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Phosphorothioate substitution identifies phosphate groups important for pre-mRNA splicing

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ABSTRACT

Substitution of pre-mRNA in vitro splicing substrates with α-phosphorothioate ribonucleotide analogs has multiple effects on the processes of spliceosome formation and splicing. A major effect of substitution is on the splicing cleavage/ligation reactions. Substitution at the 5′ splice junction blocks the first cleavage/ligation reaction while substitution at the 3′ splice junction blocks the second cleavage/ligation reaction. A second effect of phosphorothioate substitution is the inhibition of spliceosome formation. A substitution/interference assay was used to determine positions where substitution inhibits spliceosome formation or splicing. Substitution in the 3′ splice site polypyrimidine tract was found to inhibit spliceosome formation and splicing. This effect was enhanced with multiple substitutions in the region. No sites of substitution within the exons were found which affected spliceosome formation or splicing.

INTRODUCTION

The process of pre-mRNA splicing in eukaryotic cells requires the recognition of splice sites in the pre-mRNA by cellular factors to form an active spliceosome followed by the cleavage and ligation of the pre-mRNA to produce the mature spliced mRNA. A great deal of effort has gone into the evaluation of the sequences in the pre-mRNA that are required for various steps in the splicing reaction. These efforts can be broadly divided into two categories: 1) alterations of the sequence or chemical structure of the pre-mRNA to observe the effect on splicing and 2) detection of the sequences protected from chemical or enzymatic attack by the formation of splicing complexes. Included in the first approach are the analysis of mutant splice site sequences whether naturally occurring or produced in vitro as well as modification/interference experiments which probe for residues whose chemical modification is incompatible with one or another step in splicing (1, 2). The second approach includes RNase protection (3–6) and chemical footprinting investigations (7). These two types of experiments produce complementary information on the constraints placed on the sequence and structure of the pre-mRNA for it to be a substrate for splicing. The modification experiments show which residues are specifically recognized by the splicing apparatus or residues whose modification hinders these interactions. The protection experiments show which residues are contained within splicing specific structures. In general, both types of investigation have given similar results: the 5′ and 3′ splice site regions are required for splicing and spliceosome formation and are protected in the spliceosome.

A limitation of the modification approach is that the experiments have focused on changes in the nucleotide base produced either by mutation or chemical modification. The protection experiments, on the other hand, have focused on the accessibility of the phosphodiester backbone to the cleaving agent i.e. RNases (3–6) or hydroxyl radical (7). Here we present an investigation of modification of the phosphodiester backbone and its effect on splicing and spliceosome assembly using phosphorothioate substitution of the pre-mRNA substrate.

Phosphorothioate analogs of ribonucleoside triphosphates are incorporated by RNA polymerases into RNA. The location of substitution can be somewhat controlled by using only a single modified nucleoside triphosphate in the transcription reaction. The extent of modification can also be regulated by using mixtures of normal and α-phosphorothio nucleoside triphosphates.

Many enzymatic reactions involving RNA are affected by the presence of phosphorothioate linkages in the RNA substrate. Examples include cleavage by ribonucleases (8, 9) and ribozyme mediated cleavage (10) and splicing reactions (11, 12). It is likely that pre-mRNA splicing would not be an exception.

Two consequences of interference with the pre-mRNA splicing reaction by the presence of phosphorothioate in the pre-mRNA would likely be the inhibition of spliceosome formation or splicing and the exclusion of RNA molecules containing specific modified residues from the splicing pathway. The fact that phosphorothioate linkages are sensitive to cleavage by mild alkylating reagents (13) allows the identification of positions at which substitution inhibits splicing. Both types of analysis are presented below.

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MATERIALS AND METHODS

Preparation of RNA Substrates

The RNA substrates were transcribed using SP6 RNA polymerase (Ambion) from DNA templates containing a portion of the rabbit β-globin gene (pSPAL-5) or a portion of the Adenovirus 2 major late transcript leader region (pRSP-2) (7). Each of these DNAs contains two exons and one intron. The pSPAL-5 template was linearized with Sca I restriction endonuclease and the run-off transcript was 426 nucleotides long. The pRSP-2 template was linearized with Bgl I and the run-off transcript was 389 nucleotides long.

The transcription reactions for the phosphorothioate substituted RNA contained normal ribonucleoside triphosphates from Pharmacia-LKB and α-phosphorothioate triphosphates from Dupont/NEN. Conditions for preparing capped RNA transcripts were otherwise as described previously (14). RNA was labeled with either α-[^{32}P] GTP or α-[^{32}P] CTP (Dupont/NEN). Following transcription, the RNA was purified by denaturing polyacrylamide gel electrophoresis. The RNA was eluted from the gel in 500 mM ammonium acetate, 1% SDS, 1 mM EDTA and 5 mM DTT. Following ethanol precipitation, the RNA was resuspended and stored in 10 mM Tris pH 7.5, 1 mM EDTA and 5 mM DTT. DTT is added to prevent oxidation of the thiophosphate groups.

RNA labeled at the 3′ end was prepared by transcription of the pSPAL-5 template in the absence of labeled triphosphates. The transcription reaction was passed through a column of Sephadex G-50 to remove free nucleotides and the RNA was precipitated with ethanol. The RNA was labeled in a 15 μl reaction containing 50 mM HEPES pH 7.6, 20 mM $MgCl_2$, 10 μg BSA, 6 μM ATP, 3 mM DTT, 10% dimethylsulfoxide, 50 μCi of 3′, 5′ [5′^{32}P] cytidine bisphosphate (3000 Ci/mmol, Dupont/NEN) and 6 units of T4 RNA ligase (New England Biolabs). The labeling reaction was incubated for 16 hours at 4°C. The labeled RNA was then gel purified as above.

Splicing Reactions

In vitro splicing reactions contained 1.5 mM ATP, 5 mM creatine phosphate, 2.75 mM $MgCl_2$, 5×10^4 cpm of RNA and 50% HeLa cell nuclear extract prepared by the procedure of Dignam et al. (15). Splicing reactions were incubated at 30°C for 90 minutes and the RNA was extracted and analyzed on 8% polyacrylamide, 8.3 M urea gels. Autoradiographs were scanned and quantitated using a Molecular Dynamics Laser Scanning Densitometer.

Splicing Complex Reactions

Spliceosome assembly was analyzed as described by Konarska and Sharp (16). Splicing reactions as above were incubated for 30 minutes at 30°C and stopped by the addition of 1 μl of 50mg/ml heparin. The samples were analyzed on 4% nondenaturing polyacrylamide gels as described (16). Autoradiographs were scanned and quantitated as above.

Splicing Interference Assays

To analyse the effect of substitution at the 3′ splice junction, 1×10^8 cpm of 3′ end labeled pSPAL-5 RNA transcript substituted with 10% of CTPαS was spliced in a 500 μl reaction as above except that the incubation time was increased to 3 hours. The RNA products of the reaction were extracted and separated on a preparative 8% denaturing polyacrylamide gel. RNAs bands corresponding to the precursor RNA and the spliced product RNA were cut out and eluted. 1×10^4 cpm of each RNA in 5 μl H_2O was mixed with 4 μl of 10 mM EDTA, 7% iodoethanol and 88% formamide (13). The samples were incubated at 90°C for 5 minutes to allow alkylation and cleavage of the phosphorothioate linkages and then separated by electrophoresis on a high resolution 8% polyacrylamide, 8.3 M urea gel.

To analyse the effect of substitution at the 5′ splice junction, RNA was transcribed from the Bgl I cleaved pRSP-2 template in the presence of either α-[^{32}P] GTP or α-[^{35}S] GTPαS as above and purified by gel electrophoresis. The RNA was spliced in 500 μl reactions for 3 hours and the product RNAs were separated by denaturing gel electrophoresis. Purified lariat intermediate and lariat product RNAs were digested with 1 unit of RNase T1 in 20 μl of 10 mM Tris pH 7.5, 1 mM EDTA for 2 hours at 37°C. The RNase T1 oligonucleotides were separated on a denaturing 15% polyacrylamide gel and the 14 long oligonucleotide containing the branch site was isolated. The analogous 14 long RNase T1 oligonucleotide from unspliced RNA was also isolated. (The branched and unbranched forms of this oligonucleotide migrate differently in this gel system (17, 18).) These oligonucleotides were then digested with 1 μg RNase A in 10 μl 10 mM Tris pH 7.5, 1 mM EDTA for 1 hour at 37°C. The RNase A digests were spotted onto a DEAE thin layer chromatography plate and separated in 0.3 M ammonium formate, 9 M urea, 1 mM EDTA. The ^{32}P-labeled products were visualized by autoradiography and the ^{35}S-labeled products were visualized by spraying the plate with Enhance (Dupont/NEN) followed by autofluorography at −80°C.

To analyse the effect of substitution on spliceosome formation, RNA was transcribed from the Bgl I cleaved pSPAL-5 template in the presence of 10% of each of the four α-phosphorothioate triphosphates. This RNA was 3′ end labeled and assembled into spliceosomes as described above. After non-denaturing polyacrylamide gel separation, the RNA from the various complexes was eluted, cleaved with iodoethanol as above and analyzed on an 8% polyacrylamide 8.3M urea gel.

RESULTS AND DISCUSSION

Effects of phosphorothioate substitution on splicing and spliceosome formation

In order to determine if pre-mRNA splicing was affected by the presence of phosphorothioate linkages in the RNA substrate, an in vitro RNA splicing substrate derived from the rabbit β-globin second intron region was synthesized in the presence of various ratios of normal and α-phosphorothio ribonucleoside triphosphates (NTPαS). These RNAs were then incubated in a HeLa cell nuclear extract in vitro splicing system and the products were resolved by denaturing polyacrylamide gel electrophoresis. The efficiency of splicing was determined by comparing the amount of lariat intermediate and lariat intron product RNAs produced in each reaction. As shown in Figure 1A, substitution of pre-mRNA with phosphorothioate residues has a profound inhibitory effect on splicing. A 50% decrease in the amount of lariat intron product is seen when fewer than 10% of the residues have been substituted. The amount of lariat intermediate RNA species formed in these reactions shows that the first cleavage/ligation reaction in splicing is also inhibited but to a slightly lesser extent than the complete reaction. When similar splicing reactions were analyzed by non-denaturing gel electrophoresis to measure the formation of splicing complexes,

an inhibition was also apparent (Figure 1B). The formation of the pre-spliceosome complex A and the spliceosome complex B were both inhibited by phosphorothioate substitution. Figure 1C shows the quantitative effects of NTPαS substitution on the various steps of splicing and spliceosome formation. Complex B formation was reduced in parallel with lariat product formation while complex A formation was the least affected of the steps analyzed. These results show that all of the major steps of the splicing reaction are inhibited by substitution.

The steep slopes of the curves in Figure 1C suggest that pre-mRNA splicing and spliceosome formation are sensitive to substitution by phosphorothioates at several residues in the pre-mRNA. Model calculations suggest that the data for spliced product formation best fits a curve predicted for 6 critical substitution sites. However, this analysis assumes that the inhibition due to substitution at each site is complete and that the effect at each site is independent of all other sites. Neither assumption is likely to be completely true.

The effects of substitution on the various steps in the splicing reaction were also examined using substitution of only one of the four ribotriphosphates during transcription. If the inhibition of splicing observed in Figure 1 was due to a non-specific effect of the phosphorothioate modification, the use of individual nucleotides should have very similar effects. As shown in Figure 2A, however, substitution with ATPαS alone produces results distinct from those observed when all four nucleotides were substituted. Note that 100% substitution with ATPαS is equivalent to about 25% total substitution. ATPαS substitution has almost no effect on the formation of complex A while complex B levels are reduced by about 50% with total substitution. Similarly, the amount of spliced product is reduced by little more than half at 100% substitution. The amount of lariat intermediate is unaffected or slightly elevated by ATPαS substitution. These results show that substitution of any single A residue or all A residues is compatible with splicing and spliceosome formation.

A somewhat different result is obtained when GTPαS substitution is examined (Figure 2B). Here, while the formation of the spliceosome complexes A and B are relatively unaffected, major effects are seen on the cleavage/ligation reactions which produce the lariat intermediate and lariat product RNAs. The first cleavage/ligation reaction appears to be a major point of interference since there is no accumulation of lariat intermediate RNA as occurs if the second step is preferentially inhibited (see below). The inhibition of the splicing reaction is fairly linear with substitution suggesting that the effect may be due to substitution at a single critical residue.

Another pattern is seen when CTP is substituted by CTPαS (Figure 2C). Unlike the result with ATPαS and GTPαS substitution, CTPαS substitution has a significant effect on

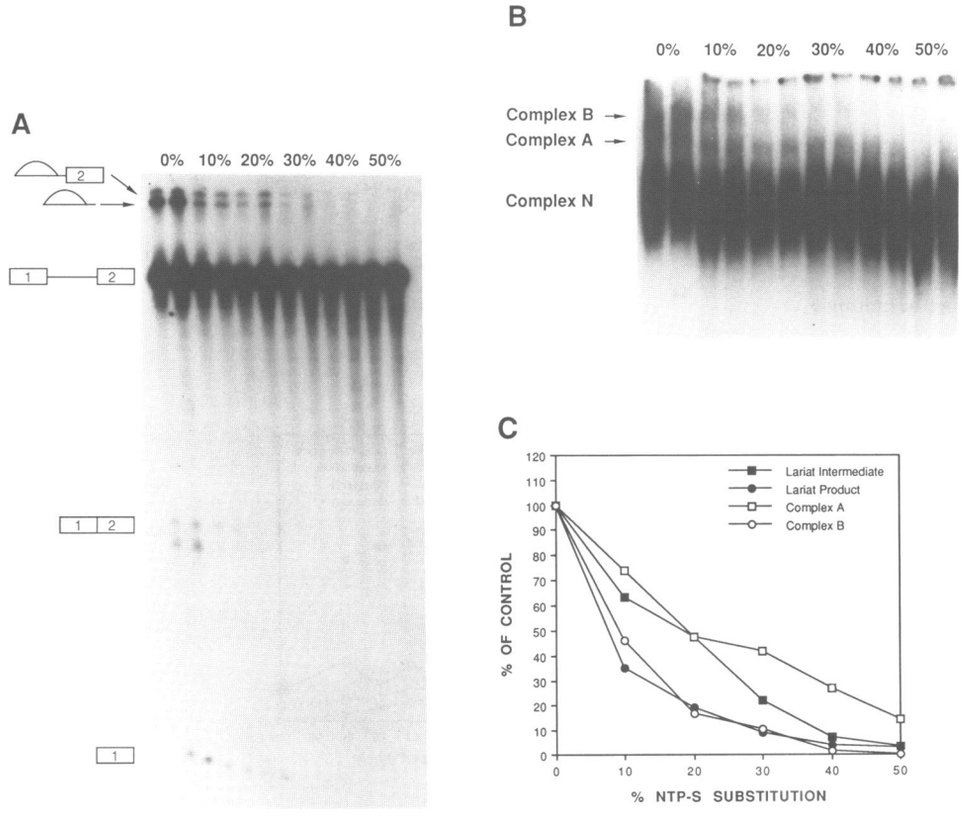


Figure 1. Effect of phosphorothioate substitution on splicing and spliceosome formation. RNA was transcribed from pSPAL-5 DNA in the presence of various levels of all four α-phosphorothioate ribonucleoside triphosphates and α-[^{32}P] GTP. **A.** The RNAs were spliced in duplicate reactions and analyzed by denaturing polyacrylamide gel electrophoresis. The positions of the precursor, splicing intermediates and products are indicated diagrammatically at left. **B.** The RNAs were assembled into splicing complexes in duplicate reactions and analyzed by non-denaturing polyacrylamide gel electrophoresis. The positions of the spliceosome complex B, the pre-spliceosome complex A and the heterogeneous complexes N are marked at left. **C.** Quantitation of the effects of substitution on the various steps analyzed in A and B expressed as a percentage of the value obtained using unsubstituted RNA.

spliceosome assembly. At 100% substitution with CTPαS, formation of both complexes A and B is reduced by 75%. Complex B appears to be more sensitive to substitution at intermediate levels of substitution. CTPαS substitution has a biphasic effect on the level of lariat intermediate which accumulates to 2.5 times the control level at 20% substitution and then decreases rapidly as substitution increases. The amount of spliced product decreases to an almost undetectable level by 70% substitution. This pattern of inhibition suggests that CTPαS substitution has two effects on splicing: inhibition of spliceosome assembly and blockage of the second cleavage/ligation reaction. At low levels of substitution, the effect on the cleavage/ligation reaction predominates and the lariat intermediate accumulates. At higher levels, the effect on spliceosome assembly leads to a decrease in the amount of lariat intermediate.

The effects on splicing and spliceosome assembly of substitution with UTPαS are shown in Figure 2D. The levels of complexes A and B are not greatly affected while both cleavage/ligation reactions are inhibited by high levels of UTPαS substitution. Lariat intermediate accumulates to about twice the control level at moderate substitution levels suggesting that the second cleavage/ligation reaction is affected preferentially at this level of substitution. The shape of the curve of lariat product formation in particular suggests the possibility that multiple substitutions are required to inhibit this step. An obvious candidate for the location of such substitutions is the polypyrimidine tract at the 3′ splice site. Evidence shown below demonstrates that multiple substitutions in this region inhibit spliceosome formation.

To investigate the generality of some of these effects of phosphorothioate substitution on splicing and spliceosome formation, a second pre-mRNA substrate was examined. This RNA was derived from the adenovirus 2 major late transcription unit and was transcribed in vitro in the presence of various levels of CTPαS or UTPαS. Figure 2E shows that CTPαS substitution leads to an accumulation of lariat intermediate RNA and a decrease in the amount of spliced product RNA. In contrast to the effect on the globin pre-mRNA, even at 100% CTPαS substitution, significant amounts of lariat intermediate RNA is produced. The pattern of complex A and B formation is similar in the two pre-mRNAs.

Figure 2E shows the results of UTPαS substitution of the adenovirus pre-mRNA. Again, the results are similar to those seen with the globin pre-mRNA. One difference is that the levels of complexes A and B are more depressed at high levels of substitution using the adenovirus pre-mRNA compared to the globin pre-mRNA. As noted above, the 3′ splice site

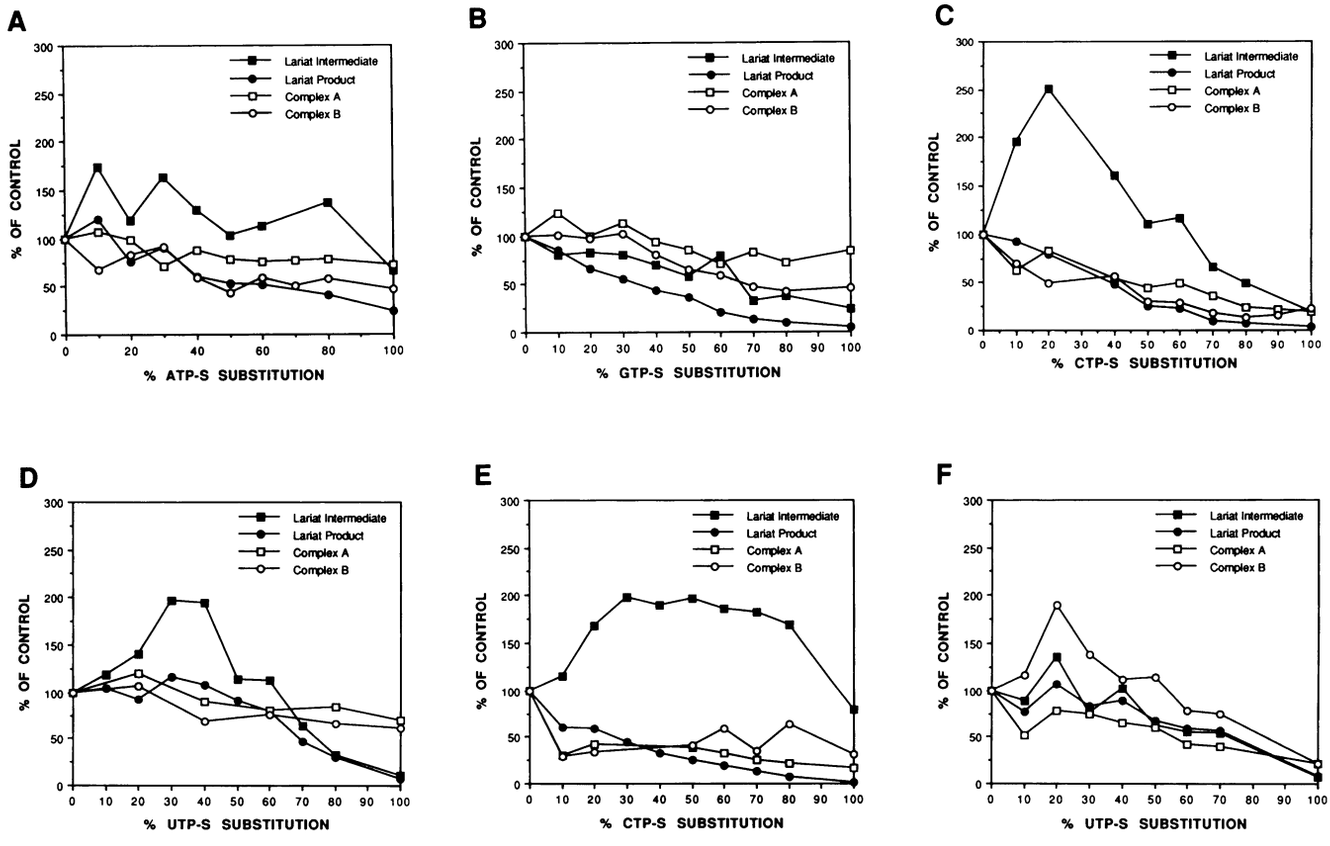


Figure 2. Effect of substitution of individual nucleotides with phosphorothioate analogs on splicing and spliceosome formation. RNA was transcribed from either the globin DNA template pSPAL-5 (panels A–D) or the adenovirus DNA template pRSP-2 (panels E and F) in the presence of various levels of a single α-phosphorothioate ribonucleoside triphosphate and α-[^{32}P] GTP. Splicing and spliceosome formation were assayed as in Figure 1 and the results were quantitated. The results are presented as the percentage of each product relative to the amount obtained using unsubstituted RNA. **A.** Globin RNA substituted with ATPαS. **B.** Globin RNA substituted with GTPαS. **C.** Globin RNA substituted with CTPαS. **D.** Globin RNA substituted with UTPαS. **E.** Adenovirus RNA substituted with CTPαS. **F.** Adenovirus RNA substituted with UTPαS. The splice site and branch site sequences of the adenovirus substrate are ... UGGG:GUGAGU ... UACUUAU ... UUCCACAG:CUC ... and the globin sequences are ... CAGG:GUGAGU ... UGCUAAC ... UCCUACAG:CUC ... where the colons denote the splice sites and the underlined A residues are the branch sites.

polypyrimidine tract is a prime candidate for the site of interference of CTPαS and UTPαS. A difference between the two pre-mRNAs tested is that the adenovirus polypyrimidine tract is more U rich and has a longer stretch of consecutive U residues than does the globin polypyrimidine tract.

Effect of a C to A mutation at the 3′ splice junction

In both pre-mRNAs tested, CTPαS substitution leads to a nearly linear decrease in the amount of lariat product RNA and an accumulation of lariat intermediate RNA. This suggests that substitution at a single C residue could block the second cleavage/ligation reaction. A likely site for this residue is at the 3′ splice junction where in both pre-mRNAs a C residue begins the second exon and so provides the phosphate used in the second step of splicing. Thus, CTPαS substitution would place a phosphorothioate in this critical bond. Similarly, GTPαS substitution would place a phosphorothioate in the 5′ splice site bond that is used in the first step of splicing. As shown above (Figure 2B), GTPαS substitution leads to an almost linear decrease in the amount of lariat product RNA without an accumulation of lariat intermediate RNA.

One way to test this possibility is to alter the pre-mRNA substrate such that a different residue supplies the phosphate at the 3′ splice junction. For this experiment, the wild type 3′ splice junction of AG:C was changed to AG:A in the globin pSPAL-5 template. This change was chosen because an A residue at the beginning of the downstream exon is frequently observed in splice sites (19) and the effect of ATP substitution in the wild type pre-mRNA is minimal at low to moderate levels of substitution (Figure 2A). Figure 3 shows that this single nucleotide change alters the pattern of lariat intermediate accumulation when the RNA is substituted with ATPαS or CTPαS. There is a dramatic increase in the amount of lariat intermediate RNA in the mutant pre-mRNA substituted with ATPαS (compare lanes 2 and 3 to lanes 7 and 8). There is also a reduction in the accumulation of lariat intermediate RNA in the mutant pre-mRNA substituted with CTPαS (compare lane 4 to lane 9).

These effects are shown quantitatively in Figure 4. The effects on the amount of spliced intron product parallel the effects on the lariat intermediate with CTPαS having a lesser effect in the mutant than in the wild type and ATPαS having a greater effect in the mutant than in the wild type. Thus, these data show that this one residue in the pre-mRNA is a major site of inhibition by phosphorothioates. At least one other C residue is sensitive to substitution since splicing is still inhibited in the C to A mutant.

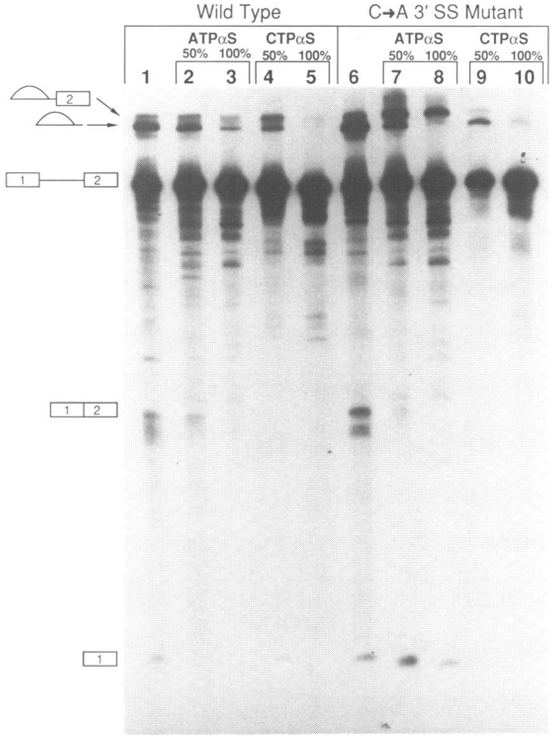


Figure 3. Effect of a C to A mutation at the 3′ splice junction on inhibition of splicing by ATPαS and CTPαS. RNA was transcribed in the presence of α-[^{32}P] GTP from either wild type pSPAL-5 DNA (lanes 1–5) or a mutant DNA containing a C to A change at the first nucleotide of the second exon (lanes 6–10). The transcription reactions contained in addition either no substituted triphosphate (lanes 1 and 6) or the indicated amounts of ATPαS (lanes 2, 3, 7 and 8) or CTPαS (lanes 4, 5, 9 and 10). The RNAs were spliced in identical reactions and analyzed by denaturing polyacrylamide gel electrophoresis. The positions of the precursor, splicing intermediate and splicing product RNAs are shown at left.

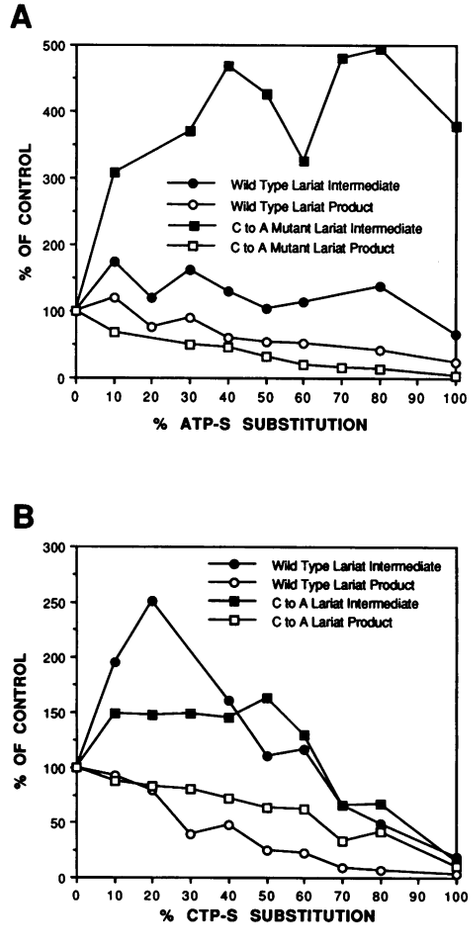


Figure 4. Comparison of the effects on splicing of ATPαS and CTPαS substitution between wild type and mutant pre-mRNAs. **A.** Quantitation of the effects of ATPαS substitution on lariat intermediate accumulation using either wild type or C to A mutant pSPAL-5 pre-mRNAs. **B.** Quantitation of the effects of CTPαS substitution on lariat intermediate accumulation using either wild type or C to A mutant pSPAL-5 pre-mRNAs.

Unlike the C at the 3′ splice site, it is not possible to alter the G at the 5′ splice site to carry out a similar analysis. It is quite likely, however, that the inhibition of splicing by GTPαS substitution is due in large part to this residue. Other experiments described below show this effect directly.

Substitution/interference analysis of splicing

Inhibition of cleavage at the 3′ splice junction. A useful feature of phosphorothioate substitution of phosphodiester bonds is that they are sensitive to alkylation under mild conditions which can lead to cleavage of the RNA at the site of substitution. Thus, phosphorothioate substituted RNA can be used for substitution/interference analyses similar to RNA modification/interference analyses (1, 2). In this analysis, substituted, end-labeled pre-mRNA is spliced in vitro and either the splicing products are separated from unspliced RNA or spliceosome complexes are separated from uncomplexed RNA. The RNA is then alkylated to effect cleavage and the cleavage patterns of the separated RNAs are compared to locate phosphates whose phosphorothioate substitution affects splicing or spliceosome formation. This procedure has been previously used to locate phosphates critical for the splicing of the Tetrahymena Group I rRNA intron (20) and for the self-cleavage of hammerhead type RNAs (10).

As noted above, it appears likely that phosphorothioate substitution of the phosphodiester bonds at either the 5′ or 3′ splice junctions blocks the cleavage/ligation reaction at that junction. To confirm this idea, substituted pre-mRNAs were spliced and the products analyzed to determine if a phosphorothioate can appear in the phosphodiester bonds formed during the splicing reaction. These experiments are performed using pre-mRNA substituted at a low (10−20%) level where the efficiency of splicing is not greatly diminished.

To examine the effect of phosphorothioate substitution at the 3′ splice junction, pSPAL-5 globin pre-mRNA was substituted with CTPαS at 10%, labeled at the 3′ end, spliced in vitro and the spliced exon product RNA was isolated. Since the phosphate used in the phosphodiester bond that links the two exons in the spliced exon product RNA is derived from the phosphodiester bond at the 3′ splice junction (17), the substitution state of this bond will show whether a phosphorothioate at the 3′ splice junction can be used in the second cleavage/ligation reaction of splicing. Figure 5 shows the result of cleaving the CTPαS substituted spliced exon product RNA by alkylation with iodoethanol (lane 2). This RNA is compared to the unspliced pre-mRNA (lane 1) and to a similarly substituted transcript of a globin cDNA (lane 3). As indicated by the asterisk, the C residue at the junction of the two exons is not cleaved whereas all other C residues are cleaved indicating that those pre-mRNA molecules which contained a phosphorothioate substitution at this C residue did not participate in the splicing reaction. This experiment provides further evidence that a phosphorothioate at the 3′ splice junction is incompatible with splicing. The analogous experiment using the pre-mRNA substrate with the C to A mutation substituted with ATPαS also showed that a phosphorothioate bond at the 3′ splice site was not used in splicing (data not shown).

In the experiment in Figure 5, the only substituted C residue in the spliced exon product RNA which interfered with splicing was at the 3′ splice junction. To identify other positions at which substitution is incompatible with splicing, spliced exon product RNAs substituted with ATPαS, GTPαS or UTPαS and labeled at either the 5′ or 3′ end were cleaved and compared to unspliced RNA. These experiments failed to detect any other positions in the spliced exon product where substitution of individual nucleotides was incompatible with splicing (data not shown).

Inhibition of cleavage at the 5′ splice junction. To examine the effect of phosphorothioate substitution at the 5′ splice junction, a somewhat different approach was employed. The phosphate at the 5′ splice junction becomes the phosphate in the 2′-5′ phosphodiester bond in the branch structure formed in the first cleavage/ligation reaction of splicing (18). Thus, analysis of the intron lariat is necessary to assay this phosphate.

For this experiment, the pre-mRNA was labeled with [α^{35}S] GTPαS (1.8% substitution), spliced in vitro and the intron lariat RNA was isolated. RNA similarly labeled with [α^{32}P] GTP was also spliced as a control. The RNase T1 oligonucleotide containing the branch structure was purified from the intron lariat RNA and further digested with RNase A. As shown in Figure 6A, RNase A digestion of the ^{32}P-labeled branch-containing oligonucleotide yields a labeled branch trinucleotide core structure and a labeled Up from the 3′ end of the oligonucleotide. The branch core structure has a nominal charge

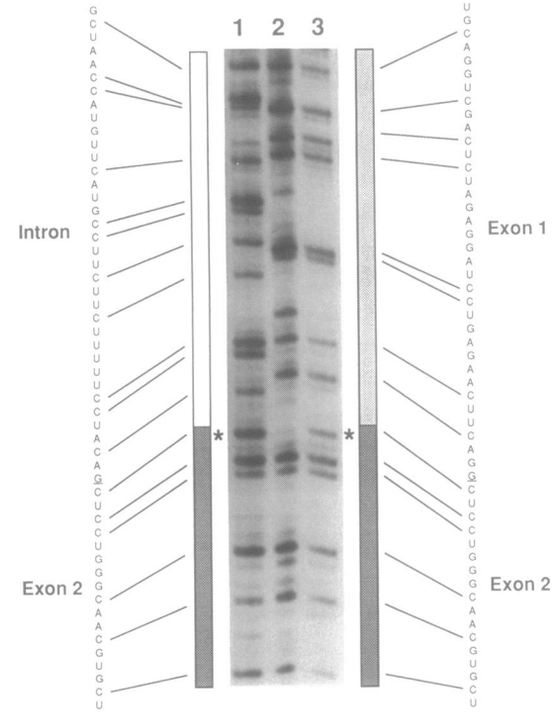
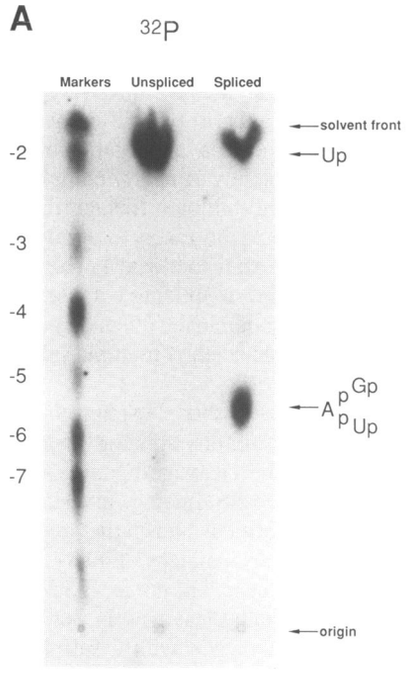


Figure 5. Substitution/interference analysis of spliced product RNA substituted with CTPαS. RNA was transcribed from pSPAL-5 DNA in the presence of 10% CTPαS and labeled with ^{32}P at the 3′ end. This RNA was spliced and the spliced exon product RNA was isolated and cleaved at the substituted positions by iodoethanol. The cleavage products were analyzed by denaturing polyacrylamide gel electrophoresis. Lane 1 contains unspliced substituted pre-mRNA cleaved with iodoethanol. Lane 2 contains spliced substituted exon product RNA cleaved with iodoethanol. Lane 3 contains substituted RNA transcribed from a cDNA clone of the exons contained in pSPAL-5 cleaved with iodoethanol. The position of the C residue at the splice junction is marked with an asterisk. Some additional, artifactual cleavages at A residues are also seen in lane 2 that are not seen in lanes 1 and 3.



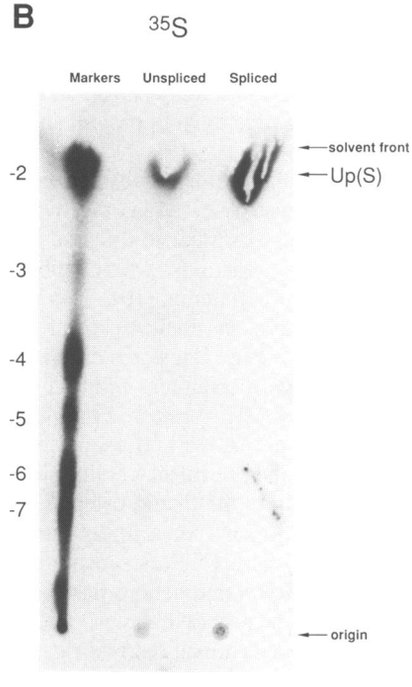


Figure 6. Substitution/interference analysis of the 2′-5′ phosphodiester bond at the branch site. RNA was transcribed from pRSP-2 DNA in the presence of either α-[^{32}P] GTP or α-[^{35}S]-GTPαS and spliced in vitro. The branched and unbranched RNase T1 oligonucleotides spanning the branch site were isolated, digested with RNase A and the products separated by charge on a DEAE thin layer chromatography plate. **A.** Analysis of the ^{32}P labeled oligonucleotide digested with RNase A. Lane 1, ^{32}P-labeled charge standards; lane 2, unbranched RNase T1 oligonucleotide; lane 3, branched RNase T1 oligonucleotide. **B.** Analysis of the ^{35}S labeled oligonucleotide digested with RNase A. Lane 1, ^{35}S-labeled charge standards; lane 2, unbranched RNase T1 oligonucleotide; lane 3, branched RNase T1 oligonucleotide. The partial quenching of the Up(S) signal is due to UV-absorbing impurities that concentrate at the solvent front during chromatography. No impurities were seen in the position of the branch core structure.

of −6 which can be easily separated from the Up mononucleotide of charge −2 by DEAE thin layer chromatography. Similarly, RNase A digestion of the ^{35}S-labeled substituted branch-containing oligonucleotide will give a labeled −2 charged Up(S) from the 3′ end of the RNase T1 oligonucleotide. However, the presence or absence of a −6 charged branch core structure will be diagnostic for the ability of a phosphorothioate at the 5′ splice junction to participate in the reaction. As can be seen in Figure 6B, no −6 charged material is present in the RNase A digest of the ^{35}S-labeled branched oligonucleotide. Since the branched and unbranched forms of this RNase T1 oligonucleotide migrate differently in the preparative separation (17), the RNA analyzed in this experiment was branched. The absence of ^{35}S label in the branch core shows that a phosphorothioate substitution at the 5′ splice junction is incompatible with the first cleavage/ligation reaction.

The results of the above experiments show that both cleavage/ligation steps in the pathway of pre-mRNA splicing cannot proceed when the bond to be broken contains a phosphorothioate group. It should be noted, however, that the phosphorothioate structure is chiral and that only the Rp diastereomer can be tested in these types of experiments. This is because RNA polymerase can only use the Sp diastereomer

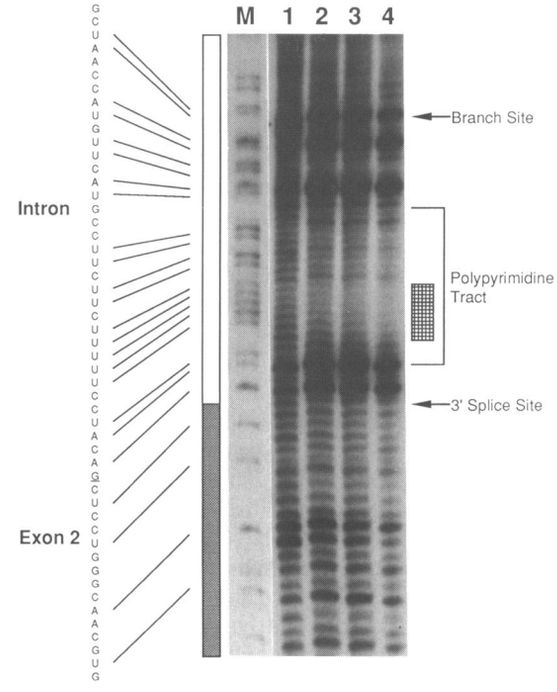


Figure 7. Substitution/interference analysis of spliceosome formation in the 3′ splice site region. RNA was transcribed from pSPAL-5 in the presence of 20% of all four α-phosphorothioate ribonucleoside triphosphates and labeled at the 3′ end. The RNA was then assembled into splicing complexes which were resolved by non-denaturing polyacrylamide gel electrophoresis into non-specific complexes (N), pre-spliceosome complex A and spliceosome complex B. RNA was isolated from these complexes, cleaved at sites of phosphorothioate modification by treatment with iodoethanol and analyzed by denaturing polyacrylamide gel electrophoresis. M, markers produced by iodoethanol cleavage of UTPαS substituted RNA; lane 1, unreacted pre-mRNA; lane 2, RNA from Complex N; lane 3, RNA from Complex A; lane 4, RNA from Complex B. Positions of the 3′ splice site, branch site and polypyrimidine tract are shown. The region of substitution/interference is indicated by the crosshatched box.

in RNA synthesis during which the configuration is inverted to Rp in the product RNA. This stereoselectivity is frequently seen in both enzymatic (8) and RNA catalyzed reactions (21). Similarly, the inversion of stereochemical configuration by the polymerase is a common feature of reactions involving phosphorus (8).

Substitution/interference analysis of spliceosome formation

As shown above, the formation of spliceosome complexes is also inhibited by phosphorothioate substitution. In particular, UTPαS and CTPαS appear to exert some or most of their effects at the level of spliceosome assembly. As noted above, a likely region of the pre-mRNA where these effects may be felt is in the polypyrimidine tract at the 3′ splice site. To investigate this possibility, a substitution/interference approach was used. Pre-mRNA substituted with 20% of all four NTPαS nucleotides was 3′ end labeled and incubated in vitro to form splicing specific complexes. The A, B and N complexes were excised from the non-denaturing gel and the RNA extracted, alkylated to cleave at sites of substitution and analyzed by denaturing polyacrylamide gel electrophoresis. As shown in Figure 7, the extent of cleavage in a subregion of the polypyrimidine tract is reduced in complexes B and A and to a lesser extent in complex N. The interference is centered on a U_5 sequence within the larger polypyrimidine tract. This is the same region of this 3′ splice site that is most protected from hydroxyl radical cleavage in splicing complexes (7). Substitution in two other protected regions detected by hydroxyl radical footprinting (at the 3′ splice junction and between the branch site and the polypyrimidine tract) does not appear to affect complex formation.

The polypyrimidine tract at the 3′ splice site has been implicated in the binding of several proteins to the pre-mRNA (22−27). Phosphorothioate substitution of RNA has been shown to affect RNA-protein interactions (28) where individual substitutions can decrease or increase the affinity of protein for RNA. The results of the present substitution/interference analysis show that substitution of a small region of the polypyrimidine tract inhibits splicing complex formation, probably due to interference with the binding of a required splicing factor to this region.

The data in Figure 2 suggested that the effect of UTPαS substitution might be cooperative such that multiple substitutions in a single RNA molecule were required to inhibit the splicing of that RNA. However, this effect is seen with the lariat intermediate and product while the amount of complex B was not greatly reduced even at 100% substitution with UTPαS. Thus the interference might not be expected to appear at the level of spliceosome formation. It should be noted, however, that the splicing complexes are transient intermediates in the splicing pathway and thus the levels of complexes observed at a single time point do not necessarily reflect the rate of flux through the pathway. It is possible that UTPαS substitution in the polypyrimidine tract slows both the entry of RNA into the spliceosome assembly pathway and also the subsequent splicing of the RNA. The exclusion of substituted residues in the polypyrimidine tract of RNA assembled into splicing complexes is consistent with this idea.

Substituted pre-mRNA labeled at the 5′ end and assembled into complexes A and B was also analyzed in a similar manner to the 3′ end labeled RNA shown above. This analysis showed no sites of interference in the upstream exon, the 5′ splice site region or the first 30 nucleotides of the intron (data not shown).

CONCLUSIONS

We have shown that substitution of phosphodiester bonds with phosphorothioate bonds in the pre-mRNA splicing substrate affects the process of splicing in two different ways. The most dramatic effect is that the cleavage/ligation reactions of pre-mRNA splicing are completely or nearly completely blocked by the presence of a phosphorothioate linkage in the bond to be broken. (This result should be taken to apply only to the Rp configuration of the phosphorothioate.) The other effect of substitution is an inhibition of splicing complex formation (and thus of splicing) by substitution in the 3′ splice site polypyrimidine tract. This inhibition is enhanced by multiple substitutions within this region.

Other workers have shown that phosphorothioate substitution in pre-mRNA blocks or inhibits splicing in vitro (29). These workers only analyzed the cleavage/ligation steps and not the spliceosome formation steps in splicing. In addition, they did not attempt to determine the critical sites in the pre-mRNA at which substitution affects the various steps. Finally, they reported a splicing-related cleavage near, but not at, the 3′ splice junction. We have not observed such cleavages in our experiments.

The substitution of an oxygen with a sulfur atom in a non-bridging position of a phosphodiester bond is a relatively minor chemical change. The geometry, bond length and atomic sizes are all similar. The major differences appear to be an altered charge distribution with more negative charge being localized on the sulfur and a change in the affinities of different counterions. Nevertheless, these differences lead to alterations in protein binding to substituted residues and changes in the sensitivity of substituted bonds to cleavage by both protein and RNA enzymes (8, 10, 28).

The effects of phosphorothioate substitution on pre-mRNA splicing are similar to those observed in other systems. The block of the cleavage reactions is similar to the case for hammerhead ribozyme self-cleavage reactions where substitution blocks cleavage under standard conditions (10). This effect is thought to be due to differences in divalent metal ion binding as substitution of Mn^{++} for Mg^{++} restores cleavage of substituted hammerhead RNAs (30). Neither addition of Mn^{++} nor substitution of Mn^{++} for Mg^{++} restored pre-mRNA splicing of substituted RNA (data not shown). It should be noted that the effect of substitution at the pre-mRNA splice sites appears to be at the cleavage step and not the ligation step as no RNAs of the sizes predicted for free exon 1 or a linear intron-second exon intermediate were seen with GTPαS substitution.

Inhibition of protein-RNA interactions has also been observed in other systems (28). The precise chemical interactions that are affected have not been determined but hydrogen bonding, ionic interactions and metal coordination could all be affected. The region of the globin 3′ splice site polypyrimidine tract where substitution blocks splicing complex formation has been previously shown to be protected from solvent in splicing complexes (7). This suggests that this region is tightly bound to splicing factors. The substitution data further suggest that some of the major contacts in this area are through the phosphate groups in the RNA. Other protected regions do not show a substitution/interference effect perhaps because the phosphate contacts are less important or because the chemistry of the contacts is not affected by phosphorothioate substitution. Until the identity of the factors binding to these regions has been determined, a detailed understanding of these interactions will not be possible.

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