Catalytic activity is retained in the *Tetrahymena* group I intron despite removal of the large extension of element P5

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ABSTRACT

We have made sizeable internal deletions within the self-splicing group I intron of *Tetrahymena thermophila*. Deletions were made in a piecewise manner in order to remove secondary structural elements thought to be extraneous to the catalytic center of the molecule. The resulting deletion mutants retain self-splicing activity, albeit under modified reaction conditions that enhance duplex stability. Considering those portions of the molecule that can be deleted without a loss of catalytic activity, one is left with a catalytic center of approximately 130 nucleotides that is solely responsible for the molecule's activity.

INTRODUCTION

Group I introns are characterized by the presence of several short highly-conserved sequence elements that result in characteristic features of local secondary structure (1-3). In the *Tetrahymena* group I intron the conserved sequence elements account for 49 nucleotides out of a total of 413 (Fig. 1). It has proven to be difficult to introduce substitutions or deletions within the conserved regions (P3, P4, P6, P7, P8) without destroying the molecule's catalytic activity (4-9). This has led to the notion that the catalytic center of the molecule resides largely within these conserved regions (10,11). On the other hand, sizeable deletions have been made within certain nonconserved regions, including removal of elements P1/L1/P2/L2 (12,13), elements P6b/L6b (12), and elements P9/L9/P9.1/P9.1a/L9.1a/P9.2/P9.2a/L9.2a (14,7), in each case resulting in a molecule that retains catalytic activity. In the present study we add nonconserved elements P2.1/L2.1 and P5a/P5b/L5b/P5c/L5c to the list of those regions that can be deleted. Thus the catalytic center resides entirely within elements P3/P4/P5/P6/P7/P8.

We focus in particular on sequence elements P5a/P5b/L5b/P5c/L5c since there is phylogenetic evidence to suggest that this region has some functional role in the large subclass of group I introns referred to as IB introns (2,15). The other large subclass of group I introns, the IA introns, do not have the large extension of element P5, but instead have extra stem-loop structures between elements P3 and P7 (2). It has been suggested that these large extensions play some role in stabilizing the overall structure of the molecule, either directly or indirectly through their

interaction with a *trans*-acting factor *in vivo* (15). In the present study we show that although elements P5a/P5b/L5b/P5c/L5c can be deleted without destroying self-splicing activity, the molecule has in some sense become destabilized since it requires increased Mg^{2+} concentration and/or the addition of spermidine in order to retain self-splicing activity.

MATERIALS AND METHODS

Nucleotides and Enzymes

Unlabeled nucleoside triphosphates, deoxynucleoside triphosphates, and dideoxynucleoside triphosphates were purchased from Sigma. [α ^{32}P] GTP and [γ ^{32}P] ATP were from ICN Radiochemicals and [^{3}H] UTP was from New England Nuclear. Synthetic oligodeoxynucleotides were obtained from Operon Technologies and were purified by polyacrylamide gel electrophoresis and chromatography on Sephadex G-10. Restriction enzymes were from New England Biolabs, T4 polynucleotide kinase, T7 gene 6 exonuclease, T4 DNA polymerase, and T4 DNA ligase from U.S. Biochemical, and AMV reverse transcriptase from Life Sciences. T7 RNA polymerase was prepared as previously described (16), and purified according to a procedure originally developed for SP6 RNA polymerase (17). Plasmid pT7TT1A3, which contains a 533 base-pair fragment of *Tetrahymena* rDNA (18) was provided by T.R. Cech.

Preparation of Mutant RNAs

Mutant RNAs were prepared directly from pT7TT1A3 DNA using an *in vitro* mutagenesis technique as previously described (19). The technique involves exonuclease digestion of the template (coding) strand of plasmid DNA, reconstruction of the template strand with inclusion of a mutagenic oligodeoxynucleotide, and subsequent transcription of the partially-mismatched double-stranded DNA using T7 RNA polymerase. Mutant RNAs were isolated by electrophoresis in a 5% polyacrylamide / 8 M urea gel, eluted from the gel, and purified by ethanol precipitation and chromatography on Sephadex G-50. ΔP2.1 was prepared using a mutagenic oligodeoxynucleotide 5'- TTT GAC GGT CTT GCT ACC AGG TG -3', ΔP2/2.1 using 5'- TTT GAC GGT CTT GTA TTT CCC TCC AAA GG -3', ΔP5abc using 5'- TTA GGA CCA TGT CTG TTG ACC CCT TT -3', ΔP5abc + A using 5'- TTA GGA TCC ATG TCT GTT GAC CCC TTT -3', and ΔP5 → T4 using 5'- CTT GGC TGC GTG GTT ATC TAT TAG AGA CTT TCC CGC AAT TTG A -3'.

Sequencing of Mutant RNAs

The mutant RNAs were sequenced by primer extension analysis using reverse transcriptase in the presence of dideoxynucleotides (20). 1.0 pmol of [5'-^{32}P]-labeled synthetic DNA primer was annealed with 0.3 pmol of mutant RNA by incubating at 65 °C for 5 min and then cooling to 30 °C over 35 min. The primer-extended cDNA products were analyzed on a 10% polyacrylamide / 8 M urea sequencing gel.

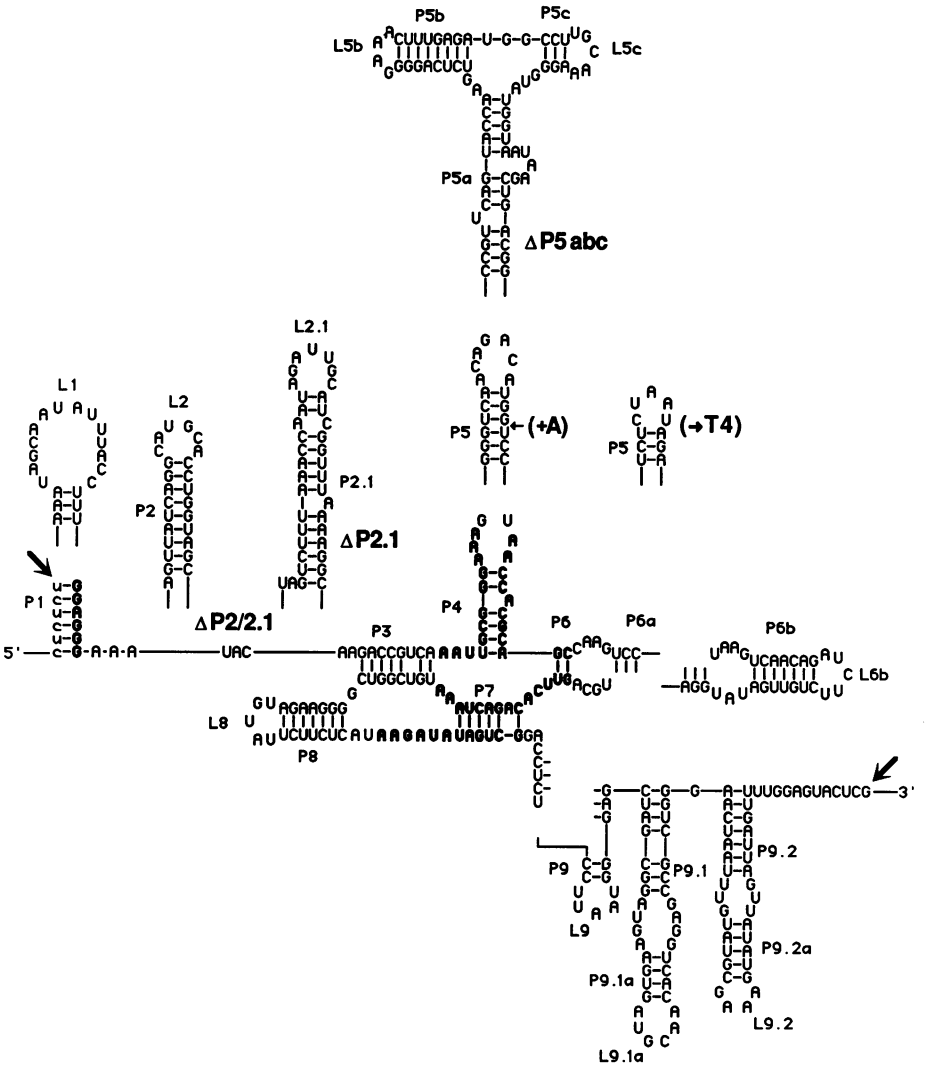


Figure 1: Secondary structure of the *Tetrahymena* ribozyme labeled according to the standard nomenclature for group I introns (29). 5′ and 3′ splice sites are indicated by a large arrow. Phylogenetically conserved sequence elements are shown in bold. The internal guide sequence, which pairs with the 3′ end of the 5′ exon, is also shown in bold. Sites of deletion corresponding to the ΔP2.1, ΔP2/P2.1, ΔP5abc, ΔP5abc + A, and ΔP5 → T4 mutants are labeled. Previous studies have shown that elements P1/L1, P6a/P6b/L6b, and P9/L9/P9.1/P9.1a/L9.1a/P9.2/P9.2a/L9.2a can also be deleted (see text); in the present study these regions were left intact.

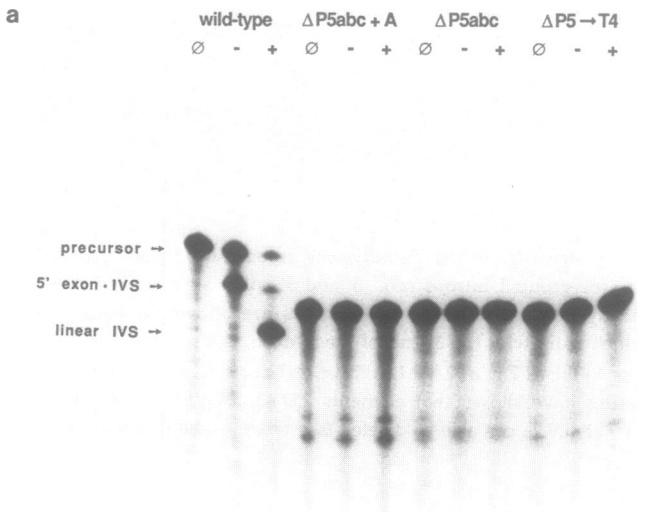
Assay of Catalytic Activity

Mutant RNAs were tested for self-splicing activity by incubating at 30 °C for 2 h under standard splicing conditions. 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 50 mM EPPS (pH 7.5), and 200 μM GTP (21). The mutant RNAs were tested under a variety of other conditions, including the use of 5-100 mM $MgCl_2$, the addition of 2 mM spermidine, the absence of $(NH_4)_2SO_4$, pHs over the range 7.5-9.0, and temperatures over the range 30-50 °C. Accuracy of the self-splicing reaction was confirmed by isolating the ligated exons in a 10% polyacrylamide / 8 M urea gel, purifying them by ethanol precipitation and chromatography on Sephadex G-50, and sequencing them (through the ligation junction) by primer extension analysis using reverse transcriptase as described above.

RESULTS

Design of the Deletion Mutants

We recently developed a technique for the rapid preparation of mutant RNAs (19). The technique, which is performed entirely *in vitro*, was used to produce all of the deletion mutants in this study. The site of deletion was determined by consideration of the known secondary structure of the *Tetrahymena* group I intron (Fig. 1). Deletions were made in such a way as to be structurally neutral, that is, symmetric with regard to stem-loop structures that are to be removed and not disruptive of those structures that are allowed to remain. ΔP2.1 results in deletion of elements P2.1/L2.1, with the site of deletion occurring at the base of the P2.1 stem. ΔP2/2.1 deletes both P2/L2 and P2.1/L2.1 in a similar manner. Our choice of deletion site within the extended P5 region was based on comparison of the group IA and IB introns. The *Tetrahymena* ribozyme is a IB intron, characterized by a long extension of the loop that closes element P5. We simply rounded



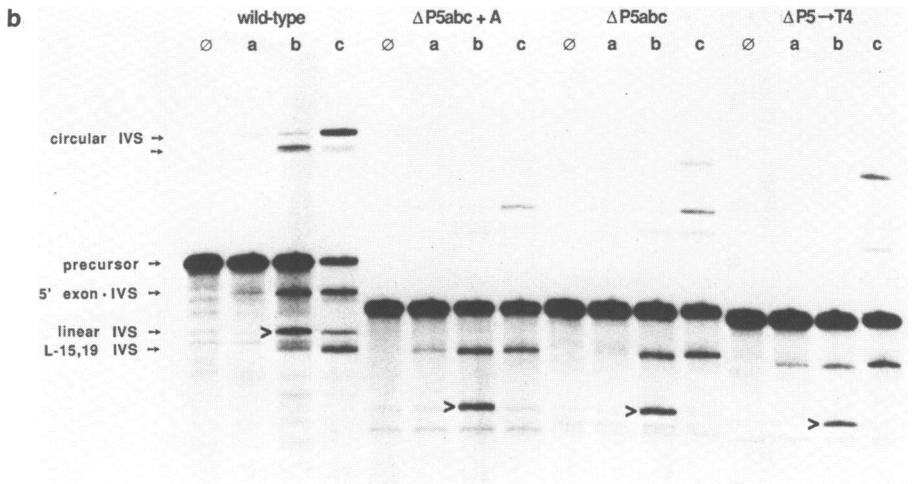
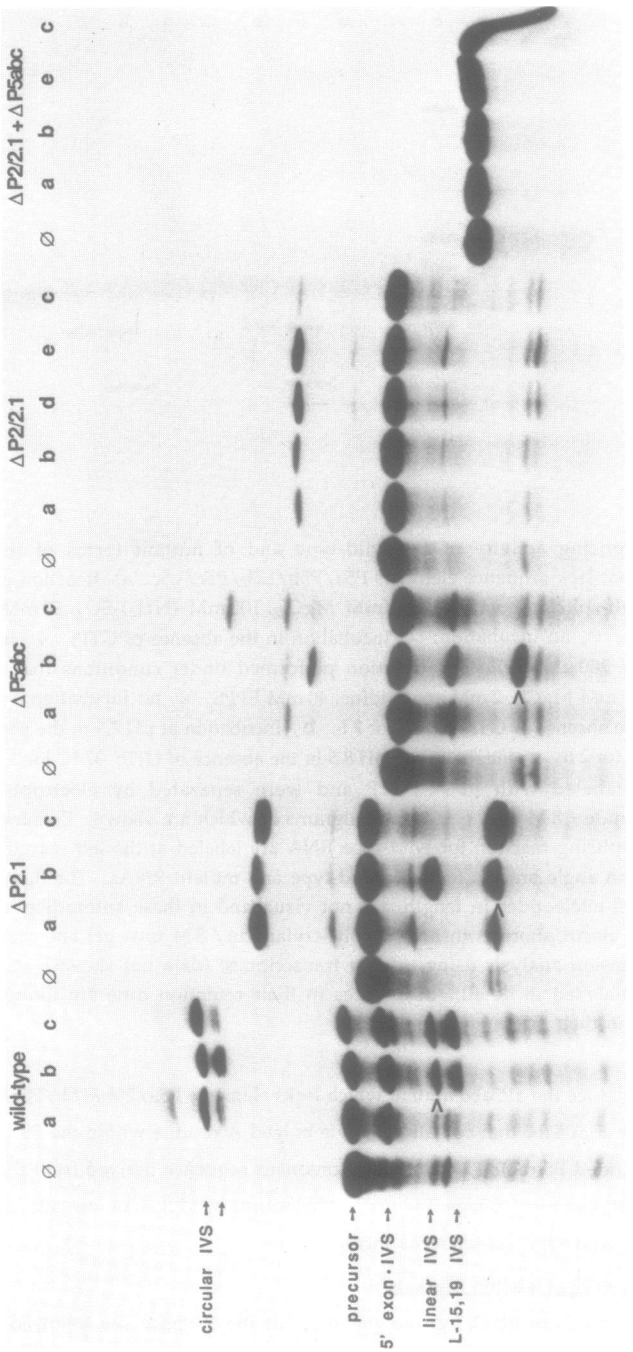


Figure 2: Self-splicing activity of the wild-type and of mutant forms of the *Tetrahymena* ribozyme that lack sequence elements P5a/P5b/L5b/P5c/L5c. a) Reaction performed under standard self-splicing conditions: 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 50 mM EPPS (pH 7.5); 30 °C for 2 h; ∅, no incubation; **-**, incubation in the absence of GTP; **+**, incubation in the presence of 200 µM GTP. b) Reaction performed under conditions that enhance duplex stability: 15 mM $MgCl_2$, 2 mM spermidine, 40 mM EPPS; ∅, no incubation; **a**, incubation at pH 7.5 in the absence of GTP; 30 °C for 2 h; **b**, incubation at pH 7.5 in the presence of 200 µM GTP; 30 °C for 2 h; **c**, incubation at pH 8.5 in the absence of GTP; 37 °C for 1.5 h. RNAs were uniformly labeled with [α^{32}P] GTP and were separated by electrophoresis in a 5% polyacrylamide / 8 M urea gel, autoradiograms of which are shown. Precursor and products of the self-splicing reaction for wild-type RNA are labeled at the left margin Linear IVS is labeled by an angle bracket for both wild-type and mutant RNAs. The ligated exons, which are only 135 nucleotides in length, are not visualized in these autoradiograms. They were isolated by electrophoresis in a 10% polyacrylamide / 8 M urea gel and were sequenced by primer extension analysis using reverse transcriptase (data not shown). Circular products were not analyzed in detail; differences in their retention time are thought to be due to differences in their size and conformation.

off the loop to produce the ΔP5abc mutant which lacks elements P5a/P5b/L5b/P5c/L5c. ΔP5abc + A is a slight variant of ΔP5abc that contains a single bulged A residue within the P5 stem. Finally, we replaced the extended P5 region with a short consensus sequence derived from the three group IA introns (*td*, *nrdB*, *sunY*) of phage T4 (22). The resulting ΔP5 → T4 mutant is 77 nucleotides smaller than the wild-type *Tetrahymena* intron.

Catalytic Activity of the Deletion Mutants

Self-splicing involves attack by free guanosine at the 5′ splice site followed by attack of the released 5′ exon at the 3′ splice site to produce the ligated exons (21,23). The *Tetrahymena*


wild-type
ΔP2.1
ΔP5abc
ΔP2/2.1
ΔP2/2.1 + ΔP5abc
∅ a b c ∅ a b c ∅ a b c ∅ a b d e c ∅ a b e c
circular IVS →
precursor →
5' exon · IVS →
linear IVS →
L-15,19 IVS →

ribozyme is able to catalyze a variety of site-specific cleavage/ligation reactions that are equivalent to either the first or second step of splicing (24-26). We tested the deletion mutants for catalytic activity in the self-splicing reaction and in various splicing-related reactions.

The three deletion mutants that lack the extended P5 region were tested for catalytic activity under standard self-splicing conditions (21). In contrast to the wild-type which undergoes self-splicing in the presence of 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, and 200 µM GTP, the three mutants showed no activity (Fig. 2a). In the absence of GTP, the wild-type *Tetrahymena* ribozyme undergoes hydrolysis at the 3′ splice site to yield the two-thirds molecule 5′ exon • IVS (25). Under the same conditions the ΔP5abc, ΔP5abc + A, and ΔP5 → T4 mutants again showed no activity (Fig. 2a). We found that by raising the $MgCl_2$ concentration to 15 mM and adding 2 mM spermidine to the reaction mixture, all three mutants became active in the GTP-dependent self-splicing reaction and the GTP-independent hydrolysis reaction (Fig. 2b). Under these modified conditions the catalytic activity of the wild-type is significantly reduced.

Most of the known group I introns do not contain elements P2.1/L2.1. The ΔP2.1 mutant, which lacks these elements, was found to retain catalytic activity in the GTP-dependent self-splicing reaction and the 3′ splice-site hydrolysis reaction when carried out in the presence of 15 mM $MgCl_2$ and 2 mM spermidine (Fig. 3). Under these conditions the level of catalytic activity exceeds that of the wild-type and that of the ΔP5abc mutant described above.

Previous studies have shown that partial deletions of the P2/L2 region are tolerated, while a deletion that spans the entire P2/L2 region results in a complete loss of self-splicing activity (12). The ΔP2/2.1 mutant was designed to produce a precise deletion of elements P2/L2 (as well as P2.1/L2.1) without disrupting the surrounding sequences. This mutant retains a low level of 3′ splice-site hydrolysis activity, but showed no activity in the GTP-dependent self-splicing reaction (Fig. 3). Increasing the $MgCl_2$ concentration from 15 to 25 mM and increasing the temperature

Figure 3: Catalytic activity of the wild-type and mutant forms of the *Tetrahymena* ribozyme under various reaction conditions. ∅, no incubation; **a**, incubation in the presence of 15 mM $MgCl_2$, 2 mM spermidine, 40 mM EPPS (pH 7.5); 37 °C for 2 h; **b**, incubation in the presence of 200 µM GTP, 15 mM $MgCl_2$, 2 mM spermidine, 40 mM EPPS (pH 7.5); 37 °C for 2 h; **c**, incubation in the presence of 15 mM $MgCl_2$, 2 mM spermidine, 40 mM EPPS (pH 8.5); 37 °C for 2 h; **d**, incubation in the presence of 200 µM GTP, 15 mM $MgCl_2$, 2 mM spermidine, 40 mM EPPS (pH 7.5); 42 °C for 1.5 h; **e**, incubation in the presence of 200 µM GTP, 25 mM $MgCl_2$, 2 mM spermidine, 40 mM EPPS (pH 7.5); 42 °C for 1.5 h. RNAs were uniformly labeled with [α ^{32}P] GTP and were separated by electrophoresis in a 5% polyacrylamide / 8 M urea gel, an autoradiogram of which is shown. Precursor and products of the self-splicing reaction for wild-type RNA are labeled at the left margin Linear IVS is labeled by an angle bracket for both wild-type and mutant RNAs. The ligated exons, which are only 135 nucleotides in length, are not visualized in this autoradiogram. Circular products were not analyzed in detail. The prominent band which appears between 5′ exon • IVS and linear IVS in ΔP2.1 - lane b corresponds to the splicing intermediate G • IVS • 3′ exon (25).

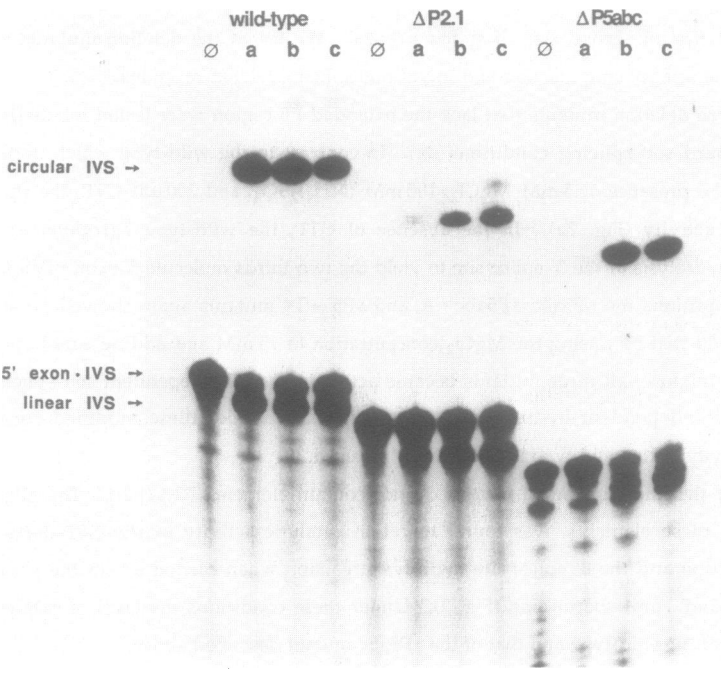


Figure 4: Cyclization activity of wild-type and mutant forms of the *Tetrahymena* ribozyme in the presence of varying amounts of $MgCl_2$. All reactions were carried out using the two-thirds molecule 5' exon • IVS, which was incubated in the presence of 50 mM EPPS (pH 7.5) at 37 °C for 1 h. ∅, no incubation; **a**, incubation in the presence of 10 mM $MgCl_2$ and 200 mM NaCl; **b**, incubation in the presence of 25 mM $MgCl_2$ and 200 mM NaCl; **c**, incubation in the presence of 50 mM $MgCl_2$ alone. RNAs were uniformly labeled with [α ^{32}P] GTP and were separated by electrophoresis in a 5% polyacrylamide / 8 M urea gel, an autoradiogram of which is shown. Reactants and products of the cyclization reaction for wild-type RNA are labeled at the left margin.

from 37 °C to 42 °C resulted in enhancement of the hydrolysis activity, but did not lead to any detectable self-splicing activity (Fig. 3). We tested $MgCl_2$ concentrations as high as 100 mM, in either the presence or absence of spermidine, and while hydrolysis activity was detected in all cases, we were unable to detect any products of the self-splicing reaction (data not shown). The double deletion mutant ΔP2/2.1 + ΔP5abc showed no activity, even in the 3' splice-site hydrolysis reaction, under any of the conditions that we tested (Fig. 3).

Hydrolysis at the 3' splice site involves attack by free OH^- to yield 5' exon • IVS, which in turn undergoes a transesterification reaction to produce a circular form of the IVS (25). In order to study the cyclization reaction in isolation, we began with the two-thirds molecule 5' exon • IVS and incubated it in the presence of 10 mM $MgCl_2$ and 200 mM NaCl at 42 °C for 1 h (24). Under these

conditions the wild-type underwent efficient cyclization, while the ΔP2.1 and ΔP5abc mutants showed only a very low level of activity. When the concentration of $MgCl_2$ was increased to 25 mM in the presence of NaCl or to 50 mM in the absence of NaCl, both mutants showed efficient cyclization activity while the activity of the wild-type was somewhat decreased (Fig. 4). The circular form of the IVS undergoes hydrolysis at the cyclization junction to produce a linear molecule (27). This reaction was also observed for the ΔP2.1 and ΔP5abc mutants when the reaction was carried out in the presence of 25 or 50 mM $MgCl_2$ (Fig. 4).

DISCUSSION

As with all data concerning the catalytic behavior of mutant enzymes, it should be emphasized that a positive result, that is, a deletion mutant which retains catalytic activity, is far more significant than a negative result. If one produces a deletion mutant that no longer has catalytic activity, it is difficult to say whether the loss of activity is due to the removal of some essential portion of the molecule or simply the result of disruption of the molecule's secondary and tertiary structure. For this reason we tried to construct deletion mutants that are neutral with regard to the secondary structure of the *Tetrahymena* IVS (28,29). However, since the molecule's tertiary structure is not known, it is impossible for us to predict what the overall effect of a deletion will be.

We have shown that nonconserved elements P2.1/L2.1 and P5a/P5b/L5b/P5c/L5c are not required for self-splicing activity and do not influence the choice of 5′ or 3′ splice site. The fact that P2.1/L2.1 can be deleted is not surprising since this region is present in only a minority of group I introns. Removal of the large extension of the P5 region comes as more of a surprise. This extension is a characteristic feature of IB introns and is thought to have some function *in vivo*, possibly through its interaction with a *trans*-acting factor (15). We find that removal of elements P5a/P5b/L5b/P5c/L5c results in a molecule that has in some sense become destabilized because conditions that enhance duplex stability are required for it to retain self-splicing activity. This is consistent with the hypothesis that the large extension of the P5 region has a role in stabilizing the overall structure of IB introns but is not part of the catalytic center and is not required for determining the 5′ or 3′ splice site. One would predict that a structurally-neutral deletion of elements P7.1/L7.1/P7.2/L7.2, which are thought to play a comparable role in IA introns, would result in a molecule that similarly is destabilized but retains self-splicing activity. In this context the IA and IB introns represent two divergent evolutionary pathways leading to two different solutions to the problem of structural stabilization.

The present deletion study is consistent with emerging structural models of the catalytic center of group I introns (10,11,30,31). The core consists of the phylogenetically conserved sequence elements (shown in bold in Fig. 1) supported by an inner shell of duplex structures (elements P4, P6 and P7). This in turn is supported by an outer shell of duplex structures (elements P3, P5, and

P8), with extensions toward the 5' and 3' splice sites. The inner and outer shells are responsible for catalytic activity in general. Self-splicing activity in particular depends on the precise alignment of the two splice sites within the catalytic center and is likely to place additional constraints on the outer shell and on the region surrounding the splice sites.

Considering not just self-splicing activity, but transesterification activity in general, we find that elements P2/L2 are also nonessential for catalytic activity. Summing over all deletions that are known to be tolerated by the *Tetrahymena* IVS, one is left with a core structure of 132 nucleotides consisting of the internal guide sequence and elements P3/P4/P5/P6/P7/P8 (Fig. 1). The remainder of the molecule is superfluous with regard to catalytic function. While it is possible to combine deletion of elements P5a/P5b/L5b/P5c/L5c and P9/L9/P9.1/P9.1a/L9.1a/P9.2/P9.2a/L9.2a (data not shown), we were unable to combine deletion of elements P5a/P5b/L5b/P5c/L5c and P2.1/L2.1. If our results are any indication, it will not be easy to combine the various deletions to produce a naked core structure that retains catalytic activity.

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