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Minimum Secondary Structure Requirements for Catalytic Activity of a Self-Splicing Group I Intron†

Amber A. Beaudry and Gerald F. Joyce*
Department of Chemistry and Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

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ABSTRACT: We have completed a comprehensive deletion analysis of the *Tetrahymena* ribozyme in order to define the minimum secondary structure requirements for phosphoester transfer activity of a self-splicing group I intron. A total of 299 nucleotides were removed in a piecewise fashion, leaving a catalytic core of 114 nucleotides that form 7 base-paired structural elements. Among the various deletion mutants are a 300-nucleotide single-deletion mutant and a 281-nucleotide double-deletion mutant whose activity exceeds that of the wild type when tested under physiologic conditions. Consideration of those structural elements that are essential for catalytic activity leads to a simplified secondary structure model of the catalytic core of a group I intron.

The *Tetrahymena* ribozyme catalyzes sequence-specific phosphoester transfer reactions involving nucleic acid substrates (Zaug & Cech, 1985; Kay & Inoue, 1987). The specificity of this reaction is determined by binding of an oligopyrimidine substrate to a sequence of purines located near the 5′ end of the enzyme (Been & Cech, 1986; Waring et al., 1986) and by binding of a guanosine substrate to a G·C base pair located within the central portion of the molecule (Michel et al., 1989). The detailed chemistry of the reaction is not known, but is thought to proceed by an S_N2 (P) mechanism involving inversion of configuration at phosphorus (McSwiggen & Cech, 1989; Rajagopal et al., 1989). A 3′-terminal guanosine residue, for example, can act as a nucleophile to attack a phosphodiester bond following a sequence of pyrimidines. The products of the reaction are guanosine joined to whatever residues lie downstream from the target phosphodiester and oligopyrimidine with a free 2′- and 3′-hydroxyl. We have used this reaction as an assay to study the structural basis of catalytic activity of the *Tetrahymena* ribozyme and to develop variant forms of the molecule that retain catalytic activity.

The *Tetrahymena* ribozyme is categorized as a group I intron based on the presence of several short highly conserved sequence elements that result in characteristic features of local secondary structure (Davies et al., 1982; Michel et al., 1982;

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* Author to whom correspondence should be addressed.

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Michel & Dujon, 1983). There is a set of nine paired regions, designated P1–P9, that are common to group I introns in general and a number of other paired regions that are variably present in one or more specific instances. Previous studies with the *Tetrahymena* ribozyme have shown that the P2 and P9 regions and all of the variably present regions can be deleted in a piecewise fashion without a loss of catalytic activity (Price et al., 1985; Szostak, 1986; Joyce & Inoue, 1987; Barfod & Cech, 1988; Joyce et al., 1989). In the present study, we complete a comprehensive deletion analysis of the *Tetrahymena* ribozyme in order to define the minimum secondary structure requirements for catalytic activity of a self-splicing group I intron.

Materials and Methods

Nucleotides and Enzymes. Unlabeled nucleoside triphosphates, deoxynucleoside triphosphates, and dideoxynucleoside triphosphates were purchased from Pharmacia. [α-^{32}P]GTP, [γ-^{32}P]ATP, and [^{3}H]UTP were from ICN Radiochemicals. Synthetic oligodeoxynucleotides were either obtained from Operon Technologies or prepared by using a Biotix DNA synthesizer and were purified by polyacrylamide gel electrophoresis and chromatography on Sephadex G-25. Restriction enzymes, T4 RNA ligase, and T4 polynucleotide kinase were from New England Biolabs, DNA polymerase I (Klenow fragment) and calf intestine phosphatase from Boehringer, T4 DNA polymerase and T4 DNA ligase from U.S. Biochemical, AMV reverse transcriptase from Life Sciences, and sequence-grade RNases from BRL. T7 RNA polymerase was prepared as previously described (Davanloo et al., 1984), and purified according to a procedure originally developed for SP6 RNA polymerase (Butler & Chamberlin, 1982).

DNA Construct. A bacteriophage M13 construct was prepared such that the single-strand (infectious) form contains the nontemplate (noncoding) strand of the gene for the *Tetrahymena* ribozyme located immediately downstream from the nontemplate (minus) strand of the promoter for T7 RNA polymerase. Plasmid pT7L-21 (Zaug et al., 1988) was digested with *Eco*RI and *Hin*dIII restriction endonucleases, and the 443 base pair fragment containing the T7 promoter, residues 22–414 of the *Tetrahymena* IVS, and residues 1–25 of the 3′ exon was isolated. This fragment was subcloned into M13mp18 which had been digested with *Eco*RI and *Hin*dIII. The resulting M13 T7L-21 construct was transformed into bacterial cells. Single-stranded M13 T7L-21 DNA was prepared, and its sequence was confirmed by primer extension analysis using *Escherichia coli* DNA polymerase I (Klenow fragment) in the presence of dideoxynucleotides (Sanger et al., 1977).

Preparation of Wild-Type and Mutant Ribozymes. Wild-type and mutant RNAs were prepared directly from single-stranded M13 T7L-21 DNA using an in vitro mutagenesis technique as previously described (Joyce & Inoue, 1989). The technique involves construction of a template strand that optionally includes one or more mutagenic oligodeoxynucleotides. The resulting partially mismatched double-stranded DNA is transcribed directly using T7 RNA polymerase. The ΔP2 mutant was prepared by using a mutagenic oligodeoxynucleotide, 5′-TTT GAC GGT CTT GTT CCC TCC TAT AGT GAG-3′, partially randomized ΔP2 using 5′-TTT GAC GGT CTN NNN CCC TCC TAT AGT GAG-3′, ΔP5 using either 5′-TGC GTG GTT ACT TTC CCG CAA-3′ or 5′-GGA CTT GGC TGC GTG GTT ACT TTC CCG CAA-3′, ΔP6 using 5′-TTT AGT CTG TGA ACT CTT GGC-3′, ΔP6b using 5′-TCT GTG AAC TGC ATC CAA GCT TAG GAC TTG G-3′, and ΔP9 using 5′-GGC TAC CTT ACG AGT ACT CCG ACT ATA TCT TAT-3′. Wild-type and mutant RNAs other than those containing the ΔP9 deletion were defined at their 3′ end by the oligodeoxynucleotide 5′-CGA GTA CTC CAA AAC-3′. Mutants containing the ΔP9 deletion were defined by the ΔP9 mutagenic oligo which directs a transcript that includes 10 nucleotides of the 3′ exon. The 3′ exon sequence was removed by RNA-catalyzed site-specific hydrolysis (Inoue et al., 1986): the RNA was incubated in the presence of 50 mM CHES (pH 9.0) and 10 mM $MgCl_2$ at 42 °C for 1 h. The ΔP6/P9 double mutant, which did not undergo site-specific hydrolysis, was prepared by using a mutagenic oligodeoxynucleotide, 5′-CGA GTA CTC CGA CTA TAT C-3′, that introduces the ΔP9 deletion and concomitantly defines the 3′ end. Wild-type and mutant RNAs were isolated by electrophoresis in a 5% polyacrylamide/8 M urea gel, eluted from the gel, and purified by affinity chromatography on Dupont Nensorb. RNAs were sequenced by primer extension analysis using AMV reverse transcriptase in the presence of dideoxynucleotides (Sanger et al., 1977), except for those containing the ΔP9 deletion, which were sequenced from the 3′ end by partial RNase digestion (Donis-Keller et al., 1977).

Preparation of Substrate RNA. The RNA substrate 5′-GGC CCU CUA_{13}-3′ was prepared by in vitro transcription using a partially single-stranded synthetic DNA template (Milligan et al., 1987). The template contains both strands of the promoter for T7 RNA polymerase (positions −17 through +1) followed by the single-stranded template sequence 3′-CGG GAG AT_{10}-5′. Run-off transcripts of the form 5′-GGC CCU CUA_n-3′, where n = 9–16, were obtained. The products were separated by electrophoresis in a 20% polyacrylamide/8 M urea gel, eluted from the gel, purified by affinity chromatography on Dupont Nensorb, and sequenced by partial RNase digestion (Donis-Keller et al., 1977). RNA substrates having the sequence 5′-GGC CCU CUA_{13}-3′ were used throughout this study.

Assay of Catalytic Activity. The 3′-terminal G_{OH} of the *Tetrahymena* ribozyme attacks a phosphodiester bond following a sequence of pyrimidines located within an RNA substrate (Joyce, 1989). The product of the reaction is the ribozyme joined to the substrate sequence that lies downstream from the target phosphodiester. This intermolecular addition reaction was performed by using 1 μM ribozyme (uniformly labeled with 1 μCi/nmol of [α-^{32}P]GTP), 1 μM GGCCCU-CU·A_{13} substrate, 30 mM EPPS (pH 7.5), 10 or 50 mM $MgCl_2$, 0 or 2 mM spermidine, and 0 or 10% DMSO, which was incubated at either 37 or 50 °C for 1 h. Reaction mixtures were analyzed by electrophoresis in a 5% polyacrylamide/8 M urea gel. Ribozyme·A_{13} products appear approximately 0.5–1 cm closer to the origin than ribozyme starting materials.

Results

Design of the Deletion Mutants. We wished to determine the minimum secondary structure requirements for phosphoester transfer activity of the *Tetrahymena* ribozyme. Phylogenetic analysis and site-directed mutagenesis studies suggest that paired regions P1, P3, P4, P6, P7, and P8 form the catalytic core of the enzyme [for recent reviews, see Burke (1988) and Cech (1988)]. A minimum representation of these pairings together with the single-stranded regions that join them is shown in Figure 1. Deletions were made in a piecewise fashion based on this structural model.

The ΔP2 deletion removes 63 nucleotides that comprise structural elements P2 and P2.1. This is similar to a 60-nucleotide deletion that was made in a previous study, resulting

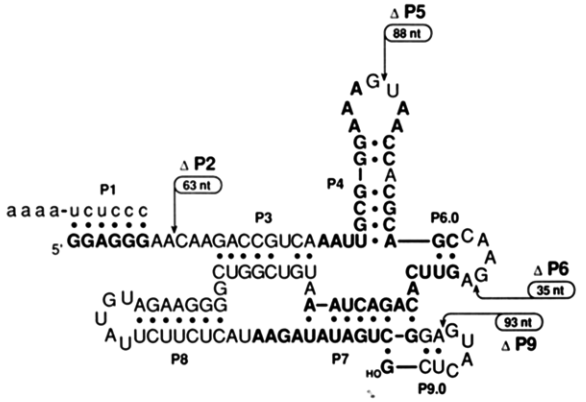

FIGURE 1: Secondary structure of the catalytic core of the *Tetrahymena* ribozyme labeled according to the standard nomenclature for group I introns (Burke et al., 1987). Phylogenetically conserved sequence elements are shown in boldface type. Arrows indicate the sites at which internal deletions were made. The oligopyrimidine substrate (shown in lower case) is bound to a sequence of purines (shown in boldface type) located at the 5′ end of the molecule.

in a molecule that retains 3′ splice site hydrolysis activity but lacks phosphoester transfer activity (Joyce et al., 1989). The present ΔP2 deletion was designed by considering the structure of the *sunY* ribozyme of bacteriophage T4 (Shub et al., 1988). *SunY* is the only known self-splicing group I intron that lacks the P2 region entirely, P1 and P3 being joined by the sequence UAAAA. In *Tetrahymena*, we joined P1 and P3 by the sequence AACAA.

The ΔP5 deletion removes 88 nucleotides that comprise structural elements P5, P5a, P5b, and P5c. Previous studies have shown that elements P5a, P5b, and P5c can be deleted without loss of phosphoester transfer activity (Joyce et al., 1989). The decision to eliminate P5, even though this region is present in all known group I introns, was based on recent Fe(II)-EDTA cleavage data suggesting that the region connecting P4 and P5 does not lie within the catalytic core of the ribozyme (Latham & Cech, 1989). We rounded off the P4 stem with the hairpin loop AAAGUAA, thus preserving the conserved residues that lie adjacent to P4.

The ΔP6 deletion removes 35 nucleotides that comprise structural elements P6a and P6b. In a previous study, it was shown that 29 nucleotides can be removed from the P6a/P6b region to produce a molecule that retains self-splicing activity, albeit at a substantially reduced level compared to wild type (Price et al., 1985). In the present study, we produced a symmetric deletion at the base of the P6a stem by rounding off P6 with the hairpin loop CAAGA. This loop is expected to have a free energy (at 25 °C) of approximately +4–5 kcal (Tinoco et al., 1973; Freier et al., 1986).

Finally, the ΔP9 deletion removes 93 nucleotides that comprise structural elements P9, P9.1, P9.2, and P9.2a. A survey of 3′-truncated forms of the *Tetrahymena* ribozyme has shown that these elements are not required for phosphoester transfer activity (Joyce & Inoue, 1987). Recent work by Michel et al. (1989) demonstrates that the guanosine binding site of the ribozyme is supported by two base pairs (termed P9.0 to distinguish it from P9) that lie immediately adjacent to the P7 stem. We rounded off the P9.0 stem using the hairpin loop GUAC. This loop is expected to have a free energy (at 25 °C) of about +6 kcal (Tinoco et al., 1973; Freier et al., 1986).

Activity of Single-Deletion Mutants. The wild-type *Tetrahymena* ribozyme and each of the four single-deletion mutants described above were tested for catalytic activity in a phosphoester transfer reaction using the substrate

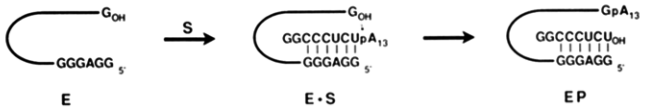

FIGURE 2: RNA-catalyzed intermolecular addition reaction used to assay phosphoester transfer activity of wild-type and mutant forms of the *Tetrahymena* ribozyme. The ribozyme (E) binds an oligopyrimidine-containing RNA substrate (S) by complementary pairing. The 3′-terminal G_{OH} of the ribozyme attacks the phosphodiester bond following a sequence of pyrimidines, resulting in transfer of the 3′ portion of the substrate to the 3′ end of the ribozyme.

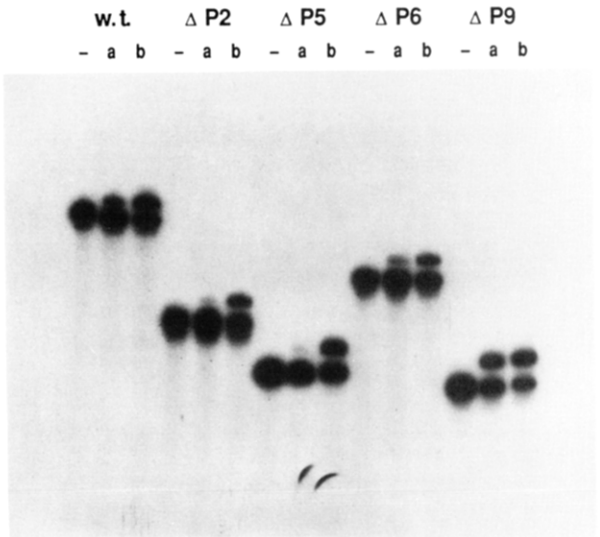

FIGURE 3: Phosphoester transfer activity of wild-type and single-deletion mutant forms of the *Tetrahymena* ribozyme. (–) No incubation; (a) incubation in the presence of 1 μM substrate, 10 mM $MgCl_2$, and 30 mM EPPS (pH 7.5) at 37 °C for 1 h; (b) incubation in the presence of 1 μM substrate, 50 mM $MgCl_2$, 2 mM spermidine, and 30 mM EPPS (pH 7.5) at 50 °C for 1 h. Ribozymes were uniformly labeled with [α-^{32}P]GTP; substrate RNA was unlabeled. Products were separated by electrophoresis in a 5% polyacrylmide/8 M urea gel, an autoradiogram of which is shown. Ribozyme·A_{13} products appear approximately 0.5–1 cm closer to the origin than ribozyme starting materials.

$GGCCCUCU{\cdot}A_{13}$. The products of the reaction are $GGCCCUCU_{OH}$ and the ribozyme joined via its 3′-terminal guanosine to A_{13} (Joyce, 1989) (Figure 2). The "L-21 form" of the ribozyme was used throughout these studies; this form lacks the first 21 nucleotides of the intron and as a result is unable to react with itself to form a circular molecule (Kay & Inoue, 1987).

Figure 3 shows the results of phosphoester transfer reactions performed under physiologic conditions (10 mM $MgCl_2$, pH 7.5, 37 °C) and under conditions that enhance reactivity (50 mM $MgCl_2$, 2 mM spermidine, pH 7.5, 50 °C). All four single-deletion mutants are reactive under both sets of conditions, although the activity of the ΔP2 and ΔP5 mutants at low $MgCl_2$ concentration and low temperature is quite poor. Surprisingly, the ΔP9 deletion mutant is more reactive than the wild type, even at low $MgCl_2$ concentration and low temperature. This is the most reactive form of the *Tetrahymena* ribozyme that we have encountered to date.

We tested the various deletion mutants under a wide range of reaction conditions, including 10–100 mM $MgCl_2$, substitution of spermine for spermidine, 30–70 °C, pH 6.5–9.0, 0–0.25 M NaCl, 0–0.5 M KCl, 0–0.5 M $(NH_4)_2SO_4$, 0–20% DMSO, 0–20% poly(ethylene glycol) (MW 8000), ±20% EtOH, ±2 mM $MnCl_2$, ±2 mM $CuSO_4$, and ±1 mg/mL protamine chloride. The only conditions that resulted in appreciable enhancement of phosphoester transfer activity were

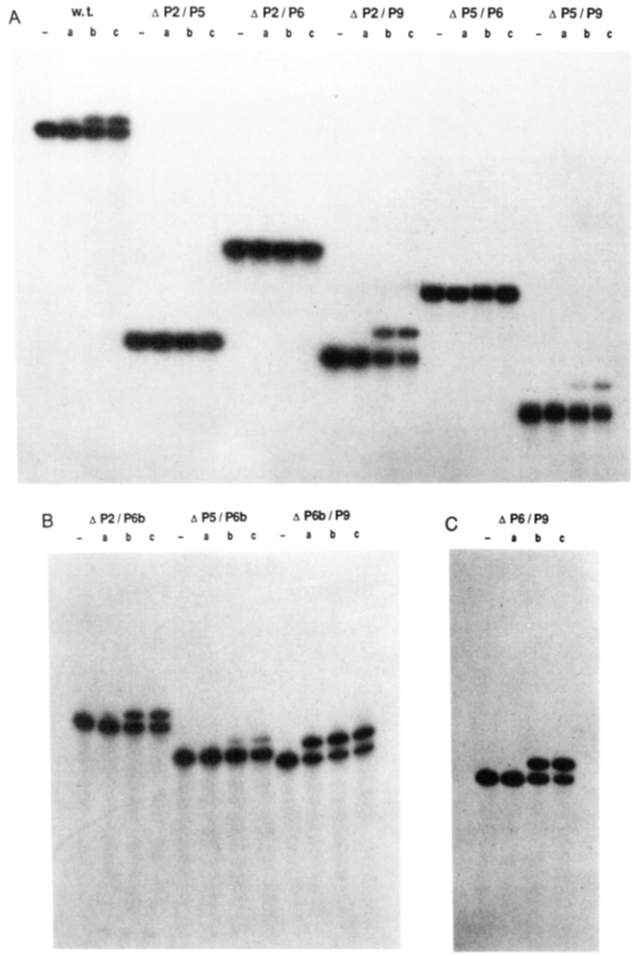

FIGURE 4: Phosphoester transfer activity of wild-type and double-deletion mutant forms of the *Tetrahymena* ribozyme. (A) Activity of double-deletion mutants representing combinations of the single-deletion mutants shown in Figure 3. (B) Activity of three double-deletion mutants that contain the ΔP6b deletion. (C) Activity of the ΔP6/P9 double-deletion mutant, prepared as a run-off transcript. (–) No incubation; (a) incubation in the presence of 1 μM substrate, 10 mM $MgCl_2$, and 30 mM EPPS (pH 7.5) at 37 °C for 1 h; (b) incubation in the presence of 1 μM substrate, 50 mM $MgCl_2$, 2 mM spermidine, and 30 mM EPPS (pH 7.5) at 50 °C for 1 h; (c) incubation in the presence of 1 μM substrate, 50 mM $MgCl_2$, 2 mM spermidine, 10% DMSO, and 30 mM EPPS (pH 7.5) at 50 °C for 1 h. Ribozymes were uniformly labeled with [α-^{32}P]GTP; substrate RNA was unlabeled. Products were separated by electrophoresis in a 5% polyacrylamide/8 M urea gel, an autoradiogram of which is shown.

those involving addition of KCl or DMSO (data not shown).

Activity of Double-Deletion Mutants. We prepared the six double-deletion mutants representing all possible combinations of the four single-deletion mutants described above. The ΔP6/P9 mutant, which was transcribed as a molecule containing a short 3′ exon (see Materials and Methods), did not undergo hydrolysis at the 3′ splice site. As a result, it was excluded from this initial survey of double-deletion mutants.

Figure 4A shows the results of intermolecular addition reactions performed under three sets of reaction conditions using wild type and the various double-deletion forms of the *Tetrahymena* ribozyme. Although all four single-deletion mutants are reactive (see above), only the ΔP2/P9 and ΔP5/P9 double-deletion mutants exhibit phosphoester transfer activity. The lack of activity of the ΔP2/P5 mutant is not surprising since the more conservative ΔP2/P5abc double-deletion mutant is already known to be inactive (Joyce et al., 1989). The fact that ΔP2/P6 and ΔP5/P6 are both inactive suggests that a P6 region supported by only two base pairs (G·U and G·C) is of marginal stability with respect to the catalytic core of the ribozyme.

We increased the stability of the P6 region by making the more conservative ΔP6b deletion. This deletion removes 19 nucleotides that comprise structural element P6b, but retains the P6a region that lies immediately adjacent to P6. All three double-deletion mutants, ΔP2/P6b, ΔP5/P6b, and ΔP6b/P9, exhibit catalytic activity in the intermolecular addition reaction (Figure 4B). The ΔP6b/P9 mutant is the most reactive of all double-deletion mutants that we have studied. Its activity exceeds that of the wild type, even under physiologic conditions. The ΔP2/P6b and ΔP5/P6b mutants, like the ΔP2/P9 and ΔP5/P9 mutants, are reactive only at high $MgCl_2$ concentration and high temperature. Their activity is enhanced somewhat by the addition of 10% DMSO (Figure 4A,B).

The ΔP6/P9 mutant, which was inactive in the site-specific hydrolysis reaction used to prepare all other ΔP9-containing mutants, was prepared directly as a run-off transcript (see Materials and Methods). It was found to have a high level of catalytic activity, comparable to that of ΔP6b/P9, when tested at high $MgCl_2$ concentration and high temperature. However, unlike ΔP6b/P9, the ΔP6/P9 mutant is not active under physiologic conditions (Figure 4C). This is consistent with the notion that a P6 region supported by two base pairs provides only marginal stability to the ribozyme.

The ΔP2/P5 double-deletion mutant was not reactive under any conditions that we tested, including the more unusual conditions described at the end of the previous section. We suspected that our choice of the sequence AACAA to join paired regions P1 and P3 may have been inappropriate with respect to the structure of the catalytic core. Accordingly, we used a partially randomized mutagenic oligonucleotide (see Materials and Methods) to test all 256 possible joining sequences of the form NNNNA, where N = G, A, C, or U. The majority of these sequences result in phosphoester transfer activity in the context of the ΔP2 deletion alone, but none of these sequences results in catalytic activity in the context of the ΔP2/P5 double deletion (data not shown).

It is possible that the ΔP2/P5 mutant is indeed active in the intermolecular addition reaction but that it subsequently undergoes rapid site-specific hydrolysis to lose the 3′-terminal A_{13} sequence and thus appear to be inactive in our standard assay. To exclude this possibility, we tested the ΔP2/P5 mutant using a 5′-^{32}P-labeled substrate. The reaction mixture contained 0.5 μM ribozyme, 0.01 μM substrate (1 μCi/pmol), 30 mM EPPS (pH 7.5), 50 mM $MgCl_2$, and 2 mM spermidine, which was incubated at 50 °C for 1 h. Although the wild-type ribozyme cleaves [5′-^{32}P]GGCCCUCU·A_{13} to produce [5′-^{32}P]GGCCCUCU$_{OH}$ (yield = 68%), the ΔP2/P5 mutant is unable to carry out this reaction (yield <1%).

The ΔP2/P6 and ΔP5/P6 mutants, which also appeared to be inactive in our standard assay, were able to cleave [5′-^{32}P]GGCCCUCU·A_{13} under the reaction conditions described above (yield = 6% and 21%, respectively). It should be noted, however, that the yield of cleaved substrate reflects the sum of phosphoester transfer events involving nucleophilic attack by the 3′-terminal G_{OH} of the ribozyme and site-specific hydrolysis events involving attack by a free OH^- at the phosphodiester bond following a sequence of pyrimidines (Inoue et al., 1986).

Discussion

Minimum Secondary Structure Requirements. In the present study, we sought to make piecewise deletions of all secondary structural elements of the *Tetrahymena* ribozyme that are not required for phosphoester transfer activity.

Previously, it was shown that structural elements P2 and P2.1 can be deleted to produce a molecule that retains 3′ splice site hydrolysis activity but lacks phosphoester transfer activity (Joyce et al., 1989). The present ΔP2 deletion removes an additional three nucleotides and results in a molecule that retains phosphoester transfer activity. At high $MgCl_2$ concentration and high temperature, its activity is comparable to that of the wild type (Figure 3).

Our ability to delete the entire P5 region is perhaps the most surprising result in this study. Structural element P5, which is present in all known group I introns, lies immediately adjacent to the highly conserved P4 region (comprised of conserved sequence elements P and Q; Davies et al., 1982). Mutations that disrupt the integrity of the P4 stem are known to reduce or destroy self-splicing activity [for a review, see Burke (1988)]. Apparently the ΔP5 deletion that we have made is structurally neutral with respect to the adjacent P4 region. The ribozyme lacking the P5 region has a low level of activity at low $MgCl_2$ concentration and low temperature; at high $MgCl_2$ concentration and high temperature, its activity is comparable to that of the wild type (Figure 3). This is consistent with the notion that the P5 region, although outside of the catalytic core, makes tertiary contacts with the catalytic core that help stabilize the ribozyme in an active conformation (Latham & Cech, 1989; Joyce et al., 1989).

In the P6 and P9 regions, we sought to retain only those base pairs that are considered to be part of the catalytic core. A minimum P6 region consists of a two base pair stem (which we designate P6.0) closed by a small loop. Likewise, a minimum P9 region consists of a two base pair stem (designated P9.0; Michel et al., 1989), again closed by a small loop. The ΔP9 mutant is particularly interesting because its activity exceeds that of the wild type under all of the reaction conditions that we tested (Figure 3). We suspect that the shortened P9 region raises the effective concentration of the 3′-terminal guanosine residue with respect to the adjacent G binding site.

Taking into account the four large internal deletions that we have made, one is left with a core structure of 114 nucleotides that form seven base-paired structural elements (P1, P3, P4, P6.0, P7, P8, and P9.0). Site-directed mutagenesis studies have shown that each of these pairings is required for phosphoester transfer activity [for reviews, see Burke (1988) and Cech (1988)]. Accordingly, we believe that these paired elements, together with the single-stranded regions that join them, constitute the minimum secondary structure requirements for catalytic activity of a self-splicing group I intron. It is conceivable that some of the paired elements could be shortened by a base pair or two, particularly the P8 pairing and possibly the P3 pairing in conjunction with a single-base deletion in the region joining P7 and P8. However, it is very unlikely that the paired element P1, P3, P4, P6.0, P7, P8, or P9.0 could be removed in its entirety without significantly redesigning the catalytic core.

Combining the findings reported in this deletion study with the wealth of information available from site-directed mutagenesis studies, we arrive at a simplified secondary structure model of the catalytic core of the *Tetrahymena* ribozyme (Figure 5). This model is based on consideration of the seven fundamental pairings discussed above, together with the need to place the 3′-terminal guanosine and oligopyrimidine substrate in close proximity within the active site of the molecule. We have included eight presumed nonstandard base pairs in the model. Recent work has established that the 3′-terminal guanosine is bound via an asymmetric pairing to a conserved

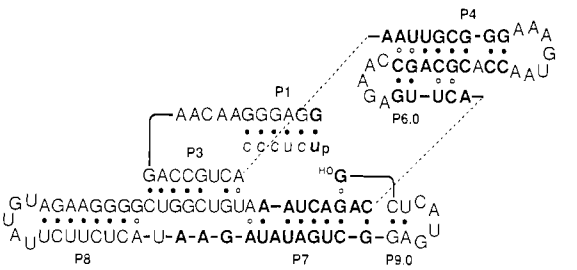

FIGURE 5: Secondary structure model of the catalytic core of the *Tetrahymena* ribozyme. Closed circles indicate Watson–Crick pairs; open circles indicate nonstandard pairs. Phylogenetically conserved sequence elements are shown in boldface type. The oligopyrimidine substrate is shown in lower case. Solid and dashed lines are added for clarity.

guanosine residue that lies within the 5′ portion of the P7 stem (Michel et al., 1989). Kim and Cech (1987) have proposed that a nonstandard pair exists at the end of the P3, P7, and P8 stems. Mutational analysis supports the existence of a symmetric G·A pair at the end of P8 (J. Szostak, personal communication), while the existence of an A·U pair at the end of P3 and an A·A pair at the end of P7 is more controversial. Finally, there is mutational evidence to suggest that the U and C residues lying immediately downstream from P6 are involved in base triples with residues in the P4 stem and less conclusive evidence to indicate that the A and U residues lying immediately upstream from P4 are likewise involved in base triples with residues in the P6 stem (J. Szostak, personal communication).

The picture that emerges is similar to the three-dimensional model proposed by Kim and Cech (1987). There are three principal helical domains: (1) a short duplex region (P1) consisting of the oligopyrimidine substrate bound to a sequence of purines; (2) a region of triple helix (P4/P6) that in the wild type is extended at both ends; (3) a long duplex region (P3/P7/P8) that includes the guanosine binding site. Kim and Cech (1987) have proposed that the P3, P7, and P8 helices stack colinearly, although there is at present no physical evidence to support this suggestion. Two other important unresolved structural questions are, first, how is the P1 region held in position so that the target phosphodiester lies in close proximity to the 3′-terminal G_{OH} and, second, how is the P4/P6 region "hinged" off the P3/P7/P8 region so as to make appropriate contacts with the remainder of the molecule. We suspect that these two questions will have a similar answer.

The Modular Ribozyme. It is remarkable that 13 secondary structural elements, comprising nearly 75% of the *Tetrahymena* ribozyme, can be deleted in a piecewise fashion without destroying the molecule's catalytic activity. The most likely role for the regions encompassed by the ΔP2, ΔP5, and ΔP6 deletions is in structural stabilization since each of these deletions results in a molecule that retains full activity only at high $MgCl_2$ concentration in the presence of a polyamine. It is not known whether this structural stabilization operates locally through the support of adjacent stem–loop structures or globally through tertiary interactions with the catalytic core. The ΔP9 mutant provides the only example of a large internal deletion that results in enhanced phosphoester transfer activity compared to the wild type, even at low $MgCl_2$ concentration in the absence of a polyamine. Apparently the region encompassed by the ΔP9 deletion does not contribute to the stability of the adjacent P9.0 stem or to the integrity of the catalytic core.

We have made available a family of reactive structural variants of the *Tetrahymena* ribozyme: ΔP2, ΔP5, ΔP6, ΔP9,

ΔP2/P9, ΔP5/P9, ΔP6/P9, ΔP2/P6b, ΔP5/P6b, and ΔP6b/P9. Each of these molecules contains the core structure, comprised of structural elements P1, P3, P4, P6.0, P7, P8, and P9.0, together with two or three supporting structural domains. The core itself can be separated into two parts, one containing the P1 pairing and the other containing elements P3, P4, P6.0, P7, P8, and P9.0 (Doudna & Szostak, 1989). It is conceivable that the core could be further subdivided by separating elements P4 and P6.0 from elements P3, P7, P8, and P9.0, as suggested by Figure 5. The disassembled ribozyme could then be reassembled, either covalently or noncovalently, by choosing an appropriate combination of the various structural modules.

The value of having a number of alternative forms of the ribozyme is threefold. First, it focuses attention on those elements of commonality that are likely to be responsible for the molecule's catalytic activity. Second, it provides a set of distinct starting points from which to begin an evolutionary search for random variants that have novel catalytic function (Robertson & Joyce, 1990). Third, it furnishes a structurally diverse array of materials with differing shapes and hence with differing packing properties, one of which might prove amenable to crystallization. We feel that we have reached the limit of what can be achieved by deletion analysis based on secondary structure considerations. Detailed understanding of the structural basis of phosphoester transfer activity of a self-splicing group I intron awaits the application of biophysical methods.

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