Ribozyme-Catalyzed Primer Extension by Trinucleotides: A Model for the RNA-Catalyzed Replication of RNA†

Jennifer A. Doudna,‡ Nassim Usman,§ and Jack W. Szostak*

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

Received October 2, 1992; Revised Manuscript Received November 25, 1992

ABSTRACT: The existence of RNA enzymes that catalyze phosphodiester transfer reactions suggests that RNA-catalyzed RNA replication might be possible. Indeed, it has been shown that the *Tetrahymena* and *sunY* self-splicing introns will catalyze the template-directed ligation of RNA oligonucleotides (Doudna et al., 1989, 1991). We have sought to develop a more general RNA replication system in which arbitrary template sequences could be copied by the assembly of a limited set of short oligonucleotides. Here we examine the use of tetranucleotides as substrates for a primer-extension reaction in which the 5′-nucleoside of the tetranucleotide is the leaving group, and the primer is extended by the remaining three nucleotides. When the 5′-nucleoside is guanosine, the reaction is quite efficient, but a number of competing side reactions occur, in which inappropriate guanosine residues in the primer or the template occupy the guanosine binding site of the ribozyme. We have blocked these side reactions by using the guanosine analogue 2-aminopurine riboside (2AP) as the leaving group, in a reaction catalyzed by a mutant *sunY* ribozyme that binds efficiently to 2AP and not to guanosine. We have begun to address the issue of the fidelity of the primer-extension reaction by measuring reaction rates with a number of different triplet/template combinations. Our results provide the basis for the further development of an RNA-catalyzed RNA replication system using short oligonucleotide substrates with novel leaving groups.

The discovery of several classes of catalytic RNA molecules in recent years has led to speculation about the role of RNA in the origin of life. One intriguing scenario is that an RNA-dependent RNA polymerase, or replicase, arose from polynucleotides synthesized under prebiotic conditions (Cech, 1986; Sharp, 1986; Gilbert, 1986; Joyce, 1989; Benner et al., 1989). Through cycles of replication and natural selection, this replicase might have evolved into an efficient enzyme which formed the basis for early cellular life.

We are interested in demonstrating the feasibility of such a scenario by developing an RNA replicase in the laboratory. Toward this end, we have chosen the group I self-splicing introns for study because they catalyze phosphodiester exchange reactions between RNA substrates (Cech, 1987, 1990). We found that the *Tetrahymena* group I intron could be modified such that it catalyzed the ligation of oligonucleotides on separate template molecules to generate full-length complementary strands of RNA (Doudna & Szostak, 1989). In principle, such a mechanism could be used to assemble copies of the ribozyme itself.

The *sunY* self-splicing intron from bacteriophage T4 (Shub et al., 1988) has advantages over the *Tetrahymena* ribozyme with regard to the design of a replicase. Excluding its open reading frame, it is approximately half the size of the *Tetrahymena* intron, and thus would require fewer cycles of ligation to copy. In addition, the *sunY* intron appears to be more efficient at using long double-stranded RNAs as substrates, resulting in higher yields of full-length cRNA (Doudna et al., 1991). The *sunY* intron can also be separated into fragments that form a multisubunit ribozyme, which may facilitate ribozyme unfolding to form templates (Doudna et al., 1991).

A modified version of the *sunY* intron (Doudna et al., 1991) was used in these experiments and is shown in Figure 1A. Our initial results indicated that this ribozyme efficiently catalyzed the ligation of RNA oligonucleotides in a template-dependent manner (Figure 1B). During the reaction, the 3′-hydroxyl of one oligonucleotide attacks the phosphate between the 5′-guanosine and the second residue of an adjacent oligomer, resulting in ligation of the two oligomers and release of the 5′-guanosine. This reaction resembles the exon ligation step of the self-splicing series of reactions catalyzed by the complete intron.

Previous experiments involved the use of oligonucleotide substrates 6–10 residues in length for ligation. While oligomers in this size range are ligated efficiently, their use as substrates for template replication is artificial in that a set of oligomers complementary to the template of interest must be supplied in the reaction. Because of their length, oligonucleotides of all possible sequences cannot be supplied, at least in reasonable concentrations, and therefore simple base substitution, insertion, and deletion mutations cannot arise. This constraint makes it impossible for the template sequence to evolve in response to selective pressures. We have therefore become interested in exploring the use of shorter oligonucleotides as substrates for primer extension, since a complete set of short oligonucleotides could be assembled into the complement to any possible template sequence. Such a set of short substrates would form the basis for a more general RNA replication system.

We have therefore used oligonucleotides two to six nucleotides in length as substrates in template-directed ligation reactions catalyzed by the *sunY* ribozyme. We found that

† This work was supported by a grant from Hoechst AG to the Massachusetts General Hospital and by a fellowship from the Lucille P. Markey Charitable Trust to J.A.D. J.A.D. is a Lucille P. Markey Scholar in the Biomedical Sciences.

* To whom correspondence should be addressed. Phone: (617) 726-5981. FAX: (617) 726-6893.

‡ Present address: Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309-0215.

§ Present address: Ribozyme Pharmaceuticals, Inc., Boulder, CO 80309.

0006-2960/93/0432-2111$04.00/0

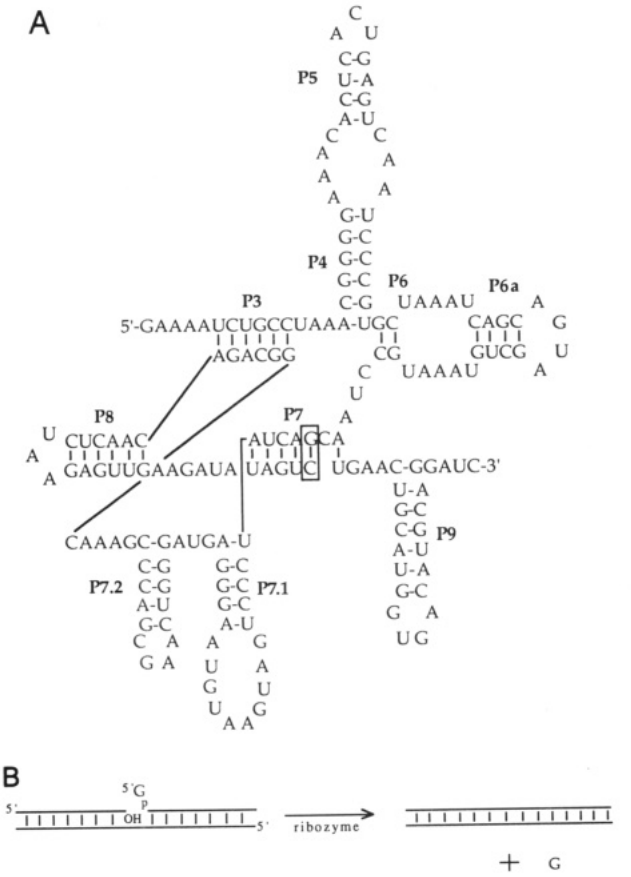
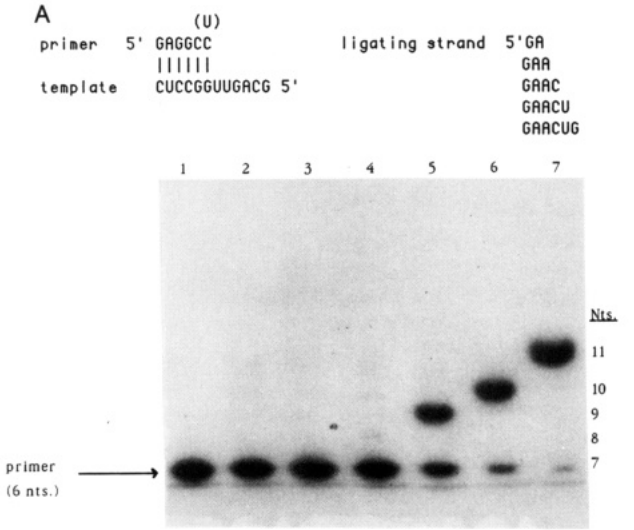


FIGURE 1: (A) Secondary structure of the modified *sunY* intron used in these experiments. The construction and characterization of this molecule have been described previously (Doudna et al., 1991). This ribozyme has been modified in several respects from the wild-type intron: stem–loops P9.1 and P9.2 have been deleted, the second and third base pairs in stem P4 and the second residue joining segment J6/7 have been changed to the sequences found in the related intron *td* (Michel et al., 1990), and the P1 stem has been removed. This ribozyme contains no internal cleavage sites. The boxed base pair is the site of guanosine binding; when it is changed to an A:U base pair, the resulting ribozyme binds 2-aminopurine riboside instead of guanosine (Michel et al., 1989). For nomenclature, see Burke et al. (1987). (B) Diagram of the template-directed oligonucleotide ligation reaction catalyzed by the ribozyme shown above. See text for details.



B **Primer ending in U**

Ligator	initial rate (pmol/min)
GA	0.6
GAA	0.7
GAAC	5
GAACU	16
GAACUG	23

Primer ending in C

Ligator	initial rate (pmol/min)
GA	<0.1
GAA	<0.1
GAAC	0.9
GAACU	5
GAACUG	7

FIGURE 2: (A) Autoradiogram of a 20% polyacrylamide/7 M urea gel showing the extent of ligation of the primer to an oligonucleotide of varying length after a 10-min incubation with the ribozyme. The sequences of the primer, template, and substrates are shown. Ribozyme concentration was 0.5 μM, primer and template concentrations were 10 μM, and oligonucleotide substrate concentration was 20 μM. The primer was radioactively labeled at its 5′ end as described; reaction conditions were as described under Materials and Methods. Lane 1, primer and template incubated without ribozyme (no substrate oligonucleotide); lane 2, primer and template incubated with ribozyme (no substrate oligonucleotide); lane 3, dinucleotide substrate added to reaction; lane 4, trinucleotide substrate added; lane 5, tetranucleotide substrate added; lane 6, pentanucleotide substrate added; lane 7, hexanucleotide substrate added. (B) Initial rates for each reaction were determined by plotting ligated product accumulation vs time. Initial rates are shown for ligation of each oligomer with primers ending in U or C. The values shown are the average of three experiments.

tetranucleotides beginning with G maintained a resonable rate of primer extension but that a number of undesired side reactions occurred. The use of 2-aminopurine riboside as the leaving group for the ligation reaction, in combination with a mutant ribozyme that bound this analogue in preference to G, resulted in enhancement of reaction specificity. These results suggest a mode of RNA-catalyzed polymerization in which a pool of all 64 possible triplet sequences could be supplied as substrates for template replication.

MATERIALS AND METHODS

Materials. A, C, G, and U ribonucleoside phosphoramidites were purchased from Milligen/Biosearch; 2-aminopurine ribonucleoside phosphoramidite was synthesized as described (Doudna et al., 1990). RNA and DNA oligonucleotides were made by solid-phase synthesis on a Biosearch 8750 oligonucleotide synthesizer. RNA oligomers were deprotected overnight at 55 °C in ethanol/30% NH_4OH (3:1), followed by removal of the silyl protecting groups by treatment with tetrabutylammonium fluoride in THF overnight at room temperature [see, e.g., Wu et al. (1989) and Scaringe et al. (1990)]. Purification of oligonucleotides was by HPLC using a Dionex NA100 ion-exchange column in 10% acetonitrile with a gradient of ammonium acetate from 10 mM to 1 M in 1 h. Following elution from the column, peak fractions were lyophilized repeatedly to remove residual salt and then resuspended in deionized water. Oligonucleotide purity was assayed as described (Doudna et al., 1990).

RNA oligomers were 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (purchased from New England Nuclear). Labeled RNA was purified by polyacrylamide gel electrophoresis (PAGE); gel slices were eluted into 0.3 M NaCl, phenol-extracted, and ethanol-precipitated. The dried pellets were resuspended in deionized water.

DNA fragments encoding *sunY* ribozyme derivatives under the control of a T7 RNA polymerase promoter were prepared by polymerase chain reaction amplification of the wild-type *sunY* DNA template using appropriate DNA oligonucleotide primers (Mullis & Faloona, 1987). The resulting fragments were cloned into a pBR322-derived plasmid, and sequences

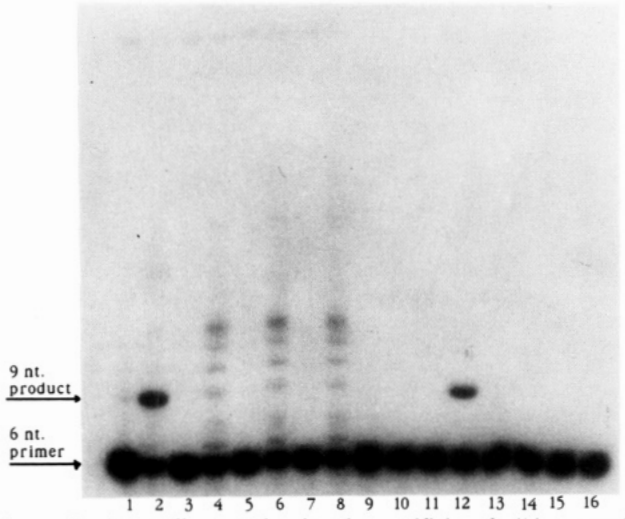


FIGURE 3: Autoradiogram showing the specificity of wild-type and altered ribozymes for tetranucleotide substrates. Primer sequence, 5′GAGGCC3′; template sequence, 5′GUUGGCCUC3′. Each reaction contained 0.5 μM ribozyme, 10 μM primer and template, and 20 μM tetramer substrate. The primer was radioactively labeled as described under Materials and Methods. Lanes 1–8, wild-type ribozyme; lanes 9–16, ribozyme containing G:C to A:U base pair change in P7 (as indicated in Figure 1A). Odd-numbered lanes, 0-min incubation; even-numbered lanes, 60-min incubation. Lanes 1, 2, 9, and 10, GAAC substrate; lanes 3, 4, 11, and 12, 2AP-AAC substrates; lanes 5, 6, 13, and 14, UAAC substrate; lanes 7, 8, 15, and 16, no substrate oligonucleotide added.

were confirmed by dideoxy sequencing (Sequenase system, U.S. Biochemicals). Plasmid templates digested with the restriction enzyme *Bam*HI were transcribed using T7 RNA polymerase purchased from New England Biolabs. RNA was purified by PAGE, eluted, precipitated, and resuspended in deionized water.

Primer-Extension Reactions. Reactions were carried out at 45 °C in 30 mM Tris-HCl (pH 7.4 at 25 °C), 20 mM $MgCl_2$, 10 mM NH_4Cl, and 1 mM aurintricarboxylic acid. Reactions were initiated by addition of the oligonucleotide substrates after a 20-min preincubation of the ribozyme, $MgCl_2$, buffer, NH_4Cl, aurin-TCA, primer, and template at 45 °C. Following incubation, reactions were quenched by the addition of an equal volume (5–10 μL) of 50 mM EDTA in 90% formamide with 0.05% each bromophenol blue and xylene cyanol, and 10 mM Tris, pH 7.4. Reaction products were analyzed by electrophoresis on 20% polyacrylamide/7 M urea gels and quantitated with use of a Betagen radioanalytic scanner. The ribozyme concentration was 0.5 μM in all experiments; primer and template concentrations were 10 μM; the tetramer concentration was 20–50 μM, as indicated in the figure legends.

RESULTS AND DISCUSSION

Tetramer Oligonucleotides Are Efficiently Ligated. In order to explore the effect of oligomer length on reaction efficiency, a set of substrates was synthesized which included a template, primer, and five oligonucleotide substrates (derived by successive shortening of one sequence) ranging from two to six nucleotides in length (Figure 2A). Template-directed ligation reactions were carried out between the primer and each oligomer substrate individually in the presence of a low concentration of the ribozyme (see Materials and Methods). As shown in the autoradiogram (Figure 2A) and quantitated in Figure 2B, the rate of the reaction decreases as the length of the oligomer substrate decreases. However, the most dramatic decrease (7-fold) occurs in going from a substrate

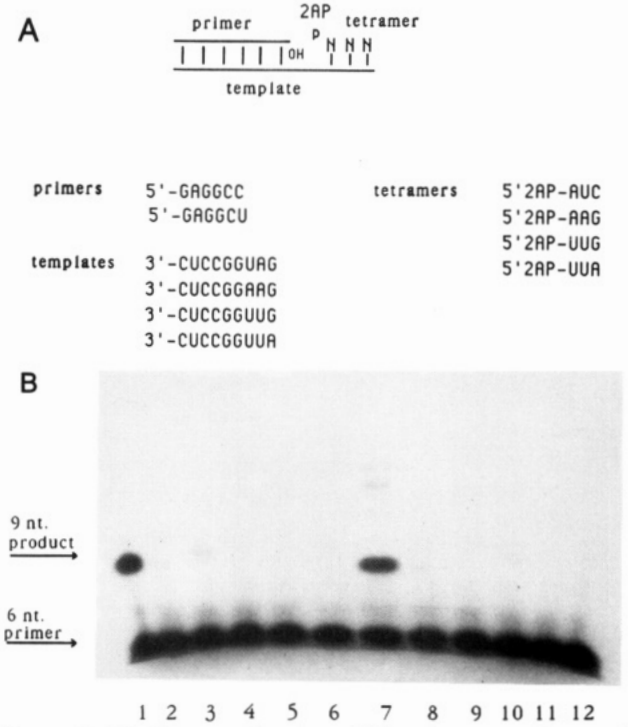


FIGURE 4: (A) Diagram of a subset of the primers, templates, and tetranucleotides which were synthesized to test the fidelity of triplet ligation. All oligonucleotides were synthesized using solid-phase phosphoramidite chemistry and purified by HPLC (see Materials and Methods). (B) Autoradiogram showing primer extension by triplet ligation. The ribozyme concentration was 0.5 μM; primer and template concentrations were 10 μM; tetranucleotide concentrations were 20 μM. The primer was radioactively labeled as described under Materials and Method. All reactions were for 60 min at 45 °C. Lanes 1–6, primer sequence 5′GAGGCU3′, template sequence 3′CUCCGGUAG5′. Lane 1, tetramer 2AP-AUC; lane 2, tetramer 2AP-UUC; lane 3, tetramer 2AP-AAC; lane 4, 2AP-GUA; lane 5, 2AP-UAC; lane 6, 2AP-AAG. Lanes 7–12, primer sequence 5′GAGGCU3′, template sequence 3′CUCCGGAAG5′. Lane 7, tetramer 2AP-UUC; lane 8, tetramer 2AP-AUC; lane 9, 2AP-UAC; lane 10, 2AP-GAC; lane 11, 2AP-GUA; lane 12, 2AP-AAG.

4 nts in length (which forms 3 base pairs with the template) to one 3 nts in length (2 base pairs with the template). This could be explained at least in part by the loss of a G:C base pair in going from 4 to 3 nts with this particular sequence. In addition, it is possible that the ribozyme normally contacts the substrate backbone at a position three residues 3′ of the ligation site. In the case, the reduction in reactivity of the trimer substrate would be due to the loss of a contact between it and the ribozyme. It appears from this experiment that tetranucleotide substrates are a reasonable compromise between shorter substrate length and acceptable reaction rate.

Specificity Enhanced Using the 2AP Leaving Group. Side reactions often occurred during reactions of the type described above (Figure 3). Products resulting from these aberrant ligations seem to result from binding of internal G residues in the substrate, primer, or template oligonucleotides by the ribozyme. Binding of these internal G nucleotides results in transesterification between the primer 3′-OH and the phosphate adjacent to the bound G. These alternate products could often be minimized by reducing the concentration of the primer or template, or competed away by increasing the concentration of the substrate strand. However, such side reactions lead to complicated mixtures of reaction products, and reduce the overall yield of the desired full-length copy of the template. Furthermore, even transient occupancy of the G binding site by inappropriate G residues competitively

Table I: Relative Rates of Triplet Addition after C:G Base Pair[a]

template	temp (°C)	initial rate, match (pmol/min)	relative rate, match	relative rates, mismatches					
UAG		AUC			UUC	AAC	GAC	AGU	
	0	0.02 ± 0.001	1.0		0.02	0.01	0.01	0.01	
	25	0.1 ± 0.05	1.0		0.01	0.001	0.001	0.001	
	45	0.4 ± 0.02	1.0		0.04	0.01	0.05	0.05	
AAG		UUC			AUC	UGC	GAC	UCA	
	0	0.04 ± 0.002	1.0		0.05	0.03	0.02	0.07	
	25	0.2 ± 0.01	1.0		0.03	0.01	0.01	0.02	
	45	0.9 ± 0.06	1.0		0.01	0.01	0.05	0.01	
UUG		AAC		GAC	UAC	AUC	AAG	UUC	AGU
	0	0.08 ± 0.005	1.0	0.8	0.07	0.1	0.05	0.1	0.07
	25	0.2 ± 0.01	1.0	0.7	0.04	0.05	0.01	0.01	0.03
	45	1.1 ± 0.03	1.0	0.7	0.03	0.1	0.03	0.03	0.13
UUA		AAU			UAU	AGU	AAC	GAC	AUC
	0	0.006 ± 0.002	1.0		0.2	0.2	0.2	3.0	0.1
	25	0.04 ± 0.002	1.0		0.3	0.3	0.5	0.2	0.1
	45	0.1 ● 0.001	1.0		0.4	0.4	0.4	0.1	0.1

[a] Initial rates of triplet ligation were measured for the indicated combinations of tetramers and templates, using the system diagrammed in Figure 4A. Rates were normalized to that observed for the completely matched substrate tetramer. Experimental conditions were as indicated in Figure 4B and under Materials and Methods. Values shown are the average of three determinations.

Table II: Relative Rates of Triplet Addition after U:G Base Pair[a]

template	temp (°C)	initial rate, match (pmol/min)	relative rate, match	relative rates, mismatches		
UAG		AUC		UUC	UAC	GUA
	0	0.15 ± 0.02	1.0	0.03	0.01	0.01
	25	0.6 ± 0.07	1.0	0.04	0.05	0.05
	45	3.6 ± 0.4	1.0	0.2	0.05	0.05
AAG		UUC		AUC	GUA	UCA
	0	0.03 ± 0.01	1.0	0.04	0.01	0.01
	25	0.6 ± 0.1	1.0	0.05	0.05	0.02
	45	1.9 ± 0.2	1.0	0.08	0.08	0.04
UUG		AAC		GAC	AUC	
	0	0.2 ± 0.02	1.0	2.5	0.03	
	25	0.6 ± 0.1	1.0	1.9	0.05	
	45	4.5 ± 0.5	1.0	1.1	0.03	
UUA		AAU		AGU	GAC	AUC
	0	0.02 ± 0.005	1.0	0.4	0.4	0.2
	25	0.06 ± 0.01	1.0	0.3	0.9	0.3
	45	0.6 ± 0.1	1.0	0.3	1.0	0.4

[a] Initial rates of triplet ligation were measured and normalized to that observed for the matched substrate. Values shown are the average of three determinations.

inhibits the correct reactions. Thus, it was important to find a way to eliminate this problem.

All known group I introns share a common reaction mechanism which is characterized by guanosine binding, and all have a G:C base pair in stem P7 (Figure 1A) which is a major component of the guanosine binding site. A base pair change from G:C to A:U in the guanosine binding site of the *Tetrahymena* intron greatly reduces that intron's affinity for guanosine, and instead allows it to bind the guanosine analogue 2-aminopurine riboside (2AP) (Michel et al., 1989). The basis for this specificity switch is an altered hydrogen-bonding scheme in the proposed interaction between the base pair at the G binding site and the incoming nucleoside substrate. We expected that a base pair change from G:C to A:U at the guanosine binding site in the *sunY* intron would have a similar effect to that demonstrated in *Tetrahymena*, namely, that G affinity would be reduced and 2AP affinity enhanced.

In order to test this, a derivative of the *sunY* ribozyme containing the G:C to A:U change was synthesized. Substrate tetranucleotides were synthesized containing a 2-aminopurine riboside moiety at their 5′ ends. Figure 3 shows a comparison of the reactivities of substrate tetramers in ligation reactions catalyzed by both the original and the A:U mutant ribozymes. As shown in the autoradiogram, the parent *sunY* ribozyme catalyzes ligation of the substrate having a 5′ G residue, but not of substrates having either a 5′ 2AP or a U residue. However, several bands are seen in all lanes due to side reactions of the type described above (i.e., alternate G residues entering the G binding site). In contrast, the A:U mutant ribozyme only catalyzes the ligation of the substrate containing a 2AP moiety at the 5′ end. In the case, the reaction is much more specific, and other products are not observed.

Fidelity of Single-Cycle Triplet Extension. We next wanted to examine the fidelity of template-directed triplet ligation reactions catalyzed by the A:U mutant *sunY* ribozyme. Initial results showed that K_ms vary for different triplets on a given template (i.e., completely matched versus one or more mismatches). Given these K_m differences, fidelity will be optimal when tetramers are tested at subsaturating (k_{cat}/K_m) conditions. Such conditions best approximate the fidelity that would be seen if tetramers were allowed to compete for binding. Testing tetramer substrates individually rather than in competition allows for straightforward product quantitation.

Apparent K_ms were measured for tetranucleotides of the sequence GAAC and 2AP-AAC using a primer of sequence 5'GAGGCU and template 5'GUUGGCCUC. While the observed K_m for the GAAC substrate was ~10 μM, that for the 2AP-AAC substrate was greater than 50 μM. This is consistent with the observation that in the *Tetrahymena* ribozyme, the P7 mutant has lower affinity for 2AP than the wild type does for guanosine (Michel et al., 1989).

Several different nine-nucleotide templates and complementary six-nucleotide primers were synthesized, as shown in Figure 4A. Reactions of different primer-template combinations with various tetramers were performed as described under Materials and Methods, with each tetranucleotide at a concentration of 20 μM. Our goal was not to explore all possible template-tetramer combinations, but rather to obtain an indication of the fidelity of the triplet extension reaction as catalyzed by the *sunY* ribozyme. In general, tetramers tested with each template were chosen either to be completely matched or to have one or two mismatches with the template.

A comparison was also done between primers ending in U (resulting in a U:G pair at the end of the primer-template helix) or ending in C (resulting in a C:G pair at that position). It has been shown that helix initiation and propagation are influenced by the base pair onto which an adjacent or incoming base stacks [e.g., see Freier et al. (1986) and SantaLucia et al. (1991)]. While a U:G base pair is found at the splice site of nearly all wild-type group I introns, a C:G is also reactive (Cech, 1990). We explored the effect of temperature on the rates and fidelities of these reactions by comparing rates of ligation at 0, 25, and 45 °C. In previous work using dinucleotides as substrates for ligation by the *Tetrahymena* intron, greater fidelity was achieved at low temperatures (Bartel et al., 1991).

An autoradiogram showing typical results is shown in Figure 4B, and the results are compiled in Tables I and II. It is interesting to note that a wide range of fidelities was observed for the tested templates. For ligation following a C:G base pair, the UAG and AAG template sequences were copied faithfully. A single mismatch between the triplet and the template was enough to reduce the reaction rate by 2–3 orders of magnitude. Fidelity with the UUG template was similar, except in the case of a triplet that formed a G:U wobble pair with it. In this case, ligation rates were similar to those observed with the completely matched substrate, and for the primer ending in U, ligation occurred faster than with the matched substrate. The UUA template was the least efficiently and the least faithfully copied, perhaps due to the inherently weak binding of the matched triplet. Ligation rates for the template sequence paired with its matched substrate were lower than for the other substrate–template combinations that contained one or more G:C base pairs. Again, a G:U wobble pair between the incoming triplet and the template resulted in enhanced ligation rates. Reaction rates correlated with the order of observed stabilities of mismatches within helices (SantaLucia et al., 1991).

There was no pronounced effect on fidelity for ligation after a C:G versus a U:G base pair. Overall rates of reaction were reduced 5–10-fold compared to those following a U:G pair, but relative rates were comparable. Although lower reaction rates were observed at lower temperatures, temperature did not have a large effect on fidelity.

Neither the efficiency nor the fidelity of the triplet extension reaction is currently high enough to make the replication of longer templates feasible in practice. However, it may be possible to select for ribozymes with enhanced affinity for the leaving group 2-aminopurine riboside, or for other more suitable leaving groups, using methods of in vitro selection [for a review, see Szostak (1992)]. In addition, selection could be used to identify ribozymes with increased binding affinity for the primer-template–substrate helix. The increased specificity obtained through use of tetranucleotides containing non-G leaving groups, together with enhanced reaction efficiency, should enable development of a simple RNA-catalyzed RNA polymerization system, at which point selection methods could be applied to the task of improving the fidelity of the replication process.

ACKNOWLEDGMENT

We thank Anna Pyle and Dan Herschlag for helpful discussions and review of the manuscript.

REFERENCES

Bartel, D. P., Doudna, J. A., Usman, N., & Szostak, J. W. (1991) *Mol. Cell. Biol. 11*, 3390–3394.
Benner, S. A., Ellington, A. D., & Tauer, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7054–7058.
Burke, J., Cech, T. R., Inoue, T., Szostak, J. W., & Tabak, H. (1987) *Nucleic Acids Res. 15*, 7217–7221.
Cech, T. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4360–4364.
Cech, T. R. (1987) *Science 236*, 1532–1539.
Cech, T. R. (1990) *Annu. Rev. Biochem. 59*, 543–568.
Doudna, J. A., & Szostak, J. W. (1989) *Nature 339*, 519–522.
Doudna, J. A., Szostak, J. W., Rich, A., & Usman, N. (1990) *J. Org. Chem. 55*, 5547–5549.
Doudna, J. A., Couture, S., & Szostak, J. W. (1991) *Science 251*, 1605–1608.
Freier, S. M., Kierzek, R., Caruthers, M. H., Nielson, T., & Turner, D. H. (1986) *Biochemistry 25*, 3209–3213.
Gilbert, W. (1986) *Nature 319*, 618.
Joyce, G. F. (1989) *Nature 338*, 217–224.
Michel, F., Hanna, M., Green, R., Bartel, D. P., & Szostak, J. W. (1989) *Nature 342*, 391–395.
Michel, F., Ellington, A. D., Couture, S., & Szostak, J. W. (1990) *Nature 347*, 578–581.
Mullis, K., & Faloona, F. A. (1987) *Methods Enzymol. 255*, 335–350.
SantaLucia, J., Kierzek, R., & Turner, D. H. (1991) *Biochemistry 30*, 8242–8251.
Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res. 18*, 5433–5441.
Sharp, P. A. (1985) *Cell 42*, 397–400.
Shub, D. A., Gott, J. M., Xu, M.-Q., Lang, B. F., Michel, F., Tomaschewski, J., Pederson-Lane, J., & Belfort, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1151–1155.
Szostak, J. W. (1992) *Trends Biochem. Sci. 17*, 89–93.
Turner, D. H., Sugimoto, N., & Freier, S. M. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 167–191.
Wu, T., Ogilvie, K. K., & Pon, R. T. (1989) *Nucleic Acids Res. 17*, 3501–3517.