer would more closely resemble subsequent reactions with regard to the relative amounts of the two members of a cross-replicating pair (fig. S1B). The enzyme E12’ formed a (5’-UAUG-3’)(5’-AUAC-3’) mismatch with the A12 substrate, but there was no mismatch between E12 and B12’.

In order to prepare the products of a serial transfer experiment for cloning and sequencing, the E and E’ molecules were purified by PAGE, then 3’-polyadenylated, reverse transcribed, and tailed at the 3’ end of the cDNA using terminal transferase. The polyadenylation reactions contained ~0.4 µM E (or E’), 0.1 U/µL poly(A) polymerase, 0.5 mM ATP, 10 mM MgCl₂, 250 mM NaCl, and 50 mM Tris-HCl (pH 8.0), which was incubated for 2 h at 37 °C. The polymerase was extracted with phenol/chloroform, the mixture was desalted using a NAP column (GE Healthcare, Piscataway, NJ), and the extended RNAs were reverse transcribed as described above, using a DNA primer having the sequence 5’-T₂₄V-3’ (V = A, C or G). Full-length cDNAs were purified by PAGE, then extended in a reaction mixture containing ~0.2 µM cDNA, 8 U/µL terminal transferase, 1 mM dGTP, 2.5 mM CoCl₂, 200 mM potassium cacodylate, 0.25 mg/ml BSA, and 25 mM Tris-HCl (pH 6.6), which was incubated at 37 °C for 2 h. The proteins were extracted with phenol/chloroform, the mixture was desalted using a NAP column, and the extended cDNAs were PCR amplified using primers having the sequence 5’-GACAGATCAGT₂₄V-3’ and 5’-GGCTAACACGAC₁₄G-3’. The PCR products were cloned and sequenced, as described above.

Kinetic analysis. RNA-catalyzed RNA ligation was carried out in a reaction mixture containing 5 µM E (or E’), 0.1 µM [5’-³²P]-labeled A’ (or A), 6 µM B’ (or B), 15 or 25 mM MgCl₂, and 50 mM EPPS (pH 8.5), which was incubated at 30 °C. The reaction was initiated by mixing equal volumes of two solutions, one containing the enzymes and substrates, and the other containing the MgCl₂ and EPPS buffer. Aliquots
were taken at various times and quenched by adding an equal volume of gel-loading buffer containing 25 mM Na₂EDTA and 18 M urea. The products were separated by PAGE and quantitated using a PharosFX molecular imager (Bio-Rad, Hercules, CA). The data were fit to the equation:

\[ F_t = a \left(1 - e^{-kt}\right) + b \]

where \( F_t \) is the fraction reacted at time \( t \), \( a \) is the maximum extent of the reaction (typically 0.88–0.92), \( k \) is the observed rate of product formation, and \( b \) is the calculated extent at \( t = 0 \) (typically 0.01–0.03).

Reactions catalyzed by E7´, E11, and E11´ were so slow that the data instead were fit to the linear equation: \( F_t = at + b \).

Cross-catalytic exponential amplification was carried out in a reaction mixture containing 0.1 µM each of E and E´, 5 µM each of [5´-32P]-labeled A and A´, 5 µM each of B and B´, 15 or 25 mM MgCl₂, and 50 mM EPPS (pH 8.5), which was incubated at 42 °C. The reaction was initiated as described above. Aliquots were taken at various times, quenched, and the amounts of newly-synthesized E and E´ were quantitated as described above. The data were fit to the logistic growth equation, as described in the main text. This equation is commonly used in population ecology to model the exponential growth of organisms subject to the carrying capacity of the local environment.

### Additional Biochemical Data

**Catalytic properties of the starting and evolved enzymes.** In the trimolecular reaction (with two separate substrates), the parental R3C ligase operates with a \( k_{\text{cat}} \) of 0.2 min⁻¹, \( K_m \) of 0.4 µM for the 3´-hydroxyl-terminated substrate, and \( K_m \) of 0.1 µM for the 5´-triphosphate-terminated substrate, measured in the presence of 25 mM MgCl₂ at pH 8.5 and 23 °C (S7). This molecule was converted to an autocatalytic format that enabled limited self-replication (S8). For the self-replicating enzyme, the substrates A and B have substantial complementarity, resulting in formation of a non-productive A•B complex. This complex was observed by gel-shift studies employing non-denaturing polyacrylamide gels (S8). Formation of the non-productive complex gives rise to biphasic kinetics, with an initial fast phase of exponential amplification, followed by a slow phase of linear growth. The amplitude of the exponential phase can be increased by increasing the concentration of A relative to B, or by controlling the order of addition, such that A is added to a mixture already containing B and E (S8).

Gel-shift analysis revealed that for E concentrations of 0.1–100 µM, most of the enzyme molecules exist as a monomer, rather than an E•E dimer or higher-order complex (measured in the presence of 10 mM MgCl₂ at pH 8.5 and 23 °C) (S8). The availability of free E enables exponential growth with product turnover until the supply of free substrates is exhausted. The behavior of the self-replicating enzyme during the exponential phase can be described by the equation:
(d[E]/dt)_{init} = k_a [E_0]^p + k_b \), where [E_0] is the starting concentration of E, \( k_a \) is the autocatalytic (E-dependent) rate constant, \( k_b \) is the non-autocatalytic (E-independent) rate constant, and \( p \) is the reaction order.

In the presence of 2 \( \mu \)M each of A and B and 25 mM MgCl\(_2\) at pH 8.5 and 23 °C, \( k_a = 0.011 \text{ min}^{-1} \), \( k_b = 3.3 \times 10^{-11} \text{ M} \cdot \text{min}^{-1} \), and \( p = 1.0 \). Under these conditions, the amplitude of the exponential phase is \( \sim 5\% \) \((S8)\).

The original cross-replicating enzyme has nearly identical sequence compared to the self-replicating enzyme, except for five altered nucleotides in the pairing regions at the 5´ and 3´ ends, and three base pairs added to the central stem to provide a size difference between E and E´ \((S9)\). In the trimolecular reaction, the original E operates with a rate constant of 0.034 min\(^{-1}\) and amplitude of 20% in the fast phase, followed by a slow phase with a rate of \( 5.0 \times 10^{-4} \text{ min}^{-1} \), while E´ operates with a rate constant of 0.026 min\(^{-1}\) and amplitude of 11% in the fast phase, followed by a slow phase with a rate of \( 4.0 \times 10^{-4} \text{ min}^{-1} \) (measured in the presence of 1 \( \mu \)M E or E´, 2 \( \mu \)M A or A´, 2 \( \mu \)M B or B´, and 25 mM MgCl\(_2\) at pH 8.5 and 23 °C).

Pulse-chase experiments were carried out to determine the dissociation rate of the E•E´ complex at various temperatures, revealing a rate of 0.09 min\(^{-1}\) at 23 °C, 0.14 min\(^{-1}\) at 33 °C, and 0.18 min\(^{-1}\) at 43 °C \((S9)\). These rates are faster than the rate constant for the individual RNA-catalyzed ligation reactions. When the reactions catalyzed by E and E´ are carried out in a common reaction mixture (employing 1 \( \mu \)M each of E and E´, 2 \( \mu \)M each of A, A´, B, and B´), E has a rate constant of \( 6.1 \times 10^{-3} \text{ min}^{-1} \) and amplitude of 15% in the fast phase, followed by a slow phase with a rate of \( 5.4 \times 10^{-5} \text{ min}^{-1} \), while E´ has a rate constant of \( 6.2 \times 10^{-3} \text{ min}^{-1} \) and amplitude of 8% in the fast phase, followed by a slow phase with a rate of \( 5.1 \times 10^{-5} \text{ min}^{-1} \) \((S9)\).

Kim and colleagues \((S10)\) carried out temperature cycling experiments using a slightly modified form of the original cross-replicating enzyme that contains an extra G•C pair in each of the two pairing regions. These molecules exhibited similar behavior in the individual RNA-catalyzed reactions compared to the molecules described above. When the two reactions were carried out in a common reaction mixture at a constant temperature of 23 °C (employing 1 \( \mu \)M each of E and E´, 2 \( \mu \)M each of A, A´, B, and B´, and 25 mM MgCl\(_2\) at pH 8.5), the maximum extent was only 1% and 3% for reactions catalyzed by E and E´, respectively. However, this increased to 9% and 13%, respectively, when the temperature was raised to 55 °C every 30 min over a total reaction period of 6.5 h \((S10)\).

The optimized cross-replicating enzyme obtained in the present study has substantially improved catalytic properties compared to the previous version. Prior to initiating \textit{in vitro} evolution, the sequence of the central stem (the portion of E that binds the 3´ end of A´, and reciprocally for E´ and A) was changed from \((5´-UAUA-3´)\•\((5´-UAUA-3´)\) to \((5´-UUUA-3´)\•\((5´-UUUA-3´)\). This change was made to disrupt the palindrome of the central stem in an effort to reduce formation of non-productive complexes. It
improved the maximum extent of reaction to 60% and 15% for E and E’, respectively. The maximum extent could not be significantly improved by increasing the concentration of enzyme, suggesting that there is an inherent limitation in one or more of the substrates.

The four substrates were evaluated individually by allowing the reaction to proceed to maximum extent in the presence of 1–3 µM enzyme, 1–3 nM of the substrate being tested, 1–3 µM of the partner substrate, and 25 mM MgCl₂, incubating at pH 8.5 and 30 °C for 24 h. The tested substrate molecules that did not react were purified by PAGE and used in a second RNA-catalyzed reaction. The maximum extents of the two successive reactions were as follows:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>1st reaction</th>
<th>2nd reaction</th>
<th>Total extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A´</td>
<td>71%</td>
<td>0%</td>
<td>71%</td>
</tr>
<tr>
<td>B´</td>
<td>48%</td>
<td>3%</td>
<td>51%</td>
</tr>
<tr>
<td>A</td>
<td>51%</td>
<td>17%</td>
<td>68%</td>
</tr>
<tr>
<td>B</td>
<td>44%</td>
<td>6%</td>
<td>50%</td>
</tr>
</tbody>
</table>

This indicated that a substantial fraction of the substrates have compositional defects, as well as conformational defects in the case of the A molecules. Accordingly, A and A´ were prepared as extended length transcripts and cleaved using *E. coli* M1 RNA to generate precise 3´ termini (see Materials and Methods). This improved the maximum extent of reaction to ~90%.

The $k_{cat}$ and $K_m$ were determined for each of the four substrates in the presence of a saturating concentration of their partner substrate and 25 mM MgCl₂ at pH 8.5 and 30 °C. Reactions were performed using various concentrations of E or E´ and trace amounts of the substrate being evaluated. The data fit well to the Michaelis-Menten equation, which was used to obtain the following catalytic parameters:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A´</td>
<td>0.03</td>
<td>0.3</td>
</tr>
<tr>
<td>B´</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>A</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>0.03</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*In vitro* evolution was carried out as described in the Material and Methods, resulting in optimized cross-replicating enzymes with the critical wobble pairs in the central stem (Fig. 1B). In order to achieve a high maximum extent, it still was necessary to employ M1 RNA to prepare A and A´ molecules with precise 3´ termini. In the trimolecular reaction, the optimized enzyme E operates with a rate constant of 1.3 min⁻¹ and maximum extent of 92%, while E´ operates with a rate constant of 0.3 min⁻¹ and maximum extent of 88%, measured in the presence of 5 µM E or E´, 0.1 µM [5´-³²P]-labeled A´ or A, 6 µM B´ or B, and 25 mM MgCl₂ at pH 8.5 and 30 °C. Both reactions exhibit monophasic kinetics. The reactions require
Mg²⁺, but the rate constant is unchanged over MgCl₂ concentrations of 5–35 mM. The rate constant increases with increasing pH over the range of 6.5–9.0, although at pH 9.0 (and especially at 42 °C) the amount of RNA degradation is substantial.

Cross-catalytic replication was carried out with the optimized enzymes, comparing reactions performed in the presence of either 15 or 25 mM MgCl₂ and at either 30 or 42 °C (always at pH 8.5). Exponential amplification is approximately two-fold faster in the presence of 25 compared to 15 mM MgCl₂, suggesting that dissociation of the E•E´ complex is not rate limiting. The higher MgCl₂ concentration was adopted for the initial test of the E1 replicator (Fig. 2), but the lower concentration was used in all subsequent experiments in order to reduce the use of mismatched substrates in mixtures of multiple cross-replicators and to render the RNA less susceptible to hydrolysis. Amplification is about four-fold faster at 42 compared to 30 °C. An initial serial transfer experiment was performed at 30 °C, involving six successive reactions of 16 h duration and transferring 1/25th of the material from one reaction mixture to the next (data not shown). However, the same amount of amplification could be achieved in about 4 h at 42 °C, so the higher temperature was used in all subsequent experiments.

Cross-replicators with swapped pairing domains.
The choice of sequence within the paired regions of the 12 cross-replicating RNA enzymes was arbitrarily related to a particular sequence within the corresponding catalytic core. For simplicity, the same catalytic core sequence was associated with both members of a cross-replicating pair, although this need not be the case. Also arbitrarily, the pairing sequences at the 5´ and 3´ ends of each enzyme were chosen to be identical when one is read in the 5´→3´ and the other in the 3´→5´ direction. This is a convenient way to ensure that the two ends are not complementary.

Variant forms of the E1, E1´, E4, and E4´ enzymes were prepared in which the paired regions within E1 and E1´ were exchanged for those within E4 and E4´, respectively. This was done to assess the independent contributions of the pairing regions and catalytic core to the behavior of the enzyme. For the original and swapped versions of each enzyme, the rate constant was determined in the trimolecular reaction, measured in the presence of 5 µM E or E´, 0.1 µM [5´,32P]-labeled A´ or A, 6 µM B´ or B, and 15 mM MgCl₂ at pH 8.5 and 30 °C. In addition, the exponential growth rate and fold amplification after 5 h was determined for each pair of cross replicators, measured in the presence of 0.1 µM each of E and E´, 5 µM each of A´, A, B´ and B, and 15 mM MgCl₂ at pH 8.5 and 42 °C. These data are summarized below.

The E1 and E4 enzymes have a similar catalytic rate constant, and swapping their catalytic cores had little effect on their behavior in the individual RNA-catalyzed reactions. The E1´ and E4´ enzymes have more disparate rate constants, with E1´ being much faster than E1, and E4´ being much slower than E4. Thus swapping the catalytic cores of E1´ and E4´ reduced activity of the former and increased activity of the latter. Exponential amplification depends on the reciprocal activity of both members of a cross-replicating pair. All of the enzymes exhibited robust exponential amplification, with the E1 and E1´ pair
performing best regardless of the choice of catalytic core, and the E4 and E4’ pair performing worst when fitted with the catalytic core originally associated with E1 and E1’.

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References

**Figure Legends**

**Fig. S1.** Catalytic activity and exponential amplification of 12 pairs of cross-replicating RNA enzymes (Fig. 1C). (A) For each pair, the observed rate of E (black) and E´ (gray) was measured in a reaction mixture containing 5 µM E (or E´), 0.1 µM [5´-32P]-labeled A´ (or A), 6 µM B´ (or B), 15 mM MgCl₂, and 50 mM EPPS (pH 8.5), which was incubated at 30 °C. Values for $k_{obs}$ were determined as described in the Materials and Methods. (B) For exponential amplification, the yield of newly-synthesized E and E´ relative to the starting amount of each enzyme was determined following incubation at 42 °C for 5 h in a reaction mixture containing 0.1 µM each of E and E´, 5 µM each of [5´-32P]-labeled A and A´, 5 µM each of B and B´, 15 mM MgCl₂, and 50 mM EPPS (pH 8.5).

**Fig. S2.** Serial transfer experiment initiated by cross-replicating RNA enzymes E1–E4 and their partners E1´–E4´ (Fig. 1C). (A) Amplification was sustained for 16 successive rounds of ∼20-fold amplification and 20-fold dilution. The concentrations of all E (black) and E´ (gray) molecules were measured at the end of each incubation. (B) Observed genotypes among 25 E´ clones that were sequenced following the last incubation. (C) Estimated ΔΔG values for binding of each possible combination of A•B´, A•B, A´•B´, A´•B pairings relative to the corresponding matched interaction (dashes). It is difficult to calculate ΔG values in the context of the enzyme-substrate complex, but ΔΔG values only consider relative predicted binding energy for the paired region, based on values obtained from the mfold web server at Rensselaer Polytechnic Institute ($S11, S12$). ΔΔG values that are <3.5 kcal/mol are highlighted in red. (D) Preferred pathways for mutation among B (and B´) substrates and among A´ substrates, corresponding to the most favorable ΔΔG values for mismatched pairings shown in fig. S2C.
Figure S1

A

B

[k_{obs} (min^{-1})]

[0.01, 0.10, 1.00]

[0.01, 0.10, 1.00]

[E or E']

[E or E']

Fold amplification

[k_{obs} (min^{-1})]

[0.01, 0.10, 1.00]

[0.01, 0.10, 1.00]

[E or E']

[E or E']
Figure S2

A

![Graph showing time (h) vs. [E] or [E'] (µM)]

B

25 clones

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C

ΔΔG values (kcal/mol)

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D

Diagram showing interactions between B1', B2', B3', B4', A1', A2', A3', and A4'.