46. Visible platform training: The cues were removed and the location of the platform was made visible by a 6-inch-high mast topped with a circular blue cylinder (5 cm tall by 4 cm diameter). This flag was equally visible from all pool locations. The same mice used in the hidden platform training were trained to swim toward the platform at random locations with six consecutive trainings for eight consecutive days. They were allowed to swim for a maximum of 60 s and to remain on the platform for 30 s.
47. The cerebral cortices fixed in paraformaldehyde were pressed flat in 30 percent sucrose–phosphate-buffered saline (PBS) for 24 hours. The parietal region was freeze mounted and cryostat sectioned (15 μM). The slides were incubated in cytochrome oxidase solution (0.5 mg/ml diaminobenzidine, 0.3 mg/ml cytochrome C, 0.2 mg/ml catalase) overnight at room temperature.
48. Paraformaldehyde-fixed brains were cryosectioned (15 μM) and stained with cresyl violet (0.5 percent).
49. Mice were perfused first with 0.36 percent sodium sulfide in phosphate buffer (10 min) and then neutral buffered formalin (15 min) and processed according to R. S. Sloviter [*Brain Res. Bull.* **8**, 771 (1982)].
50. The acetylcholinesterase histochemistry was carried out on 50 μM vibratome sections; M. J. Karnovsky and L. J. Roots, *Histochem. Cytochem.* **12**, 219 (1969).
51. Brains fixed with paraformaldehyde (4 percent) were sectioned with a vibratome (50 μM) and transferred to 24-well culture dishes. After hydrogen peroxide (0.3 percent) incubation (15 minutes), sections were washed and then blocked in 10 percent goat serum, 0.1 percent Triton X-100 in PBS for 1 hour. Sections were incubated with the MAP1 mouse monoclonal antibody (Sigma) (1:100 dilution) overnight at 4°C. After washing, the sections were incubated in horseradish peroxidase–conjugated antibody to mouse immunoglobulin G (Boehringer) diluted 1:200 in ×0.5 block for 1 to 2 hours. Three further washings preceded DAB development with glucose oxidase.
52. A P2 fraction was prepared from six wild-type mouse brains according to J. W. Gurd, P. Gordon-Weeks, and W. H. Evans [*J. Neurochem.* **39**, 1117 (1982)] Synaptosomes were prepared with the Percoll gradient method of P. R. Dunkley, P. E. Jarvic, J. W. Heath, G. J. Kidd, and J. A. P. Rostas [*Brain Res.* **372**, 115 (1986)] and lysed in tris-acetate (5 mM, pH 8.0) and centrifuged at 30,000*g* for 30 minutes. The supernatant was concentrated by ultrafiltration to 2 ml (synaptosome soluble). The sedimented material was resuspended in 1.5 ml of tris-acetate (synaptic junctional complex), and 50 mM Hepes (pH 7.5) with protease inhibitors and Triton X-100 was added to 10 ml and 1 percent, respectively. After motor homogenization, gentle agitation for 20 minutes and rehomogenization, the extract was centrifuged at 100,000*g* for 30 minutes. The supernatant (synaptic plasma membranes) was concentrated to 4 ml by ultrafiltration, and the sedimented material (postsynaptic density) was resuspended in 1 ml of Hepes. Samples containing 0.4 percent of each fraction were subject to immunoblotting with antiserum to pp59^{fyn} according to manufacturers instruction (UBI).
53. We thank R. Axel and T. Jessell for comments on earlier drafts of this manuscript; R. Hawkins, I. Kupfermann, and R. Morris for advice on behavior experiments and their statistical analyses; B. Stanfield, D. Amaral, and J. Dodd for help on the anatomy; M. Osman and D. Burton for animal care; S. Mack and C. Lam for figures; H. Ayers and A. Krawetz for preparation of the manuscript; S. Goff for *abl*$^-$ mice; and J. Bolen for *fyn* and *yes* antisera. Supported by NIMH grant MH45923, NIHA grant AG08702, and NICHD grants HD24875 and HD25326; by NIH postdoctoral fellowship HD07453 (P.L.S.); and by the Howard Hughes Medical Institute (E.R.K.; P.S., T.J.O., and S.G.N.G).

28 September 1992; 25 November 1992

Selection of a Ribozyme That Functions as a Superior Template in a Self-Copying Reaction

Rachel Green and Jack W. Szostak

The *sunY* ribozyme is derived from a self-splicing RNA group I intron. This ribozyme was chosen as a starting point for the design of a self-replicating RNA because of its small size. As a means of facilitating the self-replication process, the size of this ribozyme was decreased by the deletion of nonconserved structural domains; however, when such deletions were made, there were severe losses of enzymatic activity. In vitro genetic selection was used to identify mutations that reactivate a virtually inactive *sunY* deletion mutant. A selected mutant with five substitution mutations scattered throughout the primary sequence showed greater catalytic activity than the original ribozyme under the selection conditions. The *sunY* ribozyme and its small selected variant can both catalyze template-directed oligonucleotide assembly. The small size and reduced secondary structure of the selected variant results in an enhancement, relative to that of the original ribozyme, of its rate of self-copying. This engineered ribozyme is able to function effectively both as a catalyst and as a template in self-copying reactions.

The idea of an RNA polymerase composed of RNA is central to current theories of the origin of life, since such a molecule could replicate autocatalytically. Such a self-replicating RNA molecule is commonly referred to as an RNA replicase. Because of the similarity between the chemistry catalyzed by group I introns (phosphodiester exchange reactions) and modern-day polymerases (phosphoanhydride-phosphodiester exchange reactions), we are attempting to use this class of ribozymes as a starting point from which to engineer an RNA replicase.

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Previous studies (*1, 2*) have demonstrated that the *Tetrahymena* and *sunY* ribozymes can catalyze the ligation of oligonucleotides on exogenous templates. However, an RNA replicase would have to function efficiently as both a catalyst and a template. Conflicting requirements constrain the evolution (or design) of an RNA molecule that must play two such different roles. Maximal enzyme activity would presumably be enhanced by a strong secondary and tertiary structure, so that the three-dimensional structure required for substrate binding and catalysis will form. In contrast, elongation of the growing chain would presumably be enhanced on an unstructured (unfolded) template, so that template structure would not interfere with substrate binding. It appears that an RNA replicase must exist in a delicate balance between the folded state necessary for catalysis, and the unfolded state necessary for template activity. Furthermore, the transition between the folded and unfolded states should not be highly cooperative, so that both states can coexist over a broad range of conditions.

It was necessary to determine whether the enzymatic and template activities were mutually exclusive. Ribozymes such as the *Tetrahymena* and *sunY* introns are efficient catalysts, but their degree of structure would be likely to impede the synthesis of a complementary RNA. In contrast, small ribozyme derivatives with minimal overall structure, such as deletion derivatives generated from *sunY* (*3*), while seemingly better suited to function as templates, have thus far appeared to be poor catalysts. We have therefore applied the technique of iterative in vitro selection to the task of isolating a small but highly active ribozyme variant that can function efficiently as a template.

In vitro selection of catalytically active variants. The *sunY* intron from bacteriophage T4 is 245 nucleotides (nt) in length (without the open reading frame) and is one of the smallest known self-splicing introns (*4*). Previous efforts to further decrease the size of this intron included the removal of phylogenetically nonconserved domains, because these were unlikely to be essential to ribozyme function. While stem loops P9.1 and P9.2 (*5*) of *sunY* could be eliminated from the ribozyme with only minor losses in activity (the resulting 180-nt molecule was subsequently referred to as the "original" ribozyme, Fig. 1A) (*3, 6*),

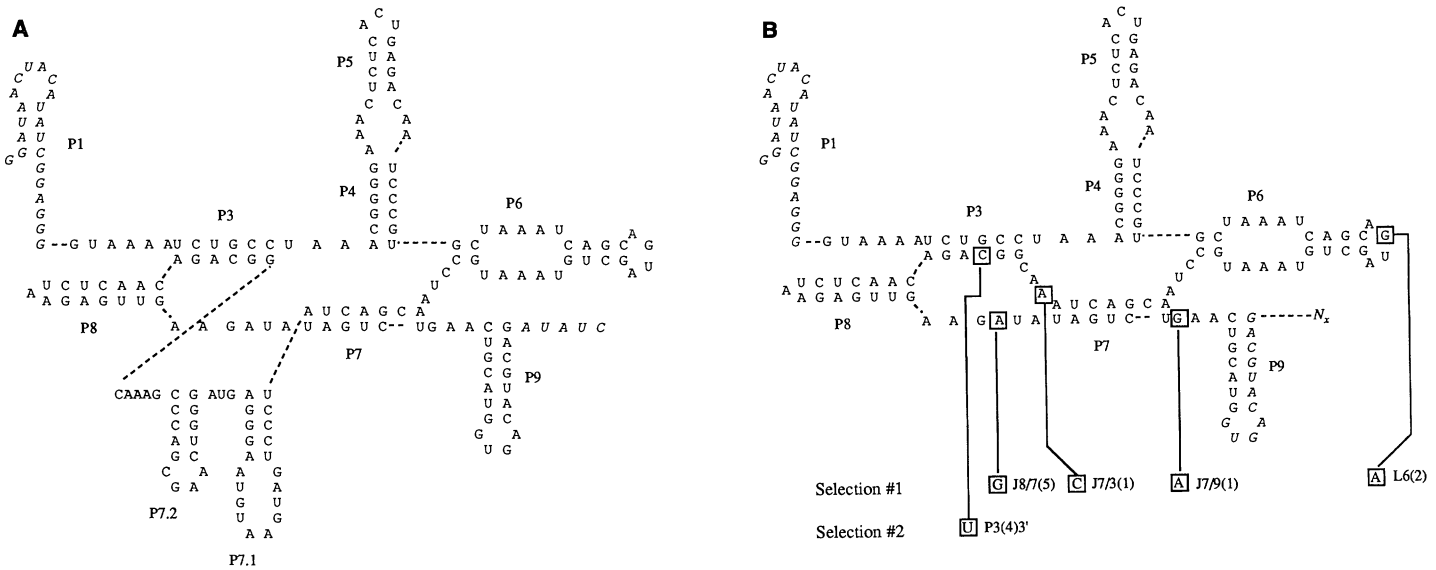

Fig. 1. Structure of original and deleted versions of the *sunY* ribozyme. (**A**) Secondary structure of original *sunY* intron derivative (*3, 4*). The 5′ and 3′ portions of the intron have been deleted and replaced with the heterologous sequences shown in italics. The partial stem loop structure, P1, represents the product of the first cleavage step in self-splicing. RNA with the partial P1 stem was used for intramolecular ligation experiments (*12*); a 5′ truncated derivative starting after P1 (GUAAA) was used for intermolecular ligation experiments (*14*). The triple helical domain of the *td* ribozyme was substituted for *sunY* sequences at P4(2), P4(3) and J6/7(2) to take advantage of the increased catalytic activity (*19*). (**B**) Secondary structure of the deleted version of *sunY* RNA with heterologous P1 sequence that was used for all intramolecular ligation experiments (*12*), including the selections; as above, a 5′ truncated molecule was used for intermolecular ligation experiments (*14*). Nucleotides in italics at the 3′ end of the intron were part of the constant region in selection 1; N_x represents the variable priming region added for each selection. The catalytic domain is identical to that of the original derivative except that P7.1 and P7.2 have been deleted leaving 5′ AAC as joining segment J7/3 (*3*). The mutations in the most active clone from each selection are indicated in boxes, with their position name indicated; the selected variant with all five mutations is referred to as the quintuple mutant or the selected ribozyme.

the deletion of stem loops P7.1 and P7.2 resulted in a 141-nt ribozyme that was much less active than the original molecule (Fig. 1B). This deletion derivative had almost no detectable activity when oligonucleotide-ligation activity was measured on an exogenous template.

We used in vitro genetic selection (*7*) to identify mutations that would functionally compensate for the loss of the stem-loop structures P7.1 and P7.2. A pool of RNA molecules was generated where 127 nt of the ribozyme domain were mutagenized (at 5 percent per position such that each variant has, on average, six mutations) by chemical synthesis (8) of a 171-nt DNA template containing a degenerate sequence (Fig. 1B) flanked by defined sequence primer binding sites. The gel-purified synthetic DNA was amplified by the polymerase chain reaction (PCR) and transcribed in vitro (9) to generate the *sunY* ribozyme core RNA flanked by primer binding sequences. We estimate that the starting pool of RNA was derived from about 2×10^{13} (*10*) independent DNA molecules. Such a pool should contain all possible single, double, triple, quadruple, and quintuple mutations within the mutagenized ribozyme domain (5.2×10^8, 9.1×10^6, 1.6×10^5, 2.8×10^3, and 4.9×10^1 of each, respectively) (*11*).

We selected from this pool of sequences those ribozymes best able to catalyze the ligation of an RNA "exon" (in this case, a synthetic oligonucleotide) to their own 5′ ends; this reaction was analogous to the reversal of the 5′ splice-site cleavage step in self-splicing and was dependent on a 5′-guanosine for catalysis. The ligated exon sequence acted as a tag that was used for recovering and amplifying active ribozymes in two sequential selection steps: oligonucleotide affinity chromatography and selec-

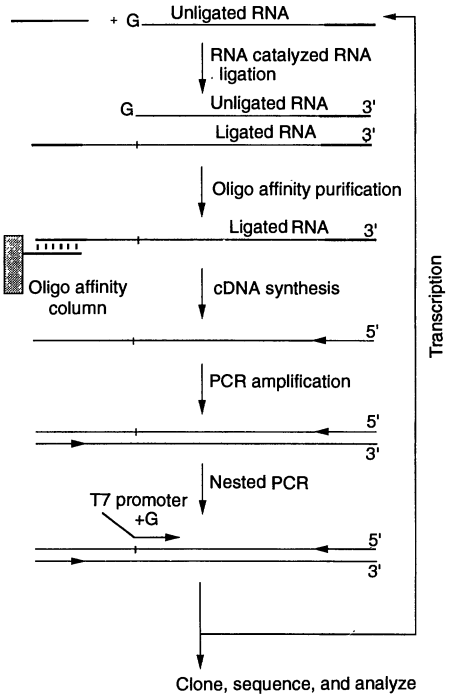

Fig. 2. Scheme for in vitro selection and amplification of active ribozymes. A pool of mutagenized RNA molecules at 0.5 μM was incubated with a short exon RNA sequence at 3 μM [r31: GGG-AGC-GAA-GGC-ACG-CCA-GCA-GGA-ACC-CUC-U or r36: GGC-GAA-U-CU-GAA-ACG-UUA-CAA-UCG-AAG-CAA-CCC-UCU, prepared by T7 transcription (*20*)] in 30 mM tris pH 7.4, 10 mM NH_4Cl, and specified concentrations of $MgCl_2$ at 40°C. Functional ribozyme variants catalyzed the ligation of the exon sequence to their 5′ end whereas nonfunctional variants remained unchanged. The RNA ligation reaction was incubated in 5 × SSC (saline sodium citrate) in 1 percent SDS with an oligonucleotide affinity column (Glen Research) with 19 bases complementary to the exon sequence (indicated with dashes). RNA molecules with the ligated exon sequence were retained by the column and eluted in water at 70°C. Eluted RNA's were amplified by reverse transcription and PCR with 3′ primers 23.25 (171-nt DNA) or 23.33 (185-nt DNA) and with 5′ primers, 34.11: GGA-CTG-CAG-GGG-AGC-GAA-GGC-ACT-CCA-GCA-GG-A-A or 31.32: GCT-CTA-GAG-GCG-AAT-CTG-AAA-CGT-TAC-AAT-C, annealing to the complement of the ligated exon sequence. A second round of PCR with a nested 5′ primer (43.12) recreated the unligated ribozyme sequence and added a T7 promoter to generate RNA for subsequent rounds of selection.

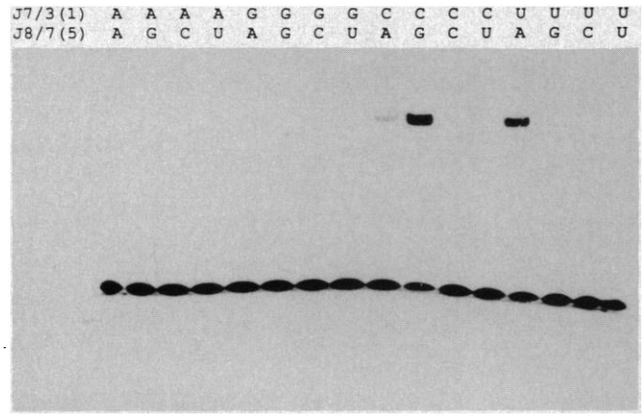

Fig. 3. Mutation-matrix establishing interaction between J7/3(1) and J8/7(5). Autoradiogram of a 20 percent acrylamide–7 M urea gel showing a single time point in the linear range for an intermolecular oligonucleotide-ligation reaction (*14*). Each lane corresponds to a different ribozyme with the designated combination of nucleotides at positions J7/3(1) and J8/7(5). Mutations were generated in the three-subunit *sunY* ribozyme (*1*) by transcribing synthetic oligonucleotides (*20*).

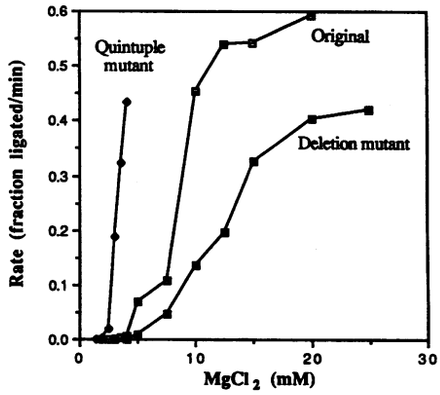

Fig. 4. Effect of Mg(II) on the intramolecular ligation activity of the different ribozyme variants. The rate of ligation (expressed as the fraction of ^{32}P end–labeled exon ligated to the ribozyme per minute) catalyzed by the different ribozymes is plotted as a function of the concentration of $MgCl_2$ (mM); each point represents a rate derived from a time course of the reaction (*12*). The reaction being catalyzed is the ligation of an oligonucleotide to the 5′ end of the ribozyme.

tive PCR. Iterated cycles of selection and amplification resulted in the purification of catalytically active species from the initial population (Fig. 2). The stringency of the selection was increased at each cycle by reducing the amount of time allowed for the ligation reaction to occur and by reducing the $MgCl_2$ concentration in the ligation buffer.

After three rounds of selection and amplification, the activity of the pool had so increased that the pool was 25 times more active than the initial deletion derivative and half as active as the original molecule when assayed in 5 mM $MgCl_2$ in a ligation reaction analogous to that used for the selection (*12*). Of 21 individual variants that were cloned and assayed for catalytic activity, the most active clone (the quadruple mutant indicated in Fig. 1B) was 100 times more active than the initial deletion derivative and twice as active as the original ribozyme.

To determine if the deletion derivative had been saturated for simple mutations that would result in increased catalytic efficiency, we performed a second selection with the quadruple mutant as the starting molecule. A 185-nt oligonucleotide (with a core domain mutagenized such that each variant had, on average, eight mutations) was chemically synthesized, gel purified, PCR amplified and transcribed in vitro as above (*13*). The 3′ primer site was changed to avoid contamination from the previous selection. We estimate that this pool was derived from 5×10^{12} molecules of DNA, and that it also contained all possible quintuple mutant combinations.

After four cycles of selection and amplification (Fig. 2), the resulting pool was 1.5 times more active than the starting quadruple mutant. Additional stringent rounds of selection did not increase the activity of the pool. Sequencing and activity analyses of 30 individual clones revealed only one mutation which resulted in a significant increase in the activity of the ribozyme. The point mutation P3(4)3′ U (Fig. 1B) results in an increase (two times greater) in activity compared to that of the starting quadruple mutant, as determined by an intramolecular ligation assay with 4 mM $MgCl_2$ (*12*). Under these conditions, the final quintuple mutant resulting from two different serial selections is 350 times more active than the original unselected deletion derivative. Because of the complexity of the RNA pool used for the second selection, the failure to recover stronger "up" mutations indicates that the intron was saturated for point substitutions (within five bases of the starting molecule of the second selection) that significantly increase intramolecular ligation activity. Further increases in activity may require more substantial changes, such as stem extensions or insertions, not encompassed by our mutational strategy.

Tertiary interaction revealed by selected mutations. Sequence analysis of the 16 most active variants from the initial selection revealed a clustering of mutations in theoooo single-stranded loop and joining regions of the intron. Most strikingly, position J7/3(1) (Fig. 1B) mutated from A in 12 of the 16 sequences as follows: nine times to U and three times to C. Furthermore, a highly suggestive covariation was observed between positions J7/3(1) and J8/7(5): when J7/3(1) changed to a C (three times), J8/7(5) changed to a G; when J7/3(1) changed to a U, no concomitant change was seen at J8/7(5). The most active RNA's examined were the three clones with the double mutation, suggesting the possibility of an interaction between these two positions. We tested this idea by generating the full matrix of nucleotide combinations at these two positions with the multisubunit form of the enzyme and by performing an intermolecular ligation assay (*14*). The increase in activity of the double mutant J7/3(1) C: J8/7(5) G variant is much greater (39 times) than the product (0.18) of the changes in activity of the two single mutants, J7/3(1) C (1.8 times) and J8/7(5) G (0.1 time) (Fig. 3). The second most active nucleotide combination, U:A, corresponds to the point substitution J7/3(1) U observed in 9 of the 16 sequences examined.

Although the two most active combinations (C·G and U·A) seem to follow Watson-Crick base pairing rules, the fact that the inverse base pairs (G·C and A·U) are not functional indicates that this interaction is more complicated than a simple canonical, coplanar base pair. Michel and Westhof (*15*) have proposed, for some group Ic introns, that a noncoplanar, homopurine hydrogen bonding interaction exists between position 6 of the purine at J7/3(1) and position 1 of the purine at J8/7(5); these introns lack P7.1 and P7.2 as does the *sunY* deletion derivative. While the covariation observed in the selected variants does not follow the same homopurine base pairing pattern, the data are consistent with the model in suggesting that these two positions are in close proximity to one another in the folded intron.

Enhanced stability of the selected quintuple mutant. If deletion of the P7.1 and P7.2 stem loops destabilizes the *sunY* ribozyme, it seems reasonable that the selected mutations would restore activity by increasing the stability of the folded state of the ribozyme. We have therefore examined the stability of the original, the deleted, and the selected variants of the ribozyme by measuring the catalytic activity as a function of Mg(II) concentration and by assaying for subunit association in a multisubunit form of the ribozyme.

Divalent metal ions have a dual role in

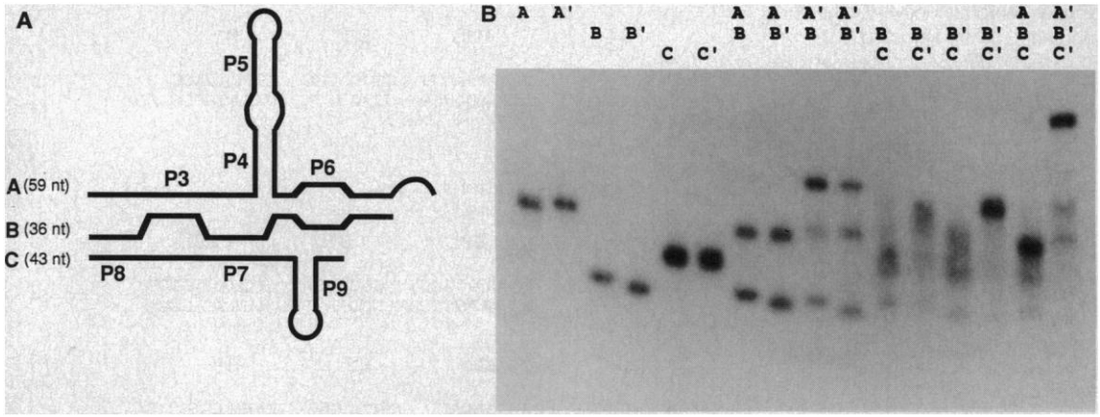

Fig. 5. Gel mobility shift assay of ribozyme subunit association. (**A**) Multisubunit ribozyme derived from the deletion derivative (Fig. 1B) with fragments A, B, and C of lengths 59, 36, and 43 nucleotides, respectively; when the five selected mutations are incorporated into these subunits, they are referred to as A′, B′, and C′. (**B**) The designated ^{32}P end-labeled subunits were combined at 0.25 μM in 100 mM tris 7.5, 0.2 mM EDTA, 10 mM NaCl, 10 mM $MgCl_2$, and 6 percent glycerol; samples were heated to 80°C and slow cooled to 40°C. Annealed samples were analyzed on an 8 percent native acrylamide (30:1 acrylamide:bis-acrylamide) gel containing 100 mM tris-Hepes 7.5, 0.1 mM EDTA, and 10 mM $MgCl_2$ (4°C at 14 V/cm) (*18*).

group I introns, being directly involved in both structural stability and catalysis (*16*). In that Mg(II) can act as a general suppressor of intron mutations and deficiencies, the Mg(II) dependence of different ribozyme variants can be used as an indicator of their relative structural and catalytic strength. We compared the relative Mg(II) dependence of the selected quintuple mutant, the unselected deletion derivative and the original ribozymes in a single turnover intramolecular ligation assay, similar to that used during selection (*12*). The selected quintuple mutant was the most active intron derivative (Fig. 4) in all Mg(II) concentrations tested and was quite active even at Mg(II) concentrations where the original ribozyme was virtually inactive. Indeed, it was difficult to measure the catalytic activity of the quintuple mutant in the presence of more than 4 mM Mg(II) because of the high reaction rate. However, the deletion mutant does not reach maximal activity until 20 mM Mg(II).

The *sunY* original ribozyme can be divided into three RNA subunits, A, B, and C, of 59, 75, and 43 nt (Fig. 5A); these fragments can associate to function effectively as a ribozyme (*1*). A deleted version of the multisubunit enzyme was constructed in which fragment B was reduced from 75 to 36 nt by the deletion of stem loops P7.1 and P7.2 (Fig. 5A). As expected, this shortened version of the multisubunit enzyme was virtually inactive. When the five selected point mutations were incorporated into the small multisubunit enzyme, its activity was increased so that it was only four times lower than that of the full-length multisubunit enzyme in an intermolecular ligation assay (*14*); this difference in activity was approximately the same as that between the original and quintuple mutant single-chain ribozymes with this assay. Furthermore, each mutation contributed to the increased activity of the three-piece ribozyme to the same extent as in the one-piece ribozyme. The J7/3(1):J8/7(5) interaction resulted in an activity approximately 50 times higher, and each of the other three mutations doubled the activity. The doubling of the activity from the L6(2) G to A loop mutation is somewhat surprising in that in the multisubunit enzyme the orientation of the P6 loop was not constrained by attachment at its 3′ end to the P6 stem. The most likely explanation for this effect is that the loop mutation improved the stacking of the L6 loop onto the P6 stem. This hypothesis is supported by the fact that a deletion of the L6 loop in the original or deleted version of the multisubunit ribozyme results in a significant decrease in catalytic activity (*17*). The similar effects of the selected mutations on the catalytic activity of the single-chain and the multisubunit ribozymes confirm the structural similarity of these ribozymes.

We used the gel mobility shift assay (*18*) to assess the effect of the selected mutations on the ability of the subunits to assemble into a complex. We initially expected that the base-paired regions P3, P6, P7, and P8 would dominate the interactions between fragments A and B, and B and C (and consequently A, B, and C). However, our results indicate that the base-pairing of these duplex regions is not sufficient for subunit association in this assay and show that the selected mutations have strong stabilizing effects on subunit association. This effect is seen in a comparison of the association of the deleted and selected versions of the subunits (Fig. 5B). The unselected fragments A, B, and C did not associate to any significant extent either in pairwise combinations, or all together. In contrast, each combination (A′B′, B′C′, and A′B′C′) of the selected mutant fragments (A′, B′, and C′) showed visible association, with the most dramatic gel shift resulting from the formation of the A′B′C′ complex.

The subunits containing the two selected mutations known to interact genetically (J7/3(1) C and J8/7(5) G) only resulted in a clear gel mobility shift when both partners of the interaction were present; that is, the B′C′ complex formed, whereas the B′C and BC′ complexes did not. The selected loop change, L6(2) A, resulted in increased association of fragment A′ with the other subunits (A′B, A′B′, and A′B′C′), in parallel with the activity data. This direct physical evidence for the increased ability of the selected ribozyme subunits to assemble into a complex suggests that these mutations stabilize the folded state of the single-chain ribozyme.

Superiority of the selected ribozyme as a template for self-copying. Having selected a revived version of the short *sunY* deletion mutant, we then examined its template properties. We expected the deletion to improve the template activity of the ribozyme by decreasing its size and, more significantly, its secondary structure (by removal of stem loops P7.1 and P7.2), but it was possible that the selected mutations, in stabilizing the folded state of the ribozyme, might have made it a correspondingly poorer template.

To test the combined enzymatic and template activities of the original and selected ribozymes, we first compared their ability to copy their own central domains (corresponding to fragment B) by template-directed oligonucleotide assembly. We focused on the region corresponding to fragment B because the deletion of P7.1 and P7.2 had both shortened and decreased the secondary structure of this part of the ribozyme. In our previous replication experiments (*1*), we had examined the copying of additional fragment C beyond that present in the ribozyme; here, there is no template other than the ribozyme itself. Under these conditions, any available template must result from unfolding of the active catalytic structure.

RNA oligonucleotides (approximately

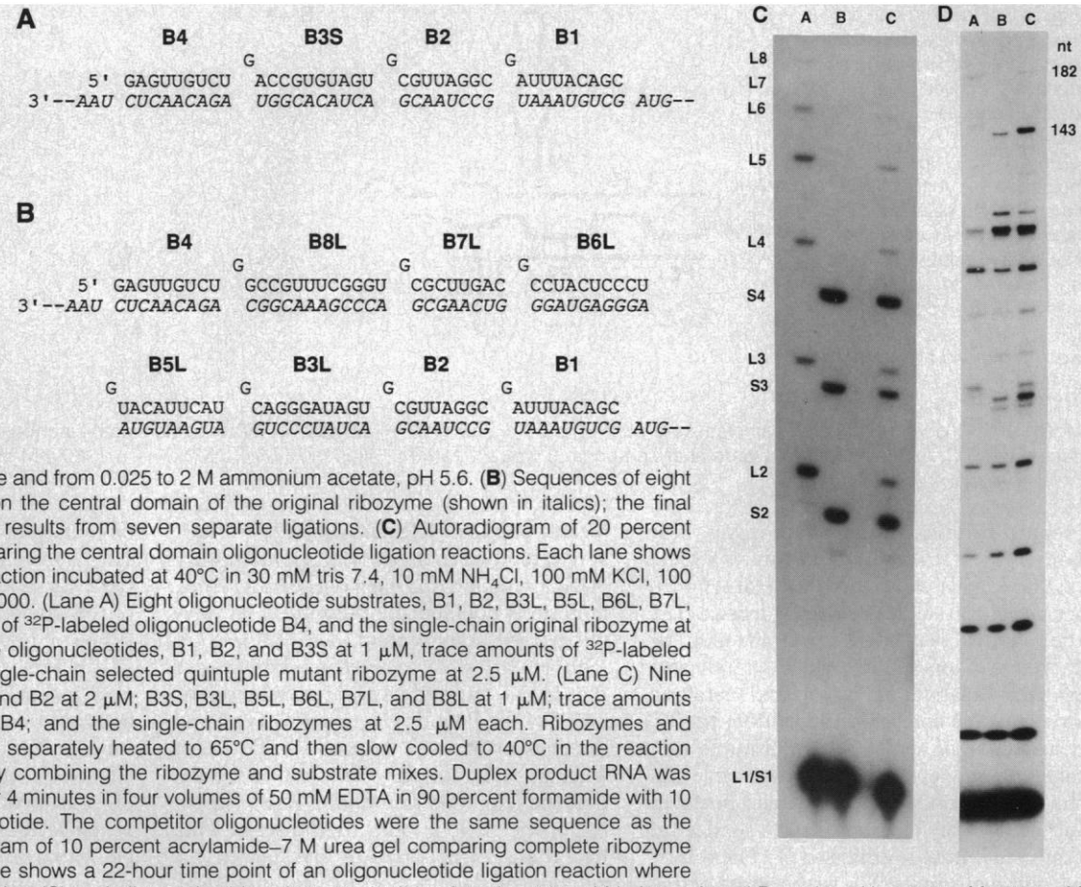

Fig. 6. Template activity of the original and selected ribozymes. (**A**) Sequences of four RNA oligonucleotides (B1 to B4) aligned on the central domain of the selected ribozyme (shown in italics); the final product is 36 nt in length and results from three separate ligations. Oligomer RNA's were synthesized chemically with phophoramidites (Peninsula, Milligen-Biosearch, and Chemgenes); after deprotection, the synthetic RNA was purified by high-performance liquid chromatography on an anion-exchange column (Dionex NA-100) with a gradient containing 10 percent acetonitrile and from 0.025 to 2 M ammonium acetate, pH 5.6. (**B**) Sequences of eight RNA oligonucleotides aligned on the central domain of the original ribozyme (shown in italics); the final product is 75 nt in length and results from seven separate ligations. (**C**) Autoradiogram of 20 percent acrylamide—7 M urea gel comparing the central domain oligonucleotide ligation reactions. Each lane shows the 15-minute time point of a reaction incubated at 40°C in 30 mM tris 7.4, 10 mM NH_4Cl, 100 mM KCl, 100 mM $MgCl_2$ and 9 percent PEG 8000. (Lane A) Eight oligonucleotide substrates, B1, B2, B3L, B5L, B6L, B7L, and B8L at 1 μM, trace amounts of ^{32}P-labeled oligonucleotide B4, and the single-chain original ribozyme at 2.5 μM. (Lane B) Four substrate oligonucleotides, B1, B2, and B3S at 1 μM, trace amounts of ^{32}P-labeled oligonucleotide B4, and the single-chain selected quintuple mutant ribozyme at 2.5 μM. (Lane C) Nine substrate oligonucleotides; B1 and B2 at 2 μM; B3S, B3L, B5L, B6L, B7L, and B8L at 1 μM; trace amounts of ^{32}P-labeled oligonucleotide B4; and the single-chain ribozymes at 2.5 μM each. Ribozymes and oligonucleotide substrates were separately heated to 65°C and then slow cooled to 40°C in the reaction buffer; reactions were started by combining the ribozyme and substrate mixes. Duplex product RNA was denatured by heating at 90°C for 4 minutes in four volumes of 50 mM EDTA in 90 percent formamide with 10 μM competitor RNA oligonucleotide. The competitor oligonucleotides were the same sequence as the ligated product. (**D**) Autoradiogram of 10 percent acrylamide–7 M urea gel comparing complete ribozyme self-copying reactions. Each lane shows a 22-hour time point of an oligonucleotide ligation reaction where buffer and ribozyme are identical to (C) and oligonucleotide substrates (*21*) are found at 0.25 μM in lanes A and B, and at either 0.25 μM or 0.5 μM in lane C; in each lane the 5′ terminal oligonucleotide substrate, C4, is ^{32}P-labeled and present in trace amounts. Lane A contains 18 oligonucleotide substrates, lane B contains 14, and lane C contains 21. The full-length ligated complementary products of both the original and selected ribozyme variants are indicated.

ten bases in length) complementary to fragment B of either the original or the small, selected quintuple mutant ribozyme were synthesized (Fig. 6, A and B). When the original (single chain) ribozyme was incubated with the eight oligonucleotides complementary to its central domain, 11 percent of the 5′ oligonucleotide was extended by at least one ligation reaction, but only ~0.4 percent was incorporated into full-length product in a 15-minute incubation. When the small selected ribozyme was incubated with the four oligonucleotides complementary to its central domain, 17 percent of the 5′ oligonucleotide was extended by at least a single ligation reaction, but approximately 20 times more full-length product (about 8 percent) was generated in the same time (Fig. 6C).

The function of these ribozymes as templates was separated from their function as enzymes in a template competition experiment; both the original and the short selected ribozyme were incubated with the complete set of nine oligonucleotides required to form complementary RNA for their central domains. In this experiment, the ribozyme activity available for the catalysis of oligonucleotide ligation on either template was equal. We observed the same 20 times higher rate of generation of full-length short (36 nt) versus long (75 nt) ligation product as in the separate reactions (Fig. 6C). This result suggests that template, and not ribozyme, availability is rate limiting under these reaction conditions.

We then investigated the contributions of template length and template structure to the difference in template activity between the selected and the original ribozymes. The effect of length was removed by comparing several four-oligonucleotide ligation reactions. Two four-oligonucleotide ligation reactions on the original template were less efficient than the four-oligonucleotide ligation on the short selected template. The ligation of four oligonucleotides (B5L, B6L, B7L, and B8L) complementary to the most structured region of the long template (P7.1 and P7.2) was at least three times less efficient than the ligation of four oligonucleotides (of the same length) on the short template (B1, B2, B3S, and B4); the ligation of four oligonucleotides (B6L, B7L, B8L, and B4) on a less structured region of the long template was 1.5 times less efficient. This analysis emphasizes the critical role that structure can play in the ability of an RNA molecule to act as a template.

Similar results were obtained when copying experiments were performed with substrate oligonucleotides complementary to the entire original and selected versions of the *sunY* ribozyme. When the original and selected versions of the single-chain ribozyme were incubated with their complementary oligonucleotides, roughly equivalent amounts of full-length product (182 nt and 143 nt, respectively) were generated in both reactions during a 22-hour incubation (Fig. 6D, lanes A and B). In a mixing experiment in which the templating abilities of these two versions of the ribozyme were compared, the small selected variant was copied into full-length product from 15 to 20 times more efficiently (Fig. 6D, lane C) than the original unselected variant, depending on the oligonucleotide substrate concentrations used.

We have shown that the selected quintuple mutant single-chain ribozyme functions as much as 20 times more effectively as a template than the equivalent unse-

lected original variant. These experiments demonstrate the self-copying capability of an RNA molecule acting as both catalyst and template. The ~10 nt long oligonucleotides that we used for these copying experiments are good substrates because they bind tightly to available template. However, this same property precluded their use as substrates for self-copying of the three-fragment enzyme, which they strongly inhibited. The use of shorter oligonucleotides (or even mononucleotides) will be essential to the development of a more general and polymerase-like copying reaction, with the potential for the replication of arbitrary template sequences (as opposed to a restricted set of template sequences complementary to the input oligonucleotides). Although the use of short oligonucleotide substrates is currently limited by their poor template binding properties, such substrates, by the same token, would not be expected to inhibit the three-fragment ribozyme. The use of in vitro selection to isolate ribozyme mutants capable of efficiently functioning with shorter oligonucleotides as substrates may therefore lead to a ribozyme capable of true self-replication.

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5. J. M. Burke *et al.*, *Nucleic Acids Res.* **15**, 7217 (1987). Paired regions are numbered and denoted as Pn (for example, P3); single-stranded regions joining two paired regions are denoted Jx/y, where x refers to the paired region of 5′ origin and y refers to the paired region of 3′ origin (for example, J7/3 joins P7 to P3); and loop regions found at the end of paired regions are denoted as Ln (for example, L6). Numbers in parentheses indicate the position within the specified region [for example, J7/3(1) is the 5′ most nucleotide found in J7/3]. In the *sunY* intron, the fourth nucleotide of J8/7 is an exception and is defined as J8/7(5) by its phylogenetic relationship to other group I introns.
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8. All oligo-deoxyribonucleotides were synthesized on a Biosearch 8750 programmable DNA synthesizer (Biosearch Inc., San Rafael, CA). Special precautions were taken during the synthesis of long mutagenized oligonucleotides (171- and 185-nt DNA's) for generating pools. Fresh reagents were placed in the machine and desiccating bags were added to the acetonitrile. The 171-nt fragment was synthesized according to a modified program that reduced the time of acid treatment used for removal of the dimethoxytrityl (DMT) protecting group. The 185-nt species was synthesized with $BF_3{\cdot}CH_3OH$ as a non-protic acid for DMT removal [M. J. Mitchell, W. Hirschowitz, F. Rastinejad, P. Lu, *Nucleic Acids Res.* **18**, 5321 (1990)] and a 7:2:1 [THF (tetrahydrofuran):pyridine:water] pulse preceded oxidation (and replaced capping) in an attempt to minimize O^6-guanosine adduct formation [R. T. Pon, N. Usman, M. J. Damha, K. K. Ogilvie, *Nucleic Acids Res.* **14**, 6453 (1986)]. Overall coupling efficiency for both oligonucleotides as assayed by trityl yield was 97 or 98 percent. After standard deprotection in NH_4OH (55°C, 16 hours), the full-length products were isolated on 6 percent acrylamide–7 M urea gels where they were visible by ultraviolet shadowing as faint bands surrounded by lower and higher molecular mass products. The final yield of gel-isolated DNA was 90 μg of the 171-nt DNA and 188 μg of the 185-nt DNA (from two separate syntheses).
9. The synthetic 171-nt species consisted of a central domain of 127 nt (nonitalicized region in Fig. 1B) mutagenized at 5 percent and flanked by constant PCR priming regions (at the 5′ end, 5′-GGA-TAA-CTA-CAT-ATC-GGA-GGG and at the 3′ end, 5′-GTG-ACA-TGC-AGG-ATC-CTC-GAA-TT). The gel-purified DNA was amplified (PCR) in 10 ml of reaction mix (divided into 100-μl samples) containing 5 μM 43.12: GGG-ATT-**AAT-ACG-ACT-CAC-TAT-AG**G-ATA-ACT-ACA-TAT-CGG-AGG-G (T7 promoter shown in boldface), 2 μM 23.25: AAT-TCG-AGG-ATC-CTG-CAT-GTC-AC, 200 μM each dATP, dCTP, dGTP and dTTP, 10 mM tris 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin and 10 units Taq/100 μl. Eight cycles of amplification were performed at the following temperatures: 45 seconds at 94°C, a 2 minute approach to 45°C followed by 2 minutes at 45°C and 3 minutes at 72°C. Five pool-equivalents of the PCR DNA (10 μg) were transcribed by T7 RNA polymerase in 50 mM tris (pH 7.9), 26 mM $MgCl_2$, 3 mM spermidine, 0.01 percent Triton X-100, 5 mM DTT (dithiothreitol), and 5 mM each (ATP, CTP, GTP, and UTP) to generate the initial RNA pool. The RNA was isolated on a 6 percent denaturing acrylamide gel.
10. The fraction of the synthetic oligonucleotide that was copied into double-stranded DNA was determined as follows. A single round of primer extension was performed with limiting (5′-^{32}P)-labeled primer (23.25 or 23.33), excess DNA (171 or 185 nt), and 2.5 units of Taq polymerase per 100 μl of reaction (in standard PCR buffer conditions). Only 2.2 percent of the 90 μg of gel-isolated 171-nt DNA could be copied into full-length complementary product, reducing total pool complexity to 2 μg (or 2×10^{13} molecules) after amplification. Approximately 0.3 percent of the 188 μg of gel- isolated 185-nt DNA could be copied into full-length complementary product, resulting in a total pool complexity of 500 ng (or 5×10^{12} molecules) after amplification. Chemical lesions generated by extensive DNA synthesis cycles were presumably responsible for the poor quality of the long synthetic oligonucleotides.
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12. The intramolecular ligation assay measured the ligation of an RNA oligonucleotide to the 5′ end of the intron containing a partial P1 stem. Each reaction contained 0.5 μM ribozyme, less than 10 nM ^{32}P end–labeled RNA primer (r31 or r36), 30 mM tris 7.4, 10 mM NH_4Cl and the designated $MgCl_2$ concentration. The ribozymes were first incubated for 10 minutes at 50°C in the buffer, and the reactions were then started by the addition of primer. During incubation at 50°C, time points were taken by stopping the reaction in 7 M urea, 10 mM tris 7.5, 25 mM EDTA, xylene cyanol (0.1 percent) and bromophenol blue (0.1 percent). The ligated products were separated from unreacted material on denaturing polyacrylamide gels and were quantitated on a Betascope 603 Blot Analyzer (Waltham, MA).
13. The synthetic 185-nt DNA contained a central domain of 139 nt mutagenized at 6 percent and flanked by constant PCR priming sites (5′ end identical to 171-nt DNA and at the 3′ end after P9, 5′-ATA-CTG-CCA-GTA-GCA-TAT-GAA-TT). Gel-purified DNA was amplified by PCR in 100-ml portions as in (*9*), except that the primers were 43.12 at 5 μM and 23.33: AAT-TCA-TAT-GCT-ACT-GGC-AGT-AT at 2 μM and 2.5 units of Taq per 100 μl. Thirteen temperature cycles were performed manually (with 7-ml portions) in water baths; 5 minutes at 96°C, 5 minutes at 42°C, and 7 minutes at 72°C. RNA was transcribed and isolated as in (*9*).
14. The intermolecular ligation assay measured the ligation of an RNA oligonucleotide to a partial stem-loop structure separate from the ribozyme (*2*). Each reaction contained each ribozyme (or ribozyme subunit at 0.5 μM), 5 μM substrate (^{32}P-labeled 6-nt fragment: GAG-GCU and hairpin loop r21: GGA-UAA-CUA-CAU-AUC-GGC-CUC), 30 mM tris 7.4, 10 mM NH_4Cl, 100 mM KCl, and 100 mM $MgCl_2$. The ribozyme was heated in the buffer to 80°C and slow cooled to 40°C. The reaction was started by resuspending the dried substrate in the ribozyme buffer mixture and incubating at 40°C. Time points were taken and quantitated as in (*12*).
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21. Eighteen substrate oligonucleotides complementary to the original ribozyme variant are listed starting from the 5′ end of the intron; A1-GAG-AUUUUAC, A2-GCCCGUUUAGGC, A3-GGAGA-GUUUC, A4-GUGUCUCAGU, A5-GUAGCACGG-GAU, A6L-GUACUGCUGAUU, B1, B2, B3L, B5L, B6L, B7L, B8L, BC1-GUUAGAGUUGUCU, C1-GCUUCAACUC, C2L-GUCAGACUAUAU, C3-GC-ACCAUGCAGU, C4-GAUAUCUGCAUGU. Fourteen substrate oligonucleotides complementary to the selected ribozyme variant are listed starting from the 5′ end of the intron; A1, A2, A3, A4, A5, A6S-GUAUUGCUGAUU-B1, B2, B3S, BC1, C1, C2S-GUUAGACUAUAC-C3, C4. In the mixing experiments, twice as much of the oligonucleotides complementary to both ribozyme variants were supplied as of oligonucleotides complementary to only one of the variants. Oligonucleotide C4 was ^{32}P end–labeled in the complete ribozyme copying experiments (Fig. 6D).

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