Destabilizing effect of an rRNA stem-loop on an attenuator hairpin in the 5' exon of the *Tetrahymena* pre-rRNA

YONG CAO¹ and SARAH A. WOODSON^{1,2}

¹Molecular and Cell Biology Program, University of Maryland, College Park, Maryland 20742-2021, USA

²Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742-2021, USA

ABSTRACT

Self-splicing of the *Tetrahymena* group I intron is attenuated by an rRNA stem-loop in the 5' exon, which competes with formation of the P1 splice site helix. The equilibrium between the P1 and P(–1) stem-loops is influenced by rRNA sequences upstream and downstream of the intron. To investigate the mechanism of this conformational switch, internal deletions and point mutations were introduced in the second rRNA stem-loop upstream of the 5' splice site. Nuclease protection, native gel electrophoresis, and self-splicing results show that this helix is important for maintaining self-splicing activity. Co-axial base stacking of adjacent helices in the 5' exon is proposed to enable exchange between inactive and active conformations of the pre-rRNA.

Keywords: group I intron; RNA structure; self-splicing

INTRODUCTION

An increasing number of examples exist in which the function of an RNA relies on the competition between two alternative conformations. Alternative secondary structures mediate the regulation of translation initiation and plasmid replication in bacteria (Altuvia et al., 1989; Spedding & Draper, 1993; Ma et al., 1994; Franch et al., 1997), and are an intrinsic feature of ribosomal and spliceosomal RNAs (reviewed in Staley & Guthrie, 1998; Wilson & Noller, 1998). In other cases, mutations that stabilize alternative secondary structures diminish the activity of autocatalytic RNAs (Séraphin et al., 1988; Fedor & Uhlenbeck, 1990), and inhibit pre-rRNA processing and assembly of RNA–protein complexes (Dammel & Noller, 1993; Fortner et al., 1994; Zavanelli et al., 1994). Self-splicing of the *Tetrahymena* pre-rRNA is attenuated by an alternative hairpin in the 5' exon (Woodson & Cech, 1991). This system provides an opportunity to study secondary structure rearrangements in large RNAs.

All group I introns, including the *Tetrahymena* intron, share a common secondary and tertiary structure (Cech, 1990). In the presence of Mg²⁺, the folded structure of the RNA provides an active site for the self-splicing reaction (Cech & Herschlag, 1996). Recognition of the

5' splice site requires base pairing between nucleotides at the end of the 5' exon and the internal guide sequence (IGS) of the intron (Been & Cech, 1986; Waring et al., 1986). The P1 splice site helix docks into a cleft formed by two helical domains of the catalytic core of the intron, P4-P6 and P3-P9 (Michel & Westhof, 1990; Cech & Herschlag, 1996). The mechanism of self-splicing involves addition of a guanosine nucleotide to the 5' splice site, followed by ligation of the 5' and 3' exons, and release of the intron as a linear RNA (Cech, 1990).

In pre-rRNAs containing natural 26S rRNA exon sequences, 5' splice site recognition is inhibited by an alternative hairpin, called P(–1), that is conserved in the mature 26S rRNA (Woodson & Cech, 1991). The 3' side of P(–1) involves the same nucleotides of the 5' exon that also base pair with the IGS (Fig. 1). As a result, P(–1) directly competes with formation of the P1 splice site helix (Woodson & Cech, 1991). This leads to two alternative pre-rRNA conformations: an inactive form that contains P(–1), and an active form that includes P1 and is competent to self-splice. The equilibrium between P(–1) and P1 depends on additional sequences in the 5' and 3' exons that comprise most of domain IV of the mature 26S rRNA (Woodson, 1992). In precursors that contain less than 146 nt of the 5' exon, P(–1) is favored and the splicing rate drops five-fold compared to longer pre-rRNAs (Woodson, 1992). In long transcripts, another alternative helix, PX, is pro-

Reprint requests to: Sarah Woodson, Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742-2021, USA; e-mail: sw74@umail.umd.edu.

FIGURE 1. Secondary structure of the *Tetrahymena* pre-rRNA. **A:** Alternative base pairings in the 5' exon. Paired regions (P) are indicated; the distal end of P(-2) is shown schematically. Upper case, exon sequences; lower case, intron (IVS); filled square, 5' splice site; box, internal guide sequence (IGS). **B:** Secondary structure of the mature 26S rRNA (Noller et al., 1981), showing the position of the intron. Sequences that are involved in alternative pairings are color coded as in A. Stem-loops on the right represent the $\Delta 17$ and $\Delta 41$ deletions in P(-2). Arrowheads indicate the limits of the pre-rRNAs: TZIVS $\Delta 12$, -20 to 29; SW020, -82 to 86; SW012, -146 to 86. Adapted from Emerick and Woodson (1993).

posed to shift the equilibrium in favor of P1 by competing with formation of P(-1) (Fig. 1; Woodson & Emerick, 1993), in a manner reminiscent of translation attenuation in the *trp* operon (Yanofsky, 1981).

In addition to PX, a conserved stem-loop upstream of P(-1), which we will call P(-2), is likely to play an

important role in stabilizing the active form of the pre-rRNA. The PX base pairing extends from P(-2), and is expected to be stabilized by direct base stacking with the end of the P(-2) helix in the splicing-competent form of the pre-rRNA. In addition, stacking between P(-2) and P(-1) in the inactive conformation could

facilitate interconversion of P(-1) and P1 by a strand-exchange mechanism. Besides promoting helix exchange, P(-2) could influence the folding of the pre-rRNA through tertiary interactions in the rRNA exons or the intron RNA. In support of this idea, a comparison of active and inactive pre-rRNA revealed differences in the susceptibility of this region to base-modifying reagents and ribonucleases (Emerick & Woodson, 1994; Pan & Woodson, 1998).

We report an investigation of the effect of P(-2) on the equilibrium between P(-1) and P1, using self-splicing assays, ribonuclease (RNase) protection experiments, thermal melting studies, and native gel electrophoresis. The results show that the P(-2) stem-loop in the rRNA promotes self-splicing of the intron by destabilizing P(-1), thereby promoting formation of the P1 substrate helix. These results provide a basis for constructing a minimal system that recapitulates the conformations of the pre-rRNA.

RESULTS

Equilibrium between P(-1) and P1

Previous work showed that the stability of P(-1) and P1 are modulated by other sequences in the pre-rRNA (Woodson & Cech, 1991; Woodson & Emerick, 1993). Accordingly, we wished to evaluate the equilibrium between these alternative hairpins in the presence and absence of exon and intron sequences. To directly compare the stability of P(-1) and P1, oligonucleotides containing only the sequences of P(-1) (20 nt) or P1 (34 nt) were prepared as described in Materials and Methods. RNase protection assays at 30 °C in 5 mM MgCl₂ confirmed that the oligomers formed the expected hairpin structures (Fig. 2). Both P(-1) and P1 RNAs migrated as one band on a native polyacrylamide gel, indicating that they most likely adopt one major conformation under these conditions (Fig. 3A).

FIGURE 2. RNase protection of P1 and P(-1). **A:** Nuclease digestion of 5' [³²P]-labeled P1 RNA was performed at 30 °C as described in Materials and Methods. Reaction mixtures were loaded on a 12% polyacrylamide sequencing gel and detected by autoradiography. Lane C, no nuclease; G, A, A + U, and C, sequencing reactions; T1, RNase T1; T2, RNase T2; B, *B. cereus* RNase; S1, nuclease S1; V1, RNase V1. **B:** Structure of P1 RNA. Circle, nuclease S1; triangle, RNase V1; cross, RNase T1; star, RNase T2; diamond, *B. cereus* RNase. For each lane, bands with greater than median intensity were considered to be strong (filled symbol), those with median or lighter intensity were considered to be weak (open symbol). In our hands, *B. cereus* RNase is only weakly specific for unpaired C's and U's. Secondary structures were determined as described in Materials and Methods. **C:** P(-1) RNA. Symbols as in B.

However, P1 RNA displayed some weak cleavages in addition to the major products (Fig. 2B), and may transiently form other secondary structures.

The secondary structure of a 46-nt RNA oligomer (R46) that can form either P(−1) or P1 was also probed using RNase protection assays. As shown in Figure 4A, the sequence ACUCUC, which forms part of either the P(−1) or P1 helix, was not digested by nucleases that are specific for single-stranded RNA, such as RNase T1, T2, and nuclease S1, but was cleaved by RNase V1, which is specific for double-stranded RNA. In contrast, sequences in the IGS or 5' exon that are complementary to ACUCUC were both cleaved by single-strand-specific enzymes, with the IGS more susceptible than the 5' exon. These data suggested that both P(−1) and P1 are present in the R46 population.

The cleavage pattern of wild-type R46 was compared to RNAs containing mutations that stabilize or destabilize P(−1) (Woodson & Cech, 1991). The double mutation −16C; −18C disrupts P(−1), and caused the RNase protection pattern to be consistent with the P1 conformation (Fig. 4B). The mutation −14C stabilizes P(−1), and the cleavage pattern of this mutant was similar to that of the 20 nt P(−1) RNA (Fig. 4C). The RNase digestion pattern of a precursor RNA with the same 20-nt 5' exon as R46 also clearly matched that of the 20-nt P(−1) RNA (Fig. 4D), consistent with its low splicing activity (Woodson & Cech, 1991). Native gel electrophoresis of R46 suggested that the P1 and P(−1) conformations are in rapid equilibrium in the wild-type oligonucleotide. The −16C; −18C and −14C oligonucleotides migrated as single bands on a 12% native gel, with −16C; −18C running more slowly than −14C (Fig. 3B). Wild-type R46 migrated as a broad band with a mobility most similar to R46:−14C.

P(−1) is thermodynamically more stable than P1

To compare the stability of the P(−1) and P1 hairpins, thermal melting transitions were obtained from the hypochromism at 260 nm. The P(−1) RNA undergoes a cooperative two-state transition, as expected for a single hairpin. The midpoint of the transition was 70 °C in 5 mM MgCl₂ (Fig. 5), and increased moderately with Mg²⁺ concentration. The free energy of the P(−1) hairpin was estimated to be −6.4 kcal/mol at 20 °C in 5 mM MgCl₂. The apparent *T_m* of the P1 RNA was 58 °C in 5 mM MgCl₂, and P(−1) was approximately 3 kcal/mol more stable than P1 over the range of Mg²⁺ concentrations tested (0–10 mM MgCl₂). However, the P1 denaturation curve was broad, and may represent two closely overlapping transitions. For both RNAs, the *T_m* values were independent of RNA concentration over the range of 0.5–2.5 μM.

FIGURE 3. Native gel electrophoresis of 5' splice site oligonucleotides. 5' [³²P]-labeled oligonucleotides were electrophoresed on a 12% native gel electrophoresis at 10–12 °C as described in Materials and Methods. Prior to loading, RNA samples were heated to 95 °C for 1 min and cooled in the presence of splicing buffer. **A:** Wild-type R46, P1, and P(−1) RNAs. **B:** Wild-type (wt), −14C, and −16C; −18C R46 RNAs, in buffer containing 0 or 5 mM MgCl₂.

Unfolding of wild-type R46 was even less cooperative than P1 RNA, also consistent with the idea that R46 undergoes active exchange between one or more conformational states. The apparent midpoint (67 °C in 5 mM MgCl₂) was intermediate between that of R46:−16C; −18C (62 °C) and R46:−14C (86 °C). A second transition at 42 °C becomes apparent in R46:−14C, which may correspond to melting of a short helix in the 3' end of the RNA (Fig. 4C).

Effect of P(−2) on the equilibrium between P(−1) and P1

Differences in the reactivity of pre-RNAs with varying exon lengths have shown that upstream sequences alter the equilibrium between P(−1) and P1 (Woodson, 1992). To obtain direct evidence for this, we prepared a 109-nt RNA (R109) that contains R46 plus the P(−2) stem-loop and a 7-nt sequence at the 5' end that forms the 5' side of the PX helix (Fig. 1). R109 can adopt two conformations: one containing PX, P(−2), and P1, corresponding to the active pre-rRNA (Woodson & Emerick, 1993), and one containing P(−2) and P(−1), corresponding to the secondary structure of the mature rRNA (Noller et al., 1981; Gutell, 1996). To determine whether the length of the P(−2) stem-loop is important, RNAs with two internal deletions that shorten P(−2) but maintain the sequence of the terminal loop (Δ17 and Δ41) were constructed. In a third variant, the entire P(−2) stem-loop was replaced with a 5-nt linker (ΔP(−2)).

As shown in Figure 6, the RNase protection pattern of the R109 RNA agreed with the predicted secondary structure of the P(−2) stem-loop in the 26S rRNA (Noller et al., 1981), although the data do not exclude other base pairing arrangements in the P(−2) stem (Clark et al., 1984). The cleavage patterns at the 3' end of the wild-type, Δ17, and ΔP(−2) RNAs were consistent with

FIGURE 4. Summary of RNase protection results for R46 RNAs. **A:** Wild-type R46. **B:** R46:-16C; -18C. **C:** R46:-14C. **D:** Precursor TZIVSΔ12. 5' Exon of TZIVSΔ12 has the same sequence as R46. Symbols as in Figure 2.

formation of P(-1), and not the P1 splice site helix (Fig. 6A,B,D). The exception was the Δ41 mutant, in which protection of the 5' end from S1 and cleavage by nuclease V1 confirmed the formation of P1 and the

alternative PX helix (Fig. 6C). These oligonucleotides migrated as one band on native polyacrylamide gels, indicating that each sequence has one major conformation (data not shown).

FIGURE 5. Optical T_m values at different concentrations of $MgCl_2$. Thermal melting curves at 260 nm were collected in 100 mM $(NH_4)_2SO_4$, 50 mM HEPES, pH 7.5, supplemented with 0–20 mM $MgCl_2$, and fit to a two-state model as described in Materials and Methods. Open circle, P1; closed circle, P(–1); filled triangles, R46; open triangles, R46:–16C; –18C; filled diamond, the high-temperature transition of R46:–14C; open diamond, low-temperature transition for R46:–14C. Values for ΔH° obtained from the fits were 44 kcal/mol for P(–1) and 23 kcal/mol for P1.

Conformation of the pre-rRNA

To determine the extent to which interactions with the intron perturb the equilibrium between P1 and P(–1), a series of pre-rRNAs with the same internal deletions of the P(–2) stem-loop were prepared. Based on the results of RNase protection experiments, the conformation of the P(–2) stem-loop was very similar in the precursor and the R109 RNAs (Fig. 7). The most significant difference was that the P1 helix was preferred in both the wild-type and $\Delta 41$ pre-rRNAs (Fig. 7A). Weak T1 cleavages in the IGS of the $\Delta 17$ pre-rRNA are consistent with a mixture of the P1 and P(–1) conformations for this RNA (Fig. 7B). Deletion of the entire P(–2) stem shifted the equilibrium back toward P(–1), indicating that P(–2) has a significant influence on the equilibrium between these alternative hairpins in the pre-rRNA, as well as in the 5' exon oligonucleotides.

Self-splicing activity

To further investigate the role of P(–2) in stabilizing the active conformation of the pre-rRNA, the self-splicing activity of transcripts containing P(–2) deletions was determined. Pre-rRNA was incubated in splicing buffer with GTP, and the accumulation of ligated exon RNA was monitored over time (Fig. 8A). The mixture of active and inactive conformers in the pre-rRNA population results in biphasic kinetics (Fig. 8B; Emerick et al., 1996). The initial burst of reaction is thought to correspond to the correctly folded RNA, whereas the rate of

the second phase is limited by slow refolding of RNAs in which the catalytic core is misfolded (Emerick et al., 1996; Pan & Woodson, 1998). As shown in Figure 8B and Table 1, deletion of the entire stem-loop ($\Delta P(–2)$) lowered the rate constant of the initial phase of splicing (λ_1) sevenfold relative to the wild-type. Deletion of 41 nt in P(–2) ($\Delta 41$) enhanced the initial rate constant of self-splicing (λ_1) about twofold, whereas $\Delta 17$ deletion had little effect (Table 1).

The amplitude (A_1) of the initial burst was similar for the wild-type and deletion mutants, suggesting that removal of P(–2) does not cause gross misfolding of the pre-rRNA. Similar results were obtained with an analogous series of pre-rRNAs that contain a shorter (82 nt) 5' exon (Table 1), although the amplitude of the fast phase is lower. The splicing activity of both series agreed well with the results of the RNase protection experiments, in which P1 was most stable in the $\Delta 41$ RNA, and least favored in the $\Delta P(–2)$ oligonucleotide. Together, the results show that the P(–2) stem-loop enhances the self-splicing activity of pre-rRNAs, most likely by shifting the conformational equilibrium in favor of the P1 splice site helix.

Intermolecular (*trans*) splicing reactions, in which a free 5' exon substrate (rGGCUCUCU) is ligated to the 3' exon, were also investigated. The 5' exon oligonucleotide competes with the intramolecular 5' exon for binding to the IGS, and RNAs containing P(–1) undergo *trans*-splicing more readily than those containing P1 (Woodson & Cech, 1991). Accordingly, $\Delta P(–2)$ pre-rRNA, which self-spliced most poorly, was *trans*-spliced most rapidly (5.0 min^{-1} with 50 μM free 5' exon) (Fig. 8C). The wild-type pre-rRNA and the other deletion mutants had similar rates of *trans*-splicing as each other (about 1.5 min^{-1}). These results support the interpretation that the change in self-splicing activity that results from deletion of P(–2) is a consequence of a shift in the equilibrium between P(–1) and P1, rather than a global change in the structure of the intron.

Native-gel electrophoresis of pre-rRNAs

The active and inactive forms of the pre-rRNA have distinct mobilities on 6% native polyacrylamide gels (Emerick & Woodson, 1994). This method was used to probe the conformational equilibrium of pre-rRNAs containing deletions in P(–2). After renaturation in the presence of Mg^{2+} , most of the wild-type, $\Delta 17$, and $\Delta 41$ pre-rRNAs migrated as the active form (band 4; Fig. 9A). Upon the addition of GTP, the active (band 4) RNA experienced a shift in mobility to band 3, which contains both precursor and spliced products (Emerick & Woodson, 1994). A smaller fraction of the RNA migrated more slowly as an inactive species (band 1), in which the catalytic core is misfolded (Pan & Woodson, 1998). For each sequence, the amount of band 4 was

FIGURE 6. RNase protection results of oligomers containing P(-2). A: Wild-type (wt) R109. B: Δ17R109. C: Δ41R109. D: ΔP(-2)R109. Symbols as in Figure 2.

FIGURE 7. RNase protection results of pre-rRNAs. Wild-type transcripts contain 146 nt of rRNA sequence in the 5' exon and a 86-nt 3' exon. RNAs were transcribed from pSW012 and its derivatives, and 5'-labeled with ^{32}P . **A:** Wild-type. **B:** $\Delta 17$. **C:** $\Delta 41$. **D:** $\Delta \text{P}(-2)$. Symbols as in Figure 2.

FIGURE 8. Intramolecular and intermolecular self-splicing. **A:** Uniformly [^{32} P]-labeled SW012 pre-rRNAs were purified by gel filtration chromatography, and splicing reactions were performed as described in Materials and Methods. Reactions were initiated with 100 μ M GTP. Aliquots were removed at the times shown above each lane (0–15 min), and applied to a 4% polyacrylamide sequencing gel. Pre-rRNA, precursor; IVS, linear intron; LE, ligated exon RNAs. **B:** Reactions similar to those in A were quantitated with a PhosphorImager, and the fraction of spliced products (f_{sp}) fit to a double-exponential equation (Table 1). Inset shows the first 5 min of the progress curve. Circle, wild-type; triangle, $\Delta 17$; diamond, $\Delta 41$; square, $\Delta P(-2)$. **C:** Intermolecular (*trans*) splicing. Reactions were initiated with 0.1–200 μ M 5' exon RNA (rGGCUCUCU), without GTP. The observed rate, k_{app} , was determined from first-order fits of f_{sp} versus time, where f_{sp} is the fraction of spliced products (Emerick et al., 1996). Error bars were taken from standard error of each fit. Curve represents fit to $k_{app} = k_{max} \cdot S / (K_M + S)$, where k_{max} is the maximum rate of *trans*-splicing, S is the concentration of 5' exon RNA, and K_M is the Michaelis constant. Symbols as in B.

qualitatively consistent with the fraction of active RNA (Table 2).

Unlike the other transcripts, $\Delta P(-2)$ RNA migrated as a mixture of band 4 and the species labeled band 2b (Fig. 9A). Band 2b is very reactive toward intermolecular exon ligation (Emerick, 1995), and is thought to represent a conformation containing P(-1) in which the catalytic core is folded (J. Pan & S. Woodson, unpubl.). This result is in agreement with the lower rate of self-splicing and the increased *trans*-splicing activity of $\Delta P(-2)$ RNA (Fig. 8B,C). It is also consistent with the RNase protection data (Fig. 7D), which indicate that deletion of P(-2) causes P(-1) to be favored over P1.

Site-directed mutagenesis of P(-2)

The observation that shortening of the P(-2) has little effect on splicing, whereas deletion of the entire stem significantly decreases activity, suggested that the base of the stem was most important. Point mutations that cause a mismatch in the first base pair of P(-2) were introduced in the wild-type pre-rRNA (Fig. 1A). The self-splicing rates of the mutated pre-rRNAs, -75C (0.23

min^{-1}) and -21G (0.04 min^{-1}), were similar to or even slower than that of the $\Delta P(-2)$ deletion mutant (0.21 min^{-1} ; Table 3). The compensatory double mutation -75C; -21G restored the rate of splicing to wild-type levels (2.29 min^{-1}). Therefore, the first base pair of P(-2) nearly accounts for all the effect of P(-2). As expected, the *trans*-splicing rates of these pre-rRNAs were opposite in trend to those of intramolecular self-splicing (Table 3). The two single mutants *trans*-spliced more rapidly than the wild-type or -75C; -21G double mutations. Point mutations at -73 and -74 that cause a mismatch in the second or third base pair of P(-2) also decreased the rate of splicing, but to a lesser extent (data not shown).

The mobility of these pre-rRNAs on native polyacrylamide gels was consistent with their self-splicing activity. As shown in Figure 9B, the -75C and -21G mutants primarily migrated as band 2b, a conformer that is active in *trans*-splicing. They also formed an increased amount of inactive (band 1) RNA. The -75C RNA appeared as a very diffuse band, with additional low-mobility species, suggesting that this mutation also promotes global misfolding of the RNA. The compen-

satory double mutation –75C; –21G reduced the fraction of band 2b and significantly increased the fraction of active (band 4) pre-rRNA, consistent with its restored splicing activity.

DISCUSSION

Model for strand exchange

Autocatalytic introns must fold into their active structure in order to achieve optimal rates of splicing (Cech, 1990). For introns in rRNA and tRNA genes, the structure of the surrounding exon sequences contributes to the stability of the folded structure, and can increase splicing activity (Woodson, 1992; Zaug et al., 1993). In other cases, alternative secondary structures impair splice-site recognition, leading to a decrease in splicing (Sraphin et al., 1988; Woodson & Cech, 1991; Semrad & Schroeder, 1998). Here, we show that the equilibrium between two alternative hairpins in the *Tetrahymena* pre-rRNA is perturbed by the presence of an upstream rRNA stem-loop, which we refer to as P(–2). The data are consistent with a simple strand exchange model for opening of the P(–1) helix, in which the base pairs of P(–1) are displaced by the upstream PX helix (Woodson & Emerick, 1993). Strand exchange between P(–1) and PX would be facilitated by co-axial stacking with P(–2), which aligns the upstream PX sequence with the base of P(–1) (Fig. 1A). A similar mechanism was proposed to account for exchange between two alternative forms of a spliced leader RNA (LeCuyer & Crothers, 1993).

Evidence in support of this model for the *Tetrahymena* pre-rRNA includes the fact that complete deletion of P(–2), which would eliminate any base stacking

FIGURE 9. Native gel electrophoresis of pre-rRNAs. Uniformly [³²P]-labeled pre-rRNAs were loaded on a 4% native gel after thermal renaturation as described in Materials and Methods. Lane (–), no GTP; (+), 100 μM GTP was added 15 s before loading. Band 1, inactive RNA; band 2b, active in *trans*-splicing only; band 4, active pre-rRNA; band 3, GTP-dependent conformer (Emerick & Woodson, 1994). **A:** Wild-type (wt) and P(–2) deletion mutants of SW012 pre-rRNA. **B:** Wild-type pre-rRNA and pre-rRNA RNA containing the single or double mutations shown in Figure 1A.

effect, drives the RNA into a conformation that contains P(–1). This lowers the initial rate of self-splicing and increases the potential for intermolecular splicing. Self-splicing is similarly inhibited by a base mismatch at the end of P(–2), which is expected to decrease the free

TABLE 1. Rate constants for self-splicing reactions.

RNA	A_1^a	λ_1 (min ^{–1}) ^a	A_2^a	λ_2 (min ^{–1}) ^a
146 nt 5' exon^b				
wt	0.52 ± 0.09 ^c	1.47 ± 0.09	0.17 ± 0.03	0.12 ± 0.05
Δ17	0.51 ± 0.01	1.26 ± 0.13	0.06 ± 0.03	0.13 ± 0.03
Δ41	0.41 ± 0.01	3.05 ± 0.01	0.19 ± 0.01	0.13 ± 0.09
ΔP(–2)	0.47 ± 0.26	0.21 ± 0.06	0.12 ± 0.10	0.09 ± 0.01
82 nt 5' exon^d				
wt	0.45 ± 0.12	1.00 ± 0.15	0.09 ± 0.04	0.05 ± 0.01
Δ17	0.36 ± 0.14	1.15 ± 0.10	0.08 ± 0.02	0.07 ± 0.01
Δ41	0.30 ± 0.05	1.73 ± 0.17	0.23 ± 0.05	0.15 ± 0.14
ΔP(–2)	0.40 ± 0.01	0.25 ± 0.12	0.22 ± 0.13	0.05 ± 0.04

^aAmplitudes (A_1 , A_2) and first-order rate constants (λ_1 , λ_2) were determined from nonlinear fits of the fraction of splice products, f_{sp} , to $f_{sp} = A_1(1 - e^{-\lambda_1 t}) + A_2(1 - e^{-\lambda_2 t})$ (Emerick et al., 1996).

^bPre-rRNAs were transcribed from pSW012 and derivatives, as described in Materials and Methods.

^cUncertainties are given as the standard deviation of values obtained from fits to 2–8 independent progress curves.

^dPre-rRNAs were transcribed from pSW020 and derivatives.

TABLE 2. Fraction of native RNA.^a

RNA ^b	Native (band 4)	G-dependent (band 3)	<i>Trans</i> -splicing (band 2b)		Inactive (band 1)	
	–GTP ^c	+GTP	–GTP	+GTP	–GTP	+GTP
wt	0.75	0.86	ND ^d	ND	0.25	0.14
Δ17	0.71	0.76	ND	ND	0.29	0.23
Δ41	0.83	0.82	ND	ND	0.17	0.18
ΔP(–2)	0.67	0.69	0.18	0.23	0.13	0.08

^aThe radioactivity in each lane of a native gel (Fig. 9A) was quantitated and the fraction of pre-rRNA in each band (1, 2b, 3, and 4) was determined relative to the sum.

^bSequence of the pre-rRNA. Wild-type (wt) RNA was transcribed from pSW012.

^cAfter annealing in splicing buffer and 2 min at 30 °C, RNAs were incubated 15 s in the absence (–) or presence (+) of 100 μM GTP at 30 °C, before loading in the well of the native gel.

^dNot detected.

TABLE 3. Splicing rates of pre-rRNAs with point mutations in P(−2).

RNA ^c	Intramolecular ^a		Intermolecular ^b	
	A_1^d	λ_1 (min ^{−1}) ^d	A_1^d	λ_1 (min ^{−1}) ^d
wt	0.52 ± 0.09	1.47 ± 0.09	0.38 ± 0.04	2.1 ± 0.4
−21C	0.57 ± 0.12	0.04 ± 0.01	0.38 ± 0.06	3.6 ± 1.8
−75G	0.63 ± 0.08	0.23 ± 0.01	0.44 ± 0.01	4.4 ± 2.0
−21C; −75G	0.54 ± 0.03	2.29 ± 0.47	0.39 ± 0.03	1.1 ± 0.4

^aIntramolecular splicing reactions contained 100 μM GTP.^bIntermolecular (*trans*) splicing reactions contained 50 μM 5′ exon substrate and no GTP.^cPre-rRNAs were transcribed from pSW012, with or without the point mutations shown.^dInitial amplitudes (A_1) and rate constants (λ_1) for the wild-type are from Table 1; values for mutant pre-rRNAs were obtained from nonlinear fits to a single first-order rate equation.

energy of stacking between P(−2) and P(−1) (Kim et al., 1996). By contrast, the Δ41 deletion, which stabilizes the P(−2) stem, increases the fraction of pre-rRNA that contains PX and P1, as judged by RNase protection assays and self-splicing activity. However, this simplistic model may not be sufficient to account for interconversion of these forms of the pre-rRNA. Strand exchange between adjacent helices is expected to occur on the second time scale (LeCuyer & Crothers, 1993; Panyutin & Hsieh, 1994). The observation that the pre-rRNA conformers are trapped in native gels at low temperatures suggests that they may be stabilized by other interactions in the RNA.

Equilibrium between P(−1) and P1

Comparison of melting temperatures and RNase protection results showed that the P(−1) hairpin is intrinsically more stable than P1 under the conditions of our self-splicing assays. Although the R46 RNA, which can form either hairpin, is conformationally dynamic, its melting temperature and native gel mobility suggest that P(−1) is still strongly favored. P(−1) is also preferred in the R109, which contains the wild-type P(−2) stem-loop. However, truncation of P(−2) in the Δ41 oligonucleotide shifts the position of this equilibrium toward P1. RNase protection experiments provide direct support for the idea that the alternative PX pairing displaces P(−1). Comparison of the relative stabilities of P(−1) and P1 among the deletion mutants shows that the stability of P(−1) follows the descending order ΔP(−2), Δ17, wt, and Δ41.

The equilibrium between these alternative secondary structures at the 5′ splice site is also influenced by other long-range interactions in the pre-rRNA. For the entire series of P(−2) variants, P1 is more stable in the pre-rRNA than in the corresponding R109 oligonucleotide. This is most evident in the wild-type sequence,

for which R109 primarily forms P(−1) and the pre-rRNA forms P1. Interactions between the 5′ and 3′ exons, which are present in the pre-rRNA but not in R109, may contribute to the stability of PX (Noller et al., 1981; Woodson, 1992). In addition, the P1 helix is stabilized by tertiary interactions with the active site of the intron core, which contribute 4–5 kcal/mol toward binding of oligonucleotide substrates by the *Tetrahymena* ribozyme at higher temperatures (Pyle & Cech, 1991; Herschlag et al., 1993). The free energy of docking, however, decreases at lower temperatures and approaches zero around 30 °C (Narlikar & Herschlag, 1996), the temperature of our self-splicing assays. As a result, docking of P1 is not sufficient to overcome competition from P(−1), in agreement with the poor splicing activity of precursors with short exons (Woodson & Cech, 1991). In conjunction with PX, however, a very small change in ΔG° could be sufficient to shift the conformational equilibrium in favor of P1.

Implications of P(−2) for the folding of active pre-rRNA

The secondary structure of domain IV, including P(−1) and the base of P(−2), is highly conserved among prokaryotic and eukaryotic large subunit (LSU) rRNAs (Noller et al., 1981; Gutell, 1996). These hairpins have not yet been assigned a specific function in the assembled ribosome. However, nucleotides in the *Escherichia coli* 23S rRNA that surround the position homologous to the *Tetrahymena* splice junction, including P(−1), are protected by P-site tRNAs and are near the decoding center in the small ribosomal subunit (Moazed & Noller, 1989; Mitchell et al., 1992; Joseph & Noller, 1996). Mutations in this region of the *E. coli* 23S rRNA inhibit association of the large and small subunits and peptidyl transferase activity of the ribosome (Levieva et al., 1995).

Curiously, group I and archaeal tRNA-like introns have been found in the rDNA of a number of organisms at positions near the splice junction of *Tetrahymena* (Woodson, 1996). In every case, the P(−1) hairpin has the potential to interfere with splice site recognition, suggesting that the mechanism for reducing the stability of the stem is common to large subunit rRNAs. In the *Tetrahymena* pre-rRNA transcripts, the adjacent P(−2) helix plays an important role in opening P(−1) and permitting folding of the RNA into a conformation that is competent to self-splice. P(−2) would also be expected to facilitate refolding of P(−1) after excision of the intron (Fig. 1A), which in turn could inhibit reverse self-splicing (Woodson & Cech, 1991). It will be of interest to know whether a similar mechanism to reduce inhibition of 5′ splice site recognition and to promote forward splicing is present in the pre-rRNA of other organisms.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis

Plasmids pTZIVSΔ12, pTZIVSΔ24, and their derivatives have been described previously (Woodson & Cech, 1991). Plasmid pSW012 encodes a precursor with 146 nt of natural rRNA sequence in the 5' exon and an 86-nt 3' exon (Woodson, 1992). Other plasmids were constructed by site-directed mutagenesis of pSW012 (Kunkel et al., 1987). pSW020 was prepared by introducing a T7 promoter at position 2216 of the natural rRNA, 82-nt upstream of the 5' splice site. Internal 17-, 41-, and 50-nt ($\Delta P(-2)$) deletions of P(-2) in pSW012 and pSW020 yielded pSW012Δ17, pSW012Δ41, pSW012ΔP(-2), pSW020Δ17, pSW020Δ41, and pSW020ΔP(-2). Point mutations shown in Figure 1A were also introduced into pSW012 by site-directed mutagenesis (Kunkel et al., 1987).

Plasmids were linearized with *EcoR* I for run-off in vitro transcription of precursor RNAs. To transcribe R46 and R109 oligomers, a *Fok* I site was introduced at position 26 in the intron through site-directed mutagenesis to give pTZIVSΔ12F (R46), pTZIVSΔ24F (P1), pSW020F (R109), pSW020Δ17F, pSW020Δ41F, and pSW020ΔP(-2)F. These plasmids were digested with *Fok* I before use in transcription reactions. Restriction enzymes were purchased from New England Biolabs; DNA primers were purchased from GIBCO BRL/Life Technologies.

Preparation of RNA

P(-1) RNA (20 nt) and the 5' exon substrate, 5'-rGGCUC UCU, were synthesized using oligodeoxynucleotide templates containing a T7 promoter (Milligan & Uhlenbeck, 1989). All other RNA oligomers and precursors were transcribed from linear plasmid DNA using T7 RNA polymerase as described previously, and purified by isolation from 4–20% polyacrylamide gels containing 8 M urea (Zaug et al., 1988; Woodson & Cech, 1991). Concentrations were determined from the absorbance at 260 nm, with an average extinction coefficient of $10 \text{ mM}^{-1} \text{ cm}^{-1} \text{ nt}^{-1}$.

5' End-labeled RNA was prepared from calf intestinal phosphatase (Promega)-treated RNA using T4 polynucleotide kinase (U.S. Biochemical) and γ -[^{32}P]-ATP (New England Nuclear), and gel purified as above (Zaug et al., 1988). Precursor RNAs used for splicing assays and for native gel electrophoresis were uniformly labeled with α -[^{32}P]-ATP during transcription by T7 polymerase, and purified with TE-100 gel filtration spin columns (Clontech) pre-equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (Emerick & Woodson, 1993). Transcription reactions were performed at 30 °C for 20–30 min to minimize splicing.

Determination of T_m

Thermal denaturation experiments were performed on an HP8452 spectrophotometer equipped with a Peltier variable temperature sample holder. The absorbance of RNA was measured at 260 nm, and temperatures were varied from 16 to 94 °C with 2° increments. Prior to the start of the experiment, purified RNA (0.2–0.5 OD or 0.2–2.5 μM) in 0.5–1 mL of 100 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM HEPES, pH 7.5, with

0–20 mM MgCl_2 , was heated to 95 °C for 3 min and placed on ice. Similar results were obtained in trials in which the temperature was decreased from 94 to 16 °C. Where possible, T_m and ΔH° values were obtained from nonlinear fits to an equation that describes a single transition with sloping upper and lower baselines, as described previously (Peterheim & Turner, 1983; Laing & Draper, 1994). In other cases, they were obtained from the first derivative of the absorbance curve.

RNase protection experiments

5' [^{32}P]-labeled RNA (5–10 pmol) in 20 mM Tris-HCl, pH 7.5, was heated to 95 °C for 1 min and cooled in the presence of 5 mM MgCl_2 . Renatured RNA was diluted fourfold in T1 buffer (200 mM NaCl, 5 mM MgCl_2 , 0.2 mg/mL tRNA, 10 mM Tris-HCl, pH 7.5) for RNase T1, RNase T2, and RNase V1; S1 buffer (200 mM NaCl, 0.1 mM ZnSO_4 , 5 mM MgCl_2 , 0.2 mg/mL tRNA, 25 mM sodium acetate, pH 5.0) for nuclease S1; or B buffer (0.8 mM EDTA, 0.5 mg/mL tRNA, 8 mM sodium citrate, pH 5.0) for *Bacillus cereus* RNase, respectively. Samples were treated 20 min at 30 °C with nuclease S1 (0.1–0.2 U/ μL), RNase T1 (0.005–0.1 U/ μL), RNase T2 (0.002–0.0005 U/ μL), RNase V1 (0.01–0.04 U/ μL), and *B. cereus* RNase (0.1–0.5 Unit/ μL) (Ehresmann et al., 1987). RNA sequencing reactions were performed as described previously (Donis-Keller, 1979, 1980). The reactions were quenched with 10 M urea loading buffer (2 $\times$), loaded on 6–12% polyacrylamide sequencing gels, and detected by autoradiography. RNase T1 cleaves after unpaired G nucleotides, U2 at A > G, Phy M at A and U, T2 and S1, at any single-stranded nucleotide, *B. cereus* at C and U, and V1 at any double-stranded or stacked nucleotide. RNase T1, T2, and U2 were purchased from Calbiochem. RNase V1, Phy M, and nuclease S1 were purchased from Pharmacia Biotech.

Secondary structure determination

RNA secondary structures with free energy values within 10% of the minimum were computed by the MFOLD program at 37 °C without constraints (Jaeger et al., 1989). The figures show the structure that agreed best with the nuclease digestion pattern for each sequence.

Self-splicing assays

Uniformly labeled precursor RNAs were heated to 95 °C and cooled rapidly in the presence of splicing buffer (100 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM HEPES, 5 mM MgCl_2 , pH 7.5) as described previously (Walstrum & Uhlenbeck, 1990; Emerick & Woodson, 1993). Splicing reactions were performed at 30 °C. Prior to the addition of 0.1 mM GTP, precursor RNA was pre-incubated at 30 °C for 2 min. Spliced products were separated on 4 or 6% polyacrylamide gels, and the radioactivity was quantitated with a Molecular Dynamics PhosphorImager. The fraction of spliced products at each time point, f_{sp} , was fit to a single first-order rate equation or a double exponential equation (Emerick et al., 1996). *Trans*-splicing reactions were performed in a similar manner, except that reactions were begun with addition of 0.1–200 μM rGGCU CUCU instead of GTP.

Native gel electrophoresis

Uniformly labeled precursor RNAs and 5' end-labeled RNA oligomers were annealed at 95 °C as for splicing reactions above, and mixed with 10% glycerol (v/v) before loading on 4–20% polyacrylamide gels containing 34 mM Tris, 66 mM HEPES, 0.1 mM EDTA, 10 mM MgCl₂, pH 7.5, at 10–12 °C as described previously (Emerick & Woodson, 1994).

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REFERENCES

- Altuvia S, Kornitzer D, Teff D, Oppenheim AB. 1989. Alternative mRNA structures of the cIII gene of bacteriophage λ determine the rate of its translation initiation. *J Mol Biol* 210:265–280.
- Been MD, Cech TR. 1986. One binding site determines sequence specificity of *Tetrahymena* pre-rRNA self-splicing, *trans*-splicing, and RNA enzyme activity. *Cell* 47:207–216.
- Cech TR. 1990. Self-splicing of group I introns. *Annu Rev Biochem* 59:543–568.
- Cech TR, Herschlag D. 1996. Group I ribozymes: Substrate recognition, catalytic strategies, and comparative mechanistic analysis. *Nucleic Acids Mol Biol* 10:1–17.
- Clark CG, Tague BW, Ware VC, Gerbi SA. 1984. *Xenopus laevis* 28S rRNA: A secondary structure model and its evolutionary and functional implications. *Nucleic Acids Res* 12:6197–6220.
- Dammel CS, Noller HF. 1993. A cold-sensitive mutation in 16S rRNA provides evidence for helical switching in ribosome assembly. *Genes & Dev* 7:660–670.
- Donis-Keller H. 1979. Site-specific enzymatic cleavage of RNA. *Nucleic Acids Res* 7:179–192.
- Donis-Keller H. 1980. Phy M: An RNase activity specific for U and A residues useful in RNA sequencing analysis. *Nucleic Acids Res* 8:3133–3142.
- Ehresmann C, Baudin F, Mougél M, Romby P, Ebel JP, Ehresmann B. 1987. Probing the structure of RNAs in solution. *Nucleic Acids Res* 15:9109–9128.
- Emerick VL. 1995. A model for RNA folding: Alternative structures in the splicing of a group I precursor RNA. [Ph.D. thesis]. College Park, Maryland: University of Maryland.
- Emerick VL, Pan J, Woodson SA. 1996. Analysis of rate-determining conformational changes during self-splicing of the *Tetrahymena* intron. *Biochemistry* 35:13469–13477.
- Emerick VL, Woodson SA. 1993. Self-splicing of the *Tetrahymena* pre-rRNA is decreased by misfolding during transcription. *Biochemistry* 32:14062–14067.
- Emerick VL, Woodson SA. 1994. Fingerprinting the folding of a group I precursor RNA. *Proc Natl Acad Sci USA* 91:9675–9679.
- Fedor MJ, Uhlenbeck OC. 1990. Substrate sequence effects on “hammerhead” RNA catalytic efficiency. *Proc Natl Acad Sci USA* 91:1668–1672.
- Fortner DM, Troy RG, Brow DA. 1994. A stem/loop in U6 RNA defines a conformational switch required for pre-mRNA splicing. *Genes & Dev* 8:221–233.
- Franch T, Gulyaev AP, Gerdes K. 1997. Programmed cell death by hok/sok of plasmid R1: Processing at the hok mRNA 3'-end triggers structural rearrangements that allow translation and anti-sense RNA binding. *J Mol Biol* 273:38–51.
- Gutell RR. 1996. Comparative sequence analysis and the structure of 16S and 23S rRNA. In: Zimmerman RA, Dahlberg AE, eds. *Ribosomal RNA: Structure, evolution, processing, and function in protein biosynthesis*. Boca Raton, Florida: CRC Press. pp 89–108.
- Herschlag D, Eckstein F, Cech TR. 1993. Contributions of 2'-hydroxyl groups of the RNA substrate to binding and catalysis by the *Tetrahymena* ribozyme. An energetic picture of an active site composed of RNA. *Biochemistry* 32:8299–8311.
- Jaeger JA, Turner DH, Zuker M. 1989. Predicting optimal and sub-optimal secondary structure for RNA. *Methods Enzymol* 183:281–306.
- Joseph S, Noller HF. 1996. Mapping the rRNA neighborhood of the acceptor end of tRNA in the ribosome. *EMBO J* 15:910–916.
- Kim J, Walter AE, Turner DH. 1996. Thermodynamics of coaxially stacked helices with GA and CC mismatches. *Biochemistry* 35:13753–13761.
- Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* 154:367–382.
- Laing LG, Draper DE. 1994. Thermodynamics of RNA folding in a conserved ribosomal RNA domain. *J Mol Biol* 237:560–576.
- LeCuyer KA, Crothers DM. 1993. The *Leptomonas collosoma* spliced leader RNA can switch between two alternate structural forms. *Biochemistry* 32:5301–5311.
- Leviev I, Levieva S, Garrett RA. 1995. Role for the highly conserved region of domain IV of 23S-like rRNA in subunit-subunit interactions at the peptidyl transferase centre. *Nucleic Acids Res* 23:1512–1517.
- Ma CK, Kolesnikow T, Rayner JC, Simons EL, Yim H, Simons RW. 1994. Control of translation by mRNA secondary structure: The importance of the kinetics of structure formation. *Mol Microbiol* 14:1033–1047.
- Michel F, Westhof E. 1990. Modelling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis. *J Mol Biol* 216:585–610.
- Milligan JF, Uhlenbeck OC. 1989. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol* 180:51–62.
- Mitchell P, Osswald M, Brimacombe R. 1992. Identification of inter-molecular RNA crosslinks at the subunit interface of the *Escherichia coli* ribosome. *Biochemistry* 31:3004–3011.
- Moazed D, Noller HF. 1989. Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. *Cell* 57:585–597.
- Narlikar GJ, Herschlag D. 1996. Isolation of a local tertiary folding transition in the context of a globally folded RNA. *Nature Struct Biol* 3:701–710.
- Noller HF, Green R, Heilek G, Hoffarth V, Huttenhofer A, Joseph S, Lee I, Lieberman K, Mankin A, Merryman C, et al. 1995. Structure and function of ribosomal RNA. *Biochem Cell Biol* 73:997–1009.
- Noller HF, Kop JA, Wheaton V, Brosius J, Gutell RR, Kopylov AM, Dohme FD, Herr W, Stahl DA, Gupta R, Woese CR. 1981. Secondary structure model for 23S ribosomal RNA. *Nucleic Acids Res* 9:6167–6189.
- Pan J, Woodson SA. 1998. Folding intermediates of a self-splicing RNA: Mispairing of the catalytic core. *J Mol Biol*. Forthcoming.
- Panyutin IG, Hsieh P. 1994. The kinetics of spontaneous DNA branch migration. *Proc Natl Acad Sci USA* 91:2021–2025.
- Petersheim M, Turner DH. 1983. Base-stacking and base-pairing contributions to helix stability: Thermodynamics of double-helix formation with CCGG, CCGGp, CCGGAp, CCGGUp, and ACCG-GUp. *Biochemistry* 22:265–263.
- Pyle AM, Cech TR. 1991. Ribozyme recognition of RNA by tertiary interactions with specific ribose 2'-OH groups. *Nature* 350:628–631.
- Semrad K, Schroeder R. 1998. A ribosomal function is necessary for efficient splicing of the T4 phage thymidylate synthase intron in vivo. *Genes & Dev* 12:1327–1337.
- Séraphin B, Simon M, Faye G. 1988. MSS18, a yeast nuclear gene involved in the splicing of intron al5b of the mitochondrial cox1 transcript. *EMBO J* 7:1455–1464.
- Spedding G, Draper DE. 1993. Allosteric mechanism for translational repression in the *Escherichia coli* α operon. *Proc Natl Acad Sci USA* 90:4399–4403.
- Staley JP, Guthrie C. 1998. Mechanical devices of the spliceosome: Motors, clocks, springs, and things. *Cell* 92:315–326.
- Walstrum S, Uhlenbeck OC. 1990. Gel purification traps the self-splicing RNA of *Tetrahymena* in a less active conformation. *Biochemistry* 29:10573–10576.

- Waring RB, Towner P, Minter SJ, Davies RW. 1986. Splice-site selection by a self-splicing RNA of *Tetrahymena*. *Nature* 321:133–139.
- Wilson KS, Noller HF. 1998. Molecular movement inside the translational engine. *Cell* 92:337–349.
- Woodson SA. 1996. Splicing and evolution of introns in ribosomal RNA. In: Green R, Schroeder R, eds. *Group I introns and ribosomal RNA*. Austin, Texas: R.G. Landes, pp 199–219.
- Woodson SA. 1992. Exon sequences distant from the splice junction are required for efficient self-splicing of the *Tetrahymena* IVS. *Nucleic Acids Res* 20:4027–4032.
- Woodson SA, Cech TR. 1991. Alternative secondary structures in the 5' exon affect both forward and reverse self-splicing of the *Tetrahymena* intervening sequence RNA. *Biochemistry* 30:2042–2050.
- Woodson SA, Emerick VL. 1993. An alternative helix in the 26S rRNA promotes excision and integration of the *Tetrahymena* intervening sequence. *Mol Cell Biol* 13:1137–1145.
- Yanofsky C. 1981. Attenuation in control of expression of bacterial operons. *Nature* 289:751–758.
- Zavanelli MI, Britton JS, Igel AH, Ares M Jr. 1994. Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. *Mol Cell Biol* 14:1689–1697.
- Zaug AJ, Grosshans CA, Cech TR. 1988. Sequence-specific endoribonuclease activity of the *Tetrahymena* ribozyme: Enhanced cleavage of certain oligonucleotide substrates that form mismatched ribozyme–substrate complexes. *Biochemistry* 27:8924–8931.
- Zaug AJ, McEvoy MM, Cech TR. 1993. Self-splicing of the group I intron from *Anabaena* pre-tRNA: Requirement for base-pairing of the exons in the anticodon stem. *Biochemistry* 32:7946–7953.