Structure of the U6 RNA intramolecular stem-loop harboring an S_P -phosphorothioate modification

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ABSTRACT

Phosphorothioate-substitution experiments are often used to elucidate functionally important metal ion-binding sites on RNA. All previous experiments with S_P -phosphorothioate-substituted RNAs have been done in the absence of structural information for this particular diastereomer. Yeast U6 RNA contains a metal ion-binding site that is essential for spliceosome function and includes the pro- S_P oxygen 5' of U₈₀. S_P -phosphorothioate substitution at this location creates spliceosomes dependent on thiophilic ions for the first step of splicing. We have determined the solution structure of the U₈₀ S_P -phosphorothioate-substituted U6 intramolecular stem-loop (ISL), and also report the refined NMR structure of the unmodified U6 ISL. Both structures were determined with inclusion of ^1H – ^{13}C residual dipolar couplings. The precision of the structures with and without phosphorothioate (RMSD = 1.05 and 0.79 Å, respectively) allows comparison of the local and long-range structural effect of the modification. We find that the U6-ISL structure is unperturbed by the phosphorothioate. Additionally, the thermodynamic stability of the U6 ISL is dependent on the protonation state of the A₇₉–C₆₇ wobble pair and is not affected by the adjacent phosphorothioate. These results indicate that a single S_P -phosphorothioate substitution can be structurally benign, and further validate the metal ion rescue experiments used to identify the essential metal-binding site(s) in the spliceosome.

Keywords: U6 snRNA; phosphorothioate; Nuclear Magnetic Resonance (NMR); metal ion; residual dipolar coupling (RDC)

INTRODUCTION

Eukaryotic genes contain introns that must be accurately spliced out of pre-messenger RNA (pre-mRNA). This splicing reaction is catalyzed by the spliceosome, a dynamic molecular assembly that requires five snRNAs (U1, U2, U4, U5, and U6) and >70 proteins to perform two successive phosphotransesterification reactions (Nilsen 1998; Burge et al. 1999; Brow 2002). U6 and U2 snRNAs are required in both steps of the reaction, directly assisting in the concerted excision of the lariat intron and the ligation of the flanking exons through a 5'–3' phosphodiester linkage (Collins and Guthrie 2000). Recently, a U2/U6 complex was shown to

catalyze a reaction similar to the first step of splicing in the absence of protein, supporting the hypothesis of an RNA active site in the spliceosome (Valadkhan and Manley 2001). In addition, inner-sphere coordination of each leaving group by a catalytic divalent metal ion is an important feature of the two chemical steps of pre-mRNA splicing (Sontheimer et al. 1997; Gordon et al. 2000).

Central to our understanding of the spliceosome is the identification of metal-binding sites and functionally important phosphate oxygens within snRNAs. Single-atom substitution studies of U6 snRNA have revealed several phosphate oxygens that are essential to splicing, revealing potential magnesium-binding sites within the spliceosome (Fabrizio and Abelson 1992; Yu et al. 1995; Yean et al. 2000). If there is a catalytically essential contact between a metal ion and a nonbridging oxygen atom, replacement of the oxygen with a sulfur atom can introduce a functional defect and loss of activity. Such a phosphorothioate substitution substantially changes the charge distribution, polarizability, bond length, and van der Waals radius of a phosphate group (Eckstein 1979; Saenger 1984). Because Mg^{2+} is a hard metal that prefers an interaction with a hard ligand (e.g., oxygen), a sulfur substitution at a critical Mg^{2+} –RNA

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Abbreviations: CT, constant time; HETCOR, heteronuclear correlation spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; ppm, parts per million; RDC, residual dipolar coupling; TOCSY, total correlation spectroscopy; RMSD, root mean square deviation.

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contact may significantly contribute to loss of activity. Conversely, more thiophilic metal ions, such as Cd^{2+} and Mn^{2+} , can restore the metal contact to a sulfur-substituted RNA, because of their strong preference or tolerance for sulfur as a ligand (Pecoraro et al. 1984).

Lin and colleagues have identified an essential and stereospecific metal ion coordination site 5' to nucleotide U_{80} in yeast U6 snRNA (Yean et al. 2000). S_p -phosphorothioate modification of nucleotide U_{80} ($\text{U6}/\text{S}_\text{p}\text{-U}_{80}$) in U6 abolishes the splicing reaction but does not interfere with spliceosome assembly. Cd^{2+} and Mn^{2+} stereospecifically rescue the $\text{U6}/\text{S}_\text{p}\text{-U}_{80}$ spliceosome, but not the corresponding R_p -phosphorothioate modification ($\text{U6}/\text{R}_\text{p}\text{-U}_{80}$) spliceosome. Furthermore, increasing concentrations of Mg^{2+} effectively compete to inhibit the Mn^{2+} reaction, halting the activity of the $\text{U6}/\text{S}_\text{p}\text{-U}_{80}$ spliceosome. These results demonstrate that the conformation of the S_p -phosphorothioate-modified spliceosome is not grossly altered and offer strong evidence for an essential RNA–metal interaction, further strengthening the hypothesis that the spliceosome may be a ribozyme.

However, interpretation of most chemical modification-interference experiments relies on the assumption that the modification does not structurally perturb functionally important regions of the molecule. In the catalytically active spliceosome, nucleotide U_{80} is located at the internal loop of the highly conserved intramolecular stem loop (ISL) of U6 RNA (Fig. 1). Recent structural studies indicate that the isolated U6-ISL RNA binds metal ions at the U_{80} S_p position in a stereospecific manner similar to the intact spliceosome (Huppler et al. 2002). However, it could be argued that a sulfur substitution in the internal loop region of the U6 ISL might alter the RNA structure and/or perturb its thermodynamic stability, in which case metal binding to the phosphorothioate may not report an essential metal ion interaction within the native spliceosome.

Previously it was demonstrated that multiple R_p -phosphorothioate modifications substantially altered the confor-

mation of an RNA hairpin containing the binding site for phage MS2 capsid protein (Smith and Nikonowicz 2000). Other crystallography and NMR (nuclear magnetic resonance) studies involving DNA and DNA:RNA hybrid duplexes have shown that R_p -phosphorothioate modifications have relatively little effect on structure, except to increase the population of a C3'-endo conformation in adjacent deoxyribose sugars (Cruse et al. 1986; Gonzalez et al. 1994; Bachelin et al. 1998). On the other hand, structural studies of S_p phosphorothioates have been hampered by the fact that they cannot be transcriptionally incorporated, as with the R_p modification. Hence S_p -phosphorothioate-substituted RNA must be synthesized chemically as a mixture of diastereomers and purified, making preparative-scale ^{13}C , ^{15}N labeling for NMR either problematic or cost prohibitive. Because of the above reasons, no complete structural data exist for S_p -phosphorothioate-containing RNAs.

To address whether the S_p -phosphorothioate substitution at U_{80} alters the conformation of the U6-ISL RNA, we have used UV and NMR spectroscopy to determine the thermodynamic properties and solution structure of the U_{80} S_p -phosphorothioate-substituted U6 ISL (hereafter referred to as S_p -ISL; Fig. 1), aided by the measurement of ^1H – ^{13}C residual dipolar couplings (RDCs) at natural ^{13}C abundance. To sufficiently determine the impact of the S_p -phosphorothioate substitution on both the local and long-range structure of the U6 ISL, we have also refined the unmodified U6-ISL structure (hereafter referred to as the WT-ISL; Huppler et al. 2002) using residual dipolar couplings. The thermodynamic stabilities of the unmodified and S_p -phosphorothioate-substituted U6-ISL RNAs were determined using temperature-controlled UV spectroscopy. These results indicate that the S_p phosphorothioate is structurally and thermodynamically benign, and further validate the use of phosphorothioate experiments to study essential metal ion interactions within the U6-ISL RNA and, potentially, other systems.

RESULTS

Thermodynamic comparison of S_p -ISL and WT-ISL RNA

The secondary structure of the U6 ISL, representing nucleotides 62–85 of *Saccharomyces cerevisiae* U6 RNA, is shown in Figure 1. Both wild-type (WT) and S_p -ISL RNAs incorporate an A_{62}G substitution, which has no effect on the growth rate of yeast at 30°C (Fortner et al. 1994) and no effect on the overall structure of the ISL (Huppler et al. 2002) but allows for optimal in vitro transcription conditions using T7 RNA polymerase.

The thermodynamic stabilities of the WT- and S_p -ISL RNAs were analyzed at pH 5.5 and pH 7.5 by temperature-dependent UV spectroscopy (Fig. 2). The T_m values of 65.8°C and 65.9°C at pH 5.5, and 63.3°C and 63.4°C at pH

FIGURE 1. Secondary structure of the U6 ISL RNA representing nucleotides 62–85 from *Saccharomyces cerevisiae*. The position of the phosphorothioate modification for the S_p -ISL construct is expanded, and the A_{62}G substitution is boxed.

FIGURE 2. Thermal stability data for the S_p - and WT-ISLs. Open circles and triangles represent S_p RNA, and closed circles and triangles represent WT RNA at pH 5.5 and pH 7.5, respectively. Data were collected in duplicate at a wavelength of 260 nm. Both pH 5.5 and pH 7.5 samples contain 1 μ M RNA, 10 mM sodium phosphate buffer, and 200 mM KCl. The curve fits to the data are indicated by solid lines. The thermodynamic parameters and melting temperature (T_m) obtained from the curve fitting from each experiment are summarized in Table 1.

7.5 were determined for the WT and S_p -ISL RNAs, respectively (Table 1). The 2.5°C higher T_m values at pH 5.5 can be assigned to protonation of the N1 atom of A₇₉ to form the C₆₇–A₇₉ wobble pair, which has an apparent pK_a of 6.5 and contributes one hydrogen bond to the internal loop structure when protonated (Huppler et al. 2002). By fitting the UV melting data, the free energy gained by protonation ($\Delta\Delta G$) is estimated to be -0.7 ± 0.3 kcal/mole (Table 1). The UV-melting curves indicate that thermodynamic stability and adenine protonation remain unchanged for the WT- and S_p -ISL RNAs, and that the S_p -ISL is thermodynamically indistinguishable from wild type.

NMR analysis of WT- and S_p -ISL RNAs

A comparison of the WT- and S_p -ISL 1D ^1H NMR imino spectra show no apparent alteration for the exchangeable protons (Fig. 3). The high degree of similarity in these spectra indicates that both stem-loop structures contain identical base pairings, with two helices separated by an internal

FIGURE 3. The 600-MHz 1D ^1H NMR spectra of the imino region. Peak assignments are indicated. The WT-ISL spectrum was acquired at pH 7.0, 10°C, 50 mM NaCl, and 1 mM RNA. The S_p -ISL spectrum was acquired at pH 6.8, 10°C, 50 mM NaCl, and 1 mM RNA.

loop and a pentaloop G₇₁–A₇₅ base pair, as previously observed (Huppler et al. 2002). This implies that the overall fold is similar for both RNAs.

Interproton distance and dihedral angle information for nonexchangeable resonances was obtained with 2D ^1H – ^1H NOESY (nuclear Overhauser effect spectroscopy) and 2D ^1H – ^1H TOCSY (total correlation spectroscopy) NMR experiments. The chemical shifts of proton and carbon resonances differ only slightly between both RNA molecules and primarily occur in the internal loop region (nucleotides 68, 69, 79–81; Figs. 4, 5). Small changes in proton chemical shifts for the phosphorothioate-adjacent nucleotides A₇₉ and U₈₀ are observed, which may reflect the different chemical and electrostatic properties introduced by the sul-

FIGURE 4. The 750-MHz 2D ^1H – ^1H TOCSY spectra (40 msec mixing time) comparing H1'–H2' and H1'–H3' ribose proton correlations between the WT-ISL (red; 1.5 mM RNA at pH 6.2, 50 mM NaCl) and the S_p -ISL (blue; 1 mM RNA at pH 6.2, 50 mM NaCl) at 30°C. Unless otherwise noted, all resonances indicate an H1'–H2' correlation.

TABLE 1. Thermodynamic parameters of wild-type (WT) and S_p -ISL RNAs derived from temperature-dependent UV spectroscopy

RNA	pH = 5.5	pH = 7.5	$\Delta\Delta G_{\text{pH } 5.5-7.5}$ (± 0.30 kcal/mole)
	T_m (°C) (± 0.05)	T_m (°C) (± 0.05)	
WT	65.9	63.3	–0.7
S_p	65.8	63.4	–0.7

FIGURE 5. (A) Representative HSQC spectrum and chemical shift comparison between the WT-ISL and the S_p -ISL. Natural abundance ^1H - ^{13}C HSQC experiment of the C1' (89–94 ppm), cytosine C5 (96–98 ppm), and uridine C5 (103–106 ppm) regions. The WT-ISL correlations are red, and the S_p -ISL correlations are blue. The pH is 6.2 ± 0.1 for both RNA samples. (B) Observed changes in chemical shift (Δ ppm) upon S_p -phosphorothioate substitution 5' to nucleotide U₈₀. Changes are plotted by residue number and include: C1' and H1' (black), C6/8 and H6/8 (white), and C5/2 and H5/2 (pink) resonances. Changes in chemical shift were calculated from the equation $\Delta \text{ ppm} = [(\Delta ^1\text{H ppm})^2 + (\Delta ^{13}\text{C ppm} - \alpha C)^2]^{0.5}$, where Δ ppm is the difference in parts per million between the chemical shifts of the WT-ISL and the S_p -ISL, and αC is the scaling factor to normalize the magnitude of the carbon chemical shift changes relative to the proton scale (Butcher et al. 2000). The αC correction factors were 0.14, 0.16, and 0.32 for C1', C6/8, and C5/2, respectively.

fur atom and possibly conformational differences. To address whether the phosphorothioate modification affects the local conformation of the backbone, the ribose sugar puckers were analyzed with a 2D ^1H - ^1H TOCSY experiment (Fig. 4). Sugar puckers that are in the S-type range (including C2'-endo) or undergo conformational averaging give rise to intraribose H1'-H2' correlations in TOCSY spectra, whereas C3'-endo sugar pucker conformations do not. The same set of ribose correlations is observed for the WT- and S_p -ISL RNAs in the TOCSY experiment (Fig. 4). These include the terminal nucleotides G₆₂ and C₈₅, A₇₉-G₈₁, and the pentaloop nucleotides 73–75. Furthermore, the peak volumes for the correlations of the WT- and S_p -ISL RNAs are nearly identical in this experiment, indicating that the S_p phosphorothioate does not alter the sugar puckers of the adjacent ribose groups. The ribose sugar of A₇₉ is closest to the phosphorothioate modification, and the A₇₉ H3' proton displays the largest change in proton chemical shift as a result of the phosphorothioate modification (Fig. 4). In both S_p - and WT-ISL RNAs, the presence of a strong H1'-H2' and weak H1'-H3' coupling for the A₇₉ ribose indicates a sugar pucker in the S-type range (Fig. 4), which was restrained to $145^\circ \pm 30^\circ$ in the structure calculations. On the other hand, U₈₀ displays intermediate H1'-H2' couplings,

indicating that the U₈₀ sugar pucker undergoes conformational averaging in both molecules (Fig. 4). Therefore, the U₈₀ sugar pucker was unrestrained for all structure calculations.

The 2D ^1H - ^{13}C HSQC (heteronuclear single quantum correlation spectroscopy) spectra of both RNAs at natural ^{13}C abundance indicate that, despite small changes in the A₇₉ and U₈₀ chemical shifts, the overall chemical shift differences between the S_p - (blue) and the WT-ISL (red) are very minor (Fig. 5A). The small change in chemical shift for the terminal G₆₂ and C₈₅ resonances is likely to be caused by the lack of a phosphate at the 5' end of the S_p -ISL, which is present on the transcriptionally produced WT-ISL (Fig. 5B). The 2D ^1H - ^1H NOESY data at different mixing times (75, 150, 250, and 300 msec) reveal no detectable differences in NOE (nuclear Overhauser effect) patterns for the two molecules (data not shown). The 2D ^1H - ^{31}P HETCOR (heteronuclear correlation spectroscopy) and 1D ^{31}P NMR experiments also reveal no detectable difference in ^{31}P chemical shifts, aside from the ~60-ppm change in chemical shift for the U₈₀ phosphorothioate (data not shown).

For the WT- and S_p -ISL RNAs, 31 and 40 ^1H - ^{13}C residual dipolar couplings (RDCs) were measured, respectively (Table 2), and incorporated into the structure calcu-

TABLE 2. Structural statistics for the U6 ISL and S_p -ISL

	U6 ISL		S_p -ISL	
	With dipolar coupling data	Without dipolar coupling data ^a	With dipolar coupling data	Without dipolar coupling data ^a
Structures				
Accepted	20	40	17	17
Calculated	100	200	100	100
NOE-derived distance restraints (per nucleotide)	506 (21.1)	521 (21.7)	322 (13.4)	322 (13.4)
Dihedral restraints	145	145	144	144
Hydrogen-bond restraints	25	25	25	25
Dipolar coupling restraints	31	—	40	—
RMSD, all heavy atoms to the mean structure (Å)				
Overall (62–71, 73, 75–85)	0.79 ± 0.30^b	1.46 ± 0.4	1.05 ± 0.3^b	2.14 ± 0.8
Internal loop (66–68, 78–81)	0.46 ± 0.2^c	0.44 ± 0.3	0.49 ± 0.2^c	0.58 ± 0.4
NOE violations (>0.5 Å)	0	0	0	0
Ave. dihedral violations ($>5.0^\circ$)	0	0	0	0
Ave. NOE RMSD (Å)	0.027	0.018	0.029	0.041
Ave. dihedral RMSD ($^\circ$)	0.640	1.270	0.710	0.761
Ave. RDC RMSD (Hz)	2.38 ± 0.11	—	2.35 ± 0.16	—

^aPreviously determined (Huppler et al. 2002).^bThe RMSD for all heavy atoms excluding bulged nucleotides (62–71, 73, 75–85) is 1.16 ± 0.4 Å between U6 ISL and S_p -ISL RNAs.^cThe internal loop (66–68, 78–81) RMSD for all heavy atoms is 0.55 ± 0.3 Å between U6 ISL and S_p -ISL RNAs.

lations. Residual dipolar couplings were measured at ^{13}C natural abundance for the S_p -ISL RNA, and these were critical for the structure determination, because only a limited NOE data set could be collected without an isotopically enriched sample (Table 2). Inclusion of the natural abundance RDC data increased the precision of the S_p -ISL structure from an RMSD (over all heavy atoms) of 2.14 ± 0.8 Å to 1.05 ± 0.3 Å (Table 2). Figure 6 demonstrates the excellent agreement between the experimentally derived and back-calculated RDCs for the two sets of structure calculations.

FIGURE 6. Comparison of all back-calculated (vertical) and experimental (horizontal) RDC values for the S_p -ISL (circles) and U6 ISL (triangles) for the lowest energy structures. The straight line indicates experimental = calculated. Correlation coefficients (R^2) of 0.96 and 0.93 were obtained for the S_p -ISL and WT-ISL, respectively.

Significantly more NOEs were assigned for the WT- than the S_p -ISL (506 vs. 322), which is reflected in the slightly lower RMSD of 0.79 ± 0.3 Å obtained for the WT-ISL (Table 2).

Superimposition of the S_p and WT-ISL structures reflects similarities in the NMR spectra (Figs. 4, 5) and reveals that the difference between the structure ensembles is close to the RMSD values for the separate ensembles. The 10 lowest energy structures from both the S_p and WT ensembles have a pairwise RMSD value to each other of 1.16 ± 0.4 Å (Table 2; Fig. 7). Both NMR ensembles adopt a near-A-helical form and consist of two helices, separated by an internal loop composed of one unpaired residue (U_{80}), capped by a GNRA-tetraloop-like conformation (Jucker et al. 1996). The S_p -ISL retains the previously observed GNR(N)A pentaloop conformation, with the U_{74} residue bulged out of the loop (Huppler et al. 2002). Additionally, both structures contain a readily protonated $C_{67}^+A_{79}$ wobble pair adjacent to an unpaired but stacked U_{80} nucleotide, also as previously observed (Huppler et al. 2002).

In the internal loop region, previous studies determined that stereoselective metal ion binding is modulated by the protonation state of the N1 adenine in the C–A wobble pair adjacent to nucleotide U_{80} (Huppler et al. 2002). The S_p -ISL RNA displays pH-dependent proton chemical shift changes within the internal loop that are similar to the WT-ISL at pH 6.2 and pH 7.5 (data not shown). The primary chemical shift differences caused by the phosphorothioate substitution in the HSQC and NOE data occur at pH 7.0 and reside around nucleotides U_{80} , A_{79} , and C_{67} (Fig. 5B). An average

FIGURE 7. Stereo view of the NMR solution structure ensemble for the S_p -ISL (A) and the WT-ISL (B). The 17 S_p -ISL and 20 U6 ISL structures represent the lowest energy structures from the structure calculations that incorporated residual dipolar couplings. The phosphate backbone is designated by the gray ribbon, guanosines are green, uridines are yellow, cytidines are blue, and adenosines are red. The RMSD over all heavy atoms is 1.05 ± 0.3 Å for the S_p -ISL (A) and 0.79 ± 0.3 Å for the WT-ISL (B). Superimposition of the 10 lowest energy structures from each ensemble (C), with S_p -ISL in blue and the U6 ISL in aquamarine, gives a direct comparison across both structures. The RMSD over all heavy atoms for these 20 structures is 1.16 ± 0.4 Å.

chemical shift change of roughly 0.20 ppm for resonances of these nucleotides indicates that a sulfur substitution does affect the chemical environment of the internal loop. How-

ever, similarities observed in backbone and ribose chemical shift and coupling information obtained from 2D ^1H - ^1H TOCSY, ^1H - ^{13}C HSQC, and ^1H - ^{31}P HETCOR NMR experiments (Figs. 4, 5; data not shown) indicate that the ISL RNA is able to accommodate an S_p -phosphorothioate substitution with no substantial change in conformation. Figure 8 reveals this through the superposition of the internal loop region (66–68, 78–81) for the S_p - and WT-ISL structures, with a local RMSD of 0.55 ± 0.3 Å. The pro- S_p oxygen atom (red) and S_p sulfur atom (gold) of U_{80} for both ensembles is directed outward toward the minor groove.

DISCUSSION

We have demonstrated that the WT- and S_p -ISL structures are substantially improved by the inclusion of the RDC data, as evident by the significantly lower RMSD values (Table 2). Large long-range structural differences are observed between the previous U6 ISL structures determined without RDC data (Huppler et al. 2002) and the structures reported here (Table 2). The long-range structural differences are particularly evident when the structures are superimposed over only the first five base pairs (Fig. 9). Whereas both structures contain the same base pairs and hydrogen bonds, the earlier structures lack the long-range order of the structures calculated with RDCs, similar to what has been observed for other RNAs (Sibille et al. 2001). Additionally, we notice that the entire ensemble of structures calculated with RDCs is much more A-form over the entire helix length, whereas the earlier ensemble does not occupy the same conformational space (Fig. 9). This result indicates that the ensemble of RNA structures calculated without RDC data is trapped in local conformational energy minima during the restrained molecular dynamic simulations, leading to significant deviations away from A-form geometry. Therefore, RDC restraints are likely to be critical for determining accurate nucleic acid solution structures.

Although fundamental chemical differences between phosphates and phosphorothioates can potentially lead to significant structural and electrostatic differences, careful interpretation of phosphorothioate-modification interference experiments has been instrumental in dissecting mechanism at the molecular level. We have demonstrated that, in certain structural contexts, a phosphorothioate substitution does not alter the overall conformation of the RNA (Figs. 7, 8). In addition to cross-validating the mainly NOE-based structure, residual dipolar couplings provide local and long-range order for the S_p - and WT-ISL solution structures, enabling a direct comparison to be made at the internal loop region and across the overall molecule. The substitution of a pro- S_p phosphoryl oxygen to a sulfur atom 5' to nucleotide U_{80} does not significantly alter the thermodynamic stability of the RNA.

The previous structure determination of the U6 ISL in the absence of RDC data (Huppler et al. 2002) accurately

FIGURE 8. Stereo superimposition of the internal loop region of the S_P -ISL structures (blue) and 10 WT-ISL structures (aquamarine). The S1P and O1P atoms 5' to nucleotide U₈₀ are gold and red, respectively. (A) Stereo superimposition of the internal loop region of the 10 lowest energy S_P -ISL and WT-ISL structures. The RMSD for all heavy atoms is 0.55 ± 0.3 Å between the internal loop (66–68, 78–81) of the U6 ISL and the S_P -ISL. (B) Stereo superimposition of the internal loop region from the single lowest energy S_P -ISL and WT-ISL structures.

defined base-pair geometries but could not precisely define the position of the U₈₀ pro- S_P oxygen. The precision of the WT- and S_P -ISL structures reported here enable us to infer how the ISL might participate in a tertiary interaction. We observe that the pro- S_P nonbridging phosphate oxygen is freely accessible in solution and angled toward the minor groove, whereas the pro- R_P position is located within the major groove, which is less accessible. Thus, it is possible that docking of the U6 ISL through its minor groove would readily position the U₈₀ pro- S_P phosphate oxygen for a tertiary interaction that is modulated or stabilized by the essential divalent metal ion (Yean et al. 2000).

MATERIALS AND METHODS

RNA synthesis and purification

The U6 ISL with the A₆₂G substitution was transcribed in vitro using purified His₆-tagged T7 RNA polymerase and synthetic DNA oligonucleotides (Integrated DNA technologies, Inc.) as previously described (Milligan and Uhlenbeck 1989; Huppler et al. 2002). All chemical reagents and unlabeled nucleoside triphosphates (NTPs) except the His₆-tagged T7 RNA polymerase were purchased from Sigma Chemical. Phosphorothioate-containing RNAs (Dharmacon, Inc.) were deprotected according to the manufacturer's instructions. The U6 ISL phosphorothioate diastereomers were separated by HPLC purification using a 4.6×250 -mm C18 reverse phase Adsorbosphere HS packed column (Alltech, Inc.). The column was equilibrated with 0.1 M ammonium

acetate (pH 6.8); 0.3 μ mole was the maximum amount of RNA that could be injected onto the column at one time, and a shallow 0%–10% acetonitrile elution gradient allowed for baseline separation between the diastereomers. The corresponding fractions were pooled, ethanol-precipitated, and desalted on a 15-mL G-25 gel filtration column. The purity of the isolated diastereomers was assessed by HPLC and 31 P NMR spectroscopy to be $\geq 95\%$ in all cases. Diastereomer assignments were made by analysis with snake venom phosphodiesterase (Slim and Gait 1991).

UV spectroscopy

Thermal stability studies were conducted on purified U6 ISL and S_P -ISL RNAs using a Cary Model 1 Bio UV-visible spectrophotometer equipped with a Peltier heating accessory and temperature probe. All samples at pH 5.5 and pH 7.5 contained 10 mM sodium phosphate buffer, 200 mM KCl, and ~ 1 μ M RNA. Samples were heated to 90°C and cooled to 20°C at a rate of 2°C/min while absorbance data were collected at 260 nm in 1°C increments. Two scans were taken for each RNA. Thermodynamic values and

transition temperatures (T_m) were calculated from normalized data using SigmaPlot v8.0 (SPSS Science). For the process of unfolding a stem-loop composed of a single RNA molecule ($F \rightarrow U$), where $\Delta C_p = 0$:

$$K = U/F = \theta/(1 - \theta) = \exp(-\Delta H/RT + \Delta S/R)$$

and

$$\Delta S = \Delta H/T_m,$$

where θ is the fraction of RNA in the unfolded state, R is the gas constant, and T is the temperature in kelvin. To fit a curve to the absorbance data for the U6 ISL and S_P -ISL RNAs, the pretransitional and posttransitional baselines of each thermal stability curve were first determined by a linear curve fit. These baselines were used to find the fraction of unfolded RNA as a function of temperature and subsequently the values of ΔH , ΔS , and T_m . The free energy difference ($\Delta\Delta G$) of each RNA at both pH values was calculated from their ΔH and ΔS values (Table 1).

NMR spectroscopy

All spectra were acquired at the National Magnetic Resonance Facility at Madison (NMRFAM) on Bruker DMX 400, 500, 600, and 750 MHz spectrometers. Natural abundance data collection was performed using a cold (cryoprobe, Bruker) single Z-axis gradient HCN probe and a Bruker QNP (quadrupole nucleus probe) tuned to phosphorous with a Z-axis gradient. All NMR data were processed with XWINNMR (Bruker) and analyzed with Felix-98 (MSI) and the NMR assignment program Sparky (<http://www.cgl.ucsf.edu/home/sparky/>).

Imino proton resonances were assigned by reference to a 2D NOESY (150 msec mixing time) in 90% H₂O/10% D₂O at 283 K.

FIGURE 9. Superimposition of an ideal A-form helix (green) with the 10 lowest-energy +RDCs (blue) and -RDCs (red) S_p -thiosubstituted ISL RNA structures fit over the lower helix. The A-form RNA model duplex has the sequence r(GGUUCAACCU/AGGUUGAACCC), which is identical in its first five base pairs to the U6 ISL sequence.

A 1–1 spin-echo pulse sequence water suppression scheme was performed for all samples in 90% H_2O /10% D_2O . Nonexchangeable resonances were assigned by reference to 2D 1H – 1H NOESY spectra (75, 150, 250, and 300 msec mixing times), and 2D 1H – 1H TOCSY, 1H – ^{13}C HSQC, and 1H – ^{31}P HETCOR spectra were collected in 99.99% D_2O at 303 K. The 1D ^{31}P spectra were acquired at 202 MHz (500 MHz 1H) with a 5-mm quadrupole nucleus probe (QNP, Bruker). For all experiments in 99.99% D_2O , the HDO resonance was suppressed using a low-power presaturation pulse. All NMR experiments for the U6 ISL and the S_p -ISL RNA contained 0.8–1.5 mM RNA and 50 mM NaCl at pH 6.2, pH 7.0, or pH 7.5. All 1H , ^{13}C heteronuclear spectra for the S_p -ISL were taken at natural abundance, and the U6 ISL was assigned using standard homo- and heteronuclear methods (Dieckmann and Feigon 1997).

Partially aligned samples contained 17 mg/mL Pf1 filamentous bacteriophage (ASLA Ltd.) and allowed for measurable internuclear residual dipolar couplings for 1H – ^{13}C aromatic and sugar resonances (Hansen et al. 2000). Natural abundance 1H – ^{13}C HSQC spectra enabled the measurement (± 2.0 Hz) of RDCs in the proton (F2) and carbon (F1) dimensions for the U6 ISL and S_p -ISL samples. Final sample conditions before and after the addition of the phage were 0.8–1.0 mM RNA in 300 μ L (pH 7.4), using a thin-wall Shigemi microcell (Shigemi Inc.).

Distance, torsion angle, and RDC constraints

Assigned NOE peak volumes were fit and integrated using the Gaussian peak-fitting function in Sparky. To assess the relative

intensity of the NOE, distances were calibrated by setting the average integrated volume of the pyrimidine H5–H6 NOEs to 2.4 Å, using the r^{-6} distance relationship and the CALIBA macro in DYANA (Güntert et al. 1997). This calibration was then used to group NOEs into three classes: strong (1.8–3.0 Å), medium (1.8–4.5 Å), and weak (3.0–6.0 Å). NOE distances for exchangeable protons were qualitatively assigned to one of these three classes.

1H – ^{31}P HETCOR and 1D ^{31}P reveal that all ^{31}P chemical shifts reside between –3 and –5 ppm, except for the S_p and R_p diastereomers (59.5 and 57.5 ppm, respectively). Therefore, α and ζ torsion angles were set to exclude the *trans* range ($0^\circ \pm 120^\circ$; Allain and Varani 1995). Except for the S_p diastereomer, it was observed that no phosphate resonances differ by more than 0.2 ppm between the U6 ISL and the S_p sulfur-substituted RNA. Nucleotides with strong H1'–H2' couplings (C_{72} , U_{74} , A_{79}), as observed in a 40-msec mixing time 2D 1H – 1H TOCSY (Fig. 4), were constrained as C2'-endo (S-type; $145^\circ \pm 30^\circ$), whereas all other nucleotides were either constrained as C3'-endo or unrestrained. Intranucleotide H1' to aromatic NOEs from a 75-msec 2D NOESY indicated that all nucleotides fell into the *anti* range, and were thus constrained with a χ value of $-160^\circ \pm 20^\circ$. χ values of nucleotides A_{79} and U_{80} were left unrestrained. Other backbone torsion angles (β , γ , ϵ) were set to standard A-form values ($\pm 40^\circ$) only in the helical regions of the structure known to be A-form Watson-Crick helices from NOE and dihedral information.

Analysis of residual dipolar couplings was performed using XWINNMR (Bruker) software by measuring the difference between the 1H and ^{13}C coupling for isotropic and partially aligned samples. The values of the axial (D_a) and rhombic (R) components of the alignment tensor were determined by analyzing the powder pattern distribution for each experimental RDC data set and further refined by following a grid search procedure that calculates a series of structures with different rhombicities (Clare et al. 1998a,b). In addition, prediction of the alignment tensor from the converged, lowest energy structures in the presence and absence of RDCs was estimated by PALES (Zweckstetter and Bax 2000; <http://spin.niddk.nih.gov/bax/software/PALES>). An axial component (D_a) of –14.0 Hz and a rhombic component of 0.22 were found to be the experimentally determined values for the S_p -ISL structures in the CNS structure calculations. Similarly, a D_a value of –14.0 Hz and an R value of 0.21 were determined for the refined U6 ISL RDC structure. These final values reflect the best agreement between the structure and the RDCs data set, as observed through the low-energy, no violation, and overall convergence rate of the structure calculation. The experimental RDCs from the final converged structures were plotted against the back-calculated predicted RDC values from CNS and were found to be in good agreement (Fig. 6).

Structure calculation and refinement

For the sulfur-substituted U_{80} nucleotide, a separate residue (SSU) was created in the topology files and was identical to the uridine (URI) residue with the exception of the pro- S_p atom at the O1P phosphoryl oxygen position, which was named S1P to represent the S_p sulfur substitution. The U_{80} S1P atom was created to be structurally distinct from that of the R_p phosphoryl oxygen atom (O2P), in the following manner: the P–S bond was set to 1.80 Å, and the van der Waals radius of the sulfur atom to 1.90 Å. This allows the sulfur to project outward almost 0.8 Å more than the

oxygen (Saenger 1984). The charge distribution parameters for the O2P atom (-0.865 esu) and the S1P atom (-0.772 esu) for SSU were set to previously determined potentials (Jaroszewski et al. 1992). This enabled the incorporation of a sulfur atom into the topology and parameter files and generated a distinct structure file for the S_p -ISL RNA.

CNS 1.1 (Brunger et al. 1998; <http://cns.csb.yale.edu/v1.1>) was used to calculate structures using NOE distances, dihedral restraints, and residual dipolar couplings. An improved version of CNS was implemented that includes an improved harmonic potential that corrects the susceptibility anisotropy (RDC) protocol (Warren and Moore 2001). For the CNS structure calculations, an extended, unfolded structure was initially generated, from which 100 starting structures were calculated from random initial velocities. An initial 60 psec of restrained molecular dynamics (MD) in torsion angle space, with 15-fsec time steps, was followed by a 90-psec slow-cooling process for initial structures. At the final stage of the calculation, 30 psec (with 5-fsec time steps) of restrained molecular dynamics was performed in Cartesian coordinate space. After this initial calculation, structures were evaluated for convergence and accepted on the basis of their overall energy potentials and the number of NOE (>0.5 Å) and dihedral ($>5.0^\circ$) violations. To observe the effect of RDCs, the S_p structure calculations were also performed in the absence of RDCs.

The lowest-energy structures were selected and subjected to a gentle refinement (1 psec of restrained molecular dynamics at 300 K) in XPLOR v. 3.843 (Brunger 1992). The purpose of this refinement was to maintain the proper chirality of phosphate oxygens and amino protons, which can be reversed in Cartesian space refinement protocols (Schultze and Feigon 1997). This refinement also assisted in the planarity of amino functional groups. The final ensemble was comprised of 17 structures for the S_p -ISL structures both in the presence and absence of RDCs. The 20 WT-ISL RDC structures were also determined using this calculation protocol. The software program Insight II (Biosym) was used to generate the idealized A-form helix with the sequence r(GGUUCAACCU-AG GUUGAACCC), which is identical in its first five base pairs to the WT-ISL sequence. All structures were viewed and analyzed using MOLMOL (Koradi et al. 1996).

[Note: Chemical shift data for the S_p -ISL RNA have been deposited in BioMagResBank (<http://www.bmrb.wisc.edu>), entry code 5703. Coordinates for the WT-ISL and S_p -ISL structures calculated with RDCs have been deposited in the Protein Data Bank (<http://www.rcsb.org>), WT accession code 1NYZ S_p accession code 1NZ1.]

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REFERENCES

- Allain, F.H. and Varani, G. 1995. Divalent metal ion binding to a conserved wobble pair defining the upstream site of cleavage of group I self-splicing introns. *Nucleic Acids Res.* **23**: 341–350.
- Bachelin, M., Hessler, G., Kurz, G., Hacia, J.G., Dervan, P.B., and Kessler, H. 1998. Structure of a stereoregular phosphorothioate DNA/RNA duplex. *Nat. Struct. Biol.* **5**: 271–276.
- Brow, D.A. 2002. Allosteric cascade of spliceosome activation. *Annu. Rev. Genet.* **36**: 333–360.
- Brunger, A.T. 1992. *X-PLOR (version 3.1) manual*. Yale University Press, New Haven, CT.
- Brunger, A.T., Adams, P.D., Clore, G.M., Delano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.-S., Kuszewski, J., Nilges, N., Pannu, N.S., et al. 1998. Crystallography and NMR system (CNS): A new software system for macromolecular structure determination. *Acta Cryst. D* **54**: 905–921.
- Burge, C.B., Tuschl, T.A., and Sharp, P.A. 1999. Splicing of precursors to mRNAs by the spliceosomes. In *RNA world* (eds. R.F. Gesteland et al.), Vol. II, pp. 525–560. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Butcher, S.E., Allain, F.H.T., and Feigon, J. 2000. Determination of metal ion binding sites within the hairpin ribozyme domains by NMR. *Biochemistry* **39**: 2174–2182.
- Clore, G.M., Gronenborn, A.M., and Bax, A. 1998a. A robust method for determining the magnitude of the fully asymmetric alignment tensor of oriented macromolecules in the absence of structural information. *J. Magn. Reson.* **133**: 216–221.
- Clore, G.M., Gronenborn, A.M., and Tjandra, N. 1998b. Direct structure refinement against residual dipolar couplings in the presence of rhombicity of unknown magnitude. *J. Magn. Reson.* **131**: 159–162.
- Collins, C.A. and Guthrie, C. 2000. The question remains: Is the spliceosome a ribozyme? *Nat. Struct. Biol.* **7**: 850–854.
- Cruse, W., Salisbury, S., Brown, T., Cosstick, R., Eckstein, F., and Kennard, O. 1986. Chiral phosphorothioate analogues of B-DNA. The crystal structure of Rp-d[Gp(s)CpGp(s)CpGp(s)C]. *J. Mol. Biol.* **192**: 891–905.
- Dieckmann, T. and Feigon, J. 1997. Assignment methodology for larger RNA oligonucleotides: Application to an ATP-binding RNA aptamer. *J. Biomol. NMR* **9**: 259–272.
- Eckstein, F. 1979. Phosphorothioate analogues of nucleotides. *Acc. Chem. Res.* **12**: 204–210.
- Fabrizio, P. and Abelson, J. 1992. Thiophosphates in yeast U6 snRNA specifically affect pre-mRNA splicing in vitro. *Nucleic Acids Res.* **20**: 3659–3664.
- Fortner, D.M., Troy, R.G., and Brow, D.A. 1994. A stem/loop in U6 RNA defines a conformational switch required for pre-mRNA splicing. *Genes & Dev.* **8**: 221–233.
- Gonzalez, C., Stec, W., Kobylanska, A., Hogrefe, R.I., Reynolds, M., and James, T.L. 1994. Structural study of a DNA·RNA hybrid duplex with a chiral phosphorothioate moiety by NMR: Extraction of distance and torsion angle constraints and imino proton exchange rates. *Biochemistry* **33**: 11062–11072.
- Gordon, P.M., Sontheimer, E.J., and Piccirilli, J.A. 2000. Metal ion catalysis during exon-ligation step of nuclear pre-mRNA splicing: Extending the parallels between the spliceosome and group II introns. *RNA* **6**: 199–205.

- Guntert, P., Mumenthaler, C., and Wuthrich, K. 1997. Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* **273**: 283–298.
- Hansen, M.R., Hanson, P., and Pardi, A. 2000. Filamentous bacteriophage for aligning RNA, DNA, and proteins for measurement of nuclear magnetic resonance dipolar coupling interactions. *Methods Enzymol.* **317**: 220–240.
- Huppler, A., Nikstad, L.J., Allmann, A.M., Brow, D.A., and Butcher, S.E. 2002. Metal binding and base ionization in the U6 RNA intramolecular stem-loop structure. *Nat. Struct. Biol.* **9**: 431–435.
- Jaroszewski, J.W., Syi, J.-L., Maizel, J., and Cohen, J.S. 1992. Towards rational design of antisense DNA: Molecular modelling of phosphorothioate DNA analogues. *Anti-Cancer Drug Design* **7**: 253–262.
- Jucker, F.M., Heus, H.A., Yip, P.F., Moors, E.H.M., and Pardi, A. 1996. A network of heterogeneous hydrogen bonds in GNRA tetraloops. *J. Mol. Biol.* **264**: 968–980.
- Koradi, R., Billeter, M., and Wuthrich, K. 1996. MOLMOL: A program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14**: 51–55, 29–32.
- Milligan, J.F. and Uhlenbeck, O.C. 1989. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol.* **180**: 51–62.
- Nilsen, T.W. 1998. RNA–RNA interactions in nuclear pre-mRNA splicing. In *RNA structure and function* (eds. R. Simons and M. Grunberg-Manago), pp. 279–307. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Pecoraro, V.L., Hermes, J.D., and Cleland, W.W. 1984. Stability constants of Mg^{2+} and Cd^{2+} complexes of adenine nucleotides and thionucleotides and rate constants for formation and dissociation of MgATP and MgADP. *Biochemistry* **23**: 5262–5271.
- Saenger, W. 1984. *Principles of nucleic acid structure*. Springer-Verlag, New York.
- Schultze, P. and Feigon, J. 1997. Chirality errors in nucleic structures. *Nature* **387**: 668.
- Sibille, N., Pardi, A., Simorre, J.P., and Blackledge, M. 2001. Refinement of local and long-range structural order in theophylline-binding RNA using ^{13}C – 1H residual dipolar couplings and restrained molecular dynamics. *JACS* **123**: 12135–12146.
- Slim, G. and Gait, M.J. 1991. Configurationally defined phosphorothioate-containing oligoribonucleotides in the study of the mechanism of cleavage of hammerhead ribozymes. *Nucleic Acids Res.* **19**: 1183–1188.
- Smith, J.S. and Nikonowicz, E.P. 2000. Phosphorothioate substitution can substantially alter RNA conformation. *Biochemistry* **39**: 5642–5652.
- Sontheimer, E.J., Sun, S.G., and Piccirilli, J.A. 1997. Metal ion catalysis during splicing of pre-messenger RNA. *Nature* **388**: 801–805.
- Valadkhan, S. and Manley, J.L. 2001. Splicing-related catalysis by protein-free snRNAs. *Nature* **413**: 701–707.
- Warren, J.J. and Moore, P.B. 2001. A maximum likelihood method for determining DaPQ and R for sets of dipolar coupling data. *J. Magn. Reson.* **149**: 271–275.
- Yean, S.L., Wuenschell, G., Termini, J., and Lin, R.J. 2000. Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome. *Nature* **408**: 881–884.
- Yu, Y.T., Maroney, P.A., Darzynkiewicz, E., and Nilsen, T.W. 1995. U6 snRNA function in nuclear pre-mRNA splicing—A phosphorothioate interference analysis of the U6 phosphate backbone. *RNA* **1**: 46–54.
- Zweckstetter, M. and Bax, A. 2000. Prediction of sterically induced alignment in a dilute liquid crystalline phase: Aid to protein structure determination by NMR. *J. Am. Chem. Soc.* **122**: 3791–3792.

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