Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2465–2469, March 1995
Biochemistry

Total chemical synthesis of a ribozyme derived from a group I intron

SUSAN K. WHORISKEY*†, NASSIM USMAN‡§, AND JACK W. SZOSTAK*¶

*Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114; and ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02138

Communicated by Paul Schimmel, Massachusetts Institute of Technology, Cambridge, MA, December 9, 1994

ABSTRACT **We describe the complete chemical synthesis of a ribozyme that catalyzes template-directed oligonucleotide ligation. The specific activity of the synthetic ribozyme is nearly identical to that of the same enzyme generated by *in vitro* transcription with T7 RNA polymerase. The ribozyme is derived from a group I intron and consists of three RNA fragments of 36, 43, and 59 nt that self-assemble to form a catalytically active complex. We have site-specifically substituted ribonucleotide analogs into this enzyme and have identified two 2′-hydroxyl groups that are required for full catalytic activity. In contrast, neither the 2′-hydroxyl nor the exocyclic amino group of the conserved guanosine in the guanosine binding site is necessary for catalysis. By allowing the ribozyme to be modified as easily as its substrates, this synthetic ribozyme system should be useful for testing specific hypotheses concerning ribozyme–substrate interactions and tertiary interactions within the ribozyme.**

The chemical synthesis of RNA has several advantages over synthesis by transcription, primarily the potential for "atomic mutagenesis" in which individual atoms or chemical groups are altered or removed by the site-specific incorporation of nucleotide analogs. Advances in the solid-phase chemical synthesis of RNA (1) have allowed the complete chemical synthesis of small ribozymes, including 19- and 35-nt hammerhead ribozymes (1, 2) and a 59-nt hairpin ribozyme (3). The roles of individual 2′-hydroxyl groups, exocyclic amino groups, and N-7s in the hammerhead ribozyme have been examined by the synthesis of RNAs containing deoxynucleotides (1, 4), purine riboside (5), inosine (6), and 7-deazaadenosine (7). Other synthetic RNAs have also been reported in which analogs such as 2-aminopurine (8), inosine (9), and 2′-fluoro (10, 11), 2′-amino (12, 13), and 2′-*O*-methyl nucleotides (14) have been incorporated. The largest chemically synthesized RNA that has been reported is the 77-nt $tRNA^{Met}$; however, its methionine-acceptance activity was only 11%. Incompletely deprotected species may have contributed to this low biological activity (15).

The group I and group II self-splicing introns and the M RNA of RNase P are larger ribozymes, typically >200 nt long. These ribozymes are beyond the reach of current synthetic methods and have generally been synthesized by the transcription of DNA templates by T7 RNA polymerase. However, certain intrinsic properties of T7 RNA polymerase are undesirable (16) including (*i*) a strongly preferred 5′ sequence, (*ii*) the addition of nontemplated bases at the 3′ end of the transcript, and (*iii*) the inability of the polymerase to incorporate ribonucleoside analogs into specified positions in a sequence [although this last limitation has been partially relieved by the methods described by Moore and Sharp (17)].

One solution to the problem of the chemical synthesis of the larger ribozymes would be to generate multisubunit versions of these ribozymes, composed of sets of smaller RNA fragments. We have reported the design of a ribozyme based upon the *sunY* group I intron that is composed of three RNA fragments generated by T7 transcription that anneal to form an active enzyme (18). This ribozyme consists of fragments of 43, 59, and 75 nt; the chemical synthesis of these fragments would still be technically difficult. In a series of experiments directed toward the design of an RNA replicase, we have generated a highly active smaller version of this three-subunit ribozyme composed of RNA fragments of 36, 43, and 59 nt (19). Here, we report the chemical synthesis of these RNA fragments, procedures for their purification, and their assembly to form an active ribozyme. We also describe derivatives of these fragments with ribonucleotide analog substitutions at critical positions within the catalytic core and the effects of these substitutions on the activity and stability of the corresponding ribozymes.

MATERIALS AND METHODS

Automated Chemical Synthesis. RNA fragments were synthesized by using ribonucleoside phosphoramidites made by the method of Scaringe *et al.* (2) or using phosphoramidites purchased from Chem-Genes (Waltham, MA) or Milligen/Biosearch. Automated chemical synthesis was conducted on a Pharmacia Gene Assembler or on a Milligen/Biosearch Cyclone on a 1.0-μmol scale by using 1000-Å (pore size) CPG supports. A standard DNA synthesis protocol was used for RNA synthesis except that the coupling time was extended to 12 min due to the bulky silyl groups (2). An average coupling efficiency of 98% was obtained during the chemical synthesis of each of the fragments. The oligoribonucleotides were cleaved from the CPG support by incubating the beads in 3 ml of ethanolic ammonia at 55°C for 16 h. This step also removed the *N*-acyl and β-cyanoethyl protecting groups. To remove the silyl protecting groups, the ethanolic ammonia supernatant was evaporated to dryness, resuspended in 50 μl of ethanol/water, 1:1 (vol/vol), with 20 μl of 1 M tetrabutylammonium fluoride per silyl group, and incubated at room temperature for 24 h. The oligoribonucleotides were desalted on a Qiagen 500 anion-exchange cartridge (Qiagen, Chatsworth, CA.). The column was prewashed with 10 ml of 50 mM triethylammonium bicarbonate (pH 7.0) and the sample was loaded. After a 10-ml wash with 50 mM triethylammonium bicarbonate (pH 7.0), the RNA was eluted with 2 M triethylammonium bicarbonate (pH 7.5) and dried repeatedly.

HPLC Purification. After deprotection, the RNAs were purified by gel electrophoresis on a 6% polyacrylamide/8 M urea gel. UV shadowing was used to visualize a major band of the expected size. Depending upon how much material was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†Present address: Cubist Pharmaceuticals, Inc., 24 Emily Street, Cambridge, MA 02139.
§Present address: Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Place, Boulder, CO 80301.
¶To whom reprint requests should be addressed.

loaded on the gel and the quality of the synthesis and deprotection, a ladder of smaller molecules could be seen. The major band was cut out of the gel, labeled with polynucleotide kinase and [γ-^{32}P]ATP, electrophoresed on a 6% polyacrylamide/8 M urea gel, and exposed for autoradiography. By this analysis, the labeled material appeared to be a single band. However, HPLC analysis revealed many species (multiple peaks) that probably represent incompletely deprotected and/or less than full-length molecules that the previous gel electrophoresis step was unable to remove. After investigating a number of HPLC purification protocols, the use of a Dionex OmniPac NA100 column with the following buffer system appeared to give optimal results. Buffer A was 0.2 M NaCl/25 mM NaOH and buffer B was 2.0 M NaCl/25 mM NaOH. Samples were loaded in buffer A and eluted with a gradient of 0–100% B in 20 min. An alkaline buffer system was used to ensure that the RNAs were fully denatured during chromatography, since RNA–RNA interactions may impede purification. The eluted RNAs were rapidly neutralized by dilution into Tris·HCl to minimize alkaline hydrolysis and were then desalted and concentrated by ethanol precipitation. A trial elution was first done to determine the retention time of the molecule on the column. For the trial run, 2–10 μg of the gel-purified material was loaded; for purification, 100–150 μg of gel-purified material was loaded. The central fraction of the major peak was collected, leaving behind the leading and trailing edges. There was variation from molecule to molecule but generally the major peak was $\approx$40% of the total area of all the peaks.

Partial Hydrolysis. HPLC-purified fragments were 5′-end-labeled with T4 polynucleotide kinase and hydrolyzed in 10 μl containing 5 μg of tRNA and 50 mM sodium bicarbonate at 90°C for 10 min. The reactions were stopped by adding an equal volume of 90% (vol/vol) formamide/0.05% bromophenol blue, heated for 1 min at 90°C, and analyzed by electrophoresis on a 7 M urea/15% polyacrylamide gel.

Ligation Assay. The 6-nt RNA primer was chemically synthesized and radioactively labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP. The 28-mer substrate RNA and all enzymatically synthesized ribozyme fragments were prepared by using T7 RNA polymerase (16). Ribozyme reaction mixtures contained 0.5 μM enzyme (each of the three fragments at 5 μM) and 5.0 μM substrate in 30 mM Tris·HCl,

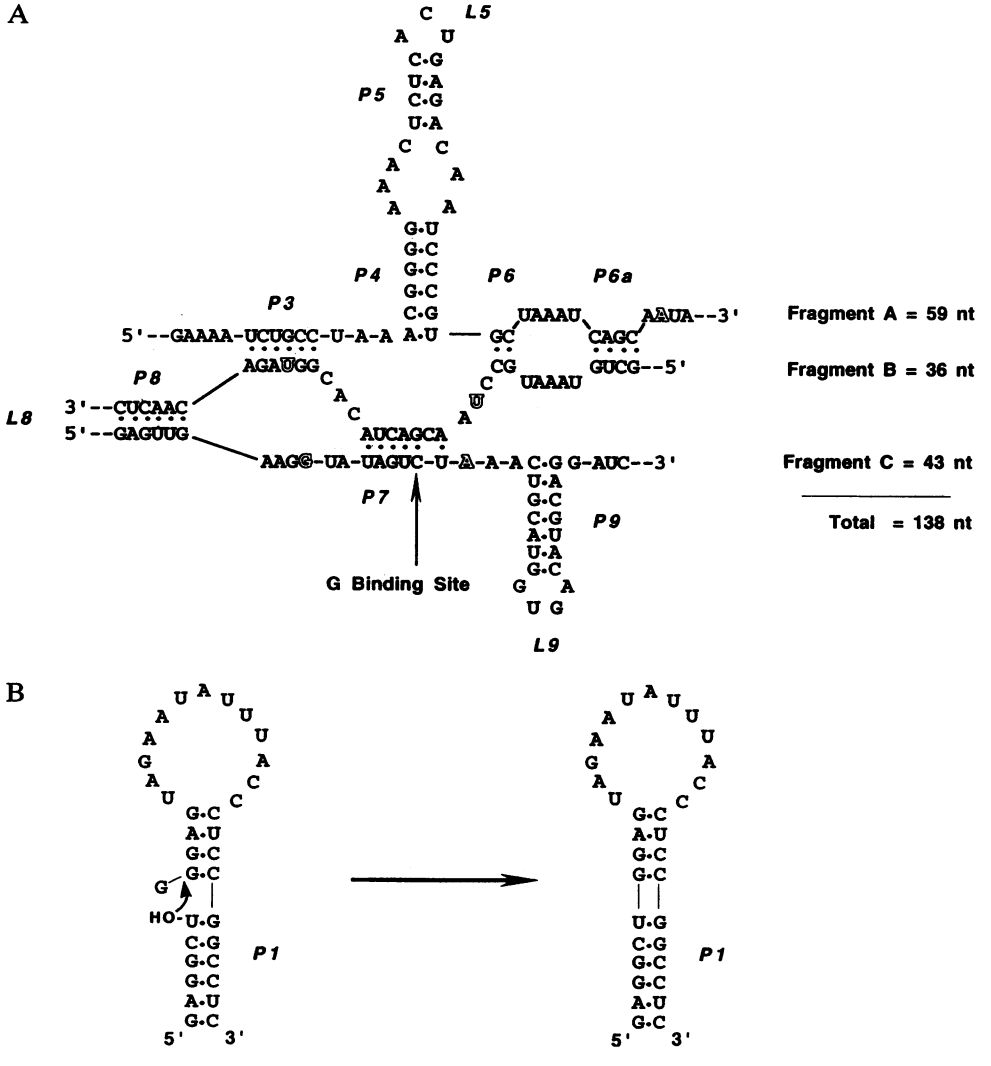

FIG. 1. Multisubunit *sunY* ribozyme and its substrate. (*A*) Sequence and secondary structure of the three-subunit *sunY* ribozyme. The conserved core of the enzyme minus the P7.1 and P7.2 regions of the molecule was synthesized as three fragments (termed fragments A, B, and C) of 59, 36, and 43 nt, respectively, that have short regions of complementarity as shown. The sequence of this ribozyme differs at 10 positions from the wild-type *sunY* ribozyme; 5 changes correspond to bases found in the bacteriophage *td* intron that result in enhanced splicing (21) and ligation (18) activity. The 5 bases shown in outline type are mutations resulting from an *in vitro* selection for increased activity of the small *sunY* enzyme (19). (*B*) The RNA ligation substrate and products. The 6-nt RNA primer is 5′-end-labeled with ^{32}P and is annealed to a 28-nt RNA to form an interrupted stem–loop structure. The ribozyme catalyzes a phosphodiester exchange reaction resulting in the release of guanosine and the formation of a 33-nt uninterrupted product.

pH 7.4/10 mM NH_4Cl/150 mM $MgCl_2$/0.4 M KCl/10% (wt/vol) polyethylene glycol, M_r 8000. The enzyme was incubated in buffer at 58°C and slow-cooled to 45°C. The reaction was started by the addition of substrate RNA. To stop the reactions, 2.5 μl was removed at the desired time points and added to 2.5 μl of 90% formamide/10 mM Tris·HCl, pH 7.4/1 mM EDTA/0.1% bromophenol blue/0.1% xylene cyanol. After denaturing gel electrophoresis on a 20% polyacrylamide/7 M urea gel, the products were analyzed by autoradiography and quantitated on a Betagen (Waltham, MA) β scanner.

Measurement of Dissociation Constants. Binding constants were determined by a slight modification of a published procedure (20). To measure the dissociation constants of the fragment A–B or B–C complexes, samples containing trace concentrations (≈0.5 nM) of 5′-end-labeled fragment B were titrated with increasing concentrations of unlabeled A or C. To measure the dissociation constant of fragment A with the B–C complex, trace concentrations of labeled fragment B were completely driven into B–C complex with 1.5 μM fragment C, and the resulting labeled B–C complex was titrated with increasing concentrations of unlabeled fragment A. Samples were heated for 5 min at 85°C and cooled to 4°C over 40 min in 50 mM Tris·HCl, pH 7.5/0.1 mM EDTA/10 mM $MgCl_2$/6.0% (vol/vol) glycerol/0.05% xylene cyanol. Samples were loaded on a native 8.0% polyacrylamide gel [30:1 (wt/wt) acrylamide to *N,N′*-methylenebisacrylamide] and electrophoresed at 4°C. The running buffer was 100 mM Tris·Hepes, pH 7.5/0.1 mM EDTA/10 mM $MgCl_2$. The gel was quantitated on a Betagen Betascope and the data were analyzed by using CRICKETGRAPH and KALEIDOGRAPH software.

RESULTS

Catalytic Activity of the Synthetic Ribozyme. RNA sequences corresponding to fragments A, B, and C of the three-subunit ribozyme (Fig. 1) were synthesized by T7 RNA polymerase transcription and by chemical synthesis. The fragments were purified as described above and annealed to allow assembly into the complete ribozyme. We compared the catalytic activity of the chemically and enzymatically synthesized ribozymes (Fig. 2) for template-directed oligonucleotide ligation (Fig. 1*B*). The ribozymes were examined with a subsaturating substrate concentration of 5 μM. Under our standard assay conditions, the HPLC-purified chemically synthesized ribozyme catalyzed the ligation reaction at an initial rate of 0.020 min^{-1}, corresponding to a k_{cat}/K_m of 4.0×10^3 $min^{-1} \cdot M^{-1}$. This compares with an initial rate of 0.022 min^{-1} (k_{cat}/K_m of 4.4×10^3 $min^{-1} \cdot M^{-1}$) for the enzymatically synthesized ribozyme (Fig. 3). When the concentrations of $MgCl_2$ and KCl were reduced to 50 mM, the chemically synthesized ribozyme was still nearly as active as the enzymatically synthesized ribozyme (data not shown). The purification of the synthetic RNAs by a combination of gel electrophoresis and HPLC was necessary for the chemically synthesized ribozyme to attain this level of catalytic activity. Crude (unpurified) synthetic RNAs had <20% of the activity of the T7 transcripts, RNAs purified only by gel electrophoresis had ≈65% of the activity of the T7 transcripts, and RNAs purified by both gel electrophoresis and HPLC had >90% of the activity of the T7 transcripts. Synthetic RNAs can contain a variety of contaminants including incompletely deprotected species, molecules containing incorrect linkages due to impurities in the starting phosphoramidites, or less than full-length molecules. Some of these contaminants may comigrate with completely deprotected molecules during electrophoresis and may account for the lower activity of the gel-purified RNA compared to the HPLC-purified material.

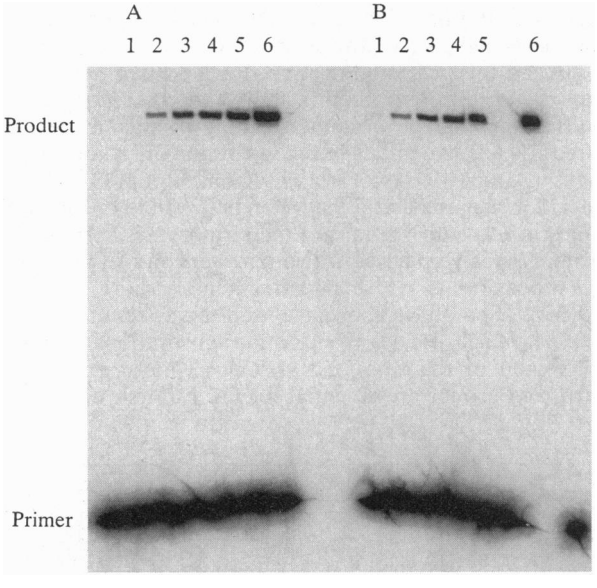

FIG. 2. Ligation activity of the three-fragment *sunY* ribozymes. Autoradiograph of the time course of ligation reactions catalyzed by the chemically (*B*) and enzymatically (*A*) synthesized ribozymes. The preannealed enzyme catalyzes ligation of a 5′-end-labeled 6-mer primer to a 28-nt RNA to yield a 33-nt product. Lanes: 1, 1 min; 2, 15 min; 3, 30 min; 4, 45 min; 5, 1 h; 6, 2 h.

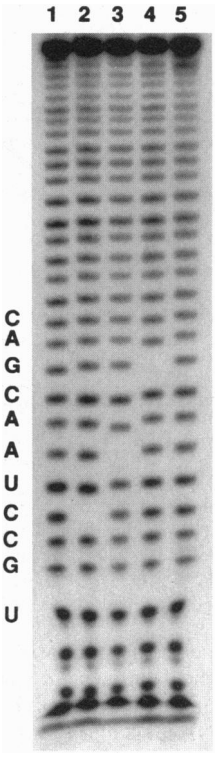

FIG. 3. Partial alkaline hydrolysis of chemically synthesized fragment B and derivatives containing ribonucleotide analogs. Autoradiograph of a 7 M urea/15% polyacrylamide gel of HPLC-purified 5′-end-labeled synthetic fragment B molecules partially hydrolyzed at 90°C in sodium bicarbonate. The molecules contain the following substitutions. Lanes: 1, P7(5′)(G2 → I); 2, J6/7(C1 → dC); 3, J6/7(A3 → dA); 4, P7(5′)(G2 → dG); 5, wild type (no substitution).

Substitutions in Fragment B. To assess the contribution of specific functional groups to the activity of the ribozyme, we incorporated ribonucleotide analogs into fragment B by chemical synthesis and assayed the catalytic activity of the corresponding ribozymes by using the ligation assay. We examined the roles of the 2′-hydroxyl group and the exocyclic amino

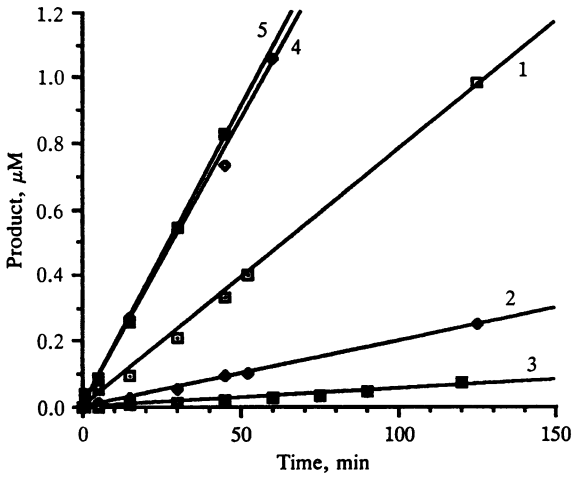

FIG. 4. Analysis of the rate of ligation by *sunY* enzymes containing substitutions in fragment B. Each data point is an average from two or more time course experiments. The enzymes contain the following substitutions. Curves: 1, P7(5′)(G2 → I); 2, J6/7(C1 → dC); 3, J6/7(A3 → dA); 4, P7(5′)(G2 → dG); 5, wild type (no substitution).

group of the guanosine in the guanosine binding site (corresponding to G264 of the *Tetrahymena* ribozyme) by replacing this nucleotide in fragment B with either deoxyguanosine or inosine, generating RNAs B-4 and B-1, respectively. The roles of the 2′-hydroxyl groups of the universally conserved adenosine at position 3 in J6/7 and the cytidine at position 1 in J6/7 (equivalent to positions 261 and 259 in the *Tetrahymena* ribozyme, respectively) were examined by the synthesis of deoxyribonucleotide substitution fragments B-3 and B-2, respectively.

Partial alkaline hydrolysis of the 5′-end-labeled HPLC-purified synthetic fragment B derivatives demonstrated that these molecules were pure and contained the expected substitutions in the correct positions (Fig. 3). Since phosphodiester bonds 3′ to a deoxyribonucleotide are resistant to alkaline hydrolysis, a deoxyribonucleotide linkage in an RNA molecule results in a gap in the hydrolysis ladder. This assay was also used to test the purity of the synthetic oligomers. Completely pure molecules showed a single discrete ladder of bands, whereas molecules that were incompletely deprotected or otherwise impure displayed a smeared pattern with several background shadow bands. Such patterns correlated with decreased catalytic activity (data not shown).

Activity of Substituted Ribozymes. Catalytic activities of the wild-type and substituted enzymes were assayed under high salt conditions, which stabilizes the assembly of the three-subunit ribozyme (Fig. 4). Under these conditions, the ribozymes containing the C → dC substitution in the first position of J6/7 [J6/7(C1 → dC)], and the A → dA substitution in the third position of J6/7 [J6/7(A3 → dA)], were 9- and 32-fold less active than the wild type. In contrast the enzyme with the G → dG substitution in the guanosine binding site retained full wild-type activity (Table 1). The enzyme with a G → I substitution at that position was consistently 2-fold less active than wild type. These results show that specific 2′-hydroxyl groups in the core of the enzyme are critical for ribozyme function.

We wanted to determine whether the reduced catalytic activity observed for two of the deoxynucleotide-substituted ribozymes resulted from a decreased ability of the fragments to associate into an active complex. We used a gel-mobility shift assay (20) to determine the binding constants of the substituted fragment B molecules for fragments A and C. This method has been used to detect tertiary interactions between a group I intron and its substrate (4). We find that all of the substituted fragment B derivatives bind to fragments A and C with affinities similar to that of the wild-type fragment B (Table 1). Similarly, the affinity of fragment A for the preformed B–C complex was not affected by any of the substitutions, suggesting that the 2′-hydroxyl groups of C1 and A3 in J6/7 may be involved in substrate binding or catalysis, rather than ribozyme folding or structure.

DISCUSSION

The difficulty of synthesizing long RNA molecules in sufficient quantity and purity has been an impediment to analyzing the role of specific functional groups in the larger ribozymes, such as the group I and group II introns and RNase P. Even the synthesis of shorter RNA molecules in the range of 30–70 nt has been difficult due to the accumulation of lesions and capped products during chemical RNA synthesis, the presence of incompletely deprotected molecules, and problems with purification. Our experiments demonstrate that a ribozyme derived from a group I intron can be made by automated chemical synthesis and that the catalytic activity of the synthetic ribozyme can approach that of enzymatically synthesized RNA, provided that the synthetic RNA is purified away from contaminating species by a combination of gel electrophoresis and HPLC. The efficiency and reproducibility of RNA purification by HPLC are enhanced by the use of strongly denaturing conditions to prevent the formation of aggregates and secondary structures that lead to spurious peaks.

The assembly of the ribozyme from multiple small RNA fragments greatly simplifies the synthesis of modified ribozymes containing ribonucleoside analogs at specific positions, since only one relatively short RNA fragment must be synthesized for each modification. Furthermore, many combinations of different modifications or of mutations and modifications can be examined simply by mixing different altered RNA fragments. The reconstitution of group II intron activity (22) and RNase P activity from multiple RNA fragments (23) suggests that it may soon be possible to chemically synthesize and alter versions of these ribozymes. Caprara and Waring (24) have devised a two-part version of the *Tetrahymena* ribozyme, in which one fragment is short enough to synthesize chemically. They have used this system to examine the effect of specific deoxynucleotide substitutions within the J8/7 region of the ribozyme and have shown that certain 2′-hydroxyl groups are essential for full catalytic activity.

Table 1. Kinetic parameters for the SunY enzymes

Molecule	Substitution	k_{cat}/K_m, $min^{-1}·M^{-1}$	K_d, nM		
			A–B	B–C	A–BC
1	G → I in guanosine binding site	3.1×10^3	43 ± 6	12 ± 2	104 ± 15
2	J6/7(C1 → dC)	8.0×10^2	54 ± 6	8 ± 1	72 ± 6
3	J6/7(A3 → dA)	2.2×10^2	70 ± 11	3 ± 0.4	86 ± 10
4	G → dG in guanosine binding site	6.8×10^3	62 ± 7	3 ± 0.3	74 ± 7
5	Wild type (unsubstituted)	7.2×10^3	100 ± 11	8 ± 2	88 ± 9

k_{cat}/K_m values are averages derived from three time course studies. The difference in rates of a given enzyme assayed on different days with the same reagents was <2.0-fold. All K_d values were determined at least twice.

We have used our three-part synthetic ribozyme to examine the effect of a base analog substitution at one position and the effects of deoxynucleotide substitutions at three positions. The positions tested were chosen on the basis of their known functional importance. The first of these positions is the guanosine residue (G264 in *Tetrahymena*) that forms part of the guanosine substrate binding site. The G → I substitution in the G·C base pair results in the loss of one hydrogen bond. The slight effect of this change on the rate of catalysis can be explained by the fact that the I·C base pair can still form, although with two instead of three hydrogen bonds. We were particularly interested in testing the activity of a dG substitution at this position because of the availability of more phosphoramidites of base analogs of dG than of ribonucleotide guanosine. Retention of activity with dG at this position would facilitate the exploration of the guanosine binding site by the chemical synthesis of several modified forms of the ribozyme. Since the dG-substituted ribozyme retains full activity, it should now be possible to chemically synthesize variant ribozymes with modified bases at this position and to test the effects of these substitutions on the specificity of the guanosine binding site.

The second and third positions at which we examined deoxynucleotide substitutions are in the J6/7 region of the ribozyme, a 3-base connecting region between the P6 and P7 stems. The first two bases of J6/7 have been proposed to form part of a triple helix with the P4 stem (21), while the third residue in J6/7 is a phylogenetically invariant adenosine that has been modeled as a single connecting nucleotide between the top of the triple helix and P7 (25). Since deoxynucleotide substitutions at two of these positions have strongly deleterious effects on catalysis, the 2′-hydroxyl groups of these residues must play an important role either in generating or maintaining the correct structure of the ribozyme, in substrate binding, or in catalysis. These 2′-hydroxyl groups, which are on the B fragment of our ribozyme, do not appear to be involved in energetically favorable interactions with functional groups on the other fragments, since their loss does not affect the association of the subunits into the ribozyme complex as observed by native gel electrophoresis. One possibility is that the complex observed in the gel is not the fully assembled active complex, but rather a simpler complex, stabilized largely by secondary structure interactions. A second possibility is that intramolecular tertiary interactions involving the 2′-hydroxyl groups and positions elsewhere in fragment B occur that decrease activity but do not affect the annealing of fragment B to fragment A or C. A third and more interesting possibility is that the 2′-hydroxyl groups interact directly with the RNA substrate of the ribozyme or participate in catalysis (for example, by acting as ligands for a critical metal ion). This three-piece synthetic system should simplify searching for and mapping the functional groups that interact with these important 2′-hydroxyl groups.

We thank the members of the Szostak laboratory for helpful discussions and Drs. Douglas Barker and Karen Chapman for critical reading of the manuscript. This work was supported by National Institutes of Health Grant GM45315 to J.W.S.

1. Perrault, J. P., Wu, T., Cousineau, B., Ogilvie, K. K. & Cedergren, R. (1990) *Nature (London)* **344,** 565–567.
2. Scaringe, S. A., Francklyn, C. & Usman, N. (1990) *Nucleic Acids Res.* **18,** 5433–5441.
3. Vinayak, R., Anderson, P., McCollum, C. & Hampel, A. (1992) *Nucleic Acids Res.* **20,** 1265–1269.
4. Yang, J. H., Usman, H., Chartrand, P. & Cedergren, R. J. (1992) *Biochemistry* **31,** 5005–5009.
5. Odai, D., Hiroaki, H., Sakata, T., Tanaka, T. & Uesugi, S. (1990) *FEBS Lett.* **267,** 150–152.
6. Koizumi, M. & Ohtsuka, E. (1991) *Biochemistry* **30,** 5145–5150.
7. Fu, D. J. & McLaughlin, L. W. (1992) *Biochemistry* **31,** 10941–10949.
8. Doudna, J. A., Szostak, J. W., Rich, A. & Usman, N. (1990) *J. Org. Chem.* **55,** 5547–5549.
9. Green, R., Szostak, J. W., Benner, S. A., Rich, A. & Usman, N. (1991) *Nucleic Acids Res.* **19,** 4161–4166.
10. Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H. & Eckstein, F. (1991) *Science* **253,** 314–316.
11. Williams, D. M., Pieken, W. A. & Eckstein, F. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 918–921.
12. Olsen, D. B., Benseler, F., Aurup, H. & Eckstein, F. (1991) *Biochemistry* **30,** 9735–9741.
13. Williams, D. M., Pieken, W. A. & Eckstein, F. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 918–921.
14. Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H. & Ohtsuka, E. (1989) *Nucleic Acids Res.* **17,** 7059–7070.
15. Usman, N., Nicoghosian, K., Cedegren, R. J. & Ogilvie, K. K. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5764–5768.
16. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. (1987) *Nucleic Acids Res.* **15,** 8783–8798.
17. Moore, M. & Sharp, P. A. (1992) *Science* **256,** 992–997.
18. Doudna, J. A., Couture, S. & Szostak, J. W. (1991) *Science* **251,** 1605–1608.
19. Green, R. & Szostak, J. W. (1992) *Science* **258,** 1910–1915.
20. Pyle, A. M., McSwiggen, J. A. & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8187–8191.
21. Michel, F. R., Ellington, A. D., Couture, S. & Szostak, J. W. (1990) *Nature (London)* **347,** 578–580.
22. Jarrell, K., Dietrich, R. C. & Perlman, P. S. (1988) *Mol. Cell. Biol.* **8,** 2361–2366.
23. Guerrier-Takada, C. & Altman, S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1266–1270.
24. Caprara, M. G. & Waring (1993) *Biochemistry* **32,** 3604–3610.
25. Michel, F. & Westholf, E. (1990) *J. Mol. Biol.* **216,** 585–610.