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2′-Hydroxyl Groups Important for Exon Polymerization and Reverse Exon Ligation Reactions Catalyzed by a Group I Ribozyme†

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ABSTRACT: The functional importance of ribose moieties in both exons and in intron sequences proximal to the 3′ splice site of a group I intron has been analyzed using a novel exon polymerization reaction. The ribozyme is a modified version of a self-splicing bacterial tRNA intron (I) that attacks a 20-nucleotide synthetic ligated exon substrate (E1·E2), yielding E1 and I·E2 by reverse exon ligation. A series of repetitive reactions then polymerize E2 on the 3′ end of the intron; attack by E1 subsequently generates E1·$(E2)_n$. Systematic deoxyribonucleotide substitution within E1·E2 was used to probe the function of 2′-hydroxyl groups in each exon and the 3′-terminal nucleotides of the intron. We find that ribose at the splice junction (U_{-1}) and at the two adjacent positions with E1 (A_{-2}, C_{-3}) is important for reverse exon ligation. Within E2, deletion of 2′-hydroxyl groups of the nucleotides that form P10 does not affect reactivity. In contrast, ribose at the 3′ end of the intron is essential for reverse exon ligation, and the presence of a 2′-OH group in each of the nucleotides comprising P9.0[3′] contributes to reaction efficiency. These results support a model in which specific 2′-hydroxyl groups at and adjacent to the reaction sites form tertiary contacts that serve to stabilize interactions with the catalytic core of the ribozyme. Furthermore, they suggest that the mechanism by which guanosine at the 3′ end of the intron is activated for reverse exon ligation is the same as that by which guanosine mononucleotide is activated in the first step of splicing.

The use of modified ribonucleoside phosphoramidites for solid-phase RNA synthesis has led to important insights into the structure and function of RNA enzymes [for review, see Usman and Cedergren (1992)]. Modified phosphoramidites allow the investigator to introduce more than the four naturally occurring bases into RNA molecules while retaining complete site selectivity. These studies are important for the analysis of ribozyme structure and function, for elucidating the catalytic mechanism, and for the construction of nuclease-resistant ribozymes for the targeted cleavage of cellular RNA molecules.

The efficiency of solid-phase RNA synthesis has limited the practical use of modified ribonucleoside phosphoramidites to polymers of about 40 nucleotides (nt) or fewer. The introduction of modified nucleotides into short ribozyme substrates has been used to study several ribozymes, including the hammerhead ribozyme (Yang *et al.*, 1990), the hairpin ribozyme (Feldstein *et al.*, 1990; Chowrira & Burke, 1991; Chowrira *et al.*, 1991), ribonuclease P (Perrault & Altman, 1992), and group I introns (Herschlag & Cech, 1990; Robertson & Joyce, 1990; Pyle & Cech, 1991; Sugimoto *et al.*, 1989; Bevilacqua & Turner, 1991; Beaudry & Joyce, 1992; Pyle *et al.*, 1992). These studies have shown that 2′-hydroxyl groups in ribozyme substrates are important for binding and, in some cases, catalytic function. Pyle *et al.* (1992) have

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shown that a hydrogen bond exists between a highly conserved base in the catalytic core of the *Tetrahymena* group I intron (A_{302}) and a 2′-hydroxyl group in the 5′ exon (U_{-3}). Although this tertiary contact contributes binding energy for the first step of splicing in the *Tetrahymena* system, it is not known whether it is a universal feature of group I introns or rather is an idiosyncratic feature of this particular intron.

For experiments involving the introduction of modified nucleotides into ribozymes, the hammerhead ribozyme (Long & Uhlenbeck, 1993) has been the molecule of choice because, at ca. 35 nt, it can be completely synthesized using solid-phase RNA chemistry (Perreault *et al.*, 1990, 1991; Olsen *et al.*, 1991; Pieken *et al.*, 1991; Fu & McLaughlin, 1992a,b; Yang *et al.*, 1990). Applications of synthetic RNA to larger ribozymes have been problematic. For the 50-nt hairpin ribozyme, we have developed a bimolecular construct that retains full catalytic activity while permitting the assembly of the ribozyme from two relatively short oligoribonucleotides (Chowrira & Burke, 1992). Using this system, we have identified four 2′-hydroxyl groups within the hairpin ribozyme that are necessary for catalytic function (Chowrira *et al.*, 1993a). The introduction of modified nucleotides into long RNA molecules has proven to be much more difficult. Moore and Sharp (1992) have devised a method to covalently link synthetic oligomers into long RNA molecules using T4 polynucleotide ligase. Recently, two groups have developed a method for noncovalently reconstituting enzymatic activity of the *Tetrahymena* group I intron from multiple RNA molecules, including those short enough to be synthesized chemically (Doudna *et al.*, 1991; Caprara & Waring, 1993).

Here, we describe the application of a novel ribozyme-catalyzed reaction to introduce synthetic nucleic acids into the 3′ end of a group I intron. This method (Chowrira *et al.*, 1993b) is based on reverse splicing (Woodson & Cech, 1989) of a modified small (205-nt) self-splicing tRNA intron from the bacterium *Azoarcus* (Reinhold-Hurek & Shub, 1992) and results in the polymerization of exon 2 (E2) on the 3′ end of the intron (I) and, subsequently, on the 3′ end of exon 1 (E1). The reaction scheme is outlined in Figure 1. The substrate for the reaction is a 20-nt analog of the ligated exons (E1·E2). First, the intron attacks the splice junction of E1·E2 to generate I·E2 and liberate E1. This reaction represents the reverse of the second step of splicing (exon ligation). Second, the newly formed 3′ splice site dissociates from the active site of the ribozyme and is replaced by the 3′ end of E2, which functions as a structural analog of the 3′ end of the intron. Third, I·E2 attacks a second E1·E2 molecule to generate I·E2·E2. Subsequent rounds of reaction generate intermediates of the structure I·$(E2)_n$, where $n \leq 18$. Fourth, attack of E1 on these intermediates generates products of the structure E1·$(E2)_m$, where $m \leq n$.

A key feature of reverse exon ligation and exon polymerization is that nucleotides on the 3′ end of the synthetic RNA substrate are brought into the active site to function as the 3′ end of the intron. In separate experiments, we have shown that exon polymerization is dependent upon the sequences known to be essential for exon ligation, P1, P9.0, P10, and the 3′-terminal G (B. M. Chowrira, A. Berzal-Herranz, and J. M. Burke, unpublished results). Therefore, reverse exon ligation and exon polymerization are valid assays for the functional importance of RNA structures essential for 3′ splice site reactions. Here, we describe the use of these reactions to study the effects of incorporating 2′-deoxyribonucleotides into E1, E2, and the 3′ end of the intron on the 3′ splice site reactions of the *Azoarcus* group I intron.

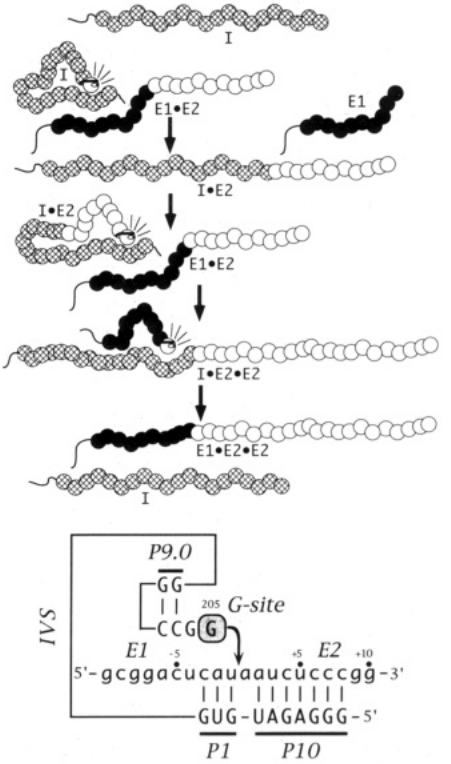

FIGURE 1: (A, top) Reaction pathway for reverse exon ligation and exon polymerization catalyzed by the modified *Azoarcus* group I intron (Chowrira *et al.*, 1993b). The intron (I) attacks a 20-nt analog of the ligated exons (E1·E2) in a reaction that is the reverse of the exon ligation step of splicing, covalently linking the intron to E2 (I·E2) and generating free E1. The 3′ end of the substrate is identical to the 3′ terminus of the intron RNA (CCGG). The I·E2 molecule translocates so that the new 3′ terminus is brought into the active site and is then used as the attacking nucleophile in the next reaction. Polymerization occurs via a series of intermediates of the structure I·$(E2)_n$, where $n = 1$ to ≤ 18. The 5′ exon accumulates during the course of this repetitive reaction and attacks the covalent intermediates to produce elongation products of structure E1·$(E2)_m$, where $m \leq n$. In this manner, the ribozyme converts 20-nt oligoribonucleotides into polyribonucleotides up to at least 180 nt by 10-nt increments. (B, bottom) Alignment of the reactive species for reverse exon ligation: uppercase letters, intron sequences; lowercase letters, exon sequences. Nucleotides corresponding to the exon sequences are numbered relative to the splice junction; nucleotides in exon 1 have negative numbers, while those in exon 2 have positive numbers. Nucleotides participating in P9.0 follow the assignments of Reinhold-Hurek and Shub (1992).

MATERIALS AND METHODS

Synthesis of Nucleic Acids. The DNA template for synthesis of the modified *Azoarcus* intron was prepared using PCR methods as described (Chowrira *et al.*, 1993b). Briefly, positions 1–5 at the 5′ end of the intron were deleted, and an 18-nt unrelated sequence was substituted that permitted the extension of P10 from 3 to 7 base pairs (bp). A promoter for T7 RNA polymerase was incorporated at the 5′ end of the template. The modified intron was obtained by runoff transcription of the PCR template and was gel purified as described (Chowrira *et al.*, 1993b).

All E1·E2 substrates and modified substrates were synthesized using DNA and RNA phosphoramidite chemistry on an Applied Biosystems Model 392 DNA/RNA synthesizer. Oligonucleotides were deprotected and gel purified as described (Scaringe *et al.*, 1990; Chowrira & Burke, 1991). Ribonucleoside phosphoramidites were obtained from ChemGenes, Inc. (Cambridge, MA).

3′ End Labeling Reactions. Substrate analogs containing a ribonucleotide at the 3′ end were partially labeled by RNA

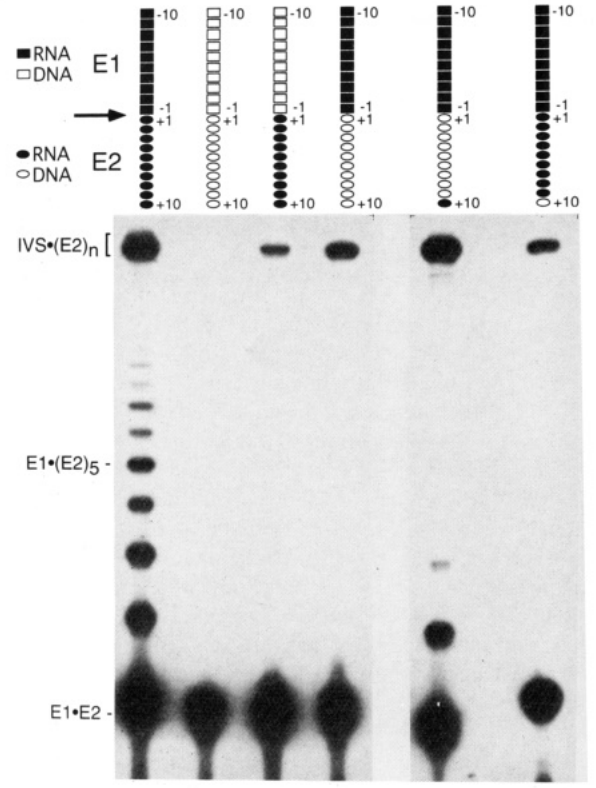

FIGURE 2: Block substitution of exons with 2′-deoxyribonucleotides and analysis of G_{+10}. The ability of the ribozyme to react with 20-nt substrate analogs containing extensive 2′-deoxyribonucleotide substitutions was tested. Reaction conditions are described under Materials and Methods. In these reactions, 0.4 μM unlabeled ribozyme and 10 μM 3′ end labeled E1·E2 substrate analogs were used. Abbreviations: E1·E2, ligated exons; E1·$(E2)_5$, exon 1 covalently linked to five tandem repeats of exon 2; IVS·$(E2)_n$, ribozyme covalently linked to n tandem repeats of exon 2; E1 and E2, exon 1 and exon 2, respectively. E1 is represented by squares; E2, by ovals. Black indicates RNA and white indicates DNA. Arrow indicates junction between E1 and E2.

ligase (Pharmacia) in the presence of [5′-^{32}P]pCp at 4 °C for 120 min (England *et al.*, 1980). Substrate analogs containing a deoxyribonucleotide at the 3′ end were labeled by terminal deoxynucleotidyltransferase (U.S. Biochemicals) in the presence of cordycepin 5′-[α-^{32}P]triphosphate at 37 °C for 60 min (Tu & Cohen, 1980). Following the labeling reaction, oligonucleotides were purified on a 20% denaturing polyacrylamide gel, visualized by UV shadowing, and eluted as described (Chowrira *et al.*, 1993b).

3′ Splice Site Reactions. Reverse exon ligation and polymerization reactions were as described (Chowrira *et al.*, 1993b). Briefly, 0.4 μM unlabeled modified ribozyme and 10 μM 3′ end labeled E1·E2 substrate were denatured at 95 °C for 1 min in a buffer containing 40 mM Tris-HCl (pH 7.5) and 100 mM $(NH_4)_2SO_4$. Reactions were initiated by the addition of $MgCl_2$ (30 mM) and were incubated at 50 °C for 60 min. Reactions were quenched by addition of an equal volume of formamide loading buffer and freezing on dry ice. Samples were resolved on 10% denaturing polyacrylamide gels. Dried gels were quantified by radioanalytic imaging with a Betascan instrument (Betagen).

RESULTS

Reactivity of DNA Exons. We first tested the reactivity of the *Azoarcus* intron against a DNA analog of the ligated exons (dE1·dE2); no detectable reaction occurred (Figure 2). To determine whether ribose moieties in E1 or E2, or both, were necessary, we synthesized two substrates (rE1·dE2 and dE1·rE2) in which a single exon was fully substituted with deoxyribonucleotides. Each of these molecules was found to function in reverse exon ligation, generating I·rE2 and I·dE2; however, polymerization products were not observed. Quantitation of the data in Figure 2 indicated that four times as much product was obtained from rE1·dE2 than for dE1·rE2, suggesting that ribonucleotides in E1 are relatively more important than in E2. Together with the results obtained with dE1·dE2, we conclude that no specific ribose moieties are absolutely required for reverse exon ligation of the *Azoarcus* intron, although ribose contributes significantly to reaction efficiency. The lack of reactivity with dE1·dE2 may result from the relative instability of RNA–DNA heteroduplexes [see, for example, Chowrira and Burke (1991)].

The 2′-Hydroxyl Group of the Intron-Terminal Guanosine Is Required for Reverse Exon Ligation and Exon Polymerization. Exon polymerization requires the 3′ end of E2 to be functionally equivalent to the 3′ end of the intron. Previously, we showed that changing the guanosine at the 3′ end of E2 (G_{+10}) to adenosine blocked exon polymerization although, as expected, it did not interfere with reverse exon ligation (Chowrira et al., 1993b). Substitution of G_{+10} with 2′-deoxyguanosine in a substrate that is otherwise entirely RNA has the same effect as riboadenosine substitution; I·E2 is produced, but no chain elongation occurs (Figure 2, lane 5). In contrast, a substrate molecule in which the only ribonucleotide is G_{+10} (Figure 2, lane 6) shows reverse exon ligation and limited exon polymerization. Together, these results show that the 2′-hydroxyl group at G_{+10} is essential for the 3′ end of E2 to function as an effective nucleophile in reverse exon ligation. In addition, they indicate that 2′-hydroxy groups at other positions in E2 contribute to increased efficiency of exon polymerization but are not themselves essential.

Ribose in P9.0[3′] Is Important for 3′ Splice Site Reactions, but P10[3′] May Be DNA. We examined the contributions of ribose within E2 to the RNA structures required for optimal reactivity at the 3′ splice site. Because E2 is bifunctional in the polymerase assay, we were able to test the effect of 2′-deoxyribose substitution on both the exon nucleotides that participate in P10 and the nucleotides at the 3′ end of the intron that participate in P9.0 (Burke *et al.*, 1990; Michel *et al.*, 1990). To accomplish this, we systematically added ribonucleotides back to rE1·dE2(rG_{+10}) (Figure 3). We found that converting P9.0 (positions +7, +8, and +9) from DNA to RNA dramatically increased the efficiency of exon polymerization (Figure 3, lane 2). Deleting single 2′-hydroxyl groups at each of these three positions from the resulting molecule significantly reduced activity (data not shown). Thus, we conclude that ribose at each position within P9.0[3′], as well as the single nucleotide between P9.0 and the intron terminus, contributes to the RNA structure necessary for optimal 3′ splice site reactivity.

In contrast, a much smaller effect was observed upon addition of ribonucleotides back to the segment of E2 that forms part of P10 (Figure 3, lane 3). This very limited increase in the extent of exon polymerization indicates that recognition of 2′-hydroxyl groups does not contribute significantly to important 3′ splice site structure. However, it is important to recognize that we have artificially reinforced P10 in this construct by extending it from 3 to 10 base pairs. Therefore, we cannot rule out the possibility that 2′-hydroxyl groups in P10 may contribute to stabilizing the 3′ splice site structure in the context of splicing the tRNA precursor in vivo.

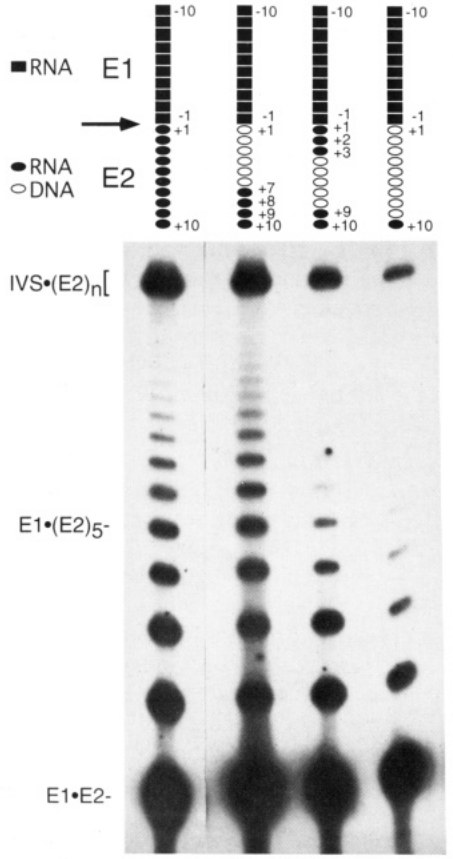

FIGURE 3: Exon polymerization reaction—deoxyribonucleotide substitutions within exon 2. Deoxyribonucleotide substitutions were introduced into exon 2 at the positions indicated. Exon 1 is comprised entirely of ribonucleotides. Reactions were carried out as described under Materials and Methods. Nomenclature is as described in the legend to Figure 2.

Functional Importance of Ribose in Exon 1. A similar approach was used to probe ribose moieties in exon 1 for function in the 3′ splice site reactions. Deleting a single 2′-hydroxyl group at the reaction site (U_{-1}) from the highly reactive rE1·rE2 substrate greatly diminished activity in both reverse splicing and exon polymerization reactions (Figure 4A, lane 1). In contrast, adding a single 2′-hydroxyl group at the same position to the dE1·rE2 molecule increased reverse exon ligation activity and restored exon polymerization activity (compare Figure 4A, lane 2 with Figure 2, lane 3). These results show that ribose at position −1 contributes more to 3′ splice site reactivity than all other ribose residues in exon 1.

Our experiments show that ribose at positions −2 and −3 in exon 1 are also important. Adding a second 2′-hydroxyl group to exon 1 at position −3 increases the yield of polymerization products (Figure 4A, lane 4), while adding a third hydroxyl at position −2 increases polymerization efficiency further (lane 5). The data shown in Figure 4B allow us to directly compare the functional importance of the ribose moieties at positions −1, −2, and −3 and show that their contributions to the extent of reaction are in the order of −1 > −3 > −2 > all others (Figure 4). These results were confirmed in a quantitative reverse exon ligation assay in a dG_{+10} background so that exon polymerization was blocked (Figure 5).

In separate experiments, we have shown that E1 can efficiently react with the splicing intermediate I·E2 to produce ligated exons but that a DNA analog of E1 cannot (data not shown). In contrast, when a single 2′-hydroxyl group was added to the DNA E1 analog with a single 2′-hydroxyl group at its 3′ end (position −1; 10 μM) and was incubated with the splicing intermediate (0.4 μM), ligated exons were obtained (data not shown). These results indicate that the presence of a 2′-hydroxyl group at position −1 is important for both the forward and reverse exon ligation reactions and suggest strongly that both the forward and the reverse exon ligation reactions have similar 2′-hydroxyl requirements.

DISCUSSION

Importance of Ribose at the Splice Junction and in the 5′ Exon. Using the *Tetrahymena* nuclear rRNA intron, Cech, Turner, and co-workers have analyzed the functional importance of 2′-hydroxyl groups in exon 1 for E1 binding (Pyle & Cech, 1991; Bevilacqua & Turner, 1991) and for guanosine attack on an oligonucleotide analog of the 5′ splice site, a reaction equivalent to the first step of splicing (Herschlag & Cech, 1990). These results showed that deletion of the 2′-hydroxyl group of U_{-3} destabilized the interaction between the 5′ exon and the intron. In particular, it was found that removing the 2′-hydroxyl group at the splice site (U_{-1}) had no significant effect on binding and reactivity. Pyle et al. (1992) went further to show that the 2′-hydroxyl group of U_{-3} forms a hydrogen bond with N1 of adenosine 302, a highly conserved nucleotide in the catalytic core of the intron. However, it is not known whether this tertiary contact occurs in all RNAs containing group I introns or whether it is important for other reactions, in particular those at the 3′ splice site.

To examine this issue, and to probe the function of ribose moieties surrounding the 3′ splice site, we have used reverse exon ligation and an exon polymerization reaction developed in our laboratory for an intron that is phylogenetically distant from the *Tetrahymena* intron. The nucleotide in the *Azoarcus* intron equivalent to A_{302} in the *Tetrahymena* intron is also adenosine (A_{167}; Reinhold-Hurek & Shub, 1992). In this bacterial intron, we found that the nucleotide at position −3 is functionally important for 3′ splice site reactions. Although we have not proven that the same tertiary contact forms in the reactions that we have studied, we believe that this is quite possible (Figure 6).

Our results showed that ribose at the splice junction (U_{-1}) was more important than at all other nucleotides in the 5′ exon. Analysis of equilibrium binding of RNA/DNA chimeric polymers to the internal guide sequence (IGS) of *Tetrahymena* ribozyme has shown that the 2′ hydroxyl group of U_{-1} does not contribute significantly toward the stabilization of the ribozyme–substrate complex. However, the 2′-hydroxyl group of U_{-1} is important for transesterification at the 5′ splice site; deoxy substitution lowers reaction efficiency by approximately 5 orders of magnitude (Herschlag & Cech, 1990; Cech *et al.*, 1992). Similar inhibition was also observed for an intron circle reopening reaction when rCpdU was used as the attacking nucleophile (Sugimoto *et al.*, 1989). These observations led to the proposal that the hydrogen at the 2′ position enhances reactivity by serving as a hydrogen bond donor to the 3′ oxygen in the transition state (Cech *et al.*, 1992). The results described here suggest that the same mechanism may be operative in activation of the reactive bonds in 3′ splice site reactions as well.

The 2′-Hydroxyl Group Is Required for Activation of the 3′-Terminal Guanosine. Our results indicate that when G_{+10} is deoxyribose, reverse exon ligation occurs, but the resulting I·E2 product cannot attack further substrate molecules. This indicates that the 2′-hydroxyl group of guanosine at the 3′ end of the molecule is required for the 3′-hydroxyl group of the

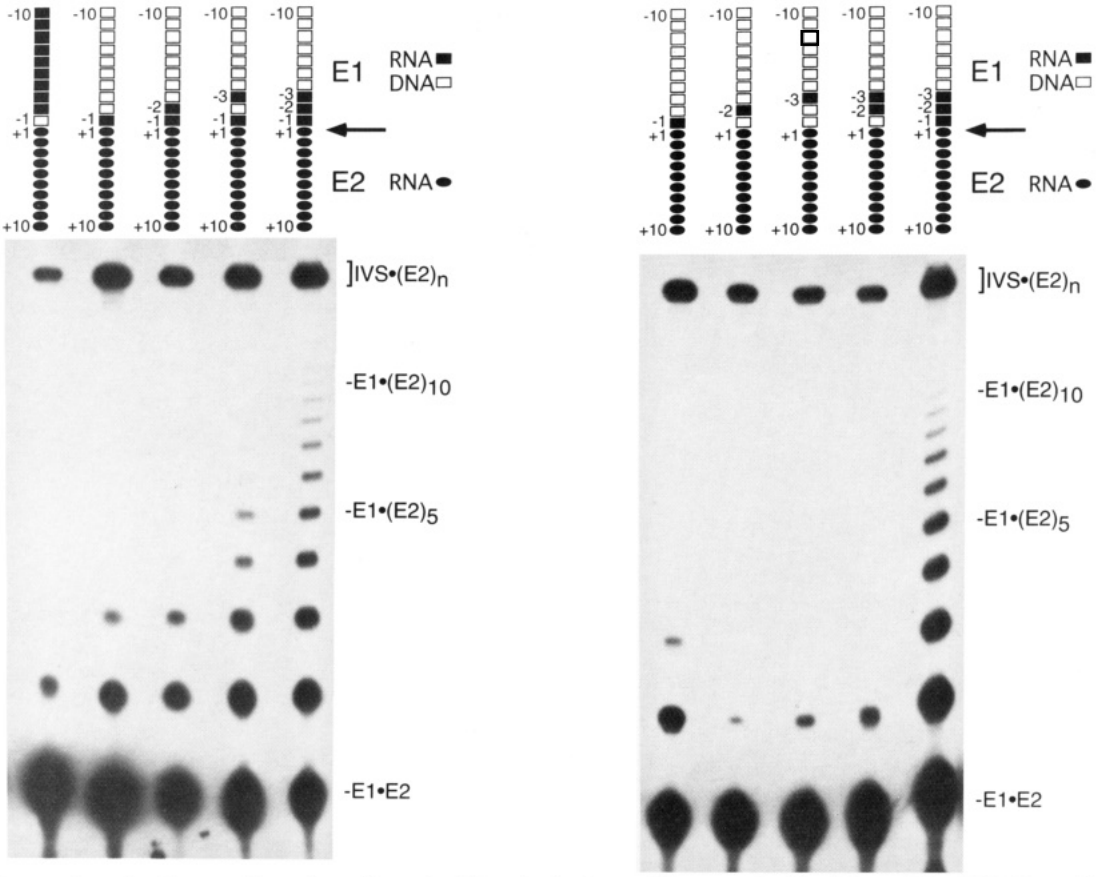

FIGURE 4: Exon polymerization reaction—deoxyribonucleotide substitutions within exon 1. (A, left, and B, right) Deoxyribonucleotide substitutions were introduced into exon 1 at the positions indicated. Exon 2 is comprised entirely of ribonucleotides. Reactions were carried out as described under Materials and Methods. Nomenclature is as described in the legend to Figure 2.

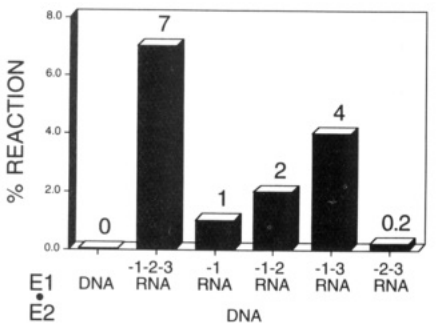

FIGURE 5: Reverse exon ligation—deoxyribonucleotide substitutions within exon 1. Results of reverse exon ligation reaction. Exon polymerization was blocked because G_{+10} is a deoxyribonucleotide. A series of substrate analogs containing deoxyribonucleotide substitutions within exon 1 were tested. Reactions were as described under Materials and Methods. Percent reaction indicates percentage of input E1·E2 that was converted into IVS·E2.

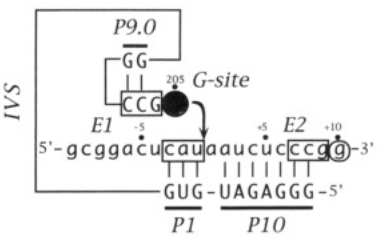

FIGURE 6: Summary—functionally important 2′-hydroxyl groups. Shadowing indicates the ribose 2′-hydroxyl group that is essential for the reverse exon ligation reaction. Ribose 2′-hydroxyl groups essential for the exon polymerization reaction are circled. Boxes indicate nucleotides at which deletion of the ribose 2′-hydroxyl inhibits, but does not arrest, the exon polymerization reaction. Boxed nucleotides at the 3′ end of the intron indicate sequences that are also important for the reverse exon ligation reaction. Nomenclature is as described in Figure 1B.

terminal guanosine to function as an effective nucleophile. This result is identical to that obtained for the 5′ splice site reaction by Bass and Cech (1984, 1986), who showed that 2′-deoxyguanosine could not replace the ribonucleotide in the first step in splicing. We have previously shown that replacement of G_{+10} with A also blocks exon polymerization (Chowrira *et al.*, 1993b).

Together, these results support a model in which the ribozyme has a single guanosine binding site that functions for binding both guanosine mononucleotide in the 5′ splice site reaction and the intron-terminal guanosine in the 3′ splice site reaction. Previously, Been and co-workers (Been & Perrotta, 1991) had provided mutational evidence that the terminal G of the *Tetrahymena* intron forms a base triple with the same base pair in P7 that interacts with guanosine in the first step of splicing (Michel *et al.*, 1989).

SUMMARY

We have described a new technique to introduce modified nucleotides at specific positions at the 3′ end of a group I ribozyme. Here, we have used it to replace specific ribonucleotides with deoxyribonucleotides. We have identified four 2′-hydroxyl groups in the 3′ end of the *Azoarcus* group Iribozyme important for 3′ splice site reactions. In addition, we have shown that three 2′-hydroxyl groups in exon 1 sequences (positions –1, –2, and –3) are important for both the forward and the reverse exon ligation reactions. These methods will enable us to further probe structure–function relationships of 3′ splice site reactions catalyzed by the group

I ribozymes.

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