The two steps of group II intron self-splicing are mechanistically distinguishable

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ABSTRACT

The two transesterification reactions catalyzed by self-splicing group II introns take place in either two active sites or two conformations of a single active site involving rearrangements of the positions of the reacting groups. We have investigated the effects on the rates of the chemical steps of the two reactions due to sulfur substitution of nonbridging oxygens at both the 5' and 3' splice sites as well as the deoxyribose substitution of the ribose 2' hydroxyl group at the 5' splice site. The data suggest that the two active sites differ in their interactions with several of these groups. Specifically, sulfur substitution of the pro-Sp nonbridging oxygen at the 5' splice site reduces the chemical rate of the step one branching reaction by at least 250-fold, whereas substitution of the pro-Sp oxygen at the 3' splice site has only a 4.5-fold effect on the chemical rate of step two. Previous work demonstrated that the Rp phosphorothioate substitutions at both the 5' and 3' splice sites reduced the rate of both steps of splicing to an undetectable level. These results suggest that either two distinct active sites catalyze the two steps or that more significant alterations must be made in a single bifunctional active site to accommodate the two different reactions.

Keywords: kinetics; metal ions; phosphorothioate; ribozyme; transesterification

INTRODUCTION

Splicing of eukaryotic nuclear pre-mRNA introns by spliceosomes and the self-splicing of group II introns share many mechanistic features, an observation that has led to frequent speculation that they are related by descent from a common source (see Nilsen, 1998 for a recent discussion). Both systems splice RNA through a two-step pathway involving sequential transesterification reactions first at the 5' splice site and then at the 3' splice site. From mechanistic and stereochemical considerations, it has been argued that both systems must have two active sites or two alternative configurations of a single active site that act sequentially to carry out the two steps (Moore & Sharp, 1993; Steitz & Steitz, 1993; Padgett et al., 1994).

Studies of a group II intron RNA have shown that modification of certain residues inhibits both steps of splicing (Chanfreau & Jacquier, 1994), whereas modi-

fication at other positions appears to affect a conformational change that occurs between the two steps to displace the products of step one with the substrates for step two (Chanfreau & Jacquier, 1996). These results were interpreted to support a single active site model. A single active site was also suggested by Steitz and Steitz (1993). In this model, two active site divalent metal ions interact with the attacking and leaving oxygens and also interact with one of the nonbridging oxygen atoms of the phosphate. Conformational shifts were proposed to exchange the reacting phosphodiester bonds in the active site and alter the stereochemistry of attack between the two steps of the splicing reaction. We have shown that a single type of RNA substrate for a *trans*-cleavage reaction can react with both a step one and a step two active site based on the stereochemical specificities of the reactions (Podar et al., 1995). That study also showed that a hydrolytic active site with the stereochemical specificity of a step two reaction was active in ribozymes that had not undergone a step one reaction, thus arguing that both the step one and step two active sites co-exist within the same RNA or that the two active site conformations can interchange on a time scale of minutes.

Recent efforts to understand the mechanistic details of the transesterification reactions of group I, group II,

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and spliceosomal introns have begun to map out the interactions between the active sites and the reacting substrates. In particular, the roles of the nonbridging oxygen atoms in the reacting phosphodiester bond have been probed by replacement with sulfur (McSwiggen & Cech, 1989; Rajagopal et al., 1989; Suh & Waring, 1992; Maschhoff & Padgett, 1993; Moore & Sharp, 1993; Padgett et al., 1994). For all splicing reactions, sulfur replacement of one but not the other nonbridging oxygen reduces the rate of the reaction by three or more orders of magnitude. This has been suggested to be evidence for a critical interaction of this oxygen with a component, usually proposed to be a divalent metal ion, within the active site. This result has also been found in the cleavage reaction of the hammerhead ribozyme (Koizumi & Ohtsuka, 1991; Slim & Gait, 1991). Thus, this arrangement appears to be a common feature of RNA-catalyzed reactions at phosphodiester bonds.

In the course of examining the stereochemistry of group II intron self-splicing (Padgett et al., 1994), we observed that one of the two phosphorothioate isomers (the Rp isomer) produced by substituting one of the nonbridging oxygens with sulfur at the 5' or the 3' splice site reduced both steps of splicing to an undetectable level (>1,000-fold reduction). The other (Sp) isomer allowed both steps of splicing to proceed, but the effect on the rate of the two steps differed. The Sp phosphorothioate at the 5' splice site slowed the rate of step one by 15–30-fold, whereas the Sp phosphorothioate at the 3' splice site had no effect on the rate of step two (Padgett et al., 1994). Previous investigations of a group I self-splicing intron showed that the active phosphorothioate isomer reduced the chemical rate of reaction by only two- to threefold, which is consistent with the magnitude of "thio effects" seen using model uncatalyzed reactions (Herschlag et al., 1991; Herschlag & Khosla, 1994). In light of this, the 15–30-fold thio effect on the step one reaction seen for the Sp phosphorothioate-substituted group II intron warranted further investigation to see if it was a chemical effect or due to binding or conformational difficulties produced by the substitution. If the large thio effect on step one were indeed due to a reduction in the chemical rate of step one, it would indicate that the pro-Sp oxygen plays a direct role in the reaction, a result that would distinguish this RNA-catalyzed reaction from the group I intron reactions and perhaps from the second step of group II intron splicing.

Up to now, it has not been possible to distinguish the first and second step reactions mechanistically; the two reactions have been characterized solely by their choice of substrates. Here we show that the reaction mechanisms of the two steps of self-splicing in a group II intron can be distinguished. This suggests that the two active sites or configurations differ from each other in important respects perhaps related to the require-

ments for activating the differing substrates of the two reactions.

RESULTS

Analysis of the effects of Sp phosphorothioate substitution at the 5' splice site on step one

In order to determine if the substantial "thio effect" of the Sp phosphorothioate substitution on step one was due to a reduction in the chemical rate of splicing, an experimental system had to be developed in which the chemical step of the reaction was rate-limiting. In our earlier investigations of the mechanisms of various *trans*-reactions catalyzed by this group II intron, we failed to find a kinetically simple reaction that fully corresponded to a pure step one reaction (Podar et al., 1995). The only step one reaction in which most of the molecules formed branches was a *cis*-splicing reaction performed in high concentrations of ammonium sulfate (Jarrell et al., 1988). Thus, we explored conditions under which the rate of this reaction could be made chemically limited.

The *cis*-branching reaction is diagramed in Figure 1A. In the complete ribozyme, the reaction proceeds through both steps to produce ligated exons and lariat intron RNAs. The rate of step two is always substantially faster than the rate of step one as evidenced by the almost complete lack of accumulation of the lariat-exon 2 intermediate during the reaction (Fig. 1B). Chin and Pyle (1995) have shown that step one is readily reversible with an equilibrium constant close to one. Thus, the step two reaction serves as a kinetic trap for the products of step one and the rate of step one can be determined by measuring the rate of production of the products of step two.

The *cis*-branching reaction conditions used here produced a biphasic reaction in which 40–60% of the RNA reacts in a fast phase and the remainder reacts with a slower k_{obs} . This biphasic kinetic behavior has been reported by others (Chin & Pyle, 1995) and appears to represent an equilibrium between conformational states. An example of such a reaction performed at pH 6.0 is shown in Figure 1B and the data are plotted in Figure 1C. The k_{obs} for this reaction is 0.29 min^{-1} . The k_{obs} at pH 7.5 is 0.8 min^{-1} (see below), which is close to the rate of the reverse step one reaction of about 1 min^{-1} reported by Chin and Pyle (1995). To allow comparison to the very much slower rates measured under different conditions and with different RNAs, we used only initial rates, which were not corrected for the fast-reacting fraction. This will lead to an underestimate of the rates by as much as a factor of two. This approximation was necessary because we could only measure the initial rates of reactions with k_{obs} values as low as $2 \times 10^{-6} \text{ min}^{-1}$ (see below).

FIGURE 1. Example of the optimized *cis*-splicing reaction for step one. **A:** Diagram of the two steps of the self-splicing reaction. Full-length self-splicing RNAs containing either a phosphodiester or a phosphorothioate in the Sp configuration at the 5' splice site (denoted by the dot in the diagram) were synthesized by oligonucleotide-mediated ligation of three RNAs as described (Padgett et al., 1994). A single ^{32}P label was positioned nine nucleotides 3' of the 5' splice site (denoted by the X in the diagram) and used to monitor the conversion of unspliced RNA to lariat intron product. **B:** Representative time course of *cis*-splicing of control unsubstituted group II intron RNA in MES buffer at pH 6.0. Locations of precursor, lariat-E2 intermediate, lariat intron, and linear intron RNAs are diagramed. **C:** Graph of data in B. The line is a fit to the early linear phase of the reaction, which gives a first-order rate of 0.29 min^{-1} .

To determine if this reaction rate measured the chemical step of the reaction, the pH dependence of the reaction rate was investigated. Because the reaction mechanism requires the abstraction of a proton from the attacking 2' hydroxyl group to generate the reactive oxyanion, the chemical rate should be inversely proportional to the H^+ concentration. Thus, the reaction should show a linear relationship between the pH of the reaction and the log of the observed reaction rate. The slope of such a line provides an indication of the number of protons involved in the rate-limiting step. Such analyses of the *Tetrahymena* group I intron (Herschlag & Khosla, 1994) and RNase P (Smith & Pace, 1993) reactions have given slopes near unity. As pH is increased, a plateau may appear due to the chemical rate becoming faster than some limiting conformational step in the reaction (Herschlag & Khosla, 1994). Alternatively, an apparent pK_a may be seen due to titration of multiple critical residues, as observed in the *Tetrahymena* group I ribozyme by Knitt and Herschlag (1996).

When the pH dependence of the group II step one reaction was investigated, we observed a complex curve as shown in Figure 2A (open circles). At pH values greater than 6.8, the reaction rate was largely independent of pH. Below pH 6.8, the reaction became pH dependent. However, the plot was nonlinear with the slope becoming progressively larger as pH decreased. These data are open to several possible interpretations with regard to which regions of the curve, if any, show chemically rate-limited behavior. To address this issue,

we investigated the pH dependence of the observed reaction rates of modified ribozymes.

Our previous experiments had suggested that the sulfur substitution of the pro-Sp oxygen at the 5' splice site greatly reduced the reaction rate (Padgett et al., 1994; Podar et al., 1995). To investigate this further, we synthesized complete *cis*-splicing RNAs containing a single, stereospecific sulfur substitution of the pro-Sp oxygen to produce the Sp phosphorothioate RNA. We then determined the pH dependence of splicing of this RNA under the same conditions used above for the unsubstituted RNA. The data are plotted in Figure 2B (open circles) and show that the Sp phosphorothioate RNA reacted substantially slower than the control RNA, in agreement with our previous results. Furthermore, unlike the control RNA, the Sp phosphorothioate RNA showed a linear dependence on pH with the slope of the line being close to 1 throughout the pH range tested.

Although not conclusive, this behavior suggests that the ribozyme with a 5' splice site Sp phosphorothioate might be reacting under chemically rate-limiting conditions. To test this possibility further, an additional modification was made in this ribozyme that would be expected to reduce the chemical rate of step one. The 2' hydroxyl group next to the 3' leaving group has been shown to play an important role in the catalytic mechanisms of group I and RNase P ribozymes where the chemical rates are reduced by around 10^3 (Herschlag et al., 1993a, 1993b; Smith & Pace, 1993; Zaug et al., 1994). In the case of the group II reaction, on the other

FIGURE 2. Rate of *cis*-splicing as a function of pH. First-order rates (k_{obs}) were determined by least-squares fitting to the early linear portions of time courses such as shown in Figure 1C. **A:** Comparison of reaction rates of control unsubstituted RNA (open circles) and RNA with a single deoxynucleotide residue at the 5' splice site (open squares) in the presence of 100 mM Mg^{+2} ; filled circles, control unsubstituted intron RNA in the presence of 90 mM Mg^{+2} and 10 mM Mn^{+2} . Inset: rates of the control RNA were divided by a factor of 50 and plotted with the deoxyribose-substituted data. Symbols are the same as in the main plot. **B:** Reaction rates of 5' splice site Sp phosphorothioate-substituted RNA (open circles) and RNA with both a 5' splice site Sp phosphorothioate and a 5' splice site deoxynucleotide substitution (open squares) in the presence of 100 mM Mg^{+2} ; filled circles, 5' splice site Sp phosphorothioate-substituted RNA in the presence of 90 mM Mg^{+2} and 10 mM Mn^{+2} . Lines are linear fits to the data (SigmaPlot) and have slopes of 1.07 (open circles), 1.17 (open squares), and 0.98 (filled circles).

hand, Griffin et al. (1995) have shown that this substitution reduces the rate of a *trans*-hydrolysis reaction by only 16-fold, suggesting that, in contrast to group I, group II ribozymes do not use this group for catalysis.

To examine this effect in the context of the complete *cis*-branching reaction, the 2' deoxyribo modification adjacent to the scissile bond was combined with the Sp phosphorothioate. As with the control and Sp phosphorothioate RNAs, this RNA reacted almost exclusively by branching (data not shown). The observed rate of reaction was reduced relative to the ribo Sp phosphorothioate RNA at all pH values tested. As shown in Figure 2B (open squares), the rate reduction was quite constant with an average value of 10-fold. This compares well with the 16-fold effect of this deoxyribo modification observed by Griffin et al. (1995). Thus, both the pH dependence and the effect of the deoxyribo modification suggest that this Sp phosphorothioate-modified ribozyme is reacting under chemically rate-limiting conditions.

The effect of the 5' splice site 2'-deoxyribo substitution on the rate of step one in the phosphodiester control RNA was also tested. As shown in Figure 2A (open squares), this modification reduced the rate of the reaction at all pH values tested. The extent of the rate reduction was a constant 50-fold. This is demonstrated in the inset to Figure 2A, where the control reaction rates have been divided by a factor of 50 and plotted with the deoxy data. The two curves superimpose, showing that the deoxy effect is constant throughout the pH range. This constancy strongly suggests that the same rate-limiting step is being measured throughout the pH range and that the curve is not a composite due to a change in rate-limiting step.

The 50-fold rate reduction due to the 2' deoxyribo modification of the phosphodiester RNA is significantly and reproducibly larger than the 10-fold effect on the Sp phosphorothioate RNA or the 16-fold effect found by Griffin et al. (1995). This, combined with the complex pH versus rate curve shown by the control ribozyme, suggests that the rate of reaction of the control ribozyme is likely to be limited by a step other than chemistry. Nevertheless, the results show that substitution of the pro-Sp oxygen with sulfur at the 5' splice site has a large effect on the chemical rate of the ribozyme reaction. The magnitude of this effect on the chemical step is at least 200–250-fold, which is the difference between the control and Sp phosphorothioate rates at pH values between 5.5 and 6.5. This is a lower limit; the actual difference could be substantially larger.

Effect of manganese ion on the step one reaction rate

Large rate reductions in reactions at phosphodiester bonds due to sulfur substitution of bridging and non-bridging oxygen atoms are frequently due to disruption

of catalytically important inner sphere metal coordination contacts (Eckstein, 1985). Magnesium ion coordinates sulfur at least four orders of magnitude weaker than oxygen, accounting for the large rate reductions often seen in reactions involving active site metal ions (Pecoraro et al., 1984). Thus, one possible interpretation of the large reduction in the group II step one reaction in the Sp phosphorothioate RNA was that an important Mg^{2+} –oxygen interaction was being disrupted. A standard test to support a specific metal–oxygen interaction is to demonstrate that addition of a more thiophilic metal ion such as Mn^{2+} , Zn^{2+} , or Cd^{2+} can substantially enhance the reaction of the sulfur-substituted RNA. We therefore tested a variety of divalent metal ions alone and in combination to determine if such a metal “rescue” could be seen.

The reaction conditions used above required Mg^{2+} at a concentration of at least 50 mM. This requirement could not be met using Mn^{2+} or Zn^{2+} alone. We therefore tested mixtures of various divalent metal ions to determine if the Sp phosphorothioate-substituted ribozyme could be specifically activated by thiophilic metals. We tested Mn^{2+} , Zn^{2+} , Pb^{2+} , and Cd^{2+} in the presence of Mg^{2+} at a total divalent ion concentration of 100 mM. The only ion that stimulated the reaction was Mn^{2+} , which gave a maximum effect at 10 mM. The effect of Mn^{2+} addition at different pH values for the control and Sp phosphorothioate RNAs was then determined and the results are shown in Figure 2A and B (filled symbols). Above pH 7.0, addition of Mn^{2+} has virtually no effect on the reaction of the control RNA (Fig. 2A). Below pH 6.0, Mn^{2+} stimulated the reaction rate by three- to fourfold. In contrast, for the Sp phosphorothioate RNA, addition of Mn^{2+} increased the reaction rate by about 10-fold at all pH values (Fig. 2B).

The effect of different divalent metals on the rate of splicing of the 5' splice site 2'-deoxyribo phosphodiester and 2'-deoxyribo Sp phosphorothioate substrates was also determined. The results were the same as for the all-ribo ribozymes, with Mn^{2+} being the only metal to show stimulation of the rate of reaction of the Sp phosphorothioate RNA. The magnitude of the Mn^{2+} stimulation of the 2'-deoxyribo Sp phosphorothioate RNA reaction was 12-fold at pH 6.0, which is similar to what was seen for the ribo Sp RNA reaction. The rate of reaction of the 2'-deoxyribo phosphodiester RNA was stimulated twofold by Mn^{2+} under similar conditions.

Analysis of effects of Sp phosphorothioate substitution at the 3' splice site on step two

In contrast to the large rate reduction in step one due to Sp phosphorothioate substitution at the 5' splice site, we previously observed no effect on the rate of step two when the pro-Sp oxygen of the 3' splice site was substituted by sulfur (Padgett et al., 1994; Podar et al., 1995). However, because this result was obtained with

the complete reaction, a substantial thio effect on step two could have been masked by the slow step one reaction. Thus, to determine if the Sp phosphorothioate had a similarly large effect on step two as on step one, a chemically rate-limited step two reaction had to be developed.

The reaction we used is similar to one used previously (Jarrell et al., 1988) and is diagramed in Figure 3A. It involves the reaction of a synthetic 17-nt exon 1 RNA with a linear RNA composed of the intron and exon 2. These RNAs resemble the authentic intermediates in the splicing reaction except that the intron does not contain the 2'-5' branch structure. Incubation of the exon 1 RNA and the intron–exon 2 RNA under the same conditions used in the step one assay led to the production of spliced exons and linear intron RNAs (Fig. 3B). The initial experiments used a 17-nt exon 1 RNA oligonucleotide (E1rC). The rate of this reaction as a function of pH was determined and found to be independent of pH and to show no effect upon Sp phosphorothioate substitution at the 3' splice site (data not shown). These results suggested that the rate of this reaction was limited by a step other than chemistry (Herschlag et al., 1991).

Because the reaction rates we obtained in the step one *cis*-assay were close to the limit of accurate measurement using manual processes and we knew that the rate of the step two *cis*-reaction was much faster than step one, we decided to design a step two assay with a slower chemical step. In the group I ribozyme reaction, the 2' hydroxyl group next to the attacking 3' hydroxyl plays a significant role in catalyzing the reaction. For example, dGTP is orders of magnitude less reactive than GTP in group I ribozyme reactions (Bass & Cech, 1984). The analogous substitution for the group II step two *trans*-reaction would be to use an exon 1 RNA with a single 2' deoxyribose residue at the 3' end (E1dC). When this RNA was used in a reaction with the intron–exon 2 RNA, correct splicing was seen as judged by comigration of the exon products from the E1rC and E1dC reactions under conditions that resolve single-nucleotide differences (data not shown). The rate of the reaction with E1dC was significantly slower than with E1rC. Furthermore, as shown in Figure 3C (open circles), the rate of the E1dC reaction was pH dependent, with a slope near 1. This behavior suggests that the step two *trans*-reaction using the E1dC oligo is occurring under chemically rate-limiting conditions.

Using the E1dC substrate, we also measured the rate of step two at various pH values with an intron–exon 2 RNA with an Sp phosphorothioate at the 3' splice site (Fig. 3C, open squares). The results show that this reaction also demonstrates a log-linear relationship between pH and rate, consistent with a chemically rate-limiting reaction. The thio effect for this reaction is between 3 and 6, with an average of 4.5.

FIGURE 3. Step two *trans*-assay and the effect of a 3' splice site Sp phosphorothioate substitution. **A:** Diagram of the reaction. A synthetic E1 RNA with a terminal deoxyribose (E1dC) and an intron-E2 RNA with either a phosphodiester or an Sp phosphorothioate at the 3' splice site (denoted by the dot in the diagram) were constructed and reacted in *trans* at various pH values. First-order rate constants (k_{obs}) were derived from least-squares fitting to the data at early time points. **B:** Representative time course of the step two assay. A single ^{32}P was located at the 3' end of E2. The locations of the intron-E2 substrate, spliced exon products, and hydrolyzed E2 RNA are diagrammed. **C:** Plot of k_{obs} versus pH. Open circles, reaction of E1dC with the control phosphodiester ribozyme in 100 mM Mg^{+2} ; filled circles, reaction of E1dC with the control phosphodiester ribozyme in 90 mM Mg^{+2} and 10 mM Mn^{+2} ; open squares, reaction of E1dC with 3' splice site Sp phosphorothioate ribozyme in 100 mM Mg^{+2} ; filled squares, reaction of E1dC with 3' splice site Sp phosphorothioate ribozyme in 90 mM Mg^{+2} and 10 mM Mn^{+2} . Lines are linear fits to the data (SigmaPlot) and have slopes of 0.86 (open circles), 1.01 (open squares), 1.01 (filled circles), and 1.00 (filled squares).

This is in the range of thio effects seen for uncatalyzed model reactions (Herschlag et al., 1991).

The reaction of both substrates was also measured in the presence of Mn^{+2} (Fig. 3C, filled symbols). Addition of Mn^{+2} ion increased the reaction rate of the control substrate an average of 1.3-fold and of the phosphorothioate substrate an average of 2.6-fold.

DISCUSSION

The starting point for these investigations was our previous finding that both phosphorothioate diastereomers at the 5' splice site of a group II intron showed

dramatic reductions in the rate of step one relative to the unsubstituted ribozyme (Padgett et al., 1994; Podar et al., 1995). The Rp isomer was essentially unreactive, whereas the Sp isomer showed substantially reduced activity. In contrast, the effects of similar substitutions at the 3' splice site on the rate of step two were different in that only one of the diastereomers, the Rp, was inactive for splicing, whereas the Sp isomer remained fully reactive. These results raised the question of whether these different responses to sulfur substitution of the nonbridging oxygens at the two splice sites reflected fundamental differences in the organization of the active site or sites of the ribozyme for the two

steps. To answer this question, we needed to determine whether the observed effects on the reaction rates reflected effects on the chemical step of the reactions or on other steps. To address this, we attempted to find conditions under which we could measure the chemical rate of each step of the group II ribozyme reaction.

Most ribozymes utilize a reaction mechanism in which the reactive phosphate bond undergoes nucleophilic attack by an oxyanion or a hydroxide ion (Narlikar & Herschlag, 1997). As a result, the observed rate of reaction is a function of the concentration of the attacking species. In hydrolytic reactions where hydroxide is the nucleophile, such as in RNase P, the concentration of the hydroxide and thus the rate of reaction is a direct function of the pH of the reaction (Smith & Pace, 1993). In cases such as the hammerhead ribozyme (Dahm & Uhlenbeck, 1991) or the *Tetrahymena* group I intron (Herschlag et al., 1991), where ribose hydroxyls are deprotonated to give the reactive oxyanions, it has been observed that this process is also governed by the pH of the reaction. Thus, these ribozymes exhibit a log-linear pH versus rate profile with a unit slope near neutrality. Group II ribozymes appear to use a similar mechanism and have been reported to exhibit such a pH-rate profile in a hydrolytic reaction (Michels & Pyle, 1995).

Step one is slowed substantially by an Sp phosphorothioate at the 5' splice site

Our results here show that, for step one of group II intron *cis*-splicing by the branching pathway and under the conditions used in this study, the reaction rate as a function of pH gives a complicated curve that does not fit simply with the above model. However, when the 5' splice site is modified to contain an Sp phosphorothioate, the rate of the reaction is greatly reduced and the pH versus rate curve becomes much more linear with a slope of 1.07 over more than two pH units. Adding the 5' splice site 2' deoxyribose substitution to the Sp phosphorothioate ribozyme further reduced the rate of reaction by about 10-fold, which agrees well with the 16-fold effect on the chemical rate measured in this same ribozyme using a different reaction (Griffin et al., 1995). These results suggest that the Sp phosphorothioate-substituted ribozyme is chemically rate-limited for step one under these conditions.

There are several possible explanations for the results seen with the unsubstituted ribozyme. Some of these are: (1) the observed rate is the chemical rate; (2) there is a change in rate-limiting step in the pH interval examined; (3) the observed rate is limited by a step other than chemistry. In the case of model 1, Knitt and Herschlag (1996) have provided a framework in which to understand complex behaviors of ribozyme pH versus rate profiles. They suggest that the combined effects of titrations of groups on many critical

RNA residues with pK_a s far removed from the pH of measurement may give rise to apparent pK_a s.

An observation that argues against model 1 is that when the pro-Sp oxygen was substituted by sulfur, the magnitude of the rate reduction was not constant throughout the pH range as might be expected for a reaction limited by chemistry. This result could be consistent with model 2, however. A change in rate-limiting step was shown to occur in the *Tetrahymena* ribozyme giving rise to an apparent pK_a (Herschlag & Khosla, 1994). Arguing against model 2 is the finding that a different modification, the deoxyribo substitution of the 2' position adjacent to the 3' leaving group in the reaction, reduced the reaction rate by a constant 50-fold at all pH values. This suggests that a change in rate-limiting step is not occurring.

We favor the third possibility listed above, in which a pH-dependent conformational step is rate-limiting. Conformational changes in RNA that are pH dependent have been observed previously (Kao & Crothers, 1980; Gluick et al., 1997). The nature of the titratable groups in the group II intron is unclear, but we can hypothesize that hydrogen bonding interactions needed to stabilize the active conformation might involve protonated bases. For example, A-C base pairs requiring a protonated adenosine have been described where the pK_a of the base is perturbed to near neutrality (Puglisi et al., 1990). Protonation of C in C⁺(G-C) base triples has also been observed (Macaya et al., 1992). The combined contributions of several such interactions could account for the steep curve of pH versus rate at low pH values. In this view, the 50-fold effect of deoxyribose substitution of the 2' hydroxyl at the 5' splice site can then be understood as being due to the disruption of a conformational or binding interaction perhaps related to bringing the scissile phosphodiester bond into the active site for step one. Once positioned, however, the role of this 2' hydroxyl in the chemical step is small, as indicated by the independent measurements of 10- and 16-fold reductions upon deoxyribose substitution (Griffin et al., 1995).

A more radical suggestion is that both the control and Sp phosphorothioate RNAs are reacting under chemically rate-limiting conditions and the different pH curves and rate reductions due to deoxyribo substitution at the splice site are due to a change in the rate-limiting chemical step in the reactions of the two substrates. This model cannot be eliminated with the data here. However, in the one other case where this group II intron has been evaluated under chemically rate-limiting conditions, both an apparent pK_a and a 16-fold deoxyribo effect were observed (Griffin et al., 1995).

Regardless of whether or not the control RNA reaction is chemically rate-limiting, the response of the rate to the Sp phosphorothioate modification is large. The magnitude of the observed thio effect varies with pH, but shows a maximum of about 250-fold around

pH 6.5. This is a much larger thio effect than is observed in the *Tetrahymena* group I intron reaction (Herschlag et al., 1991) and is consistent with a function for the pro-Sp oxygen in the catalytic mechanism of step one of group II intron splicing.

A candidate for such a function is the binding of a divalent metal ion. Sulfur substitution of an oxygen coordinated to a magnesium ion is expected to substantially reduce the strength of the interaction (Eckstein, 1985). If the interaction is important for catalysis, this should lead to a substantial reduction in the rate of the reaction in the presence of magnesium. Manganese ion binds better to sulfur than does magnesium and might be expected to restore or rescue the rate of the reaction (Pecoraro et al., 1984). When manganese was added to the group II intron reaction, it stimulated the reaction rate of the Sp phosphorothioate RNA by 10–12-fold and the control RNA reaction by less than 2-fold. This result is difficult to interpret because, as noted above, the control and Sp phosphorothioate RNA reactions may not be limited by the same step. The modest stimulation by manganese could be due to a direct binding to the Sp sulfur or to a general enhancement of the reaction. Attempts to show greater rate enhancements with more thiophilic metal ions such as Zn^{2+} or Cd^{2+} were unsuccessful, perhaps due to constraints on which metals could be accommodated in the active site.

Step two is not substantially affected by an Sp phosphorothioate at the 3' splice site

We then examined the rate effect of Sp phosphorothioate substitution at the 3' splice site on step two of group II splicing. In order to assay step two, we used a *trans*-assay in which a free exon 1 RNA is reacted with a linear intron–exon 2 RNA. In preliminary experiments, the reaction rate of an all-RNA exon 1 with the intron–exon 2 was independent of pH. This suggested that the reaction was not limited by the chemical step. In an attempt to reduce the chemical reactivity of the exon 1, the 3'-terminal ribonucleotide was substituted by the 2' deoxyribonucleotide. This substituted exon 1 (E1dC) reacted more slowly with the intron–exon 2 RNA. In addition, the reaction rate was a log-linear function of pH with a slope near 1, a behavior that is consistent with a reaction rate limited by the chemical step.

When E1dC was reacted with an intron–exon 2 RNA with an Sp phosphorothioate substitution at the 3' splice site, the rate of the reaction was reduced about 4.5-fold. This rate reduction is within the range seen in uncatalyzed hydrolytic reactions (Herschlag et al., 1991) and is further evidence that this reaction is limited by the chemical step. Thus, in contrast to step one, the step two reaction mechanism appears to be insensitive to the substitution of the pro-Sp oxygen by sulfur aside from small intrinsic effects. Both steps, however, are

blocked by substitutions of the pro-Rp oxygens at the respective splice sites (Padgett et al., 1994).

The two reactions are performed by mechanistically distinct active sites

Unlike the case in group I introns, it is clear that the two steps of group II splicing cannot be performed by a single active site. The nature of the attacking nucleophiles is different in the two steps and the stereochemical specificities for phosphorothioates are the same rather than opposite for the two steps (Padgett et al., 1994). These findings argue that at least some features of the two active sites must differ.

Beyond these geometrical arguments, which could be reconciled with a single active site by minor conformational rearrangements (Steitz & Steitz, 1993), we have shown here that the mechanisms of the two steps differ with respect to the function of the nonbridging oxygens at the two splice sites. Figure 4 diagrams the active sites for the two steps of group II splicing. Both the pro-Rp and pro-Sp oxygens of the scissile phosphodiester bond at the 5' splice site in Figure 4A are shown interacting with the ribozyme active site. The two interactions are not equivalent, however. Sulfur substitution of the pro-Rp oxygen blocks detectable splicing, whereas substitution of the pro-Sp oxygen substantially reduces the rate of the reaction but does not block it completely. Although divalent metal ions are prime candidates for the components of the active site that interact with these oxygens, there is no direct evidence for this so far. However, the case for catalytically active metal ions in group II self-splicing has been strengthened by recent work on the effect of group substitutions in the catalytically essential domain 5 substructure (Konforti et al., 1998).

For the step two active site diagramed in Figure 4B, only the pro-Rp oxygen is shown interacting with the ribozyme. Sulfur substitution of this oxygen at the 3' splice site blocks detectable splicing. However, as we have shown here, there is no evidence for a role of the pro-Sp oxygen in the step two reaction.

These mechanistic differences suggest that there are concomitant structural differences in the active sites for steps one and two. Whether these differences are accommodated by the existence of two distinct physical active sites on the ribozyme or by conformational rearrangements of a single bifunctional active site such that components are shared between the two reactions remains to be determined. For example, Figure 4 shows the pro-Rp oxygens for the two steps interacting with different sites on the ribozyme. An alternative model can also be constructed in which both pro-Rp oxygens interact with the same site in a bifunctional active site. In this case, however, the positions of the other equatorial substituents must be different in the two steps.

It is worth noting that experiments in the spliceosomal splicing system using sulfur substitution of 3' bridging oxygens at the splice sites suggest that the two steps of this splicing reaction may differ in their interactions between metal ions and the leaving groups (Sontheimer et al., 1997). Extension of this work to the group II splicing system and of our work to the spliceosome will lead to further insights into the mechanisms of both splicing systems and relationships between them.

MATERIALS AND METHODS

Construction of modified RNAs

The control and 5' splice site phosphorothioate-substituted complete self-splicing RNAs were constructed as described (Padgett et al., 1994) from a transcribed exon 1 fragment and a transcribed intron–exon 2 fragment joined by a chemically synthesized RNA oligonucleotide containing the modifications. The three RNAs were combined with a bridging DNA oligonucleotide and ligated together using T4 DNA ligase (Moore & Sharp, 1992). The all-ribo control and Sp phosphorothioate oligos were described previously (Padgett et al., 1994). The oligos with a single deoxyribose residue or a single deoxyribose plus the Sp phosphorothioate substitution were purchased from Cruachem. The phosphorothioate diastereomers were separated as described (Slim & Gait, 1991; Maschhoff & Padgett, 1993). The control and 3' splice site phosphorothioate-substituted intron–exon 2 RNAs were similarly constructed from two RNAs joined by DNA-mediated ligation. The intron portion was synthesized by T7 RNA polymerase starting with the first nucleotide of the intron and ending two nucleotides before the 3' splice site using a PCR-generated template DNA essentially as described (Padgett et al., 1994). To this was ligated a 13-nt long RNA oligonucleotide labeled at the 3' end and containing either a phosphodiester or Sp phosphorothioate at the 3' splice site as described (Padgett et al., 1994). The E1 RNAs were chemically synthesized 18-nt long oligonucleotides (Cruachem) purified by gel electrophoresis. E1rC = ACGUGGUGGGACA UUUUC; E1dC = ACGUGGUGGGACA UUUUdC.

Step one splicing reactions

Reactions were performed in 40 mM buffer, 90 mM MgSO₄, 10 mM MgCl₂ or 10 mM MnCl₂, and 0.5 M (NH₄)₂SO₄ (Aldrich). The RNA was at a concentration of about 1 nM. Buffers used were MES-K (pH 5.0–6.9), MOPS-K (pH 6.5–7.3), HEPES-K (pH 6.5–7.5), and EPPS-K (pH 7–7.6). In all cases,

FIGURE 4. Model of the active sites for the first and second steps of group II intron self-splicing showing the trigonal bipyramidal transition states. Features of the model are described in the text. Base pairing between the intron binding site of E1 (IBS1) and exon binding site of the RNA (EBS1) is shown, as are known tertiary interactions involving bases near the splice sites (Michel & Ferat, 1995; Jacquier, 1996).

the pH was determined at the reaction temperature in the presence of all reaction components except RNA. Trial reactions were performed in different buffers at the same pH where the ranges overlap to control for specific effects of the buffers. None of the buffers listed above showed specific effects. Tris-HCl buffer, however, showed a specific rate reduction and so was not used in this analysis. The RNA was preincubated in the reaction buffer without Mg^{+2} or Mn^{+2} at 42 for 10 min. The reactions were started by addition of Mg^{+2} or $Mg^{+2} + Mn^{+2}$. The reactions were quenched by dilution into gel loading solution containing 80% formamide. The RNAs were separated by denaturing PAGE and quantitated on a PhosphorImager. The reactions showed biphasic behavior, with 40–60% reacting in the fast phase, and proceeded almost exclusively via the branching pathway with negligible production of linear intron. Both these features agree with the results of others (Jarrell et al., 1988; Daniels et al., 1996). Final extents of reaction were greater than 90%, with the experimentally determined value used as a correction for the initial rate determination. First-order rate constants were determined from the initial fast phase of the reaction using the Ultrafit program (Biosoft). The variation in derived rate constants was no more than 30% between experiments performed on different days or between different RNA preparations and much less than 30% within the same experiment.

Step two splicing reactions

Reaction conditions were the same as for the step one reactions. The 3' end labeled intron–exon 2 RNA was at a concentration of 20 nM, whereas the E1rC or E1dC RNAs were used at a concentration of 20 μ M. Previous studies have shown that the K_d for E1 RNA is less than 100 nM (Chin & Pyle, 1995). Saturation of the reactions with E1rC or E1dC RNAs was confirmed by varying the concentration between 1 and 30 μ M at several pH values. The reaction rates were calculated by quantitation of labeled product (E1E2 plus free E2). Only small amounts of free E2 were generated in the E1dC reactions and its exclusion from the rate calculations had minimal effect. The pseudo first-order plots were linear for at least three half lives, but usually only the initial 10–20% of the reaction was used for calculations. The end points were 94% for O and 87% for Sp, and these values were used for correction of the reaction rates. We previously showed that the correct 3' splice site was used by the Sp phosphorothioate-substituted intron (Padgett et al., 1994) and, in these experiments, a single band of E1E2 (28 nt) was produced that comigrated with the product of the control RNA under conditions where single-nucleotide differences are readily detectable.

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