The stereochemical course of the first step of pre-mRNA splicing

Kathryn L. Maschhoff and Richard A. Padgett*

Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, Dallas, TX 75235, USA

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ABSTRACT

We have determined the effects on splicing of sulfur substitution of the non-bridging oxygens in the phosphodiester bond at the 5' splice site of a pre-mRNA intron. Pre-mRNAs containing stereochemically pure Rp and Sp phosphorothioate isomers were produced by ligation of a chemically synthesized modified RNA oligonucleotide to enzymatically synthesized RAs. When these modified pre-mRNA substrates were tested for *in vitro* splicing activity in a HeLa cell nuclear extract system, the RNA with the Rp diastereomeric phosphorothioate was not spliced while the Sp diastereomeric RNA spliced readily. The sulfur-containing branched trinucleotide was purified from the splicing reaction of the Sp RNA and analyzed by cleavage with a stereospecific nuclease. The results showed that the Sp phosphorothioate was inverted during the splicing reaction to the Rp configuration; a finding previously obtained for a Group I self-splicing RNA. This inversion of configuration is consistent with a transesterification mechanism for pre-mRNA splicing. The lack of splicing of the Rp modified RNA also suggests that the pro-Rp oxygen at the 5' splice site is involved in a critical chemical contact in the splicing mechanism. Additionally, we have found that the HeLa cell RNA debranching enzyme is inactive on branches containing an Rp phosphorothioate.

INTRODUCTION

In the process of pre-mRNA splicing, the RNA undergoes two cleavage and ligation reactions leading to the production of the ligated exons and the lariat intron (1, 2). This pathway was determined several years ago along with the origin and fate of each of the cleaved and ligated phosphate groups in the reaction (1–3). The results of these studies showed that there was a conservation of phosphodiester bond energy and of phosphate groups such that there was no obvious requirement for exogenous energy or phosphates to complete the reaction. Since this is also true of the self-splicing Group I and Group II introns, it is a widely held suspicion that pre-mRNA splicing is, at bottom, a

reaction involving two transesterification steps catalyzed by an active site(s) composed of snRNA and pre-mRNA (see for example (4)). Since many *trans* acting proteins are required for pre-mRNA splicing, it follows that these factors function to bring the pre-mRNA and snRNAs into the correct conformation for the transesterification reactions to take place.

While the above argument is attractive, there is no direct evidence that the reactions in pre-mRNA splicing are transesterifications. The inability to detect cleavage in the absence of ligation or covalent intermediates in either step of splicing argues for a transesterification reaction mechanism but this cannot be considered proof. One piece of information that has not been previously available is the stereochemical course of the cleavage/ligation reactions in pre-mRNA splicing. In the case of self-splicing Group I introns, the demonstration that the cleavage/ligation reaction proceeds through inversion of the stereochemical configuration around phosphorus gives strong support to the transesterification model (5, 6).

An alternative possible mechanism for pre-mRNA splicing is that cleavage and ligation are separate, enzymatically catalyzed reactions as is seen in pre-tRNA splicing (7). The stereochemical consequence of this mechanism is most likely to be retention of configuration via two inversions. A reaction mechanism involving the formation of a covalent intermediate would also be likely to lead to retention of configuration. Thus, the determination of the stereochemical course of the pre-mRNA splicing reaction could provide evidence for one of these models of the cleavage/ligation reactions.

The substitution of a non-bridging oxygen in a phosphodiester bond with a sulfur (a phosphorothioate) produces a chiral center at the phosphorus atom with either an Sp or an Rp configuration. This substitution is tolerated by a wide variety of enzymes and has allowed the stereochemical course of many reactions involving nucleotides to be determined (8). The finding that the self-splicing Group I Tetrahymena ribozyme splices with inversion of configuration suggests that this splicing reaction proceeds via an 'in line' Sn2 mechanism involving nucleophilic attack on phosphorus by a ribose hydroxyl group (5, 6). Here we describe a similar analysis of the stereochemical course of the first step in the pre-mRNA splicing reaction.

* To whom correspondence should be addressed

MATERIALS AND METHODS

Oligonucleotides

5' splice site RNA oligonucleotide: AGP(S)GUAAGUAC

Bridging DNA oligonucleotide: ACCAAGCTTGTATTCGTA-CTCACCTCTGGCCCTCGCA

RNA oligonucleotide synthesis

RNA oligonucleotides were synthesized and deprotected as described (9) on an Applied Biosystems Model 391 DNA Synthesizer using RNA phosphoramidites obtained from Glen Research Corp. After removal of the silyl protecting groups, the oligonucleotides were desalted by passage through an OPC cartridge (Applied Biosystems) in 0.1 M triethylammonium acetate followed by a wash with water and elution with 150 μ l of 50% acetonitrile. The oligonucleotides were dried down and resuspended in 100 μ l H₂O.

For the addition of sulfur to form the site specific phosphorothioate, the phosphoramidite addition cycle was interrupted just prior to the iodine oxidation step, the column was removed from the synthesizer and treated by syringe addition with tetraethylthiuram disulphide (Aldrich, 0.5 g in 5 ml acetonitrile) for 1 hour at room temp. and washed with 10 ml acetonitrile. The column was reattached to the machine and synthesis was continued (9).

The synthetic oligonucleotides were purified first by HPLC on a Partisil 10 SAX ion exchange column (250 $\times$ 4.6 mm, Phenomenex) using a gradient of buffer A: 1 mM KPO₄ buffer pH 6.3, 40% acetonitrile and buffer B: 300 mM KPO₄ buffer pH 6.3, 40% acetonitrile (10% B 5', 10–50% B 10', 50–100% B 15'). This separated full length oligonucleotides from failure products and separated the thio substituted oligonucleotide from unsubstituted oligonucleotide (9). The two phosphorothioate diastereomers were separated by reverse phase HPLC on a Bondclone 10 C18 column (300 mm $\times$ 3.9 mm, Phenomenex) using a gradient of buffer A: 0.2 M ammonium acetate and buffer B: 20% buffer A/80% acetonitrile (0% B 5', 0–20% B 30'). The thio substituted oligonucleotide gave two approximately equal major peaks (Figure 2A). The peaks were collected, dried down, resuspended in 0.5 M ammonium acetate and ethanol precipitated.

Preparation of pre-mRNA splicing substrates

The pre-mRNA splicing substrate was based on the first two exons and intron of 6 the adenovirus-2 major late leader RNA as modified in the pRSP-2 and pRSP-7 plasmids (10). Two RNA transcripts and the synthetic RNA oligonucleotide were ligated together as follows. The upstream RNA containing the first exon sequences was transcribed from the pRSP-2 template cut with Pvu II using SP6 RNA polymerase in the presence of α -³²P ATP and GpppG to produce capped, labeled RNA. The downstream RNA containing a truncated form of the intron and the second exon sequences was transcribed from the pRSP-7 template cut with Sca I using SP6 RNA polymerase in the presence of α -³²P ATP, 1 mM GMP and 0.1 mM GTP to produce labeled RNA with a 5' monophosphate terminus or omitting the GMP and treating the transcript with calf intestinal alkaline phosphatase (Boehringer Mannheim) followed by kinasing with T4 polynucleotide kinase and ATP. The synthetic oligonucleotides were kinased using T4 polynucleotide kinase and ATP. The two transcripts and the RNA oligonucleotide were mixed with the bridging DNA oligonucleotide and ligated together using T4 DNA ligase for 4 hours at 22°C as described (11). The

ligation products were separated on a 10% polyacrylamide, 8.3 M urea, TBE gel and the full length RNAs were excised and eluted.

Splicing reactions and analysis of products

The ligated RNAs were spliced in standard HeLa cell nuclear extract *in vitro* splicing reactions (50 μ l) for 2–3 hours at 30°C (12). The RNAs were extracted from the reaction and digested with 1 μ g nuclease P1 in 50 mM ammonium acetate pH 4.7 for 3 hours at 37°C. The nuclease digests were spotted onto a DEAE cellulose thin layer chromatography plate and developed in 0.3 M ammonium formate, 9 M urea, 1 mM EDTA (3). The branch core trinucleotides with a charge of –4 were eluted with 2 M triethanolamine bicarbonate.

The purified branch core trinucleotides were then digested with 1 μ g snake venom phosphodiesterase (Boehringer Mannheim) in 50 mM Tris pH 7.6 with 10 μ g tRNA for 1 hour at 37°C and the products separated by DEAE cellulose thin layer chromatography in 0.2 M ammonium formate, 9 M urea, 1 mM EDTA. For iodoethanol treatment, the branch core trinucleotides were heated to 95°C for 3 min in 81% formamide, 7% iodoethanol (Sigma), 10 mM EDTA, 0.1% bromophenol blue (13) and separated on a 25% polyacrylamide (19:1 acrylamide:bis), TBE gel. The gel did not contain urea as this suppresses the separation of the modified and unmodified trinucleotides.

Debranching reactions

RNA from *in vitro* splicing reactions was isolated and fractionated on a 10% polyacrylamide, 8.3 M urea, TBE gel and the lariat intron product bands were cut out and eluted. Purified lariat RNAs were treated with HeLa cell S-100 extract in 25 mM HEPES buffer pH 8.0, 12.5 mM EDTA, 5 mM DTT and 1 U/ μ l RNase inhibitor (Ambion) for 1 hour at 30°C. The RNA was isolated from the reactions and analyzed on a 10% polyacrylamide, 8.3 M urea, TBE gel.

RESULTS

In order to investigate the stereochemical fate of a phosphorothioate linkage at the pre-mRNA splice sites, it is first necessary to demonstrate that such a substitution is compatible with splicing. We have previously shown that the Rp diastereomer inserted by the action of RNA polymerase at either the 5' or 3' splice site cannot participate to any detectable degree in pre-mRNA splicing (12). Due to the stereospecificity of RNA polymerases, which will only polymerize Sp nucleoside triphosphates to yield Rp linkages in the product RNA, it was impossible to test the Sp diastereomer by enzymatic synthesis. Therefore, the approach shown in Figure 1 was used to produce RNA molecules with a single substitution at the 5' splice site in either of the two configurations. The RNA was constructed in three parts. RNA 1 (Figure 1) is an SP6 RNA polymerase transcript initiated with a 5' cap dinucleotide (GpppG) and run off just prior to the 5' splice site. RNA 3 is also an SP6 RNA polymerase transcript initiated within the intron with GMP and run off within the second exon. RNA 2 is a ten nucleotide long chemically synthesized RNA which spans the 5' splice site junction. The phosphodiester bond at the 5' splice junction in oligo 2 was modified during synthesis by addition of a sulfur atom to create a phosphorothioate linkage. The two diastereomers of this oligo were separated by reverse phase HPLC (Figure 2A).

As a control, an RNA oligonucleotide of identical sequence but with a normal phosphodiester bond was also synthesized and used in parallel with the phosphorothioate-modified oligonucleotides. RNAs 1, 2 and 3 were ligated together using DNA ligase after hybridization of the three RNAs to a bridging DNA oligonucleotide (Figure 2B) (11). Both RNAs 1 and 3 were labeled by transcription in the presence of α - 32 P ATP.

Previous results have shown that the order of elution of phosphorothioate-modified nucleotides from reverse phase HPLC is the Rp diastereomer eluting first and the Sp diastereomer eluting second (9). To confirm this order, the two oligos were separately digested with either nuclease PI or snake venom phosphodiesterase (SVPD), treated with alkaline phosphatase and kinased with γ - 32 P ATP. Nuclease PI and SVPD are stereospecific for cleavage of phosphorothioates; nuclease PI cleaves Sp linkages (14) while SVPD cleaves Rp linkages (15). The products were then analyzed by two-dimensional thin layer chromatography. The early eluting RNA produced the four normal mononucleosides when digested with SVPD but produced an additional, slower migrating species (presumed to be pGp(S)G) after digestion with nuclease PI (data not shown). The later eluting RNA gave the 9 converse results. Thus, these results confirm

Figure 1. Overall scheme for the determination of the stereochemical course of splicing. The thick lines represent exon sequences and the thin lines represent intron sequences. RNAs 1 and 3 are SP6 RNA polymerase transcripts. RNA 2 is a chemically synthesized RNA oligonucleotide containing either a normal phosphodiester bond at the 5' splice site (as a control) or a phosphorothioate in either the Sp or Rp diastereomeric forms. The three RNA molecules are prepared separately as described in the text and ligated together using T4 DNA ligase in the presence of a DNA oligonucleotide that spans the junction all three RNAs. The resulting full length pre-mRNA is spliced *in vitro*. The spliced RNA is then digested with nuclease PI to yield a labeled branch core trinucleotide containing the phosphorothioate linkage. This trinucleotide is then digested with the stereospecific snake venom phosphodiesterase which will give either a labeled dinucleotide or a labeled mononucleotide depending on the configuration of the phosphorothioate as shown.

the stereochemical assignments of the two separated oligonucleotides.

Figure 3 shows a time course of splicing of control and phosphorothioate-containing RNA transcripts in a standard HeLa nuclear extract *in vitro* splicing reaction. Both the unmodified and the Sp phosphorothioate-containing RNAs were spliced with comparable efficiency. There was no significant difference in the initial kinetics of product formation (Figures 3B and C). In fact, the Sp phosphorothioate-containing RNA accumulated more excised lariat intron than the unmodified RNA. This is probably due to the resistance of the sulfur-containing branch structure to cleavage by the HeLa debranching enzyme (see below). In contrast, the RNA with the Rp diastereomer in the phosphorothioate linkage was not efficiently spliced. This result is in agreement with our previous findings (12) and further supports our assignment of configuration to the oligonucleotides. The small amount of excised lariat intron seen in the Rp reaction could be due to either a small contamination of the Rp oligonucleotide with Sp oligonucleotide or to a low level of desulfurization of the RNA.

To determine the stereochemical course of splicing at the phosphorothioate substituted position, the product containing this bond must be recovered and analyzed. The phosphate group at the 5' splice site becomes the linking phosphate in the 2'-5' phosphodiester bond in the branch structure (3). To characterize this bond, the RNA from a splicing reaction using either a control or an Sp phosphorothioate RNA substrate was digested with nuclease PI to generate 5' monophosphate nucleotides and the branch core trinucleotide (see Figure 1) (3). Since the RNA was labeled with α - 32 P ATP, the branch core trinucleotide has a single labeled phosphate 5' to the A residue. This branch core was purified by thin layer chromatography under conditions where the digestion products are separated on the basis of charge (Figure 4) (1). Also included is a nuclease PI digestion of spliced unmodified RNA for reference. The presence of a sulfur atom

Figure 2. Purification and ligation of the chemically synthesized modified RNA oligonucleotide. (A) Reverse phase HPLC separation of the crude oligonucleotide. The two major peaks correspond to the two diastereomeric configurations of the phosphorothioate. Further digestions demonstrate that the earlier eluting peak contains the Rp diastereomer and the later eluting peak contains the Sp diastereomer. (B) Example of the analysis of a ligation reaction by denaturing polyacrylamide gel electrophoresis. RNAs are numbered as in figure 1.

in the branch core structure has almost no effect on migration in this system. The branch core with a charge of -4 separates well from the mononucleotides with a charge of -2 . To confirm that this material represents the branch core trinucleotide, samples were separated by two dimensional TLC. Both forms migrated at a position diagnostic of a branched trinucleotide of the correct composition (3, 16) (data not shown).

To demonstrate that the branch core trinucleotide from the Sp phosphorothioate splicing reaction still retained a sulfur atom, branch trinucleotides from both the control and phosphorothioate splicing reactions were separated on a 25% polyacrylamide gel. Figure 5 shows that the two trinucleotides migrate differently in this gel system. To demonstrate that this difference in migration was due to the sulfur atom, the trinucleotides were treated with iodoethanol. This reaction leads to alkylation of the sulfur and hydrolysis of the phosphorus-sulfur bond with occasional cleavage of the phosphodiester bond (13). Thus, iodoethanol treatment produces almost complete conversion of the sulfur-containing trinucleotide to the oxygen-containing trinucleotide as expected.

The -4 branch core trinucleotide from Figure 4 was then digested with snake venom phosphodiesterase (SVPD) to determine the stereochemistry of the phosphorothioate linkage. As mentioned previously, SVPD is stereospecific in its cleavage of phosphorothioates. It is much more active on Rp linkages than on Sp linkages. This specificity has been shown for both 3'-5' and 2'-5' phosphorothioate linkages (15, 17-19).

Figure 6, lane 3 shows a charge separation analysis of a SVPD digestion of the control nuclease P1 branch core trinucleotide which gives the expected labeled pA product migrating at the -2 position. Digestion of the phosphorothioate-containing nuclease P1 branch core trinucleotide would be expected to give either a -3 charged pAp(S)G dimer if the linkage were Sp or a -2 charged pA monomer if the linkage were Rp. Figure 6, lane 5 shows that SVPD digestion of the phosphorothioate-containing nuclease P1 branch structure produced the pA monomer with a -2 charge. This shows that the linkage in the branch structure was Rp and hence the splicing reaction inverted the configuration of the active Sp isomer. In control experiments, a SVPD digestion of a sample of the starting Sp phosphorothioate-containing oligonucleotide which had 70 times less RNA than in the experiment shown in Figure 6 produced the expected SVPD-resistant pGp(S)G product (data not shown). This shows that the SVPD digestion conditions used in Figure 6 were well below those necessary to cleave Sp phosphorothioate bonds.

To test the idea noted above that the Rp phosphorothioate-containing intron lariat RNA accumulated to a greater extent than

Figure 3. Time course of the *in vitro* splicing reaction containing control, Rp or Sp phosphorothioate pre-mRNAs. (A) The ligated RNAs were incubated in a HeLa nuclear extract splicing reaction for 0, 15, 30, 60 and 120 minutes, isolated and analyzed by denaturing polyacrylamide gel electrophoresis. The positions of the intermediates and products of the splicing reaction are shown at the right. The first exon portion of the RNA in this experiment was labeled at 2% of the specific activity of the intron-second exon portion so that the first exon intermediate in the splicing reaction is not seen. (B) Quantitation of the time course of spliced lariat intron accumulation using a Molecular Dynamics PhosphorImager. Open circles—unmodified RNA; closed circles—Sp phosphorothioate modified RNA. (C) Quantitation of the time course of spliced exon product accumulation. Symbols as in B.

the control intron lariat because it could not be debranched, control and Rp phosphorothioate lariat introns were isolated from splicing reactions and incubated in HeLa cell S-100 extract under conditions that promote debranching (20, 21). Figure 7 shows that the control but not the Rp phosphorothioate lariat intron RNA was efficiently debranched. Thus, the HeLa cell RNA debranching enzyme is another example of an enzyme sensitive to the substitution of oxygen by sulfur in its substrate.

DISCUSSION

The results described here, in addition to our previous work (12), establishes three facts about the chemical mechanism of pre-mRNA splicing: 1) Neither of the two cleavage/ligation reactions can proceed with a phosphorothioate linkage in the Rp configuration. 2) The first cleavage/ligation reaction can proceed with an Sp configuration at the 5' splice junction. 3) The first

cleavage/ligation reaction proceeds via inversion of configuration. These facts lead to several interpretations. First, the inversion of configuration demonstrated here supports a transesterification mechanism involving an 'in line' Sn2 nucleophilic attack on phosphorus by the 2' hydroxyl group of the branch site adenosine as diagrammed in Figure 8. This reaction would proceed through a trigonal bipyramidal transition state with the attacking and leaving groups in the axial positions followed by conversion to products with inversion of configuration. This mechanism has also been proposed to explain the inversion of configuration seen in reactions using the Tetrahymena Group I intron ribozyme (5, 6) and the self-cleavage of 'hammerhead' (9, 22) and 'hairpin' (22) ribozymes. While this result for pre-mRNA splicing is consistent with the possibility that the chemical reactions are RNA catalyzed, it is also consistent with the operation of a protein catalyst since many enzymes that catalyze phospho-transfer reactions also invert the configuration (8). The inversion result

Figure 4. Isolation of the labeled nuclease PI resistant branch core trinucleotide from *in vitro* splicing reactions containing either unmodified (Control) or Sp phosphorothioate modified (Thio) pre-mRNA. The splicing reactions were carried out in the presence or absence of ATP as indicated, digested with nuclease PI and separated on the basis of net negative charge by DEAE cellulose thin layer chromatography. The 5' phosphate mononucleotides migrate at the -2 position while the branch trinucleotides migrate at the -4 position as indicated. The markers are labeled pre-mRNA digested with RNase A.

Figure 5. Analysis of control and sulfur-containing branch core trinucleotides. Control (O) or sulfur-containing (S) branch trinucleotides were run on a 25% polyacrylamide gel with or without treatment with iodoethanol as indicated.

Figure 6. Stereochemical analysis of the branch core splicing product. Control (lanes 2 and 3) or sulfur-containing (lanes 4 and 5) branch core trinucleotides were separated on the basis of net negative charge by DEAE cellulose thin layer chromatography without (lanes 2 and 4) or with (lanes 3 and 5) digestion with snake venom phosphodiesterase. The markers are labeled pre-mRNA digested with RNase A.

Figure 7. Debranching of isolated lariat intron RNAs by treatment with HeLa cell S-100 extract. Isolated control or phosphorothioate substituted lariat RNA were treated with the indicated amount of S-100 extract and analyzed on a 10% polyacrylamide gel. Lanes marked C contain untreated RNAs. The positions of the lariat and linear forms of the RNA are indicated.

Figure 8. Diagram of a possible reaction mechanism for the first step of pre-mRNA splicing. The 2' hydroxyl of the branch site adenosine is shown attacking the phosphate at the 5' splice site with a sulfur in the Sp position. A hypothetical trigonal bipyramidal transition state is shown for a concerted transesterification mechanism with the attacking and leaving groups in the apical positions. The product is shown with the phosphorothioate in the inverted Rp configuration.

does argue against a class of two-step mechanisms involving sequential cleavage and ligation with or without a covalent intermediate. Such a mechanism would likely give two inversions leading to a net retention of configuration in the cleavage/ligation reaction. Other data that argue against such a mechanism are the conservation of phosphorus atoms in the splicing reaction and the fact that an uncoupling of cleavage and ligation has never been observed for either splicing step.

The selectivity of the splicing reaction for only one of the phosphorothioate diastereomers is striking. This type of selectivity is often seen in both enzymatic and RNA catalyzed reactions (8, 9, 23). While the replacement of oxygen with sulfur is a relatively

minor change in the structure of a phosphodiester bond, it can reduce reaction rates by more than three orders of magnitude. In some cases the effects are due to differences in the metal binding properties of sulfur and oxygen. Substitution of metal ions, for example Mn^{++} for Mg^{++} , can sometimes restore activity to substituted substrates (24, 25). Our preliminary experiments with adding Mn^{++} to the splicing reaction have not resulted in the recovery of splicing using Rp substituted pre-mRNA.

In the case of pre-mRNA splicing, the block due to Rp phosphorothioate substitution at the 5' splice site appears to be at a step following the formation of the spliceosome, but prior to the chemical cleavage step. In our previous enzymatic substitution experiments, even complete substitution of the pre-mRNA with GTP α S which produces Rp phosphorothioate linkages in the RNA, reduced the formation of the spliceosome complex B by only 50% while splicing was undetectable (12). In addition, no evidence of cleaved but unligated RNA species was found either in our previous experiments or those reported here. Thus, it is likely that the block to splicing is due to interference with the chemical step and strongly suggests that the pro-Rp oxygen of the phosphodiester bond is involved in the reaction. Likely interactions are metal ion coordination (26) or hydrogen bonding to functional groups on proteins or RNA.

A similar argument cannot be made for the pro-Sp oxygen based on the data shown here. Although there is no difference in the rates of splicing of the phosphorothioate substituted pre-mRNA versus unsubstituted control pre-mRNA, there is no evidence that the chemical step is rate limiting in this reaction. Thus the phosphorothioate substitution could cause a very large decrease in the rate of the chemical step and yet have no apparent effect on the rate of the overall reaction (27). This is especially true given the leisurely pace of the pre-mRNA splicing reaction *in vitro*. Therefore, the data shown here can neither establish nor exclude a role for the pro-Sp oxygen in the chemical step of splicing.

These data and our earlier findings (12) suggest that the second step of pre-mRNA splicing is not a simple reversal of the first step. Such a mechanism would require that the stereochemical requirements in the substrate RNA should be inverted in the two steps. However, the Sp over Rp preference in the first step is not accompanied by a preference for Rp in the second step. A possible model is that, following the first step of splicing, the active site is reconfigured to bind the 3' splice site in a geometry similar to that of the 5' splice site in the first step and the 3' hydroxyl of the first exon is oriented in a manner similar to the 2' hydroxyl of the branch site in the first step. This would explain the inactivity of the Rp phosphorothioate at the 3' splice site and predict that the Sp diastereomer would be active and would splice with inversion of configuration. Indeed, work from another group (28) has confirmed our findings for the first step of splicing and has shown that the Sp diastereomer at the 3' splice site is active and is inverted during the second step of splicing. These and other data have been used to suggest that the two steps of pre-mRNA splicing are catalyzed by two distinct, but possibly overlapping, active sites (29).

Self-splicing of Group II introns also proceeds through a two step pathway with branching to an adenosine close to the 3' splice site (30). Since this must be an entirely RNA catalyzed reaction, a similar study of the stereochemistry of Group II self-splicing introns would be quite interesting.

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REFERENCES

1. Padgett, R. A., Konarska, M. M., Grabowski, P. J., Hardy, S. F. and Sharp, P. A. (1984) *Science* **225**, 898–903.
2. Ruskin, B., Krainer, A. R., Maniatis, T. and Green, M. R. (1984) *Cell* **38**, 317–331.
3. Konarska, M. M., Grabowski, P. J., Padgett, R. A. and Sharp, P. A. (1985) *Nature* **313**, 552–557.
4. Guthrie, C. (1991) *Science* **253**, 157–163.
5. McSwiggen, J. A. and Cech, T. R. (1989) *Science* **244**, 679–683.
6. Rajagopal, J., Doudna, J. A. and Szostak, J. W. (1989) *Science* **244**, 692–694.
7. Zillmann, M., Gorovsky, M. A. and Phizicky, E. M. (1991) *Mol. Cell. Biol.* **11**, 54105416.
8. Eckstein, F. (1985) *Ann. Rev. Biochem.* **54**, 367–402.
9. Slim, G. and Gait, M. J. (1991) *Nucl. Acids Res.* **19**, 1183–1188.
10. Wang, X. and Padgett, R. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7795–7799.
11. Moore, M. J. and Sharp, P. A. (1992) *Science* **256**, 992–997.
12. Maschhoff, K. L. and Padgett, R. A. (1992) *Nucl. Acids Res.* **20**, 1949–1957.
13. Gish, G. and Eckstein, F. (1988) *Science* **240**, 1520–1523.
14. Potter, B. V. L., Connolly, B. A. and Eckstein, F. (1983) *Biochemistry* **22**, 13691377.
15. Burgers, P. M. and Eckstein, F. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4798–4800.
16. Peebles, C. L., Belcher, S. M., Zhang, M., Dietrich, R. C. and Perlman, P. S. (1993) *J. Biol. Chem.* **268**, 11929–11938.
17. Nelson, P. S., Bach, C. T. and Verheyden, J. P. H. (1984) *J. Org. Chem.* **49**, 2314–2317.
18. Griffiths, A. D., Potter, B. V. L. and Eperon, I. C. (1987) *Nucl. Acids Res.* **15**, 41454162.
19. Suhadolnik, R. J., Lee, C., Kariko, K. and Li, S. W. (1987) *Biochemistry* **26**, 7143–7149.
20. Ruskin, B. and Green, M. R. (1985) *Science* **229**, 135–229.
21. Arenas, J. and Hurwitz, J. (1987) *J. Biol. Chem.* **262**, 4274–4279.
22. van Tol, H., Buzayan, J. M., Feldstein, P. A., Eckstein, F. and Bruening, G. (1990) *Nucl. Acids Res.* **18**, 1971–1975.
23. Suh, E. and Waring, R. B. (1992) *Nucl. Acids Res.* **20**, 6303–6309.
24. Dahm, S. C. and Uhlenbeck, O. C. (1991) *Biochemistry* **30**, 9464–9469.
25. Piccirilli, J. A., Vyle, J. S., Caruthers, M. H. and Cech, T. R. (1993) *Nature* **361**, 85–88.
26. Steitz, T. A. and Steitz, J. A. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6498–6502.
27. Herschlag, D., Piccirilli, J. A. and Cech, T. R. (1991) *Biochemistry* **30**, 4844–4854.
28. Moore, M. J. and Sharp, P. A. (1993) *Nature* **365**, 364–368.
29. Moore, M. J., Query, C. C. and Sharp, P. A. (1993) In Gesteland, R. F. and Atkins, J.F. (eds.), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 303–357.
30. Perlman, P. S., Peebles, C. L. and Daniels, C. (1990) In Stone, E. M. and Schwartz, R. J. (eds.), *Intervening sequences in evolution and development*, Oxford University Press, New York, pp. 112–161.