MOLECULAR AND CELLULAR BIOLOGY, June 1991, p. 3390–3394
0270-7306/91/063390-05$02.00/0

Vol. 11, No. 6

Template-Directed Primer Extension Catalyzed by the *Tetrahymena* Ribozyme

DAVID P. BARTEL,[1] JENNIFER A. DOUDNA,[1] NASSIM USMAN,[2] AND JACK W. SZOSTAK[1]*

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114,[1] and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[2]

Received 26 December 1990/Accepted 25 March 1991

The *Tetrahymena* ribozyme has been shown to catalyze an RNA polymerase-like reaction in which an RNA primer is extended by the sequential addition of pN nucleotides derived from GpN dinucleotides, where N = A, C, or U. Here, we show that this reaction is influenced by the presence of a template; bases that can form Watson-Crick base pairs with a template add as much as 25-fold more efficiently than mismatched bases. A mutant enzyme with an altered guanosine binding site can catalyze template-directed primer extension with all four bases when supplied with dinucleotides of the form 2-aminopurine-pN.

Our interest in the possible role of RNA replicases in the origin and early evolution of life (2, 5, 8, 12, 16, 24) led us to examine the extent to which a template can direct the incorporation of mononucleotides in a primer extension reaction catalyzed by the *Tetrahymena* ribozyme (1). The primer extension reaction involves nucleophilic attack by the 3′ hydroxyl group of an oligonucleotide primer on the phosphate of a GpN (where N = A, C, or U) dinucleotide. This reaction extends the primer by one nucleotide and releases the guanosine nucleoside (Fig. 1a). Each addition is analogous to the second step of self-splicing (6), in which the primer represents the 5′ exon and is extended by a single-nucleotide 3′ exon (17). This primer extension reaction is also analogous to protein-catalyzed RNA and DNA polymerization in that the 3′ end of the growing chain is extended by successive additions of mononucleotides, with guanosine as the leaving group instead of pyrophosphate.

Template-influenced primer extension. The original primer-extension system studied by Been and Cech (1) did not include a uniquely defined template base that could interact with the incoming nucleotide. We tested the possibility that such a template base might influence the primer extension reaction by adding a template region just 5′ of the primer-binding site, as illustrated in Fig. 1a. In our scheme, the primer can anneal with the primer-binding site in only one way; since the primer cannot slide along the template, it is possible to examine the influence of a single template base.

Four ribozymes that differed in the template base were incubated with dinucleotide and labeled primer, with enzyme in large excess over primer (Fig. 1b). All four ribozymes begin with pppG, followed by the indicated template base, followed by the primer-binding site, followed by the catalytic core (bases G27 to G405 in reference 7) of the wild-type *Tetrahymena* intron. These ribozymes were generated by runoff transcription of plasmid templates that were linearized at an *Eco*RI site inserted immediately after the position corresponding to G405 of the wild-type intron. Plasmids were constructed by polymerase chain reaction mutagenesis (21), and transcription and purification of ribozymes were as previously described (19). Primer was transcribed from a single-stranded oligonucleotide template (20). Dinucleotide initiation (22) with 4 mM GpC and abortive cycling (18) were employed to increase primer yield. Primer was gel purified, end labeled, and gel purified again. Primer sequence and purity were confirmed by RNase sequencing and ion-exchange high-performance liquid chromatography (HPLC). Dinucleotides (Sigma) were purified by reverse-phase HPLC.

The variation in the rate of addition of pA, pC, and pU across from the four possible template bases (Fig. 1b) shows that the template can indeed influence the base that is added. For example, when the template base is U, the primer is extended much more efficiently in the presence of GpA than in the presence of GpC or GpU. In each case, the base that can form a Watson-Crick match with the template adds the most rapidly. Use of single-turnover conditions (enzyme in large excess over primer) simplifies the interpretation of these experiments, since the rate of product release, which would be expected to vary depending on whether a matched or mismatched nucleotide was added, can no longer affect the observed rate of the reaction.

The number of nucleotides added to the primer varies with different template and dinucleotide combinations (Fig. 1b). After pC or pU adds across from a G or A template nucleotide (to form a Watson-Crick or wobble base pair), a second pC or pU can add across from the next template base (G). However, when pA adds across from pU, a second pA is not added. In the presence of both GpA and GpC, efficient primer extension by two nucleotides is observed (Fig. 1c), presumably because of copying of the template and extension of the primer by 5′-ApC-3′.

Kinetic parameters of fidelity. To understand the observed preference for primer extension with Watson-Crick base pairs, we determined the kinetic parameters governing this reaction. This analysis is simplified by use of preformed ribozyme-primer complex (E·Primer). The observed rate of the reaction becomes first order when preformed E·Primer complexes are used, since the rate depends on the binding of only one of the substrates, the GpN dinucleotide. Saturating the primer with enzyme before and during the reaction also eliminates the possibility that GpNs might differentially affect primer binding.

The affinity of the primer for the primer-binding site of the enzymes used in our initial studies was too low to permit efficient formation of the E·Primer complex by ribozyme saturation of the primer. To increase the affinity of the

* Corresponding author.

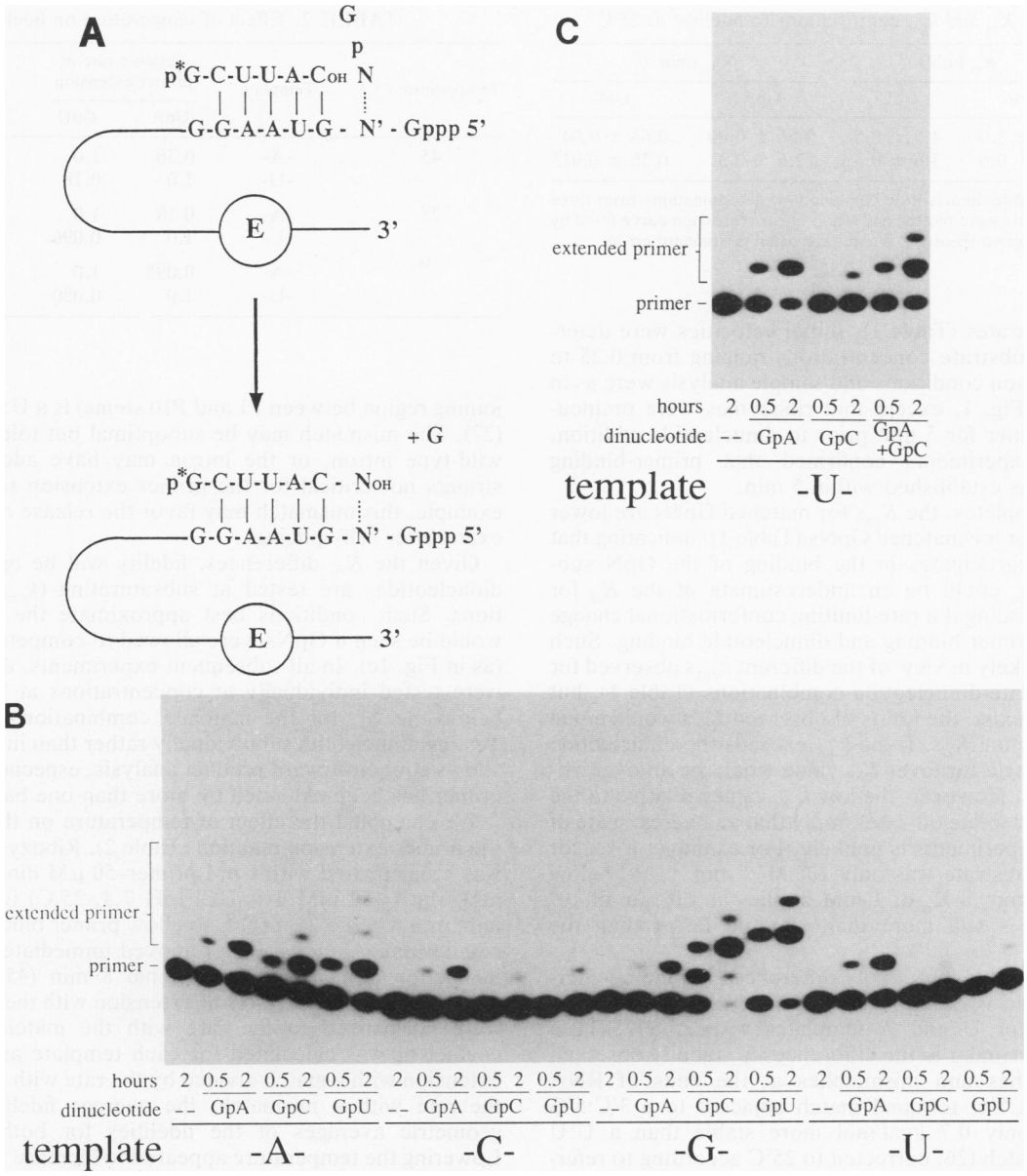

FIG. 1. Template-directed primer extension. (A) End-labeled primer (5′-p*GCUUAC) pairs with the primer-binding site (3′-GGAAUG) and is extended by a ribozyme (E)-catalyzed phosphodiester exchange reaction in which the 3′ hydroxyl group of the primer attacks the phosphate group of a GpN dinucleotide (N = A, U, or C). The dotted line represents the interaction of the N of the dinucleotide with the template base N′. (B) Differential primer extension rates with different dinucleotide-template combinations. (C) Two cycles of template-directed primer extension. Ribozymes were incubated for 30 min at 25°C in reaction buffer. Ribozyme (250 nM) with the indicated template base was then incubated at 25°C with 1 nM labeled primer–50 mM Tris·HCl (pH 7.4)–20 mM $MgCl_2$, and 1 mM GpN, when present. Reactions were stopped at 10 min, 30 min, or 120 min by the addition of 90% formamide–25 mM EDTA, and the products were analyzed by electrophoresis on 20% acrylamide, 8 M urea gels. Reaction rates were determined by direct gel scanning on a Betagen scanner.

enzyme for primer, the primer-binding site was changed from 3′-GGAAUG-5′ to 3′-CGAAUG-5′, thus increasing base pairing with the primer from five to six base pairs. This change decreases the dissociation constant (K_d) for primer to 50 nM (data not shown), well below the enzyme concentration of 500 nM during preincubation and 250 nM during the reaction. An additional G was also added to the 5′ end of the ribozyme to improve the efficiency of in vitro transcription. These changes were introduced by using polymerase chain reaction mutagenesis (21), and amplified DNA with these changes was transcribed directly. These changes do not affect the fidelity of the primer extension reaction.

Single-turnover reactions of preformed E·Primer complexes and GpN substrates simplify to the following scheme:

$$\text{E·Primer} + \text{GpN} \overset{K_m}{\leftrightarrow} \text{E·Primer·GpN} \overset{k_{cat}}{\rightarrow} \text{E·Primer-pN·G}$$

We measured K_m and k_{cat} for enzymes with A and U template bases, with both matched and mismatched GpA

TABLE 1. K_m and k_{cat} contributions to fidelity[a] at 25°C

Template	K_m (mM)		k_{cat} (min^{-1})	
	GpA	GpU	GpA	GpU
-A-	14 ± 3.0	4.5 ± 0.5	0.56 ± 0.09	0.64 ± 0.04
-U-	2.2 ± 0.6	3.6 ± 0.3	2.6 ± 0.3	0.26 ± 0.013

[a] For each template-dinucleotide combination, determinations from three separate experiments were plotted and a non-linear regression curve fitted by using the P.Fit program (Biosoft). Values are listed ± standard errors.

TABLE 2. Effect of temperature on fidelity

Temperature (°C)	Template	Relative rate of primer extension		Average fidelity
		GpA	GpU	
45	-A-	0.36	1.0	0.79
	-U-	1.0	0.18	
25	-A-	0.18	1.0	0.88
	-U-	1.0	0.096	
0	-A-	0.095	1.0	0.92
	-U-	1.0	0.080	

and GpU substrates (Table 1). Initial velocities were determined at six substrate concentrations ranging from 0.25 to 4.5 mM. Reaction conditions and sample analysis were as in the legend to Fig. 1, except that ribozymes were preincubated with primer for 5 min prior to dinucleotide addition. Pulse-chase experiments confirmed that primer-binding equilibrium was established within 5 min.

For both templates, the K_ms for matched GpNs are lower than the K_ms for mismatched GpNs (Table 1), indicating that the template participates in the binding of the GpN substrate. The K_m could be an underestimate of the K_d for dinucleotide binding if a rate-limiting conformational change lies between primer binding and dinucleotide binding. Such an effect is unlikely in view of the different k_{cat}s observed for different template-dinucleotide combinations (Table 1), but even if it does exist, the ratios of observed K_ms would equal the ratios of actual K_ds. If the k_{cat} exceeds the dinucleotide off-rate, the single-turnover K_m value would be an overestimate of the K_d. However, the low k_{cat} values relative to the expected dinucleotide off-rates imply that an overestimate of K_ds in these experiments is unlikely. For example, if k_{on} for dinucleotide substrate was only 10^6 M^{-1} min^{-1}, 10^4 below the diffusion limit, a K_d of 1 mM implies an off-rate of 10^3 min^{-1}, which is still more than 100-fold faster than the average k_{cat}.

The differences in K_ms imply differences in binding energies for matched versus mismatched substrates of −0.3 and −0.7 kcal/mol for -U- and -A- templates, respectively. These differences are similar to the differences in stability observed between matches and mismatches at the ends of RNA helices. A 3′A:5′U terminal match adjacent to a 3′C:5′G base pair is only 0.7 kcal/mol more stable than a U:U terminal mismatch (26; corrected to 25°C according to reference 11). The stability of an A:A terminal mismatch has not been measured adjacent to 3′C:5′G but is thought to be about 0.3 kcal/mol less than that of the 3′U:5′A match (25a). Although one would not necessarily expect a mismatched N of GpN to interact with the primer and template in the same way as a 3′ dangling nucleotide, the correspondence of the observed values to those calculated in reference 26 make such a model plausible.

Preferential binding of matched dinucleotide substrates does not completely explain the observed fidelity; there is a 10-fold higher k_{cat} with the GpA substrate and the -U- template than with the mismatched GpU substrate (Table 1). The different k_{cat}s point to a more efficient chemical step (or possibly stabilization of an associated conformational change) with the matched substrate. Interactions of the bound dinucleotide with the template may differentially stabilize the transition state by positioning the phosphate of the dinucleotide relative to the 3′ hydroxyl group of the primer and the catalytic groups of the enzyme. This k_{cat} contribution to fidelity is intriguing given the fact that the analogous position in the wild-type *Tetrahymena* intron (the joining region between P1 and P10 stems) is a U:U mismatch (27). The mismatch may be suboptimal but tolerated in the wild-type intron, or the intron may have additional constraints not critical for the primer extension reaction. For example, this mismatch may favor the release of the ligated exons after self-splicing.

Given the K_m differences, fidelity will be optimal when dinucleotides are tested at subsaturating (k_{cat}/K_m) conditions. Such conditions best approximate the fidelity that would be seen if GpNs were allowed to compete for binding (as in Fig. 1c). In all subsequent experiments, dinucleotides were tested individually at concentrations at least 40-fold below the K_m for the matched combinations in Table 1. Testing dinucleotides individually rather than in competition allows straightforward product analysis, especially when the primer has been extended by more than one base.

We examined the effect of temperature on the fidelity of the primer extension reaction (Table 2). Ribozyme (250 nM) was preincubated with 1 nM primer–50 μM dinucleotide–20 mM $MgCl_2$–50 mM Tris-HCl (pH 7.4, 25°C) for 4 min, 10 min, or 4 h at 45, 25, or 0°C to allow primer binding to reach equilibrium. Aliquots were removed immediately after preincubation and after an additional 8 min (45°C), 20 min (25°C), or 16 h (0°C). Rates of extension with the mismatches were normalized to the rate with the match. A fidelity coefficient was calculated for each template as the rate of extension with a match divided by the rate with a match plus the rate with a mismatch; the average fidelities are the geometric averages of the fidelities for both templates. Lowering the temperature appeared to increase fidelity. This temperature effect implies that entropically disfavored (e.g., stacking) (23, 25) interactions are critical for differentiation between GpNs.

In a search for other conditions that would increase the fidelity of the primer extension reaction, we examined a variety of reaction buffers using the -A- and -U- templates with GpU and GpA dinucleotides. Varying the Mg^+ concentration, or adding monovalent cations, polyamines, or sulfate ions did not significantly affect fidelity, though overall rates of primer extension changed.

Extension by all four nucleotides. We were unable to examine primer extension by pG in the above experiments because cleavage reactions predominate during incubation with GpG, presumably due to the 3′ rather than the 5′ G occupying the ribozyme G-binding site. However, a mutant form of the *Tetrahymena* ribozyme core binds 2-aminopurine much better than it binds G (19). Use of this mutant ribozyme together with 2-aminopurine(3′-5′)guanosine dinucleotide (XpG) allows primer extension with G. The fidelity of primer extension can thus be assayed with all four XpNs and all four template bases (Fig. 2). With each template the

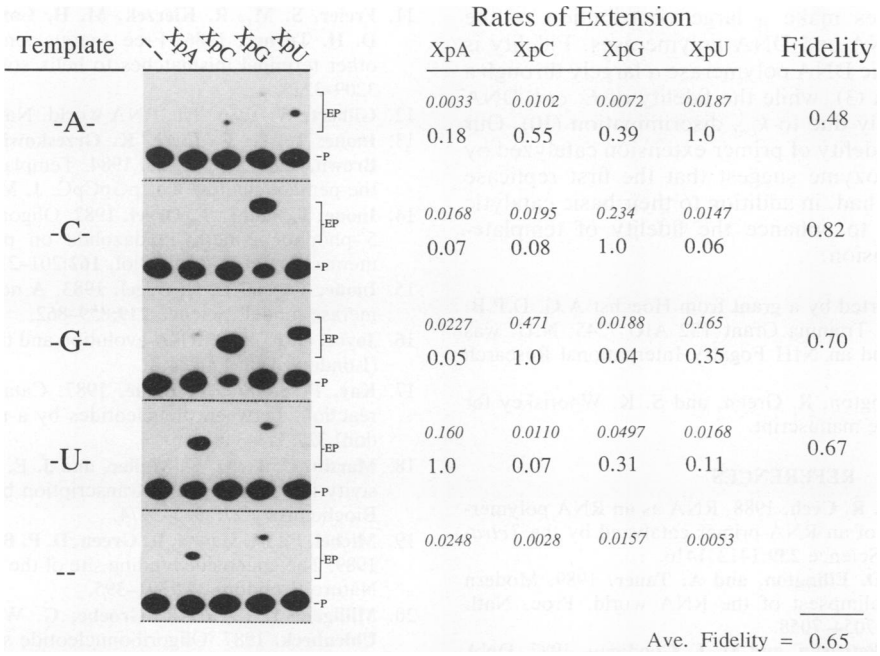

FIG. 2. Primer extension by ribozymes with altered G-binding sites and XpN dinucleotides. Ribozyme (500 nM) with the A264:U311 G-binding-site mutation (19) was incubated at 25°C with 1 nM labeled primer–20 mM $MgCl_2$–50 mM Tris-HCl (pH 7.4) and 50 μM of the indicated dinucleotide, when present. Enzyme and primer were preincubated at 25°C for 5 min prior to the reaction. Aliquots were removed from the reaction mix at 1, 3, and 6 h. The gel fragments shown are from the 3 h (C, G, and U templates) and 6 h (A template and no template) time points. Rates of extension are in italics. These rates were normalized to the rate for each Watson-Crick match, and fidelity coefficients were calculated as in Table 2. Ribozymes were generated as described in the text. The no-template ribozyme has no bases 5′ of the primer-binding site. XpNs were synthesized by coupling a protected 2-aminopurine riboside phosphoramidite (9) to a protected base on a solid support (Milligen; Biosearch). XpNs were purified by reverse-phase HPLC.

primer is preferentially extended by the dinucleotide creating the Watson-Crick match. The relative rates of primer extension show that Watson-Crick matches are preferred 3- to 25-fold over each of the nonwobble mismatches and 2- to 14-fold over the wobble mismatches. XpA and XpG are preferred with no template. Purines exhibit stronger stacking interactions than pyrimidines, so pA and pG would be expected to add fastest in the absence of a template if part of the dinucleotide binding energy comes from stacking of the 3′ nucleotide on the helix formed by the primer and the primer-binding site. The rates of extension with XpA and XpG are slower with the -A- template than with no template, indicating that the template can interact not only positively with matched XpNs but also negatively with mismatched dinucleotides.

The observed fidelity varies greatly with the identity of the template base, ranging from 0.82 with -C- as a template to 0.48 with -A- as a template. The average fidelity of the primer extension reaction calculated from the data in Fig. 2 is 0.65; i.e., if all four XpNs were available, a primer would extend with the nucleotide creating a Watson-Crick match 65% of the time and would extend with each of the three nucleotides creating mismatches about 12% of the time. Although the K_m of the AU mutant ribozyme for 2-aminopurine riboside (X) is 10-fold higher than the K_m of the wild-type ribozyme for guanosine (19), the fidelity seen with XpNs is similar to that observed with GpNs examined under the same conditions. The simplest interpretation of this result is that the binding of all XpNs is decreased uniformly relative to the binding of GpNs, so that matched XpNs have the same relative advantage over mismatched XpNs that matched GpNs have over mismatched GpNs.

In contrast to the uniform effect of the leaving group of the dinucleotide (X or G), the identity of the last primer-template base pair is likely to have a strong effect on fidelity. Model studies with oligonucleotides indicate that the average ΔΔG of helix stabilization due to the addition of a base pair as opposed to a mismatch is greatest following 3′G:5′C, less following 3′C:5′G, and least following 3′A:5′U and 3′U:5′A (26). Assuming that dinucleotide binding follows these trends, the K_m contribution to fidelity would depend on the previous primer-template base pair, with G:C > C:G > (A:U, U:A). In our experiments, the last primer-template base pair was always a C:G.

A true RNA replicase would have to be able to copy an external template (a template that is not part of the replicase molecule). We have found that primer extension on an external template, while detectable, is 10^4-fold slower than when the enzyme is linked to the template. This is presumably due to the inefficient formation of the enzyme-template-primer-dinucleotide complex. Though higher than the 0.25 fidelity of polymerization expected from random extension, the observed fidelity of primer extension catalyzed by the *Tetrahymena* ribozyme is far lower than that required for an RNA replicase. Our results are consistent with the earlier results of Orgel and colleagues (4, 13–15), who were able to demonstrate the influence of a template on the spontaneous polymerization of activated nucleotides, but not at a sufficient fidelity to generate accurate copies of long chains. Enzyme contacts with the template and substrate that favor

Watson-Crick matches make a large contribution to the fidelity of protein RNA and DNA polymerases. Fidelity is achieved by eukaryotic DNA polymerase α largely through a K_m-based mechanism (3), while the fidelity of *E. coli* DNA polymerase I is mainly due to k_{cat} discrimination (10). Our observations on the fidelity of primer extension catalyzed by the *Tetrahymena* ribozyme suggest that the first replicase molecules must have had, in addition to their basic catalytic ability, the capacity to enhance the fidelity of template-directed primer extension.

This work was supported by a grant from Hoechst A.G. D.P.B. was supported by NIH Training Grant T32 AIO 7245; N.U. was supported by NASA and an NIH Fogarty International Research Fellowship.

We thank A. D. Ellington, R. Green, and S. K. Whoriskey for helpful comments on the manuscript.

REFERENCES

1. **Been, M. D., and T. R. Cech.** 1988. RNA as an RNA polymerase: net elongation of an RNA primer catalyzed by the *Tetrahymena* ribozyme. Science **239**:1412–1416.
2. **Benner, S. A., A. D. Ellington, and A. Tauer.** 1989. Modern metabolism as a palimpsest of the RNA world. Proc. Natl. Acad. Sci. USA **86**:7054–7058.
3. **Boosalis, M. S., J. Petruska, and M. F. Goodman.** 1987. DNA polymerase insertion fidelity. J. Biol. Chem. **262**:14689–14696.
4. **Bridson, P. K., and L. E. Orgel.** 1980. Catalysis of accurate poly(C)-directed synthesis of 3′-5′-linked oligoguanylates by Zn^{2+}. J. Mol. Biol. **144**:567–577.
5. **Cech, T. R.** 1986. A model for the RNA catalyzed replication of RNA. Proc. Natl. Acad. Sci. USA **83**:4360–4364.
6. **Cech, T. R.** 1987. The chemistry of self-splicing RNA and RNA enzymes. Science **236**:1532–1539.
7. **Cech, T. R., N. K. Tanner, I. Tinoco, Jr., B. R. Weir, M. Zucker, and P. S. Perlman.** 1983. Secondary structure of the Tetrahymena ribosomal RNA intervening sequence: structural homology with fungal mitochondrial intervening sequences. Proc. Natl. Acad. Sci. USA **80**:3903–3907.
8. **Doudna, J. A., and J. W. Szostak.** 1989. RNA-catalysed synthesis of complementary-strand RNA. Nature (London) **339**:519–522.
9. **Doudna, J. A., J. W. Szostak, A. Rich, and N. Usman.** 1990. Chemical synthesis of oligoribonucleotides containing 2-aminopurine: substrates for the investigation of ribozyme function. J. Org. Chem. **55**:5547–5549.
10. **El-Deiry, W. S., A. G. So, and K. M. Downey.** 1988. Mechanisms of error discrimination by *Escherichia coli* DNA polymerase I. Biochemistry **27**:546–553.
11. **Freier, S. M., R. Kierzek, M. H. Caruthers, T. Neilson, and D. H. Turner.** 1986. Free energy contributions of G · U and other terminal mismatches to helix stability. Biochemistry **25**:3209–3213.
12. **Gilbert, W.** 1986. The RNA world. Nature (London) **319**:618.
13. **Inoue, T., G. F. Joyce, K. Grzeskowiak, L. E. Orgel, J. M. Brown, and C. B. Reese.** 1984. Template-directed synthesis on the pentanucleotide CpCpGpCpC. J. Mol. Biol. **178**:669–676.
14. **Inoue, T., and L. E. Orgel.** 1982. Oligomerization of (guanosine 5′-phophor)-2-methylimidazolide on poly(C)—an RNA polymerase model. J. Mol. Biol. **162**:201–217.
15. **Inoue, T., and L. E. Orgel.** 1983. A nonenzymatic RNA polymerase model. Science **219**:859–862.
16. **Joyce, G. F.** 1989. RNA evolution and the origins of life. Nature (London) **338**:217–224.
17. **Kay, P. S., and T. Inoue.** 1987. Catalysis of splicing-related reactions between dinucleotides by a ribozyme. Nature (London) **327**:343–346.
18. **Martin, C. T., D. K. Muller, and J. E. Coleman.** 1988. Processivity in early stages of transcription by T7 RNA polymerase. Biochemistry **27**:3966–3974.
19. **Michel, F., M. Hanna, R. Green, D. P. Bartel, and J. W. Szostak.** 1989. The guanosine binding site of the *Tetrahymena* ribozyme. Nature (London) **342**:391–395.
20. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. **15**:8783–8798.
21. **Mullis, K., and F. A. Faloona.** 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. **255**:335–350.
22. **Niyogi, S. K., and A. Stevens.** 1965. Studies of the ribonucleic acid polymerase from *Escherichia coli*. J. Biol. Chem. **240**:2593–2598.
23. **Saenger, W.** 1984. Principles of nucleic acid structure. Springer-Verlag, New York.
24. **Sharp, P. A.** 1985. On the origin of RNA splicing and introns. Cell **42**:397–400.
25. **Ts'o, P. O. P.** 1974. Bases, nucleotides, and nucleotides, p. 453–584. *In* P. O. P. Ts'o (ed.), Basic principles of nucleic acid chemistry, vol. 1. Academic Press, New York.

25a. **Turner, D.** Personal communication.

26. **Turner, D. H., N. Sugimoto, and S. M. Freier.** 1988. RNA structure prediction. Annu. Rev. Biophys. Biophys. Chem. **17**:167–191.
27. **Waring, R. B., C. Scazzocchio, T. A. Brown, and R. W. Davies.** 1983. Close relationship between certain nuclear and mitochondrial introns—implications for the mechanism of RNA splicing. J. Mol. Biol. **167**:595–605.