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Brief Communication

A Second Divalent Metal Ion in the Group II Intron Reaction Center

Peter M. Gordon,[1,4] Robert Fong,[1] and Joseph A. Piccirilli[1,2,3,*]
[1] Department of Biochemistry and Molecular Biology
[2] Howard Hughes Medical Institute
[3] Department of Chemistry
The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA
[4] Present address: Department of Pediatric Hematology/Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA.
*Correspondence: jpicciri@uchicago.edu
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SUMMARY

Group II introns are mobile genetic elements that have been implicated as agents of genetic diversity, and serve as important model systems for investigating RNA catalysis and pre-mRNA splicing. In the absence of an atomic-resolution structure of the intron, detailed understanding of its catalytic mechanism has remained elusive. Previous identification of a divalent metal ion stabilizing the leaving group in both splicing steps suggested that the group II intron may employ a "two-metal ion" mechanism, a catalytic strategy used by a number of protein phosphoester transfer enzymes. Using metal rescue experiments, we now reveal the presence of a second metal ion required for nucleophile activation in the exon-ligation step of group II intron splicing. Coupled with biochemical and structural evidence of at least two metal ions at the group I intron reaction center, these results suggest a mechanistic paradigm for describing catalysis by large ribozymes.

INTRODUCTION

Protein phosphoester transferase enzymes frequently contain two metal ions within their active sites, bound in a manner that allows spatial and electronic complementarity to the phosphoester transition state [1–5]. These catalytic metal ions putatively help to position the reactants, activate the nucleophile, and stabilize charge buildup on the leaving group and phosphoryl group in the transition state [6–8]. Biochemical evidence strongly suggests that ribozymes could mimic features of this mechanism, folding into global architectures that poise metal ions for catalysis of phosphoester transfer. Recently, a structural basis for an RNA-mediated two-metal ion mechanism has emerged from crystallographic analyses of the group I intron, showing that RNA can position two divalent metal ions 4 Å apart so as to accommodate the trigonal bipyramidal transition state [9, 10]. The relevance of this mechanism to other biologically significant splicing machineries (group II introns and the spliceosome) and other ribozymes remains unknown.

Group II introns are mobile genetic elements found in bacteria and the organelles of plants, fungi, and algae [11]. Beyond their direct biological and evolutionary significance as agents for genetic variation [12–14], group II introns provide important model systems for exploring RNA catalysis as they harbor the catalytic apparatus that promotes their own excision, frequently with the help of a protein [15, 16]. Elegant biochemical and phylogenetic analyses together with computational approaches have led to detailed models for the global architecture of the group II intron core [17–20]. However, structural information at atomic resolution remains limited [21, 22], as does our understanding of the active site configuration and mechanism of catalysis. Divalent metal ions mediate group II intron folding and catalysis, but little information exists about the specific role of the metal ions in these processes [15, 16, 23]. We showed previously that the ai5γ group II intron uses a divalent metal ion to stabilize the leaving oxyanion during both the first and second steps of splicing [24, 25]. We now report that the active site contains a second distinct divalent metal ion that activates the nucleophile in the second step of splicing. The resulting mechanistic picture for exon ligation, although still incomplete, has features in common with that for the group I intron, validating speculation that the two-metal ion mechanism may be generally applicable to describe catalysis by large ribozymes.

RESULTS

The ai5γ group II intron from *Saccharomyces cerevisiae* self-splices by two consecutive phosphotransesterification reactions that result in ligated exons and a lariat intron (for reviews, see [15, 16, 26]). In the first step of splicing, the 2′ hydroxyl group of the branch point adenosine nucleophilically attacks the 5′ splice site and generates a lariat intron/3′ exon intermediate and free 5′ exon. In the second step of splicing, the 3′ hydroxyl of the last nucleotide of the 5′ exon attacks the 3′ splice site to produce ligated

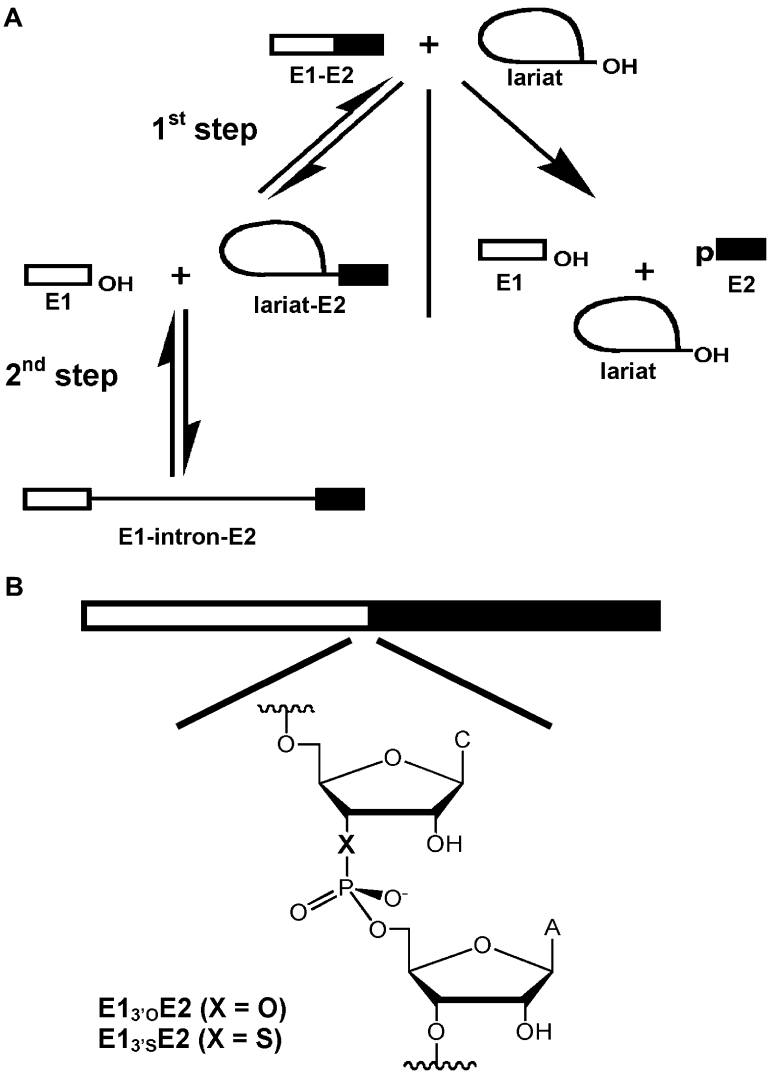

Figure 1. Probing the Group II Intron Reverse Splicing Pathway

(A) Schematic representation of the two pathways of ai5γ group II intron reverse splicing. The two pathways differ in the identity of the nucleophile during the first step of the reaction. In the spliced exons reopening reaction (SER) shown to the right, the lariat intron catalyzes the nucleophilic attack of water or hydroxide at the exon 1/exon 2 junction. This results in the generation of free exon 1 (E1) and exon 2 (E2) products. In the alternative pathway shown at left, the 3′ hydroxyl of the last nucleotide of the intron acts as the nucleophile in the first step of splicing. This results in the release of exon 1 (E1) and the generation of a lariat intron/exon 2 intermediate (lariat-E2). In the second step, the 3′ hydroxyl of the last nucleotide of exon 1 (E1) attacks the branch structure to give rise to a linear exon 1/intron/exon 2 product (E1-intron-E2).

(B) Sulfur substitution of the 3′ oxygen leaving group to generate the (E1$_{3'S}$E2) oligonucleotide. The structure of the 3′-*S*-phosphorothiolate linkage at the cleavage site is shown below the schematic of the exon 1/exon 2 substrate.

exons and a lariat intron. Both steps of splicing are reversible (Figure 1A) [27–30], which allows the lariat intron to splice back into RNA and DNA targets. Beyond the profound evolutionary implications [31], reverse splicing facilitates testing for metal ion/nucleophile interactions during group II intron self-splicing.

Metal rescue experiments with substrates that substitute the oxygen nucleophile with sulfur are unlikely to reveal a metal ion/nucleophile interaction because sulfur exhibits poor nucleophilicity toward phosphorus centers [32, 33]. However, microscopic reversibility dictates that a metal ion/leaving group interaction in the reverse reaction also must occur for a metal ion/nucleophile interaction in the forward reaction. Therefore, metal rescue experiments designed to probe for a metal ion/leaving group interaction in the reverse of the second step of splicing provide a direct test for a metal ion/nucleophile interaction during the second step of splicing.

The reverse of the second step of splicing occurs by two distinct pathways that differ in the identity of the nucleophile. In the spliced exons reopening (SER) reaction (Figure 1A, right pathway), water or hydroxide acts as the nucleophile, whereas in the reverse splicing reaction, the 3′ hydroxyl of the last nucleotide of the intron (Figure 1A, left pathway) acts as the nucleophile. Whereas the former hydrolytic reaction can be monitored by incubating an exon 1-exon 2 construct with the linear group II intron, it has been shown that the lariat structure is specifically required for efficient catalysis of the second step of splicing. In a bipartite assay for the second step of splicing, linear intron/3′ exon reacts almost 1000-fold more slowly than a lariat intron/3′ exon substrate [30]. Because we sought to reveal mechanistic features of the second step of the natural splicing pathway, we chose to examine the reverse second step reaction with a lariat, rather than linear, intron.

To test for a leaving group/metal ion interaction during the reverse second step reaction, we used chemical synthesis and enzymatic ligation to construct E1-E2 substrates with either a 3′ oxygen (E1$_{3'O}$E2) or 3′ sulfur (E1$_{3'S}$E2) leaving group at the cleavage site (Figure 1B)

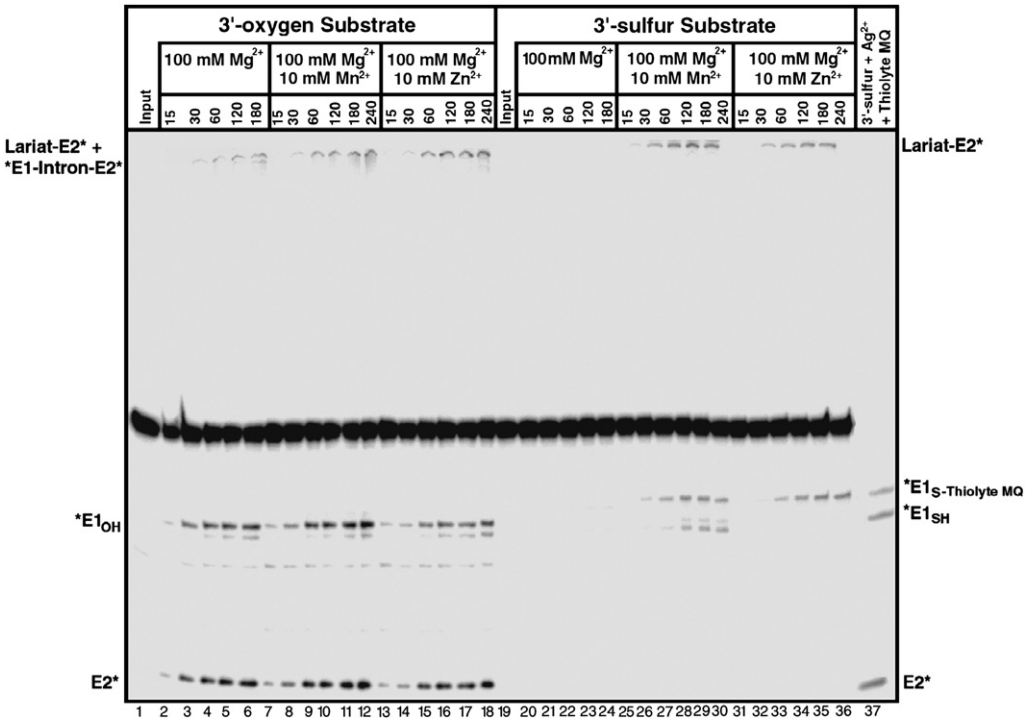

Figure 2. Sulfur Substitution at the Cleavage Site Results in a Metal Specificity Switch

Reverse splicing reactions were performed with substrates containing a 3′ oxygen ($E1_{3'O}E2$) (lanes 1–18) or a 3′ sulfur group ($E1_{3'S}E2$) (lanes 18–36) at the cleavage site. Reactions contained trace radiolabeled substrate, 400 nM lariat intron, 40 mM MOPS (pH 7.5), 0.5 M $(NH_4)_2SO_4$, and either 100 mM $MgCl_2$, 100 mM $MgCl_2$/10 mM $MnCl_2$, or 100 mM $MgCl_2$/10 mM $ZnCl_2$, as specified above each lane. Reactions were incubated at 40°C for the times (in minutes) indicated at the top of each lane. Products are labeled at the left of the gel consistent with the notation used in Figure 1, and asterisks indicate the position of radiolabels.

[24, 34]. ^{32}P radiolabels were introduced into both exon 1 and exon 2, thereby allowing all the products of reverse splicing and the SER reaction to be visualized on a single polyacrylamide gel. In the presence of lariat intron ribozyme, 40 mM MOPS (pH 7.5), 0.5 M $(NH_4)_2SO_4$, and 100 mM $MgCl_2$ at 42°C, the control substrate ($E1_{3'O}E2$) bearing the 3′ oxygen leaving group reacts via both the SER and reverse splicing pathways (Figure 2, lanes 1–5). The SER reaction generates free exon 1 (E1) and exon 2 (E2) products (Figure 1A, right pathway), whereas the reverse splicing pathway results in the release of exon 1 (E1) and the generation of lariat intron/exon 2 (lariat-E2) and subsequent exon 1/intron/exon 2 (E1-intron-E2) products that migrate more slowly during electrophoresis (Figure 1A, left pathway). Splicing also occurs efficiently when $MgCl_2$ is supplemented with 10 mM $MnCl_2$ or 10 mM $ZnCl_2$ (Figure 2, lanes 6–18).

In contrast to the control substrate, the 3′ sulfur substrate ($E1_{3'S}E2$) was unreactive in the presence of only $MgCl_2$ (Figure 2, lanes 20–24). However, reactivity was restored upon addition of the more thiophilic metal ions $MnCl_2$ and $ZnCl_2$ (Figure 2, lanes 25–36), demonstrating rescue for this reaction. The low-mobility product at the top of the gel corresponds to only the lariat intron/exon 2 intermediate, as the poor nucleophilicity of sulfur toward phosphodiester centers blocks the second step of reverse splicing (corresponding to the reverse of the first step of forward splicing), in which the terminal 3′ thiol of exon 1 would have to attack the lariat intron/exon 2 structure.

To confirm the accuracy of cleavage, each reaction was treated with a thiol-specific modifying reagent, Thiolyte MQ (Calbiochem), to test for the presence of the free 3′ thiol at the terminus of exon 1. Unreacted 3′ sulfur substrate was also treated with silver (I), which causes the specific cleavage of the sulfur-phosphorus bond of a 3′-*S*-phosphorothiolate linkage [35] and generates a standard for correct cleavage (Figure 2, lane 37). As shown in Figure 2, exon 1 released upon splicing in $MnCl_2$ and $ZnCl_2$ reacts with the Thiolyte MQ reagent to generate a product that comigrates with the standard for correct cleavage. These results confirm the identity of the cleavage site as the sulfur-phosphorus bond and rule out the possibility of miscleavage being mistaken for rescue of reactivity. The absence of any free exon 2 product indicates that the SER reaction is not occurring and suggests that rescue occurs exclusively via the reverse splicing pathway. This metal rescue provides strong evidence for a metal ion/leaving group interaction during the reverse of the second step reaction. Microscopic reversibility dictates that the forward and reverse reactions must proceed through the same transition state. We conclude that

a divalent metal ion coordinates to the nucleophile during the second step of group II intron self-splicing.

DISCUSSION

We have shown that a divalent metal ion interacts with the nucleophile during the second exon-ligation step of ai5γ group II intron splicing (Figure 3, colored in blue). This metal ion may lower the pKa of the nucleophile so as to provide a higher concentration of the oxyanion; however, this hypothesis remains untested for this or any other ribozyme. We previously obtained evidence that divalent metal ions interact with the 3′ oxyanion leaving groups during the first and second steps of splicing [24, 25], presumably stabilizing the developing negative charge. Based on the stereochemical dispositon of atoms expected from in-line nucleophilic attack, the leaving group and nucleophile for the second step of splicing must interact with distinct metal ions. Thus, the active site for exon ligation contains two divalent metal ions with functional interactions consistent with those of the so-called two-metal ion mechanism [36].

Metal rescue experiments have also implicated the 2′ hydroxyl group at the 3′ splice site as a ligand for a metal ion: Mn^{2+} rescues exon ligation of a substrate bearing 2′ aminocytidine at the 3′ splice site with the same concentration dependence as for the 3′ sulfur leaving group, suggesting that the same metal ion mediates both interactions [37]. This configuration of metal ion interactions mimics those observed structurally for the *Azoarcus* group I intron (Figure 3), in which one metal ion resides within coordination distance of the nucleophile and a second resides within coordination distance of both the 2′ and 3′ oxygens of the 3′ splice site nucleotide [9]. Both metal ions in the *Azoarcus* active site also mediate interactions with the nonbridging *pro*-R_P phosphate oxygen at the 3′ splice site. Consistent with the possibility that analogous interactions occur during group II intron splicing, sulfur substitution of the *pro*-R_P oxygen at the 3′ splice site has a deleterious effect on splicing [37]. However, this phosphorothiolate substitution has resisted rescue by softer metal ions, prohibiting its definitive assignment thus far as a metal ion ligand.

A Mechanistic Imperative?

RNA catalysts that mediate phosphoryl transfer fall into two general classes: (1) large ribozymes (RNase P and self-splicing introns) that catalyze nucleotidyl transfer from a 3′ oxygen leaving group to water or a 2′ or 3′ oxygen nucleophile, and (2) endonucleolytic ribozymes (VS, hairpin, hammerhead, HDV, and glmS) that catalyze RNA strand scission via internal 2′-O-transphosphorylation, in which a 2′ hydroxyl group nucleophilically attacks the adjacent phosphodiester to displace the 5′ oxygen and generate a 2′,3′ cyclic phosphate. Biochemical and structural investigations of the large ribozymes have shown that they use metal ions directly in catalysis, through inner sphere coordination to the scissile phosphate, the nucleophile, and/or the leaving group atoms [10, 24, 25, 37–43]. In contrast, the endonucleolytic ribozymes have no obligate requirement for divalent metal ions [44]. A divalent metal ion at the active site of the HDV ribozyme facilitates catalysis [45, 46], but does not appear to interact directly (via inner sphere coordination) with the leaving group or the nucleophile [47]. The absence of an obligate metal ion requirement in the endonucleolytic ribozymes may reflect the need for less catalytic power. The uncatalyzed internal 2′-O-transphosphorylation occurs a million-fold more rapidly than the uncatalyzed nucleotidyl transfer [48, 49] due to the proximity of the 2′ hydroxyl group. Conversely, the metal ion requirement observed for the large ribozymes may reflect the need for greater catalytic power to promote nucleotidyl transfer on a biological timescale.

Based on these considerations, our new results, and the many similarities between group II intron self-splicing and nuclear pre-mRNA splicing, it seems highly likely that the spliceosome will employ multiple divalent metal ions for catalysis. Biochemical studies have established at least one metal ion at the spliceosome active site [39].

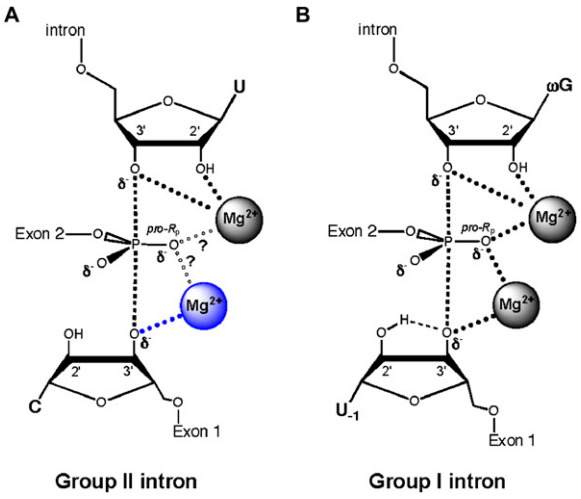

Figure 3. Metal Ion Interactions in the Transition States of Two Large Ribozymes

(A) Functional transition-state model for the exon-ligation reaction catalyzed by the group II intron. The 3′ oxyanion leaving group and the cleavage site 2′ hydroxyl group interact either with the same metal ion (as shown) or with distinct ions that bind to the ribozyme with the same affinity [24, 25]. The work described herein identifies a second metal ion that coordinates to the 3′ oxygen nucleophile at the terminus of exon 1 (E1). This metal ion and its interaction with the nucleophile are colored in blue. Based on the deleterious effect on reaction of sulfur substitution at the *pro*-R_p position [37], it is possible that either one or both of the pictured metal ions interact with this nonbridging oxygen. The inability to rescue the sulfur substitution with softer divalent metal ions has precluded definitive assignment of this oxygen as a metal ligand, however.

(B) Structure-based transition-state model for group I intron-catalyzed exon ligation. Crystallographic analysis of the *Azoarcus* group I intron with both exons shows two divalent metal ions at the active site. One metal ion resides within coordination distance of the 2′ and 3′ oxygen atoms of ωG and the nonbridging *pro*-R_p oxygen of the 3′ splice site. The second metal ion also resides within coordination distance of the *pro*-R_p oxygen of the scissile phosphate and the 3′ oxygen (the nucleophile for the exon-ligation reaction) at the terminus of the 5′ exon [10].

SIGNIFICANCE

The results of this work provide mechanistic insight into the splicing pathway of the group II intron ribozyme. Specifically, we have shown biochemically that a metal ion activates the nucleophile in the second splicing step. In conjunction with the previously identified metal ion that activates the leaving group in this reaction, these results reveal that group II intron splicing employs at least two metal ions for catalysis, analogous to many protein phosphoester transfer enzymes and the group I intron. The mechanistic parallel to the group I intron thus established suggests that the large ribozymes employ a common catalytic strategy, distinct from those of the small ribozymes. The spliceosome, a ribonucleoprotein machine that shares many similarities with group II introns, likely employs the same mechanistic strategy to catalyze eukaryotic pre-mRNA splicing.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis

Oligonucleotides were synthesized on a Millipore solid-phase DNA/RNA synthesizer. 3′-*S*-phosphoramidites were synthesized and coupled as previously described [50]. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. The following oligoribonucleotides were synthesized (the subscript $_s$ denotes a 3′-*S*-phosphorothiolate linkage): Ex1-2 (5′-ACGUGGUGGGACAUUU-3′), SER (5′-UCACUAUGUAU-3′), SES (5′-UC$_s$ACUAUGUAU-3′).

Generation and Isolation of the Lariat Intron Ribozyme

We modified the protocol described by Deme et al. [30] to obtain large quantities of unlabeled lariat intron. Transcription of the full-length ai5γ group II intron was performed in a 2 ml reaction containing 40 mM Tris-HCl (pH 7.5), 1 mM each NTP, 20 mM $MgCl_2$, 10 mM DTT, 40 ng/μl pJD20 plasmid [27] linearized with HindIII, and 0.16 μg/μl T7 RNA polymerase. After reacting for 3 hr at 37°C, the reaction was treated with DNase I to remove the plasmid and extracted with phenol/chloroform/isoamyl alcohol (50/49/1). RNA was then precipitated by adding three volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.3). The ai5γ intron was then resuspended in 500 μl of 40 mM MOPS (pH 7.5), denatured at 90°C for 1 min, and then equilibrated at 42°C for 10 min. Splicing was initiated by adding an equal volume of prewarmed splicing mix (1 M $[NH_4]_2SO_4$, 200 mM $MgCl_2$, 40 mM MOPS [pH 7.5]) and allowed to proceed for 75 min at 42°C. The RNA was desalted with a NAP 10 column (G-25 Sephadex; Amersham Pharmacia) and precipitated as described above. Finally, the lariat intron was purified by denaturing 4% polyacrylamide gel electrophoresis.

Generation of Substrate RNAs

The full-length E1$_{3'X}$E2 substrates containing either an oxygen (X = O) or sulfur (X = S) leaving group were constructed by ligating the Ex1-2 oligonucleotide to the SES and SER oligonucleotides with T4 DNA ligase and a bridging oligonucleotide [24, 34]. To incorporate a ^{32}P radiolabel into both E1 and E2, the SER/SES oligonucleotides were 3′ ^{32}P labeled with [α-^{32}P]3′-deoxyadenosine triphosphate (New England Nuclear) and yeast poly(A) polymerase and then subsequently 5′ ^{32}P phosphorylated with [γ-^{32}P]adenosine triphosphate (New England Nuclear) and T4 polynucleotide kinase. Annealing and ligation reactions were performed as previously described [24] and resulted in very good yields. Ligated RNAs were purified by denaturing 20% polyacrylamide gel electrophoresis.

Splicing Reactions

All reactions were at 40°C and contained 400 nM lariat ribozyme, trace radiolabeled substrate, 40 mM MOPS (pH 7.5), 0.5 M $(NH_4)_2SO_4$, 100 mM $MgCl_2$, 10 mM $MnCl_2$ or $ZnCl_2$, and 5 mM Tris(2-carboxyethyl)-phosphine (Strem Chemicals). Before reaction, the lariat intron was denatured at 90°C for 1 min and then allowed to equilibrate at 40°C for 10 min in splicing mix before the reaction was initiated by the addition of substrate. Aliquots of reaction mixture were removed at specified times and quenched by the addition of stop solution (8 M urea, 0.5× TBE [89 mM Tris, 89 mM borate, 2 mM EDTA], 50 mM EDTA, 0.04% xylene cyanol, 0.01% bromophenol blue). To specifically modify the free thiol group, 10 mM Thiolyte MQ reagent (Calbiochem) was included in the stop solution and the samples were incubated at room temperature for 5 min before storing at −80°C. Substrates and products were fractionated by denaturing 20% polyacrylamide gel electrophoresis.

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