

Thiophosphates in yeast U6 snRNA specifically affect pre-mRNA splicing *in vitro*

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ABSTRACT

A thorough mutational analysis of U6 RNA in combination with a functional reconstitution assay, revealed that three domains are important for U6 function in pre-mRNA splicing. In order to further analyze why these regions are so critical for splicing, we make use of phosphorothioate substituted U6 RNAs. Wild-type U6 RNA was transcribed *in vitro* with T7 RNA polymerase in the presence of either phosphorothioate (α -S) ATP, GTP, UTP or CTP. The functionality of the transcripts was monitored by *in vitro* reconstitution. While substitution with α -S ATP, GTP or UTP blocked splicing, substitution with α -S CTP had little or no effect on splicing. We made use of this α -S CTP effect in an attempt to elucidate which phosphates in the U6 RNA molecule play a role in the first or in the second step of splicing. U6 mutants in which a change of an A, G or U to C does not have any significant effect on splicing were transcribed in the presence of α -S CTP. Observed effects on splicing thus have to be attributed to the presence of the thio-substituted phosphate group rather than the nucleotide change. The results of *in vitro* reconstitution give a clear answer for at least three phosphates; two of them play a role in the first step, while one of them is involved in the second step of splicing.

INTRODUCTION

Nuclear pre-mRNA splicing takes place on a large dynamic particle, the spliceosome (1), whose function is to fold the pre-mRNA into a substrate for splicing and to catalyze the reactions. The two distinct transesterification reactions which take place within the spliceosome are mechanistically similar to those of group II self-splicing introns (2, 3). The first step involves cleavage at the 5' splice site and formation of a 2'–5' branched intermediate (lariat); in the second step the 3' splice site is cleaved and the exons are ligated.

The splice sites and catalytic sites in a group II intron are determined through intron sequence and conserved intron structure. Self-splicing *in vitro* does not require ATP and proteins. In pre-mRNA introns the splice sites are defined by minimal

sequences (4, 5). These are recognized and may be subsequently folded into a reactive conformation by numerous *trans*-acting factors. ATP is required for this process.

The *trans*-acting factors are the components of the spliceosome. They include complexes of small RNAs (the small nuclear RNAs U1, U2, U4, U5 and U6) and a set of 7–10 tightly associated proteins (6,7). These small nuclear ribonucleoprotein particles (snRNPs) are likely to mediate the catalytic events of splicing.

Both RNA and protein-based interactions can contribute to the communication between the various snRNPs and between them and the intron. However, in light of self-splicing reactions it seems possible that nuclear pre-mRNA splicing evolved from an RNA machinery (8). The archetype of recognition mediated by snRNPs is the interaction of the U1 snRNP with the 5' splice site (9, 10) and of the U2 snRNP with nucleotides surrounding the branch-point (11,12). These interactions establish the role of U1 and U2 in intron recognition, but do not imply a possible involvement in the actual cleavage and formation of covalent bonds.

The U6 snRNA sequence is remarkably conserved among eucaryotes (13). This snRNA can be found base paired with U4 snRNA in the same snRNP particle. Other snRNAs have retained only short regions of phylogenetically conserved sequence. Although the U4/U6 snRNP does not participate in splice site recognition by any obvious base-pairing, a dramatic change in the conformation of the U4/U6 snRNP occurs just before the first splicing reaction (14). The conformational change may be important for a possible function of U6 snRNA in splicing catalysis. Indeed, it has been suggested that U6 comprises the catalytic core of the spliceosome and that U4 functions as a negative regulator specifically released only just before 5' cleavage and lariat formation (15). Thus, the primary role of U4 may be to mask a catalytically active domain of U6 until other conditions of spliceosome assembly have been established (15).

We studied the function of yeast U6 snRNA in splicing using synthetic U6 RNA in a functional reconstitution assay (16). Using U6 mutants in this system we found that there are three domains in U6 at which single nucleotide substitutions affect splicing (17, and this work). While certain mutants within these three domains block splicing completely, others allow the first step of splicing to occur but block the second step. Interestingly, these domains are also hotspots for insertion of mRNA-type introns in the U6

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genes of various yeast species (18). In the fission yeast *Schizosaccharomyces pombe*, the finding of an intron similar to nuclear pre-mRNA introns inserted in one of these conserved domains, suggested that this region of U6 RNA was part of the catalytic center of the spliceosome (15, 19). One and four introns were also found in *Rhodospiridium dacryoidum* and in *Rhodotorula hasegawae* U6 genes, respectively (18). The introns of the *S.pombe* and *R.dacryoidum* U6 genes are located immediately adjacent to the nucleotides that we have shown to be essential for the second step of the splicing reaction (17). These nucleotides may be part of the catalytic center of the spliceosome. We have now used phosphorothioate substituted U6 RNA to further analyze why these nucleotides are so critical for splicing.

We transcribed wild-type U6 RNA with T7 RNA polymerase in the presence of either phosphorothioate (α -S) ATP, GTP, UTP or CTP in an attempt to elucidate whether phosphate-residues in the U6 RNA molecule play a role in the first or in the second step of splicing. In phosphorothioate RNA, phosphate groups are replaced by phosphorothioate groups, with the result that one of the peripheral oxygens of the phosphodiester bond is replaced with sulfur (20). We found that only the α -S CTP substituted U6 RNA can reconstitute splicing. We made use of this α -S CTP effect to demonstrate that at least three distinct phosphate-residues in U6 RNA are important for U6 function. These phosphates may represent binding sites for magnesium or for protein factors.

MATERIALS AND METHODS

Construction of stem II mutants and transcription

Mutants from position 67 to 80 of the U6 RNA stem II region were constructed using the strategy described (17). The oligonucleotide for site-directed mutagenesis, 5'-CatccttatgcaggGAACTGCTGATCA-3', was designed to introduce a doped nucleotide at each individual position from nucleotide 67 to 80 (small letters) using an equal proportion of the other three bases. The doped oligonucleotide was used for total U6 RNA gene synthesis together with oligonucleotides previously described (17). For the construction of the mutant G81C the oligonucleotide 5'-GATCCTTATGCAGGGGAACTGCTGATCA-3' was used instead. The U6 gene in each clone was sequenced entirely. Each clone was cut with BamHI and *in vitro* transcribed with T7 RNA polymerase in a 20 μ l reaction volume in the presence of 2 μ Ci of [α -³²P]UTP and 1 mM each of unlabeled ATP, CTP, GTP, and UTP or any of the phosphorothioate (α -S) NTPs. When (α -S)UTP was used, 2 μ Ci of [α -³²P]GTP were used instead. RNAs transcribed under these conditions have a specific activity of 7.7 cpm/fmol. Each transcript was gel purified, phenol and ether extracted and resuspended in water at a concentration of 400 fmol/ μ l. The enzymatic synthesis of unlabeled (α -S) containing RNAs was performed as described (16).

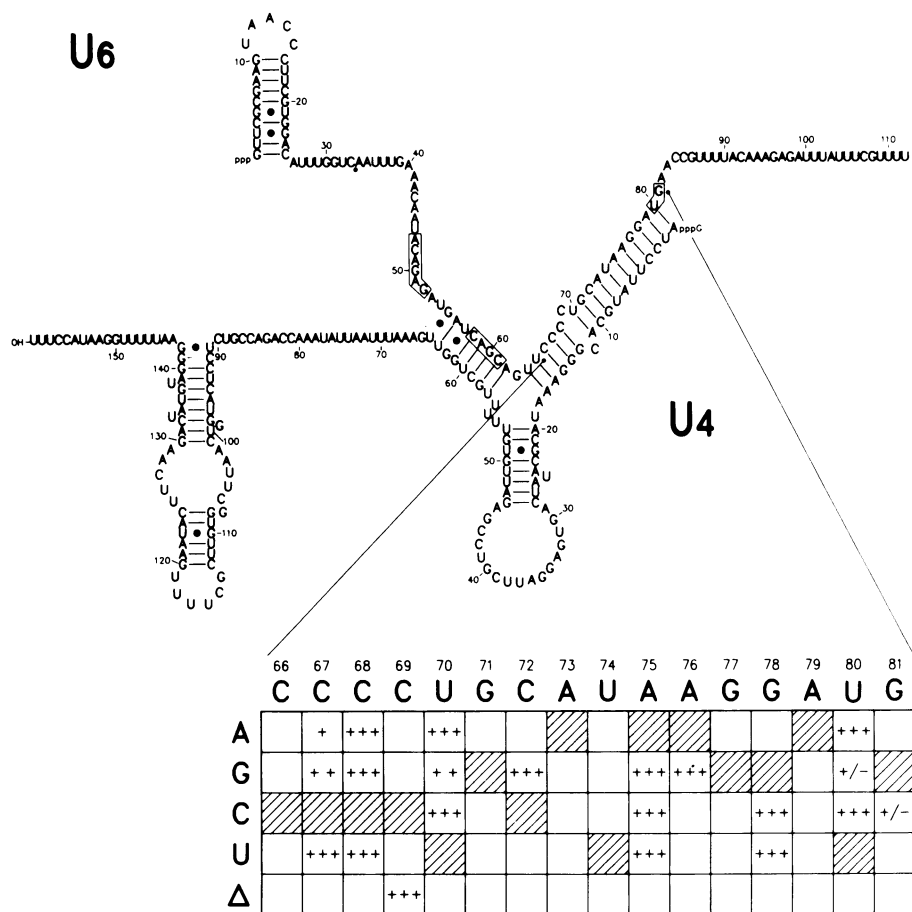


Figure 1. Summary of the effects on pre-mRNA splicing of point mutations at nucleotides 67 to 81 of U6 RNA (Stem II region). +++ = 80 to 100 percent splicing efficiency. ++ = 50 to 80 percent splicing efficiency. + = 20 to 50 percent splicing efficiency. +/- = less than 10 percent splicing efficiency. Δ = deletions of nucleotides. The three domains important for U6 function are boxed.

In vitro reconstitutions and complex formation

Yeast splicing extract (21) was first treated with 300 nM oligonucleotide d1 (16) in the presence of ATP to cleave the endogenous U6 RNA. Reconstitution was then performed as described (16, 17) with various amounts of each synthetic U6 RNA clone. Splicing activity was assayed with ³²P-labeled actin pre-mRNA (17). Splicing efficiency was quantified by Cerenkov counting of the bands excised from the dried gels. For spliceosome formation (22), reconstitution was performed with 15 nM U6 RNA at a specific activity of 800 cpm/fmol.

RESULTS

U6 mutants of the stem II region

The U4/U6 interaction domain is comprised of two regions that have been proposed to base-pair with U4 (stem I and II). Recent data from mammalian *in vitro* experiments as well as studies in *Xenopus* and yeast provide strong support for this proposed base-pairing (23, 24). To understand the importance of the stem II region of U6 RNA during *in vitro* assembly and splicing, we made point mutations in highly conserved nucleotides of the stem II region. RNA from each of the mutant genes was synthesized

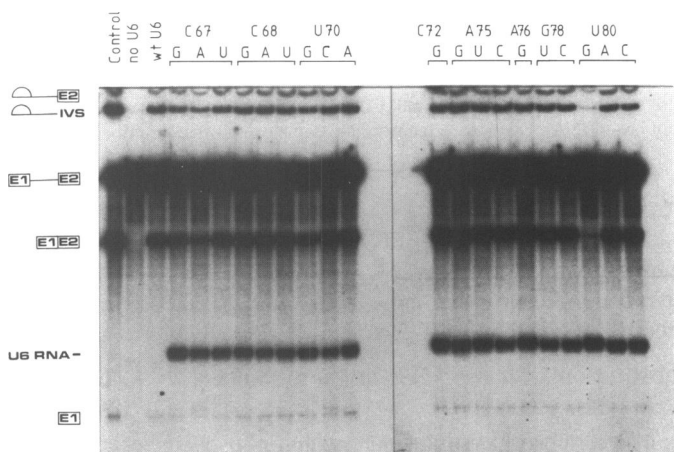


Figure 2. Mutational analysis of the *S.cerevisiae* U6 RNA stem II. U6 RNA mutants of the stem II region were used for reconstitution of splicing activity at a concentration of 40 nM. RNA species are indicated to the left of the gel by the following symbols: E1, exon 1; E2, exon 2; IVS, intervening sequence or intron. Control = mock-treated extract. The upper band of an apparent E1 doublet in lanes C67A and U70C represents a degradation product of U6 RNA instead.

and tested for activity in our *in vitro* reconstitution assay. An active splicing extract in which endogenous U6 RNA was destroyed during incubation of the extract with a deoxyoligonucleotide complementary to U6 RNA, was used for reconstitution. The results are summarized in Figure 1 and shown in Figure 2. Only the mutations of C67 to A, U80 to G and G81 to C (not shown) substantially affected splicing.

Substitution with α-S NTPs in wild-type U6 RNA blocks splicing

The effect on splicing of U6 RNA transcribed with T7 RNA polymerase in the presence of α-S ATP, CTP, GTP or UTP was monitored by *in vitro* reconstitution of the α-S substituted U6 RNA. The results are shown in Figure 3. α-S ATP substituted

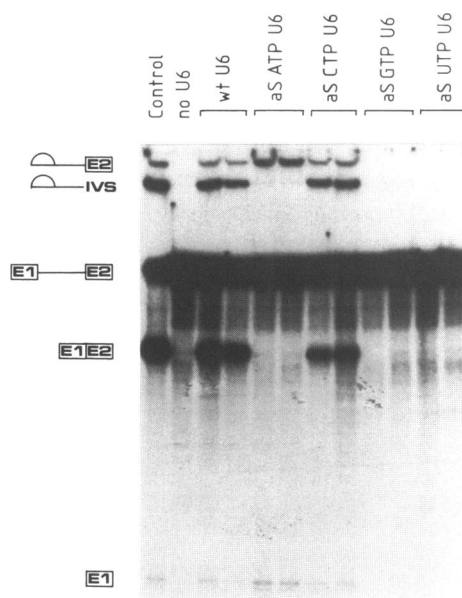


Figure 3. Substitution with phosphorothioate in U6 RNA blocks splicing. Wild-type U6 RNA was uniformly substituted with phosphorothioate nucleotides during transcription with T7 RNA polymerase. After gel purification the transcripts were used for reconstitution of splicing activity at a final concentration of 40 and 10 nM, respectively. Wt U6, wild-type unsubstituted U6 RNA; αS ATP, αS CTP, αS GTP, αS UTP U6, U6 RNA substituted with phosphorothioate ATP, CTP, GTP, and UTP, respectively. Control = mock-treated extract. RNA species are indicated to the left of the gel by the following symbols: E1, exon 1; E2, exon 2; IVS, intervening sequence or intron. The E1 doublet represents a degradation product of E1, also observed in mock-treated control experiments.

	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
	A	A	U	U	U	G	A	A	A	C	A	A	U	A	C	A	G	A	G	A	U	G	A	U	C	A	G	C	A
A	///	///	+++			+++	///	///	///		///	///		///	-	///	+	///	++	///		++	///	+++	⊖	///	+	+	///
G				+++	+++	///			+++				+++	++	+/-	+	///	⊖	///	+++		///			⊖	⊖	///	+	
C	+++		+++				+++		///					+	///	+	+	⊖	///	+++			++	++	⊖	⊖	///	+/-	+++
U			///	///	///			+++		+++		///	+++	+/-	++	+	⊖	///	+++	+++	///	+++	+++	///	++	⊖	+/-	++	+++
Δ												+++			-														

Figure 4. Summary of the mutational analysis in the central domain and stem I region of U6 RNA, respectively (17). +++ = 80 to 100 percent splicing efficiency. ++ = 50 to 80 percent splicing efficiency. + = 20 to 50 percent splicing efficiency. +/- = less than 10 percent splicing efficiency. - = no products detected. The lariat symbol signifies various amounts of block in the second step of splicing. Δ = deletions of nucleotides.

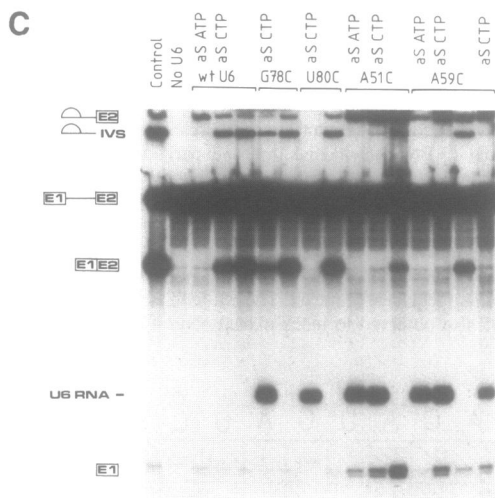
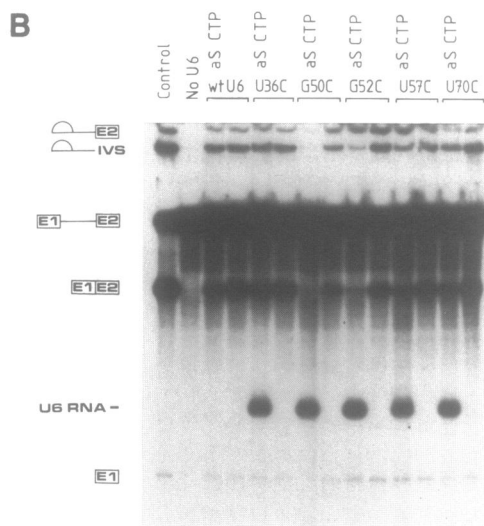
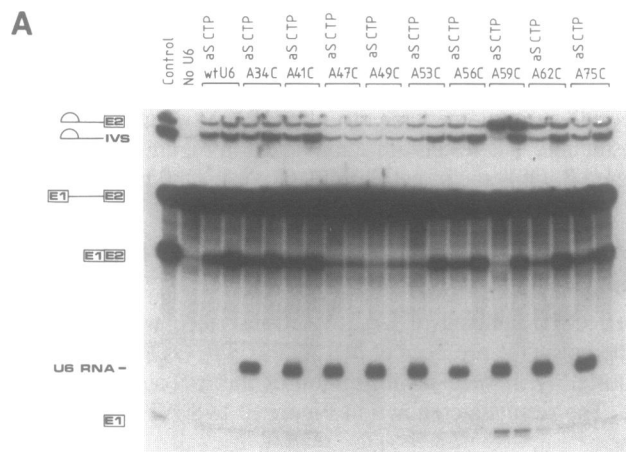


Figure 5. A, B and C. Effects of substitution with phosphorothioate CTP in A, G, and U to C U6 mutants. Reconstitution of splicing activity was done with 40 nM of wild-type and mutant U6 RNAs, substituted with phosphorothioate CTP (α S CTP) and unsubstituted, respectively. Figure 5C also shows the effects of the substitution with phosphorothioate ATP. Control = mock-treated extract. RNA species are indicated to the left of the gel by the following symbols: E1, exon 1; E2, exon 2; IVS, intervening sequence or intron. U6 RNA = U6 transcripts used for reconstitution. U6 RNAs which do not appear in the gel have been used unlabeled. No difference in reconstitution between identical amount of labeled and unlabeled U6 RNA has been observed.

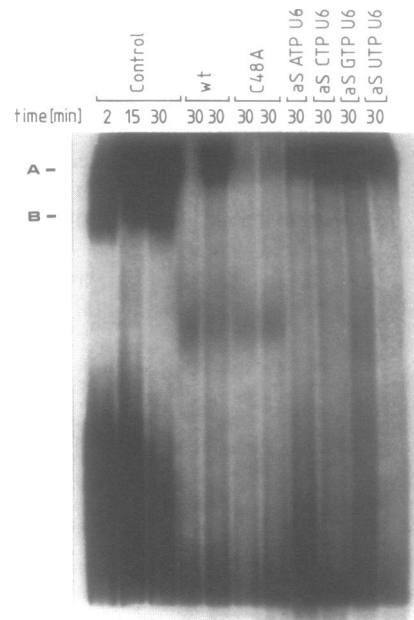


Figure 6. Phosphorothioate U6 RNA enters the spliceosome. Reconstitution was performed with 15 nM of various U6 RNA transcripts which were 32 P-labeled at a specific activity of 800 cpm/fmol. Spliceosome formation was assayed after incubation with unlabeled actin pre-mRNA, for the time indicated, except in the first lane of the wild-type reaction where precursor was not added. Complexes were isolated in a non-denaturing gel. Control = mock-treated extract with labeled actin pre-mRNA. The nomenclature of the complexes is that described in (22).

U6 RNA completely blocks splicing at the second step of the reaction. The first step of splicing is also less efficient. Substitution of C's with α -S CTP within the U6 RNA does not have a significant effect on splicing. Finally, incorporation of either α -S GTP or α -S UTP into U6 RNA totally blocks splicing.

Substitution with α -S CTP in U6 mutants with A, G, U to C changes

As a first approach to elucidate which phosphate residues are responsible for these blocks we used the following strategy. We chose among the U6 mutants of the central domain, and of stem I and stem II, those in which a change of an A, G or U to C did not have any significant effect on splicing (see Figure 4 for a summary). We transcribed these mutants in the presence of α -S CTP so that an eventual effect on splicing would have to be attributed to the presence of a phosphate residue carrying a sulfur, rather than to the base substitution itself in that particular position. The result is shown in Figure 5A, B and C. Figure 5A shows that the α -S CTP substituted A59C mutant causes a clear total block in the second step of splicing, while its unsubstituted counterpart shows only little effect. Substitution with α -S CTP in all of the other mutants shown in Figure 5A had no effect. Figure 5B shows that G50C and to a lesser extent G52C are not able to reconstitute splicing after substitution with α -S CTP. In these cases the block is at the first step of splicing. A complete block of splicing occurs also when the α -S CTP substituted U80C mutant is used in reconstitution (Figure 5C).

α -S GTP and -UTP substituted U6 RNA assembles into the spliceosome

We demonstrated that mutations in U6 RNA which block the first step of splicing, completely block the association of U6 with

the spliceosome (17). In this study, it was important to know whether U6 RNAs substituted with α -S GTP and -UTP, which block the first step of splicing completely, still allow correct spliceosome assembly. Complexes formed after reconstitution with 32 P-labeled α -S GTP and -UTP substituted U6 RNA, were separated by electrophoresis in a non-denaturing gel (Figure 6). Surprisingly, all phosphorothioate U6 RNAs associated with the spliceosome. Figure 6 also shows that α -S ATP substituted U6 RNA which completely blocks the second step of splicing is found in the spliceosome. In fact, that there is less efficient association of α -S ATP substituted U6 with the spliceosome may be the cause of its low efficiency in reconstitution experiments.

As a control, Figure 6 shows the spliceosome formation with C48A, one of the U6 mutants that blocks the first nucleolytic step of splicing and that does not participate in spliceosome formation.

DISCUSSION

One fundamental aspect of the splicing mechanism that needs to be understood is how splicing is catalyzed. The removal of introns from nuclear pre-mRNA shares properties with certain RNA self-splicing reactions. An attractive notion is that the RNA components of the snRNPs represent the primary catalyst of the two transesterification reactions which take place in the spliceosome (25). Thus, catalysis would be reduced to the interaction of the four bases, ribose sugars, phosphate groups and magnesium ions.

The first intriguing suggestion that U6 RNA functions as the catalyst in pre-mRNA splicing was made because of the extraordinary conservation of U6 sequences in evolution. This suggests that U6 is under severe size and sequence constraints and it is consistent with the presence of U6 at the physical and functional center of the splicing machine (13).

We have studied the role of many of the conserved bases of U6 RNA by mutational analysis. Although until now it has been difficult to establish a direct involvement of U6 RNA in bond cleavage and formation because a protein-free *in vitro* system is not available, there is evidence reported by us and by others that U6 RNA is or is a part of the catalytic element in pre-mRNA splicing (17, 18).

In this work we used phosphorothioate substituted U6 RNA to show that certain phosphates play an important role in splicing. In phosphorothioate RNA, phosphate groups are replaced by phosphorothioate groups. This substitution of an oxygen by a sulfur could have various consequences. Sulfur is larger and therefore could disrupt tertiary structure. However, it is important to point out that structural perturbations of U6 RNA do not or only to a minimal extent occur in the α -S CTP substituted U6 RNA. The efficiency of reconstitution of α -S CTP substituted wild-type U6 RNA is 50–70% of the unsubstituted U6 RNA. However, when various U6 mutants with nucleotide changes of A, G or U to C, which per se do not have any significant effect on splicing, are substituted with α -S CTP, a dramatic effect is noticeable. One of the α -S CTP substituted mutants, A59C, blocks splicing at the second step and two more block splicing completely: G50C and U80C. A fourth, G52C, has an intermediate effect (see Figure 5B). These effects have to be attributed to the presence of the thio-substituted phosphate group rather than the nucleotide change.

The sulfur of a phosphorothioate group binds less well to magnesium than the oxygen of the phosphate group. Substitution

of the oxygen by a sulfur could therefore alter or entirely eliminate a coordination site for this essential metal ion. Recently, a hammerhead RNA containing a single phosphorothioate linkage at the self cleavage site was shown to have a reduced rate of cleavage (26, 27). The substitution of an oxygen by a sulfur atom substantially reduced the affinity for a Mg^{++} ion necessary for efficient cleavage. The reaction proceeded normally in the presence of Mn^{++} , presumably due to its ability to coordinate equally well with both oxygen and sulfur at the cleavage site.

Therefore we tried to reverse the splicing block caused by phosphorothioate substitution of U6 by using $MnCl_2$ instead of $MgCl_2$ in the reconstitution reaction. We have not observed such an effect of Mn^{++} . We only observed a generally lower requirement of $MnCl_2$ compared to $MgCl_2$ (0.5 mM optimum for $MnCl_2$ versus 2.5 mM for $MgCl_2$, data not shown) and a slightly lower efficiency of reconstitution in the presence of Mn^{++} (not shown).

The substitution of an oxygen by a sulfur is also often found to alter the affinity of proteins for nucleic acids. Phosphorothioate substitution has been shown to either increase or decrease the affinity of a small RNA hairpin for R17 coat protein (28). Thus, the substitution of oxygen by a sulfur in certain positions of U6 RNA could alter specific protein–phosphate contacts.

Presently, only PRP24 protein is known to tightly bind the yeast U6 RNA. PRP24 was initially identified in a screen for temperature-sensitive lethal mutations that block pre-mRNA splicing (29). PRP24 is tightly associated with U6 in wild-type extracts (30). However, the exact site of interaction between PRP24 and U6 RNA is unknown. It may for example be that the phosphates of U6 RNA which have been identified as being essential for splicing, are binding sites for PRP24 or other protein factors. The most exciting possibility of course would be the direct involvement of these phosphate group in catalysis. Under this aspect it is important to recall that most of the introns which have been found inserted in the U6 genes of various yeast species locate within a conserved domain, which is strikingly similar in structure to the catalytic center of the negative strand of the satellite RNA of tobacco ring spot virus [(–)sTRSV] (18). The catalytic RNA of (–)sTRSV cleaves the 5' site of its substrate RNA at the GU sequence, which is also the consensus sequence of the 5' splice site in pre-mRNA splicing (31). Tani and Ohshima (18) have recently reported that a 49 nucleotide long synthetic RNA which has 78% sequence identity with the conserved region of *S.pombe* U6 RNA can cleave *in vitro*, after base-pairing interaction, a 15 base RNA substrate that contains GU sequences. Although a base-pairing interaction between U6 RNA and the precursor has never been reported, it may be that in pre-mRNA splicing proteins and ATP are the factors which make possible the close interactions necessary for catalysis between U6 RNA and the splice sites.

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