Supporting Information

Structure-guided engineering of the regioselectivity of RNA ligase ribozymes

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Supporting Discussion

The 2'-5' RNA World

The role of 2'-5' linked nucleic acids in the RNA world is not clear. The prevalence of 2'-5' linkages resulting from the polymerization of activated mononucleotides in solution (reviewed in ^{1,2}), their presence in the genomes of parasitic nucleic acids³ and the role of 2'-5' oligoadenylic acid as an activator in anti-viral defense mechanisms⁴, together suggest that 2'-5' linkages may have been important in the RNA world. However, the decreased thermostability of double helices containing 2'-5' linkages^{5,6}, their sensitivity to hydrolysis⁷ and their susceptibility to sequence effects⁸ would conceivably be selective barriers against any organism relying on a 2'-5' linked RNA genetic polymer. Nonetheless, the ability of a polymerase ribozyme to catalyze the polymerization of disparate substrates would likely increase the structural and functional diversity available to an RNA based organism. In fact some common RNA motifs are actually more stable when joined by 2'-5' linkages¹⁰, indicating that there is no reason, *a priori*, that an RNA based genetic system would have excluded 2'-5' linkages.

Catalytic Mechanism of Ligase Ribozymes

Protein-based nucleic acid polymerases employ a conserved two-metal ion mechanism. One divalent ion activates the attacking hydroxyl, the second coordinates the γ and β phosphates, assisting the pyrophosphate leaving group, and both metal ions shield the excess negative charge of the pentacovalent transition state¹¹. Mg²⁺ ions are critical for

activity of both the L1 and the class II ligases, but there are no data to indicate that these ions are directly involved in catalysis. In our class II ligase product-template structures, no metal ions are within 8 Å of the relevant functional groups, suggesting that any catalytic cations, if present, must be brought to the active site through tertiary interactions with peripheral elements outside the substrate-template duplex. In the L1 ligase structure, a well-ordered Mg^{2+} ion is present in the active site, nucleating a critical tertiary interaction in the docked conformer. However, the coordination geometry of this Mg^{2+} is not ideal for activating the nucleophilic 3'-OH of U71 and its role in binding the pyrophosphate leaving group is unknown¹². The structures of both the L1 and class II ligases are of product complexes of the ribozymes. It is possible that either the affinity or coordination geometry of these putative metal ion binding sites in the precursor state is different from the product state, making it difficult to deduce a mechanism based on the available structures. Nonetheless, both the L1 and the Class II ligases are obligate metalloenzymes requiring Mg^{2+} concentrations much higher than the 10-100 μM dissociation constants of free nucleoside triphosphates for Mg^{2+} , suggesting that the γ and β phosphates coordinate Mg²⁺ in the precursor. In the L1 ligase, were the complete structure of the ribozyme is known, how the nucleophilic hydroxyl is activated remains an open question¹². In this ribozyme, the non-bridging phosphate oxygen of A39, which coordinates a Mg^{2+} ion, is equidistant (2.8 Å) from the 2' and 3' OHs, but in the precursor it has been predicted to form an activated hydrogen bond to the 3' OH, owing to a well order water molecule that could quench activation of the 2'OH¹². Alternatively, it is also plausible that it is the Mg²⁺ bound by A39 that directly activates the 3' nucleophile for attack, and the proximity of this ion to the 3'-OH (2.8 Å), compared to the 2'-OH (4.1 Å), helps explain the strong 3'-5' regioselectivity we observe when U71 is mutated to C (Figure 5 C,D), which should remove many of the hydrogen bonds to the critical water molecule Robertson and Scott predict to quench the activated 2' nucleophile¹².

Modularity of RNAs and Recombinatorial Evolution?

Contemporary genetic systems can rely on heterologous recombination to drive the evolution of novel protein enzymes by the chimeric assembly of disparate genetic elements. The local folding and inherent structural flexibility of RNAs, which results from their chemically similar monomers and stereotypical structural motifs¹³, may make recombination an even greater factor in the evolution of new ribozymes¹⁴. Our experiments relied on this flexibility to aid in the rational engineering of ligase ribozymes, resulting in catalysts with simple changes in regioselectivity. However, in the RNA world, primitive genetic systems likely exploited these same traits, through recombination, to generate the diverse array of ribozymes that would have been required for life.

		Crystal form II			
Data collection					
		Crystal 1		Crystal 2	Crystal 3
Unit Cell Dimensions					
<i>a, b, c</i> (Å)	31.17,31.17, 169.09		31.55, 31.55, 171.07	30.04, 30.04, 59.67	
Space Group		<i>P</i> 4 ₁		P4 ₁	<i>P</i> 3 ₁
Wavelength (A)	0.91500	0.92004	0.91981	1.00000	1.00000
Resolution (A)	30-2.9	30-2.9	30-2.9	30-2.7	30-2.3
Reflections ^a /Redundancy	3541/9.9	2163/5.0	3544/10.0	4565/3.8	2663/5.7
R _{sym} (%)	8.3 (42.6) ^₀	6.2 (32.1)	7.3 (39.2)	4.6 (44.2)	4.2 (30.7)
<l>/<o(l)></o(l)></l>	38.8 (5.9)	31.9 (5.0)	43.3 (6.0)	29.4 (2.9)	31.3 (4.6)
Completeness (%)	98.4 (99.4)	98.4 (99.4)	98.5 (99.4)	98.7 (99.6)	99.5 (99.6)
Phasing					
IF _H I/E					
ano	0.71 (0.1)	1.89 (0.72)	2.68 (1.56)		
iso		1.78 (0.75)	2.55 (1.43)		
R _{Kraut} (%)					
ano	3.4 (4.8)	4.2 (12.6)	5.1 (13.7)		
iso		4.3 (13.0)	5.1 (13.4)		
Mean Figure of Merit		0.58 (0.32)			
Refinement					
Atoms					
(total/water/ions)				999/6/11	466/12/5
$R_{\rm work}/R_{\rm free}$				21.8/26.8	20.8/23.4
Mean B-factor				44.6	43.4
RMSD bond					
lengths/angles (Å / °)				0.005/1.05	0.005/1.2
Cross-validated oA				0.6	0.4
coordinate error (Å)					
PDB accession code				3FTM	3FS0

Table S1. Crystallographic statistics

^a Unique reflections
 ^b Values in parentheses are for highest resolution shell.

		Mutant	Rate (min ⁻¹)	σ (min⁻¹)	R	Regioselectivity (2'-5':3'-5')	Yield 2'-5' %product [♭]	σ (%)
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Class II ligase a4-11	WT	5.5 × 10 ⁻¹	-	0.991	77 : 1 ^d	100	3
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		C-1U	2.3 × 10 ⁻³	3 × 10⁻ ⁶	-	15 : 1	95	3
$ \begin{array}{c} \begin{array}{c} {\rm G49A} & 2.1 \times 10^{7} & 3 \times 10^{6} & - & 80 \cdot 1 & 104 & 7 \\ {\rm G49A,C-1U} & 1.8 \times 10^{7} & 2 \times 10^{6} & - & 84 \cdot 1 & 109 & 2 \\ {\rm G49C,C-1U} & 1.8 \times 10^{7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm G49C,C-1G} & 9.1 \times 10^{7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm G49C,C-1G} & 9.1 \times 10^{7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm G49C,C-1G} & 9.1 \times 10^{7} & - & 0.999 & - & ND & - \\ {\rm G1A} & 3.8 \times 10^{4} & 1 \times 10^{4} & - & 85 \cdot 1 & 110 & 4 \\ {\rm A48G} & 1.4 \times 10^{4} & 9 \times 10^{5} & - & 84 \cdot 1 & 109 & 7 \\ {\rm G1A,A48G} & 1.0 \times 10^{5} & 2 \times 10^{6} & - & - & ND & - \\ {\rm G2A} & 1.1 \times 10^{1} & - & 0.999 & 30 \cdot 1 & 98 & 4 \\ {\rm G2C} & 3.5 \times 10^{2} & - & 0.987 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.999 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.999 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.999 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.999 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.999 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.999 & 23 \cdot 1 & 97 & 2 \\ {\rm G2U} & 1.3 \times 10^{1} & - & 0.997 & 23 \cdot 1 & 97 & 2 \\ {\rm G47A} & 2.0 \times 10^{5} & 8 \times 10^{5} & - & 82 \cdot 1 & 107 & 0.7 \\ {\rm G47A} & 2.0 \times 10^{5} & 8 \times 10^{5} & - & 82 \cdot 1 & 107 & 0.7 \\ {\rm G47U} & 9.5 \times 10^{6} & 8 \times 10^{6} & - & 77 \cdot 1 & 100 & 18 \\ {\rm G47U} & 9.5 \times 10^{6} & 8 \times 10^{6} & - & 77 \cdot 1 & 100 & 16 \\ {\rm G47U} \cdot \Delta 3 \cdot C -1U & 1.3 \times 10^{6} & 8 \times 10^{7} & - & ND & - \\ {\rm G47U} \cdot \Delta 3 \cdot C -1U & 3.8 \times 10^{7} & 2 \times 10^{6} & - & - & ND & - \\ {\rm G47U} \cdot \Delta 3 \cdot C -1U & 3.8 \times 10^{7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm G47U} \cdot \Delta 3 \cdot C -1U & 3.8 \times 10^{7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm G47U} \cdot \Delta 3 \cdot C -1U & 3.8 \times 10^{7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm U71C} & 3.2 \times 10^{7} & 1 \times 10^{6} & - & - & ND & - \\ {\rm U50C} \cdot \Delta 10 \cdot 8 \times 10^{7/7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm U50C} \cdot \Delta 10 \cdot 8 \times 10^{7/7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm U50C} \cdot \Delta 10 \cdot 8 \times 10^{7/7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm U50C} \cdot \Delta 10 \cdot 8 \times 10^{7/7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm U50C} \cdot \Delta 10 \cdot 8 \times 10^{7/7} & 7 \times 10^{6} & - & - & ND & - \\ {\rm U50C} \cdot \Delta 10 $		C-1G	3.1 × 10 ⁻⁴	5 × 10⁻⁰	-	-	ND	-
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		G49A	2.1 × 10 ⁻²	3 × 10⁻⁵	-	80 : 1	104	7
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		G49A:C-1U	1.8 × 10⁻²	2 × 10⁻ ⁶	-	84 : 1	109	2
$ \begin{array}{c ccccc} G49C & 16\times10^4 & 7\times10^6 & - & - & ND & - \\ G49CC^-1U & 41\times10^4 & 7\times10^6 & - & - & ND & - \\ G41A & 38\times10^4 & 1\times10^4 & - & 88:1 & 110 & 4 \\ A48G & 14\times10^4 & 9\times10^5 & - & 84:1 & 109 & 7 \\ G1AA48G & 10\times10^5 & 2\times10^6 & - & - & ND & - \\ G2A & 11\times10^1 & - & 0.999 & 30:1 & 98 & 4 \\ G2C & 35\times10^4 & - & 0.987 & 23:1 & 97 & 2 \\ G2U & 13\times10^1 & - & 0.987 & 23:1 & 97 & 0.7 \\ A3G & 63\times10^4 & 5\times10^5 & - & 43:1 & 199 & 6 \\ A3G & 63\times10^4 & 5\times10^5 & - & 43:1 & 199 & 6 \\ A3U & 16\times10^6 & 3\times10^6 & - & - & ND & - \\ A3G & 63\times10^6 & 2\times10^5 & - & 43:1 & 99 & 6 \\ A3U & 16\times10^6 & 3\times10^6 & - & - & ND & - \\ A3G & 63\times10^6 & 14\times10^6 & - & - & ND & - \\ A3G & 15\times10^5 & 3\times10^6 & - & - & ND & - \\ A3G & 15\times10^6 & 8\times10^6 & - & - & ND & - \\ A3G & 15\times10^6 & 8\times10^6 & - & - & ND & - \\ G47U & 0 & 0\times10^5 & 1 & 10^6 & 0 & 1^6 & - & - \\ G47U & 13\times10^6 & 1 & 10^7 & - & - & ND & - \\ G47U A33 & 3 & 10^7 & 1 \times10^6 & - & - & ND & - \\ G47U A33 & 3 & 10^7 & 1 \times10^6 & - & - & ND & - \\ G47U A33 C 1U & 3 & 10^6 & - & - & ND & - \\ G47U A33 C 1U & 13 & 10^6 & - & - & 0 \\ 999 & 1 : 126 & 0 & 3 \\ 0 \\ 0 & 0 \\ \mathsf$		G49A:C-1G	7.9 × 10⁻⁵	5 × 10⁻ ⁶	-	-	ND	-
$ \begin{array}{c cccc} \mbox{G49C:C-1U} & 4.1 \times 10^4 & 7 \times 10^5 & - & - & ND & - \\ \mbox{G49C:C-1G} & 9.1 \times 10^2 & - & 0.999 & - & ND & - \\ \mbox{G1A} & 3.8 \times 10^4 & 1 \times 10^4 & - & 85:11 & 110 & 4 \\ \mbox{A48G} & 1.4 \times 10^4 & 9 \times 10^5 & - & 85:11 & 110 & 4 \\ \mbox{G1A,A48G} & 1.0 \times 10^5 & 2 \times 10^5 & - & - & ND & - \\ \mbox{G2A} & 1.1 \times 10^1 & - & 0.999 & 30:11 & 98 & 4 \\ \mbox{G2C} & 3.5 \times 10^2 & - & 0.987 & 30:11 & 97 & 27 \\ \mbox{G2U} & 1.3 \times 10^1 & - & 0.999 & 23:1 & 97 & 0.7 \\ \mbox{A62} & 4.4 \times 10^4 & 1 \times 10^5 & - & 2:1 & 67 & 2 \\ \mbox{A62} & 4.4 \times 10^4 & 5 \times 10^5 & - & 43:1 & 99 & 6 \\ \mbox{A3C} & 8.0 \times 10^6 & 5 \times 10^5 & - & 43:1 & 99 & 6 \\ \mbox{A3C} & 8.0 \times 10^6 & 2 \times 10^5 & - & 43:1 & 99 & 6 \\ \mbox{A33} & 4.5 \times 10^5 & 3 \times 10^5 & - & 82:1 & 107 & 0.7 \\ \mbox{G47A} & 2.0 \times 10^6 & 8 \times 10^6 & - & - & ND & - \\ \mbox{A33} & 4.5 \times 10^5 & 8 \times 10^7 & - & 77:1 & 100 & 18 \\ \mbox{G47C} & 1.9 \times 10^5 & 8 \times 10^7 & - & - & ND & - \\ \mbox{A33} & 4.5 \times 10^6 & 8 \times 10^7 & - & - & ND & - \\ \mbox{A33} & 4.5 \times 10^6 & 8 \times 10^7 & - & - & ND & - \\ \mbox{A32} & 4.5 \times 10^6 & 1 \times 10^7 & - & - & ND & - \\ \mbox{A32} & 4.5 \times 10^7 & 2 \times 10^8 & - & - & ND & - \\ \mbox{A33} & 3.5 \times 10^7 & 2 \times 10^8 & - & - & ND & - \\ \mbox{A32} & 4.5 \times 10^7 & 7 \times 10^6 & - & - & ND & - \\ \mbox{A33} & 4.5 \times 10^7 & 7 \times 10^6 & - & - & ND & - \\ \mbox{A32} & 4.5 \times 10^7 & 7 \times 10^8 & - & - & ND & - \\ \mbox{A33} & 4.5 \times 10^7 & 7 \times 10^6 & - & - & ND & - \\ \mbox{Carss II substrate-template duplex alone * \\ \mbox{U50G} & ND & - & - & ND & - \\ \mbox{U50G} & A3isertion UT71C & 7.4 \times 10^5 & - & 0.999 & 1:12400 & 0.024 & 0.022 \\ \mbox{U50G} & Jin & - & - & ND & - \\ \mbox{U50G} & A3isertion UT71C & 7.4 \times 10^5 & - & 0.999 & 1:144 & 6.6 \\ \mbox{A3isertion} & 5.1 \times 10^4 & 7 \times 10^6 & - & - & ND & - \\ \mbox{U50G} & A3isertion UT71C & 7.4 \times 10^5 & - & 0.993 & 1:14 & 6.6 \\ \mbox{U50G} & A3isertion UT71C & 7.4 \times 10^5 & - & 0.993 & 1:14 & 6.6 \\ \mbox{U50G} & A3isertion UT71C & 7.4 \times 10^5 & - & 0.993 & 1:14 & 6.6 \\ \mbox{U50G} & A3isertion UT71C & 7.4 \times 10^5 & - & - & ND & - \\ \mbox{U50G} $		G49C	1.6 × 10 ⁻⁴	7 × 10 ⁻⁶	-	-	ND	-
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		G49C:C-1U	4.1×10^{-4}	7 × 10⁻ ⁶	-	-	ND	-
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		G49C:C-1G	9.1 × 10 ⁻²	-	0.999	-	ND	-
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		G1A	3.8×10^{-4}	1 × 10 ⁻⁴	-	85 : 1	110	4
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		A48G	1.4×10^{-4}	9 × 10⁻⁵	-	84:1	109	7
$ \begin{array}{c} G2A & 1.1 \times 10^{-1} & - & 0.999 & 30 \cdot 1 & 98 & 4 \\ G2C & 3.5 \times 10^{2} & - & 0.987 & 23 \cdot 1 & 97 & 2 \\ G2U & 1.3 \times 10^{-1} & - & 0.999 & 23 \cdot 1 & 97 & 0.7 \\ AG2 & 4.4 \times 10^{4} & 1 \times 10^{5} & - & 2 \cdot 1 & 67 & 2 \\ A3G & 6.3 \times 10^{4} & 5 \times 10^{5} & - & 80 \cdot 1 & 104 & 0.7 \\ A3C & 8.0 \times 10^{6} & 2 \times 10^{5} & - & 43 \cdot 1 & 99 & 6 \\ A3U & 1.6 \times 10^{4} & 3 \times 10^{6} & - & - & ND & - \\ AA3 & 4.5 \times 10^{5} & 3 \times 10^{5} & - & 82 \cdot 1 & 107 & 0.7 \\ G47A & 2.0 \times 10^{5} & 8 \times 10^{7} & - & 77 \cdot 1 & 100 & 18 \\ G47C & 1.9 \times 10^{5} & 8 \times 10^{7} & - & 777 \cdot 1 & 100 & 18 \\ G47U & 9.5 \times 10^{6} & 6 \times 10^{7} & - & 777 \cdot 1 & 100 & 18 \\ G47U & 9.5 \times 10^{6} & 6 \times 10^{7} & - & 777 \cdot 1 & 100 & 16 \\ G47U & 9.5 \times 10^{6} & 8 \times 10^{6} & - & - & ND & - \\ AG2:C-1U & 6.0 \times 10^{6} & 1 \times 10^{7} & - & 1 \cdot 3 & 27 & 1 \\ G47U:A23:C-1U & 3.3 \times 10^{7} & 2 \times 10^{8} & - & - & ND & - \\ G47U:A33 & 3.9 \times 10^{7} & 1 \times 10^{7} & - & - & ND & - \\ G47U:A33:C-1U & 3.8 \times 10^{6} & - & 0.999 & <1 : 126 & 0.8 & 3 \\ Class II \\ substrate- template duplex \\ alone^{4} & & & & & & & & & & & \\ G47U:\Delta A3:C-1U & 4.8 \times 10^{7 c} & 7 \times 10^{8} & - & & & & & & \\ & & & & & & & & & & &$		G1A:A48G	1.0 × 10 ⁻⁵	2 × 10⁻⁵	-	-	ND	-
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		G2A	1.1 × 10 ⁻¹	-	0.999	30 : 1	98	4
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		G2C	3.5×10^{-2}	-	0.987	23 : 1	97	2
$ \begin{array}{c} \Delta G2 & 4.4 \times 10^{\circ} & 1 \times 10^{\circ} & - & 2.1 & 67 & 2 \\ \Delta 3G & 6.3 \times 10^{4} & 5 \times 10^{5} & - & 80 : 1 & 104 & 0.7 \\ \Delta 3C & 8.0 \times 10^{5} & 2 \times 10^{5} & - & 43 : 1 & 99 & 6 \\ \Delta 3U & 1.6 \times 10^{4} & 3 \times 10^{6} & - & - & ND & - \\ \Delta A3 & 4.5 \times 10^{5} & 3 \times 10^{5} & - & 82 : 1 & 107 & 0.7 \\ G47A & 2.0 \times 10^{5} & 8 \times 10^{6} & - & 77 : 1 & 100 & 18 \\ G47C & 1.9 \times 10^{5} & 8 \times 10^{6} & - & 77 : 1 & 100 & 16 \\ G47U & 9.5 \times 10^{6} & 6 \times 10^{7} & - & 77 : 1 & 100 & 16 \\ G47U & 9.5 \times 10^{6} & 6 \times 10^{7} & - & - & ND & - \\ \Delta G2:C-1U & 6.0 \times 10^{6} & 1 \times 10^{7} & - & 1 : 3 & 27 & 1 \\ G47U:\Delta 3C-1U & 3.3 \times 10^{7} & 2 \times 10^{6} & - & -16 & 14 & 6 \\ G47U:\Delta 3 & 3.9 \times 10^{7} & 2 \times 10^{6} & - & - & ND & - \\ G47U:\Delta 3:C-1U & 3.8 \times 10^{6} & - & 0.999 & <1 : 126 & 0.8 & 3 \\ \hline \\ Class II \\ substrate- template duplex \\ alone^{3} & & & & \\ dot g^{3} & & & & & \\ G47U:\Delta 3:C-1U & 4.8 \times 10^{7 c} & 7 \times 10^{8} & - & & & ND & - \\ \hline \\ UT1C & 3.2 \times 10^{4} & 1 \times 10^{6} & - & - & ND & - \\ U50G:A3insertion & 5.1 \times 10^{7} & 7 \times 10^{6} & - & & & ND & - \\ U50G:A3insertion:U71C & 7.9 \times 10^{7} & 8 \times 10^{6} & - & & & ND & - \\ \hline \\ U50G:A3insertion:U71C & 7.9 \times 10^{7} & 8 \times 10^{8} & - & & & ND & - \\ \hline \\ U50G:A3insertion:U71C & 7.9 \times 10^{7} & 8 \times 10^{8} & - & & & ND & - \\ \hline \end{array}$		G2U	1.3 × 10 ⁻		0.999	23 : 1	97	0.7
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		$\Delta G2$	4.4 × 10 ⁻⁴	1 × 10⁻°ٍ	-	2:1	67	2
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		A3G	6.3 × 10 ⁻⁴	5 × 10⁵⁵	-	80 : 1	104	0.7
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		A3C	8.0×10^{-3}	2 × 10 ⁻⁵	-	43 : 1	99	6
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		A3U	1.6×10^{-1}	3 × 10°	-	-	ND	-
$ \begin{array}{c} {\rm G47A} & 2.0 \times 10^{\circ} & 8 \times 10^{\circ} & - & 77 : 1 & 100 & 18 \\ {\rm G47C} & 1.9 \times 10^5 & 8 \times 10^7 & - & 77 : 1 & 100 & 16 \\ {\rm G47U} & 9.5 \times 10^6 & 6 \times 10^7 & - & - & {\rm ND} & - \\ {\rm G47U:C-1U} & 1.3 \times 10^6 & 8 \times 10^8 & - & - & {\rm ND} & - \\ {\rm G47U:AG2:C-1U} & 6.0 \times 10^6 & 1 \times 10^7 & - & 1 : 3 & 27 & 1 \\ {\rm G47U:AG2:C-1U} & 3.3 \times 10^7 & 2 \times 10^8 & - & <1 : 6 & 14 & 6 \\ {\rm G47U:AA3} & 3.9 \times 10^7 & 1 \times 10^7 & - & - & {\rm ND} & - \\ {\rm G47U:AA3:C-1U} & 3.8 \times 10^6 & - & 0.999 & <1 : 126 & 0.8 & 3 \\ \end{array} $		ΔΑ3	4.5×10^{-5}	3 × 10 ⁻⁵	-	82:1	107	0.7
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		G47A	2.0×10^{-5}	8 × 10 ⁻⁰	-	77:1	100	18
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		G47C	1.9×10^{-5}	8 × 10 ⁻⁷	-	77:1	100	16
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$		G47U	$9.5 \times 10^{\circ}$	6×10^{-8}	-	-	ND	-
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$		G470:C-10	1.3×10^{-6}	8 × 10 ⁻⁷	-	-	ND	-
$ \begin{array}{c} G470: \Delta G270: \Delta G270: \Delta G270: \Delta G33: \times 10^{-7} & 1 \times 10^{-7} & - & - \times 1:6 & 14 & 6 \\ G470: \Delta A3 & 3.9 \times 10^{-7} & 1 \times 10^{-7} & - & - & ND & - \\ G470: \Delta A3:C-1U & 3.8 \times 10^{-6} & - & 0.999 & <1:126 & 0.8 & 3 \\ Class II \\ substrate- \\ template duplex \\ alone^{ a} & G470: \Delta A3:C-1U & 4.8 \times 10^{-7 ^{\circ}} & 7 \times 10^{-8} & - & - & ND & - \\ G470: \Delta A3:C-1U & 4.8 \times 10^{-7 ^{\circ}} & 7 \times 10^{-8} & - & - & ND & - \\ G470: \Delta A3:C-1U & 4.8 \times 10^{-7 ^{\circ}} & 7 \times 10^{-8} & - & - & ND & - \\ G470: \Delta A3:C-1U & 4.8 \times 10^{-7 ^{\circ}} & 7 \times 10^{-8} & - & - & ND & - \\ U71C & 3.2 \times 10^{-4} & 1 \times 10^{-5} & - & -1:4300 & 0.023 & 0.02 \\ U50G & ND & - & - & ND & - \\ U50G: U71C & 3.5 \times 10^{-4} & 2 \times 10^{-5} & - & ND & - \\ U50G: A3insertion & 5.1 \times 10^{-4} & 7 \times 10^{-5} & - & ND & - \\ U50G: A3insertion: U71C & 7.4 \times 10^{-5} & - & 0.993 & 1:14 & 6.6 & 0.3 \\ L1 substrate- \\ template duplex \\ alone^{ a} & U50G: A3insertion: U71C & 7.9 \times 10^{-7} & 8 \times 10^{-8} & - & ND & - \\ \end{array}$			6.0×10^{-7}	1×10^{-8}	-	1:3	27	1
$\begin{array}{c} \text{G47U}:\Delta A3 & \text{G47U}:\Delta A3 &$		G470:AG2:C-10	3.3×10	2×10^{-7}	-	< 1:6	14	6
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			3.9×10	1 × 10	-	-	ND	-
$\begin{array}{cccccccccccccccccccccccccccccccccccc$		G470:ΔA3:C-10	3.8 × 10	-	0.999	< 1 : 126	0.8	3
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Class II substrate- template duplex	WT	7.5 × 10 ⁻⁷	2 × 10 ⁻⁸	-	-	ND	-
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	alone	G47U:∆A3:C-1U	4.8×10^{-7} c	7 × 10⁻ ⁸	-	-	ND	-
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$			2					
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	L1 Ligase L1x6C	VV I	7.3×10^{-1}	4 40-5	0.994	1:525	0.19	0.04
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		0/10	3.2 × 10	1×10^{-5}	-	< 1 : 4300	0.023	0.02
$\begin{array}{cccccccccccccccccccccccccccccccccccc$		U50G	ND	-	-	< 1 : 4300	0.024	0.02
$\begin{array}{cccccccccccccccccccccccccccccccccccc$		U50G:071C	3.5×10	2×10	-	-		-
L1 substrate- template duplex alone a° U50G:A3insertion:U71C 7.9 × 10 ⁻⁷ 8 × 10 ⁻⁸ ND -		USOG ASINSERIION	5.1×10^{-5}	7 ×10	-	-	ND 6.6	-
L1 substrate- WT $1.2 \times 10^{\circ}$ 2×10^{-7} ND - template duplex alone $^{\circ}$ U50G:A3insertion:U71C 7.9×10^{-7} 8×10^{-8} ND -		050G.ASINSettion.07 TC	7.4 × 10	-	0.995	1.14	0.0	0.5
U50G:A3insertion:U71C 7.9×10^{-7} 8×10^{-8} ND -	L1 substrate- template duplex alone ^a	WT	1.2 × 10 ⁻ ° °	2 × 10 ⁻⁷	-	-	ND	-
		U50G:A3insertion:U71C	7.9 × 10 ⁻⁷	8 × 10⁻ ⁸	-	-	ND	-

Table S2. Rates and regioselectivities of ligase ribozyme mutants

ND = Not determined WT = wildtype

^a Residues 6 - 44 of class II ligase A4-11 replaced by a three base pair stem capped by a GAAA tetraloop and residues 8 - 44 of L1 ligase L1x6c replaced by a GAAA tetraloop.

^b Percentage yield 2'-5' yields for class II mutants are normalized to that of the wildtype class II ligase a4-11¹⁵

⁶ The difference between the isolated substrate-template duplexes of the engineered class II and the wildtype L1 ligases is statistically significant. The difference in rates may reflect the increased thermostability conferred by the more stable Watson-Crick duplex 5' of the ligation junction (on the substrate strand) in the L1 substrate-template duplex since the wildtype L1 substrate-template duplex is 2 base pairs longer than the engineered class II substrate-template duplex, which only contains the mutations G47U:∆A3:C-1U relative to the class II substrate-template duplex.

^d Reference ¹



Figure S1. Experimental electron density map Portion of the density-modified experimental electron density map calculated with MAD phases from crystal 1, phase-extended to 2.7 Å using data from crystal 2, and crystal 2 structure factor amplitudes, contoured at 1.2 σ and 4 σ (blue and red mesh, respectively). Final refined models from three adjacent asymmetric units are shown in black to display pseudo-infinite helix packing in the crystal.



Figure S2. Anomalous difference Fourier map of crystal form I A portion of the anomalous difference Fourier map calculated with amplitudes from the 0.91981 Å dataset of Crystal 1 and the density-modified MAD phases, contoured at 10 σ (orange mesh). Peaks were used to locate 5-bromouridine residues in the pseudo-infinite helix prior to model building.

References for Supporting Information

- (1) Orgel, L. E. Cold Spring Harb Symp Quant Biol 1987, 52, 9-16.
- (2) Joyce, G. F. Cold Spring Harb Symp Quant Biol 1987, 52, 41-51.
- (3) Cote, F.; Levesque, D.; Perreault, J. P. *J Virol* **2001**, *75*, 19-25.
- (4) Player, M. R.; Torrence, P. F. *Pharmacol Ther* **1998**, 78, 55-113.
- (5) Kierzek, R.; He, L.; Turner, D. H. *Nucleic Acids Res* **1992**, *20*, 1685-1690.
- Wasner, M.; Arion, D.; Borkow, G.; Noronha, A.; Uddin, A. H.; Parniak, M. A.; Damha,
 M. J. *Biochemistry* 1998, *37*, 7478-7486.
- (7) Usher, D. A.; McHale, A. H. *Proc Natl Acad Sci U S A* **1976**, *73*, 1149-1153.
- Premraj, B. J.; Raja, S.; Bhavesh, N. S.; Shi, K.; Hosur, R. V.; Sundaralingam, M.;
 Yathindra, N. *Eur J Biochem* 2004, *271*, 2956-2966.
- (9) Hannoush, R. N.; Damha, M. J. J Am Chem Soc 2001, 123, 12368-12374.
- (10) Lorsch, J. R.; Bartel, D. P.; Szostak, J. W. Nucleic Acids Res 1995, 23, 2811-2814.
- (11) Steitz, T. A. *Nature* **1998**, *391*, 231-232.
- (12) Robertson, M. P.; Scott, W. G. Science 2007, 315, 1549-1553.
- (13) Leontis, N. B.; Lescoute, A.; Westhof, E. Curr Opin Struct Biol 2006, 16, 279-287.
- (14) Lehman, N.; Unrau, P. J. *J Mol Evol* **2005**, *61*, 245-252.
- (15) Ekland, E. H.; Szostak, J. W.; Bartel, D. P. Science 1995, 269, 364-370.