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A phosphorothioate at the 3′ splice-site inhibits the second splicing step in a group I intron

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ABSTRACT

RNA polymerases can synthesize RNA containing phosphorothioate linkages in which a sulfur replaces one of the nonbridging oxygens. Only the Rp isomer is generated during transcription. A Rp phosphorothioate at the 5′ splice-site of the Tetrahymena group I intron does not inhibit splicing (McSwiggen,J.A. and Cech,T.R. (1989) *Science* 244, 679). Transcription of mutants in which the first base of the 3′ exon, U_{+1}, was mutated to C or G, in the presence, respectively, of either cytosine or guanosine thiotriphosphate, introduced a phosphorothioate at the 3′ splice-site. In both cases exon ligation was blocked. In the phosphorothioate substituted $U_{+1}G$ mutant, a new 3′ splice-site was selected one base downstream of the correct site; despite the fact that the correct site was selected with very high fidelity in unsubstituted RNA. In contrast, the exon ligation reaction was successfully performed in reverse using unsubstituted intron RNA and ligated exons containing an Rp phosphorothioate at the exon junction site. Chirality was reversed during transesterification as in 5′ splice-site cleavage (*vide supra*). This suggests that one non-bridging oxygen is particularly crucial for both splicing reactions.

INTRODUCTION

The role of a nonbridging oxygen in biochemical reactions involving phosphate diester substrates of both proteins and ribozymes can be investigated by replacing an oxygen atom with a sulfur atom (1). Introduction of a single sulfur group generates a chiral center at the phosphate with either the Rp or Sp configuration. T7 and SP6 RNA polymerases only incorporate the Sp nucleoside α-thiotriphosphate into RNA and, in so doing, generate a phosphorothioate diester with the Rp configuration (2).

Phosphorothioate substitution has been used to study pre-mRNA splicing (3) and an endonuclease reaction catalyzed by a group I intron derived ribozyme (4). Substitution of an Rp phosphorothioate at the cleavage site of the hammerhead ribozyme substantially reduces cleavage (5, 6, 7, 8, 9). The inhibition is suppressed by replacing Mg^{2+} with Mn^{2+}, suggesting that a divalent ion coordinates directly to the phosphate at the cleavage site (8). By contrast, substitution with the Sp isomer has little effect (9, 10). Substitution of an Rp phosphorothioate at the cleavage site of the hairpin ribozyme has a very minor effect (6, 11).

Group I intron splicing involves two transesterification reactions. The 3′ hydroxyl of a guanosine cofactor cleaves the 5′ splice-site (5′SS) and forms a phosphodiester bond with the first nucleoside of the intron. The 3′ hydroxyl of the 5′ exon then attacks the 3′ splice-site (3′SS) to generate the ligated exons and the excised intron (12). The excised intron from the rRNA of *Tetrahymena thermophila* can circularize as a result of a third transesterification reaction in which the end of the intron attacks the phosphodiester bond between either bases 15 and 16, or 19 and 20 (13). Incorporation of the Rp phosphorothioate into the 5′SS of the Tetrahymena intron does not block 5′SS cleavage (14, 15) and slightly slows the chemical step of an analogous endonuclease reaction (3). Transesterification results in inversion of configuration to the Sp isomer, consistent with an in-line reaction mechanism (15, 16).

It has been reported that the second step of splicing is also not inhibited by the incorporation of a Rp phosphorothioate (14). We have not been able to repeat this result, but can provide a likely explanation for the earlier report. We show that substituting sulfur for the oxygen corresponding to the proR position at the 3′SS significantly inhibits exon ligation. By setting up the reverse reaction, we also show that substitution of the other non-bridging oxygen is non inhibitory, indicating that one non-bridging oxygen plays a major role in transesterification.

MATERIALS AND METHODS

Plasmid construction

DNA fragments, containing the intron and flanking exons (17), (Figure 1), were shuttled as EcoRI HindIII fragments into RNA transcription vectors: either pSP65 (Promega Corp), which uses SP6 RNA polymerase, to give pSPTT14 (18), or pIBI24 (International Biotechnologies Inc.), which uses T7 RNA polymerase, to give pTT14 (19).

Initially, all transcripts for the bimolecular reactions were generated in the SP6 transcription vector system pSP65. A silent mutation in the L8 loop (Figure 1) creates an AatII site (20). This mutation, obtained from John Burke, was inserted into

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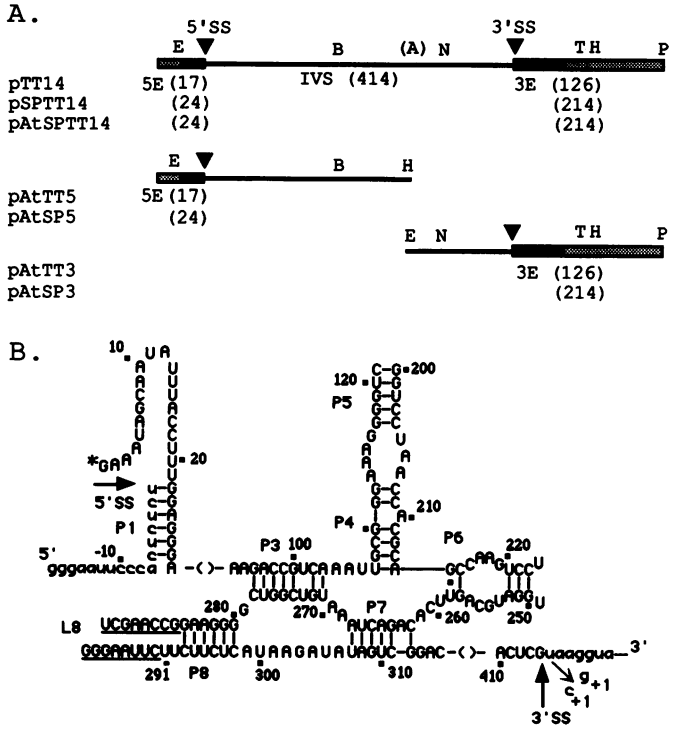

Figure 1. RNA transcription constructs and the bimolecular reaction. (A) Speckled and solid bars indicate vector and Tetrahymena exon sequence respectively. E = EcoRI, B = BglII, A = AatII, N = NheI, T = PstI, H = HindIII, P = PvuII. Arrowheads indicate splice-sites. pSPTT14, pAtSP5, pAtSP3 are based on the SP65 vector system and have 5′ and 3′ exons of 24 and 214 bases. pTT14, pAtTT5, pAtTT3 are based on the T7 system and have 5′ and 3′ exons of 17 and 126 bases. pAtSPTT14 is pSPTT14 with an AatII site. (B) The bimolecular reaction was achieved by splitting the RNA in L8. The T7 system is shown with exon sequence in lower case and vector sequence in the L8 region underlined. The 5′ exon is shown paired with its internal guide sequence, after GTP (G*) has cleaved the 5′SS, and prior to initiation of exon ligation. The location of the C_{+1} and G_{+1} mutations are indicated.

pSPTT14 to give pAtSPTT14, which was modified to produce plasmids used to provide template DNA for the 5′ (pAtSP5) and 3′ (pAtSP3) portions of the RNA precursor. pAtSP5 was generated by cleaving pAtSPTT14 with AatII and PstI to remove the 3′ region of the precursor; overhang sequences were recessed and the flush ends rejoined. pAtSP5 was cleaved with HindIII to generate a defined 3′ end for the 5′ RNA molecule. pAtSP3 was generated by cleaving pAtSPTT14 with EcoRI and AatII to remove the 5′ region of the precursor; the EcoRI 5′ overhang was filled in and the AatII 3′ overhang recessed using the Klenow fragment of DNA polymerase, and the flush ends rejoined (regenerating an EcoRI site). pAtSP3 was linearized with PvuII to generate a defined 3′ end for the 3′ RNA molecule. The 5′ and 3′ sequences were shuttled into the T7 transcription vector, pIBI24, as EcoRI HindIII fragments.

RNA transcription and splicing

Transcription plasmids were linearized with PvuII. Transcription reactions (20−100 μl) contained 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 100 μg/μl BSA, 1 u/μl SP6 (2 u/μl for T7) RNA polymerase, 1 u/μl RNasin, 50−75 μg/ml DNA, 0.02 mM (0.1 mM for T7 RNA pol.) GTP, 10 μCi ^{32}P-NTP, where N did not correspond to the NTPαS analogue, 0.4 mM remaining nucleoside triphosphates and 0.4 mM NTPαS. Incubation was at 37°C for 1 hour. The reactions were phenolized, ethanol precipitated and precursor RNA purified on 7 M urea, 5.5% polyacrylamide gels. RNA was located by autoradiography. Acrylamide strips were cut out, the RNA eluted overnight in 0.3 M Na-acetate, 20 mM tris pH 8.0, 2 mM EDTA at 4°C, and ethanol precipitated twice.

Standard splicing conditions were 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 0.2 mM GTP for 30 minutes at 30°C, unless otherwise stated. Bimolecular reactions were performed at 42°C for 1 hour with the 5′ and 3′ molecules at 20−50 nM and 50 nM respectively. Hydrolysis conditions were 50 mM Tris pH 9.0, 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, for 30 minutes at 37°C. Reactions were stopped by the addition of 1 volume of loading dye (95% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Splicing reactions were analyzed on 7 M urea, 5.5% polyacrylamide gels. All autoradiography for quantitative purposes was performed at room temperature without an intensifier screen. RNA was quantified using an UltroScan densitometer (Pharmacia).

Determination of splice-site choice in the G_{+1} mutant

Splicing reactions were loaded onto a preparative denaturing gel. Location of the ligated exon RNA was determined by autoradiography which was also used to determine the extent of the reaction. Ligated exons were purified, reverse transcribed and the cDNA amplified using PCR. The DNA was digested with EcoRI and HindIII, gel purified and mixed in a ligation reaction with EcoRI HindIII digested, phosphatase treated pIBI24 (or M13mp18) vector, the ligation mix being used to transform DH5α or JM103. Solid agar contained 0.1 mM IPTG and 2% X-gal to assay for β-galactosidase. Colonies were classified into dark blue, blue or white. The frequency of dark blue colonies having vector but no insert was less than 2%. To ensure blue colonies which correspond to insertion of correctly spliced ligated exons were not misclassified as vector containing dark blue colonies, and vice-versa, DNA from 10 non-white colonies were dot blotted onto nitrocellulose and probed with a oligonucleotide specific for the ligated exon sequence. Classification was also confirmed by sequencing DNA from a few colonies. The total number of blue colonies with correctly spliced ligated exons was inferred from the fraction present in the sample of 10 non-white colonies. If there were mostly white colonies, sequence analysis of DNA from 5−10 colonies indicated that the vast majority contained ligated exons generated by use of the G_{+1} site. In four control reactions in Table 1, white colonies were produced at less than 1% frequency. All were sequenced; all showed use of the correct 3′SS but had acquired an independent mutation (usually addition of CUCU at the end of the 5′ exon).

Purity of NTPαS compounds

It is likely that there is no significant preferential incorporation of an NTP compared to it's NTPαS analogue (2). To minimize background levels of the normal triphosphate nucleotide in transcription reactions, the following precautions were taken. DNA for template preparation was purified on CsCl gradients. All standard nucleotide triphosphates were of the highest purity available from the manufacturer (Pharmacia). An appropriate ^{32}P-NTP was used to uniformly label RNA. GTPαS, UTPαS, CTPαS were obtained from DuPont and were all reported to contain less than 0.1% of the NTP analogue. Concern has been expressed that the phosphorothioate can be oxidized back to the

Table 1. Selection of 3′SS in the G_{+1} mutant substituted with guanosine phosphorothioate

Expt[a]	Mg^{2+}/Mn^{2+} (mM)	Nucleoside	# colonies Blue	Freq. of White	% precursor that blues (%)	% precursor that reacted[b]	gave blues[b]
Uni-	1	25/−G	274	0[c]	>99	78	78
mol.		GαS	<10	875	<1.1	73<0.8	
	2	25/−G	324	0[c]	>99	75	75
	25/−	GαS	0	224	<0.4	91	<0.4
	22.5/2.5	GαS	<6	236	<2.5	77	<1.9
Bi-	1	25/−G	141	0[c]	>99	<1	<1
mol.	25/−	GαS	3	80	3.6	<1	<0.03
	22.5/2.5	G	177	0[c]	>99	<1	<1
	22.5/2.5	GαS	1	60	1.6	<1	<0.02
	2	25/−GαS	13	518	2.4	10	0.2

[a]Two independent unimolecular reaction were performed — The first and second bimolecular reactions were performed using the SP6 and T7 systems respectively.
[b]The percentage of precursor that reacted was determined from the yield of ligated exons; this value was multiplied by the percentage of blue colonies to obtain the percentage of the total precursor which reacted at the correct 3′SS.
[c]Six white colonies caused by independent mutations were excluded from this analysis.

standard phosphodiester and that NTPαS can be oxidized to NTP (15). Although we have not detected this problem, RNA was generally reacted immediately after transcription, and no significant difference was seen when the GTPαS was repurified afresh by HPLC (by DuPont).

Despite the above precautions, there will presumably be a very small fraction of RNA containing a standard phosphate at the 3′SS in substituted RNA. This background limited our ability to quantify rare utilization of the correct 3′SS. In some inefficient GαS G_{+1} reactions, blue colonies represented 2−4% of the reacted RNA but a tiny fraction when expressed as a percentage of the total starting material (last column of Table 1). Therefore these blues may not represent a low level of utilization of the phosphorothioate substituted 3′SS, but simply the background of molecules with an unsubstituted 3′SS.

Analysis of the products of the reverse reaction

A plasmid (pLE), containing the ligated exons without the intron from a pTT14 construct, was linearized at the HindIII site, transcribed with UTPαS replacing UTP, and the purified RNA 3′ endlabelled with ^{32}pCp. 50 nM ligated exon RNA was reacted with 20 nM unsubstituted, unlabelled intron RNA under standard splicing conditions at 30°C. RNA representing intron joined to 3′ exon (I3E) was purified and partially cleaved with either RNase T1 or iodoethanol (21).

RESULTS

Transcription of pSPTT14 linearized with PvuII gives rise to a precursor RNA containing 24 bases of 5′ exon, 413 bases of the Tetrahymena intron and 234 bases of 3′ exon. If the precursor is synthesized under conditions where one of the four nucleotides is completely replaced by its phosphorothioate counterpart (NαS), splicing is blocked if the nucleotide is adenosine or uridine, but not if it is guanosine or cytidine (14). Normally the first nucleotide of the 3′ exon is a uridine (U_{+1}). As introducing UαS elsewhere in the RNA blocks splicing, it is not possible to ask whether UαS at the 3′SS blocks exon ligation. Two mutations were made, G_{+1} and C_{+1}. These are almost silent, reducing the rate of splicing under standard conditions 2.0 and 2.2 fold respectively (data not shown).

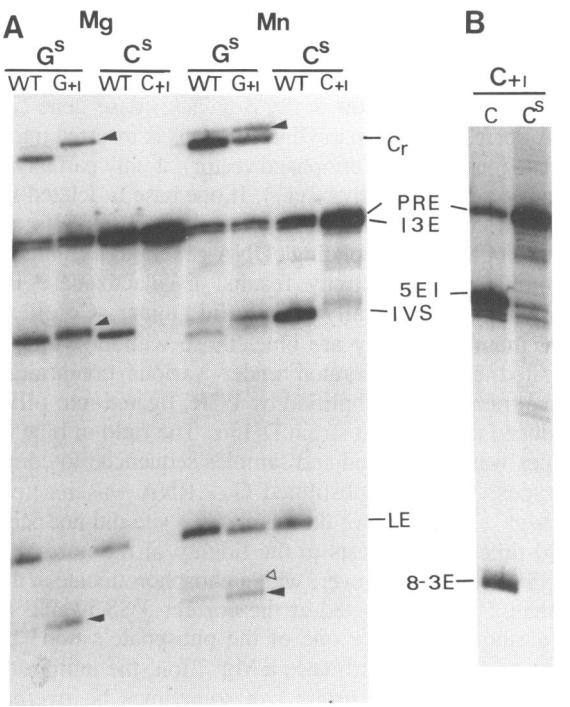

Figure 2. A phosphorothioate at the 3′SS blocks activity in the unimolecular substituted RNA. (**A**) Standard reaction conditions were used with either 25 mM Mg^{2+} (Mg) or 22.5 mM Mg^{2+}, 2.5 mM Mn^{2+} (Mn). G^S and C^S indicates that the precursor was uniformly substituted with GαS or CαS. Cr = circular IVS; PRE = precursor; I3E = intermediate of intron joined to 3′ exon; IVS = intron, LE = ligated exons. Open arrowhead = 3′ exon. Arrowheads show the products of reactions involving aborted I3E molecules in which a cryptic 5′SS at +6 in the 3′ exon (Fig. 1) is cleaved and the 'intron +6′ fragment cyclizes. (**B**) Reaction conditions were as in (A) with 25 mM Mg^{2+} plus the synthetic 5′ exon 8mer at 0.1 μM. Cleavage of the 3′SS by the 8mer produces the 5′ exon joined to the intron (5EI) and an analogue of the ligated exons (8-3E).

When wildtype precursor, transcribed in the presence of either CTPαS or GTPαS, was incubated under splicing conditions including 25 mM Mg^{2+}, both 5′SS cleavage and exon ligation were observed (Figure 2). The CαS substituted C_{+1} precursor underwent 5′SS cleavage, accumulating the intermediate of the intron attached to the 3′ exon (this molecule is only resolved in

light exposures of Figure 2 as it is only 17 bases shorter than the precursor). However, it only generated a trace (0.2%) of ligated exons; PCR analysis confirmed that the correct splice site had been used. The GαS substituted G_{+1} precursor underwent 5'SS cleavage and produced a small amount of ligated exons. To assess whether the correct 3'SS had been used, ligated exon RNA was purified, reverse transcribed and the resulting cDNA amplified by PCR and sequenced. The splicesite had shifted one nucleotide downstream (data not shown). Group I introns require a terminal G for the second splicing step. In the GαS substituted G_{+1} mutant, the phosphorothioate containing 3'SS is ignored; instead, the G_{+1} mutation is recognized as the end of the intron, with A_{+2} becoming the first base of the 3' exon.

Splice-site competition in the G_{+1} mutant

Sequence analysis of the G_{+1} GαS substituted ligated exons indicated that a new 3'SS was being utilized one base downstream, at what will be referred to as the +1 site. The following phenotypic assay was used to determine whether the normal 3'SS was being used at low frequency. pIBI24 is a plasmid which contains the α-peptide encoding portion of the β-galactosidase gene and complements an *E.coli* host strain carrying a NH_2 proximal deletion in the β-galactosidase gene (22). If a cDNA corresponding to the ligated exons is inserted into pIBI24 (or analogous M13 bacteriophage vector), it only partially reduces α-complementation activity (17). If one base is deleted from the ligated exons, as is the case when the +1 3'SS is selected, insertion of the corresponding cDNA generates a frame shift and no complementing activity results. β-galactosidase is easily assayed histochemically on solid agar. Colonies with complementing activity are blue, those without white.

Ligated exons, generated under various conditions, were reverse transcribed, amplified by PCR, ligated into pIBI24 and introduced into the host strain DH5α. The ratio of blue to white colonies was determined and samples sequenced to confirm the genotypes. When unsubstituted G_{+1} RNA was reacted in 25 mM Mg^{2+}, utilization of the cryptic +1 site did not occur once in 600 times. This attests to the fidelity of the normal process of 3'SS selection. However, with a phosphorothioate at the 3'SS, less than 1% now reacted at the correct 3'SS (Table I).

In a ribozyme where one of the phosphate's non−bridging oxygens normally coordinates a Mg^{2+} ion, the inhibitory effect of substituting a thio group can sometimes be overcome by replacing Mg^{2+} with Mn^{2+} (8). We therefore tested to see if the inclusion of Mn^{2+} would activate the thio-substituted 3'SS. Replacement of 25 mM $MgCl_2$ with 22.5 mM $MgCl_2$ and 2.5 mM $MnCl_2$ (a ratio between 10:1 and 5:1 is optimal in suppressing other internal thio substitutions) did not significantly stimulate exon ligation in either the CαS substituted C_{+1} mutant (Figure 2A) or the GαS substituted G_{+1} mutant (Table 1). The inclusion of Mn^{2+} increased the splicing activity of the phosphorothioate substituted RNA in general: at the 5'SS in the mutants and at both splicing steps in the wild-type (Figure 2A).

These results suggested that a Rp phosphorothioate at the 3'SS was very inactive. However it was possible (at least for the CαS reactions) that the catalytic step was being slowed so much that it allowed the 5' exon to dissociate from its binding site (Figure 1). before it could complete exon ligation. To test this, the reactions were performed in the presence of a synthetic 5' exon, an 8mer oligoribonucleotide (GGCUCUCU). The concentration of the 8mer was 0.1 μM, which is near saturation (23). While reaction of the CαS wild type control was extensive (> 80%),

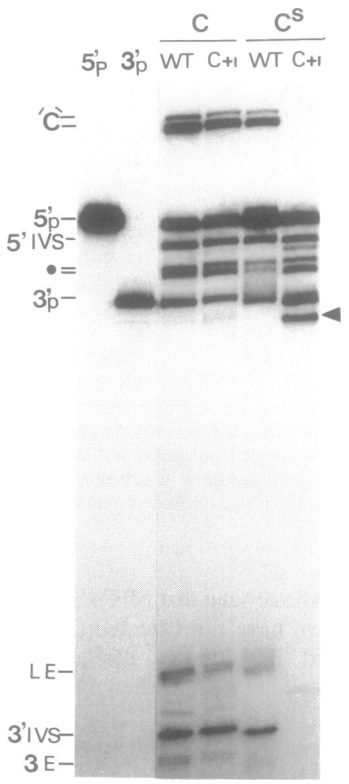

Figure 3. A phosphorothioate at the 3'SS inhibits exon ligation in a bimolecular reaction. Standard reaction conditions were used with 25 mM Mg^{2+}. The 5' RNA component of the bimolecular complex (5' P) was unsubstituted and was reacted with unsubstituted (C) or CαS substituted (C^S) wild-type or C_{+1} mutant 3' RNA (3' P) (Figure 1). 5' IVS = 5' RNA intron fragment after cleavage of the 5'SS; 3' IVS = 3' RNA intron fragment after cleavage of the 3'SS. LE = ligated exons and 3E = 3' exon (both smeared due to size heterogeneity during transcription run-off). 'C' = 'cyclized' IVS (actually linear in a bimolecular reaction) formed by the cyclization reactions (13). Arrowhead = a contaminating RNA from the C_{+1}, CαS 3' RNA preparation. • = 5' IVS minus 15 or 19 bases after 'cyclized' RNA has been reopened (13) or after direct attack of the cyclization sites by exogenous GTP (27). Note that phosphorothioate incorporation increases RNA mobility.

the CαS C_{+1} reaction produced only a very small amount of intron sized RNA, and no visible ligated exon RNA (Figure 2B).

Bimolecular reactions

Utilization of the correct but phosphorothioate substituted 3'SS could have been inhibited in the above reactions due to the presence of other phosphorothioates in the rest of the intron. To reduce the number of phosphorothioates, bimolecular reactions were set up in which the intron was split about three quarters of the way along in the L8 loop (Figure 1); the 5' part of the precursor RNA contained no phosphorothioate substitutions, while the 3' part was transcribed in the presence of GTPαS or CTPαS instead of GTP or CTP respectively.

The initial bimolecular reaction in the SP65 vector system was inefficient, even in control reactions in which the RNA was unsubstituted in both 5' and 3' molecules. Shuttling the DNA encoding the 5' and 3' fragments into pIBI24, a T7 RNA polymerase based vector (Figure 1), significantly improved the bimolecular reaction but made the ligated exons difficult to visualize because the precursor (or 3' exon) terminated at several positions. With GαS in the G_{+1} 3' fragment, the cryptic +1 site

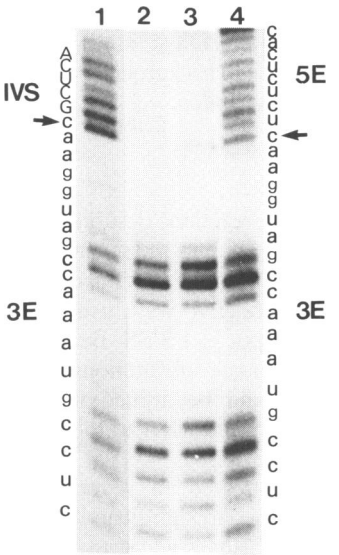

Figure 4. Bimolecular modification–interference assay of a phosphorothioate at the 3–SS. Unsubstituted unlabelled 5′ RNA from pAtTT5 (Figure 1) was reacted with 3′ RNA transcribed from pAtTT3 using a ratio of 10:1 CTP to CTPαS and then 3′ end-labelled with ^{32}pCp. Unreacted 3′ RNA (lane 1) and ligated exon RNA were purified and partially cleaved with iodoethanol (21). Control ligated exon RNA (lane 4) was prepared by transcribing a cDNA clone, pLEC$_{+1}$ (pLE with the C$_{+1}$ mutation), using CTPαS, and then 3′ end–labelling. Lanes 2 and 3 = ligated exon RNA from splicing reactions in 25 mM $MgCl_2$ and 22.5 mM $MgCl_2$/2.5 mM $MnCl_2$ respectively. As the RNAs were 3′ end labelled and as the 3′ end of the RNAs were heterogenous, each band is present as a doublet — the bottom band of each doublet is labelled. The band generated by cleavage of the phosphorothioate 5′ to C$_{+1}$, either at the 3′SS (lane 1), or at the exon junction of the transcribed cDNA ligated exon control (lane 4), is indicated by an arrow. Such a band would be present in lanes 2 and 3 if the phosphorothioate at the 3′SS of some of the 3′ molecules had reacted to yield ligated exons with a phosphorothioate at the exon junction site. No cleavage was seen in the 5′ exon of the ligated exons as the 5′ molecule was unsubstituted.

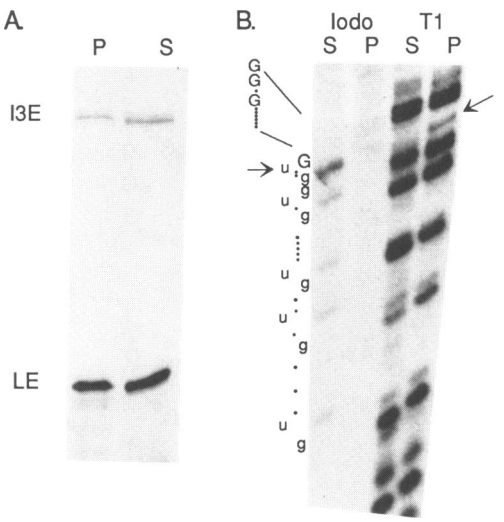

Figure 5. The exon ligation reaction in reverse. (**A**) Unsubstituted excised intron RNA was reacted with 3′ end labelled ligated exon RNA (LE) to yield the intron joined to the 3′ exon (I3E). LE RNA was unsubstituted (P) or fully substituted with UαS (S); in the latter case, this placed a phosphorothioate at the exon junction site U$_{-1}$·U$_{+1}$. (**B**) I3E RNA was gel purified and partially cleaved with RNase T1 or iodoethanol (21). Dots indicate A or C in the RNA sequence, with 3′ exon and intron sequence in lower and upper case respectively. Arrows indicate the expected position of the band produced by cleavage of the reconstituted 3′SS (G$_{414}$·U$_{+1}$), by either iodoethanol (Nps↓U), or RNase T1 (Gp↓N). No iodoethanol cleavage is seen in the intron RNA as it was unsubstituted. The reason for the weak T1 digestion at the 3′SS of the unsubstituted control RNA is not known.

was still used highly preferentially but there was about 2–3% utilization of the normal 3′SS (Table 1). With CαS in the wildtype 3′ fragment, the bimolecular reaction proceeded greater than 50% but the reaction of the C$_{+1}$ mutant proceeded less than 1% (Figure 3) — no ligated exon RNA was visible on the autoradiogram but PCR amplification confirmed that some ligated exon RNA had been generated. After completing splicing, the excised intron can cyclize (13) (a linear molecular is produced in a bimolecular reaction). No such reaction was seen in the CαS C$_{+1}$ reaction, consistent with inhibition of the second splicing step (Figure 3). Inclusion of the synthetic 8mer 5′ exon in the CαS C$_{+1}$ bimolecular reaction, to compensate for premature dissociation of the natural 5′ exon, did not stimulate utilization of the 3′SS (data not shown).

Mn^{2+} did not stimulate utilization of the normal 3′SS in the SP6 bimolecular system, but the efficiency of the reaction was very poor (Table 1). In the T7 bimolecular system, with GαS in the G$_{+1}$ 3′ RNA, Mn^{2+} resulted in the ligated exons (obtained using the +1 cryptic site) being present at a less than stoichiometric amount compared to the intron fragment of the 3′ precursor molecule. The cause of this anomaly is currently being pursued. The CαS C$_{+1}$ bimolecular reaction was not activated by addition of 2.5 mM $MnCl_2$.

The 3′SS of the precursor RNA can undergo significant hydrolysis at pH 9.0 without a preceding reaction at the 5′SS (24). Hydrolysis was also blocked by the presence of a phosphorothioate at the 3′SS in the GαS and CαS unimolecular reactions and the CαS bimolecular reaction (data not shown).

To reduce the background of phosphorothioates further, a modification interference assay (25) was performed using the bimolecular reaction. The 5′ molecule was unsubstituted. The 3′ RNA was transcribed from the C$_{+1}$ mutant in a 10:1 ratio of CTP to CTPαS so that each bimolecular complex had on average just 2.5 other phosphorothioates randomly distributed in the last quarter of the intron. If a phosphorothioate at the 3′SS is reactive, then some of the ligated exon RNA molecules should have a phosphorothioate at the exon junction site. These can be detected by cleavage with iodoethanol (4, 19, 21), which cleaves phosphorothioate but not phosphate diesters. However no cleavage at the junction site was detected (Figure 4). No difference was seen with the addition of 2.5 mM $MnCl_2$, although the aforementioned stoichiometric anomaly should be borne in mind in interpreting this result.

The reverse reaction occurs with a Rp phosphorothioate at the reactive site and inverts the configuration

The second step of splicing can be conducted in reverse by reacting excised intron with ligated exons (26). We asked whether a Rp phosphorothioate at the junction of the 5′ and 3′ exon would hinder the reverse reaction. A plasmid was constructed which contained the ligated exons without the intron of pTT14 (Figure 1). This was linearized at the HindIII site to produce an RNA transcript composed of a 17 base 5′ exon joined to a 35 base 3′ exon. A Rp phosphorothioate was incorporated at the exon junction site by substituting UTPαS for UTP during transcription. 3′ end-labelled ligated exon RNA was reacted with unsubstituted

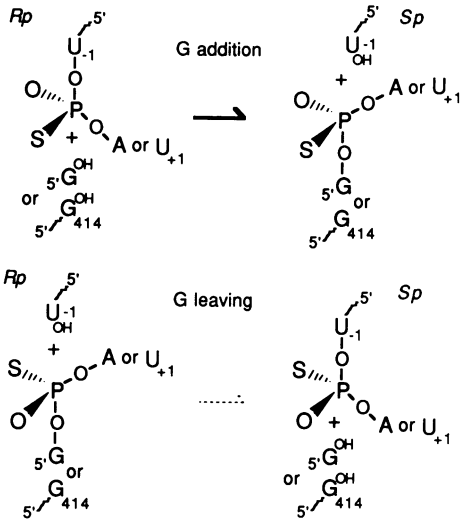

Figure 6. Similarities in the stereochemistry of the first and second step of splicing. The G addition reaction is represented by either the first splicing step, with exogenous G attacking the 5'SS (U_{-1}-A) (15), or the reverse of the second step of splicing, with G_{414} at the end of the intron attacking the exon junction site (U_{-1}-U_{+1}) (this work). The G leaving reaction is represented by either the reverse of the first splicing step, with U_{-1} at the end of the 5' exon attacking a (G-A) bond (16), or exon ligation with U_{-1} attacking the 3'SS (G_{414}-U_{+1}) (this work). Note, in each scheme, the polarity of the diester bond. The thin arrow indicates that the reaction goes very poorly due to the presence of a pro-R thio group. The reverse of the reactions, starting with Sp material, has not been tested.

excised intron RNA to give an RNA consisting of intron joined to the 3' exon (I3E) (and presumably some precursor). Although the amount of product generated was limited, UαS substituted RNA and unsubstituted control ligated exon RNA reacted to the same extent (Figure 5A), indicating that a Rp phosphorothioate at the exon junction site does not inhibit the exon ligation reaction in reverse.

We confirmed that the intron reacted with the phosphorothioate at the exon junction site, and not an alternate site, by gel purifying the product of the reaction, generating a cDNA spanning the reconstituted 3'SS, amplifying the cDNA by PCR and subcloning a region of the amplified DNA (a BglII HindIII fragment in Figure 1). On the basis of a phenotypic assay, the correct site was utilized in all of 41 randomly selected, insert containing colonies; 19 colonies were checked by sequencing.

The transcribed UαS ligated exon RNA should have a Rp phosphorothioate at the exon junction site ($U_{-1}\cdot U_{+1}$). The reverse reaction should create a 3'SS with the sequence $G_{414}\cdot U_{+1}$ which should contain the phosphorothioate originally 5' to U_{+1} in the ligated exons. GpN sequences containing a Rp phosphorothioate diester are only cleaved twice as slowly by T1 RNase as a normal phosphate diester, whereas a Sp isomer is highly resistant to cleavage (15). T1 RNase partially digested I3E molecules derived from UαS substituted and unsubstituted ligated exon RNA were compared (Figure 5B). The absence of a band at the G_{414} position indicated that a Sp isomer was now at the 3'SS and that the configuration of the phosphorothioate had inverted. Partial cleavage with iodoethanol showed that a phosphorothioate was present at the 3'SS of the great majority of I3E molecules since the 3'SS specific band (arrow in Figure 5B) was as intense as others in the UαS substituted 3' exon. This observation and the T1 cleavage data confirm that the I3E RNA was generated because the Rp isomer was indeed reactive, and not because a small percentage of the ligated exons had a phosphate at the 3'SS due to background contamination of UTPαS with UTP during transcription.

DISCUSSION

It is evident that, in the reactions described, a phosphorothioate at the 3'SS significantly inhibits the second step of splicing. This was determined, in an absolute way, using the CαS substituted C_{+1} mutant and, in a relative way, using the GαS substituted G_{+1} mutant and its cryptic +1 3'SS. Because the accumulation of internal phosphorothioate substitutions slowed the reaction of wild-type RNA, it could be argued that these substitutions diminished the ability of the RNA to tolerate the presence of a phosphorothioate at the 3'SS.

This issue was addressed in several ways. Bimolecular reactions were established to significantly reduce the number of phosphorothioates. $MnCl_2$ was added to the reaction to suppress the inhibitory effect of internal phosphorothioates. A synthetic 8mer oligoribonucleotide was added to compensate for the possible premature dissociation of the 5' exon from the splicing intermediate. The bimolecular reaction resulted in only a small number of phosphorothioates being introduced into the catalytic core of the intron since the region between 315 and 410 is not obligatory for exon ligation (Caprara and Waring, unpublished results). This leaves either C311 and 413 or G 303, 309, 311, 312 and 414. Because both GαS and CαS reactions could be conducted, this reduces further the possibility that the data can be explained as the synergistic inhibitory interaction of a specific internal phosphorothioate upon an otherwise reactive 3'SS phosphorothioate. Furthermore, the bimolecular modification interference assay reduced the phosphorothioate background to a minimal level.

It was originally reported that introduction of a phosphorothioate at the 3'SS does not block exon ligation (14). This conclusion was drawn from performing reactions with the G_{+1} mutant substituted with GαS. Though not specifically stated, the basis of this conclusion was presumably the appearance of an RNA molecule corresponding in size to the ligated exons — this is now shown to result from use of a unsubstituted 3'SS, one base downstream.

Substitution of the same non-bridging oxygen with sulfur inhibits catalysis at both splice sites

Cleavage of the 5'SS by GTP is a G addition reaction (Figure 6). A reaction analogous to this first step in splicing is barely hindered by the substitution of sulfur for the pro-R oxygen (15), even in conditions where the chemical step is rate-limiting (3) (Figure 6). In contrast, a reaction analogous to the reverse of 5'SS cleavage, a G leaving reaction, is about a thousand times slower in the presence of the Rp isomer (16). This can be explained because it is the other nonbridging oxygen which is substituted in the reverse reaction. As the reaction proceeds by inversion (15, 16), by definition, the pro-R oxygen in one direction is identical to the pro-S oxygen in the reverse reaction (Figure 6).

From the viewpoint of the stereochemical effect of substituting a non-bridging oxygen with sulfur, the second step of splicing is directly analogous to the first step: substitution of one oxygen is inhibitory, on the basis of assaying exon ligation or the reverse of 5'SS cleavage, while substitution of the other oxygen is non-

inhibitory, on the basis of assaying the reverse of exon ligation or 5'SS cleavage (Figure 6). In all the reactions described the pro-R oxygen is substituted. In both splicing steps, substitution of the pro-R oxygen is inhibitory in the G leaving reaction and permissive in the G addition reaction. This fully supports the proposal that exon ligation may be viewed as simply the reverse of the first splicing step (24): UpN + G-OH ⟷ U-OH + GpN. It also supports the view that the same G binding site (28) and the same catalytic site are used in both splicing reactions.

In pre-mRNA splicing, a Rp phosphorothioate blocks the splicing reaction at both the 5' and 3'SS (4). As this contrasts with the situation reported here, it implies that the precise mechanisms of splicing differ, despite the fact that they both probably involve two sequential transesterification reactions.

The effect of Mn^{2+} on an inhibitory phosphorothioate at the 3'SS

The strong inhibitory effect of substituting the pro-R oxygen with sulfur in a G leaving reaction implies that the oxygen atom plays a key role in catalysis, such as stabilizing the transition state of the proposed trigonal bipyramid intermediate during transesterification (29); for example, the oxygen could form a hydrogen bond with a nitrogenous base or it could coordinate a Mg^{2+} ion. Evidence for the latter type of interaction has been obtained for the hammerhead ribozyme by demonstrating that Mn^{2+} ions can suppress the inhibitory effect of a phosphorothioate (8). No striking suppressive effect by Mn^{2+} was seen in this work. However inclusion of Mn^{2+} resulted in a diminished level of ligated exons in the GαS G_{+1} mutant T7 bimolecular system. It therefore remains possible that a stimulatory effect of Mn^{2+} will be seen under the right experimental conditions.

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