Dynamics and Metal Ion Binding in the U6 RNA Intramolecular Stem–Loop as Analyzed by NMR

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The U6 RNA intramolecular stem–loop (ISL) is a conserved component of the spliceosome, and contains an essential metal ion binding site centered between a protonated adenine, A79, and U80. Correlated with protonation of A79, U80 undergoes a base-flipping conformational change accompanied by significant helical movement. We have investigated the dynamics of the U6 ISL by analyzing the power dependence of 13C NMR relaxation rates in the rotating frame. The data provide evidence that the conformational transition is centered around an exchange lifetime of 84 μs.

The U80 nucleotide displays low internal mobility on the picosecond time-scale at pH 7.0 but high internal mobility at pH 6.0, in agreement with the global transition resulting in the base of U80 adopting a looped-out conformation with increased dynamic disorder. A kinetic analysis suggests that the conformational change, rather than adenine protonation, is the rate-limiting step in the pathway of the conformational transition. Two nucleotides, U70 and U80, were found from chemical shift perturbation mapping to interact with the magnesium ion, with apparent Kd values in the micromolar to millimolar range. These nucleotides also displayed metal ion-induced elevation of R1 rates, which can be explained by a model that assumes dynamic metal ion coordination concomitant with an induced higher shielding anisotropy for the base 13C nuclei. Addition of Mg2+ shifts the conformational equilibrium toward the high-pH (base-stacked) structure, accompanied by a significant drop in the apparent pK of A79.

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Keywords: U6 snRNA; nuclear magnetic resonance (NMR); 13C relaxation; base-flipping; chemical shift anisotropy

Introduction

A growing body of data suggests that the folding, recognition, and function of RNA may often depend on dynamic conformational transitions.1–3 Folding of individual RNA helical domains, and in some cases tertiary structure formation, can occur on the 10–100 ms scale.4 RNA conformational exchange processes that occur on the sub-millisecond time-scale, however, have only been investigated for a small number of cases.5–9

Previously, we have reported that the U6 spliceosomal RNA intramolecular stem–loop (ISL) displays conformational exchange on the microsecond to millisecond time-scale.10

The spliceosome catalyzes nuclear pre-messenger RNA (pre-mRNA) splicing and consists of a dynamic assembly of five small nuclear RNAs (U1, U2, U4, U5, and U6) and approximately 70 proteins.11 The spliceosome undergoes a large conformational change after assembly, during which base-pairing between U4 and U6 is disrupted, and U2 and U6 associate.11 The association

Abbreviations used: CSA, chemical shielding anisotropy; Δσeff, effective chemical shielding anisotropy; DFT, density-functional theory; Φ, chemical exchange parameter ($Φ=\frac{p_A}{1-p_A}Δ\omega$), where $p_A$ and $p_B$ are the fractional populations of conformations A and B respectively, and $Δ\omega$ is the corresponding chemical shift difference; HSQC, heteronuclear single quantum correlation spectroscopy; ISL, intramolecular stem–loop; ppm, parts per million; $R_1$, longitudinal (spin-lattice) relaxation rate; $R_{1\text{rot}}$, rotating frame relaxation rate; $R_{1\text{rot}}$, rotating frame relaxation rate at infinite spin-lock power; RDC, residual dipolar coupling; $τ_{\text{con}}$, conformational exchange lifetime; $T_1$, longitudinal (spin-lattice) relaxation time; $T_1\text{rot}$, rotating frame relaxation time; TOCSY, total correlation spectroscopy.

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of U2 and U6 leads to formation of a highly conserved ISL structure within the U6 RNA. Metal ion binding at the U80 pro-S oxygen within the U6 ISL is required for splicing. In addition, point mutations (U80G, C67A, or C72A) within the U6 ISL RNA are lethal in yeast. A protein-free RNA preparation of U2 and U6 catalyzes a slow reaction analogous to the first step of splicing. Therefore, the U2 and U6 spliceosomal RNAs may participate directly in splicing catalysis.

We have previously determined the three-dimensional structure of the U6 ISL by NMR spectroscopy (Figure 1) and have investigated its metal binding properties. Metal binding to U80 pro-S oxygen and protonation of an adjacent C67→A79 pair (pKₐ of 6.5) are antagonistic. However, protonation of the C67→A79 wobble pair stabilizes the RNA by contributing an additional 0.7(±0.3) kcal/mol of folding free energy. Recently, we have identified the presence of a pH-dependent conformational equilibrium reaction of the U6 ISL that involves a helical movement of approximately 25° (Figure 1) accompanied by base flipping of U80. Structure refinement against ¹H–¹³C residual dipolar couplings (RDCs) and comparison of the U6 ISL structures at pH 7.0 and pH 5.7 indicate that the U80 nucleotide is in equilibrium between a stacked and a looped-out conformation, with the latter predominating at low pH. The helical movement and the concomitant U80 base flipping equilibrium are correlated with the protonation state of the adjacent A79 base, as well as changes in the sugar puckers of A79 and U80 and the stacking interactions within the internal bulge.

To further investigate the dynamics of the U6 ISL RNA, we have studied the power dependence of ¹³C NMR relaxation rates in the rotating frame (R₁ₑᵣ) under various conditions. Relaxation rates in the rotating frame reflect molecular motions on the microsecond to millisecond time-scale, which may arise from conformational exchange. The exchange lifetime parameter, τₑₓ, represents the time-scale of dynamic processes for a system exchanging between distinct conformational states, and can be extracted from the power dependence of ¹³C R₁ₑᵣ relaxation rates. For RNA labeled uniformly with ¹³C, R₁ₑᵣ measurements allow for accurate determination of transverse relaxation rates without interference from carbon–carbon couplings.

Our analysis of the ¹³C R₁ₑᵣ relaxation data indicates that globally occurring conformational transitions with an average lifetime of 84 µs exist within the U6 ISL RNA structure, in agreement with recent structural evidence for the existence of two states. The dynamics results give further insights into the time-scale of base flipping and helical bending in RNA that distinguish these states.

The location of metal binding sites in RNA can be difficult to identify in solution. Our results reveal that relaxation measurements offer a novel approach for detecting metal–RNA interactions. In the presence of Mg²⁺, the U70 and U80 nucleotides of the U6 ISL exhibit specific increases in longitudinal relaxation rates, which we interpret in terms of dynamic coordination of the metal ion to the O2 or O4 positions of the pyrimidines. Binding of Mg²⁺ at the U80 position and protonation of the A79 N1 imino group have been shown to be mutually antagonistic. Our results on the kinetic behavior of the protonation of A79, the analysis of the pH-dependent dissociation constants of Mg²⁺ binding at U80, and the results on the structural dynamics in the U6 ISL can all be accommodated in the proposed model of a conformational switch mediated by proton uptake, which in turn modulates the binding of an essential metal ion. Therefore, the conformational change in the U6 ISL RNA may potentially act as a switch for regulation of the catalytic activity of the spliceosome.

Results and Discussion

The longitudinal relaxation rate R₁ and the
relaxation rate in the rotating frame $R_{1p}$ were determined for a number of carbon resonances throughout the U6 ISL RNA. Relaxation data were acquired in the absence of Mg$^{2+}$ at pH 7.0, for which a structure is available (PDB 1SY4)\textsuperscript{19} and at pH 6.0. Additional data were obtained from a sample at pH 7.0 containing 3 mM MgCl$_2$. At pH 7.0 and in the absence of MgCl$_2$, the $R_1$ and $R_{1p}$ relaxation rates were determined for all resolved A/G-C8, U/C-C6, A-C2, and C1' resonances for a total of 42 carbon atoms throughout the molecule. At pH 7.0 in the presence of MgCl$_2$ and at pH 6.0

Table 1. Observed longitudinal and rotating frame $^{13}$C relaxation rates for the U6 ISL at 600 MHz

<table>
<thead>
<tr>
<th></th>
<th>$R_1$ (s$^{-1}$)</th>
<th>0.97 kHz</th>
<th>1.4 kHz</th>
<th>2.0 kHz</th>
<th>2.8 kHz</th>
<th>4.0 kHz</th>
<th>5.6 kHz</th>
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<td></td>
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<td>Field 2</td>
<td>Field 1</td>
<td>Field 2</td>
<td>Field 1</td>
<td>Field 2</td>
</tr>
</tbody>
</table>

Rates were determined at pH 7.0 and in the absence of Mg$^{2+}$ unless otherwise stated. $R_{1p}$ rates are reported for each applied $B_0$ field strength, given as $yR_1/2\pi$. The reported $R_{1p}$ rates are offset corrected. The uncertainties in the rates were derived by error propagation from the uncertainties in the observed $R_1$ and $R_{1p}$ rates.
Table 2. Conformational exchange parameters for the U6 ISL derived from $^{13}$C $R_{1p}$ relaxation rates at 600 MHz

<table>
<thead>
<tr>
<th>A. Conformational exchange parameters derived by three-parameter fitting of relaxation rates for individual $^{13}$C resonances</th>
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</thead>
</table>
| **Table 1.** For the U6 ISL at pH 7.0 in the absence of MgCl$_2$, relaxation rates were measured for the C6 resonances of the five uridine bases. The measured relaxation rates determined for the U6 ISL are characteristic for a system with an overall rotational correlation time $\tau_z \approx 4$ ns, thus the contribution from $^{13}$C–$^{13}$C cross-relaxation to the longitudinal relaxation is expected to be small for all sites in the U6 ISL. The application of semi-selective $^{13}$C excitation in the initial INEPT transfer of the $T_1$ experiment serves to further reduce the complicating effects of $^{13}$C–$^{13}$C cross-relaxation. A detailed analysis of the U-C6 $T_1$ data showed that the intensity of the U-C5 carbon signal to be below 10% of the intensity of the corresponding detected U-C6 carbon signal in all cases. This implies an amount of C5 carbon magnetization at the beginning of the relaxation delay that will ensure a minimal contribution from $^{13}$C–$^{13}$C cross-relaxation to the $T_1$ measurements. The relaxation decays were fitted to mono-exponential functions, with none of the fits indicating deviation from the model; we thus conclude that contributions to the extracted longitudinal relaxation rates from cross-relaxation are insignificant (less than 2%).

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**Evidence for conformational exchange**

Longitudinal relaxation rates and offset-corrected rotating frame relaxation rates are presented in Table 1. For the U6 ISL at pH 7.0 in the absence of Mg$^{2+}$, the $R_{1p}$ rates from several signals displayed a $B_1$ power dependence indicative of molecular exchange processes in the microsecond to millisecond time range. For 11 of these resonances the exchange parameters were derived from a three-parameter fit of the experimental data to equation (3) (see Materials and Methods). The $R_{1p}$ rates of an additional 11 signals showed visible signs of a spin-lock power dependence, although the data did not allow for simultaneous determination of all three exchange parameters. The results for the fitted resonances are given in Table 2A with examples of the fits shown in Figure 2. The resonances included in Table 2A all display significant exchange contributions, as reported by the relaxation dispersion parameter $\Phi$. We also note that the standard errors of the fitted parameters were sufficiently high in some cases to render the fitted exchange lifetime virtually undetermined. Although this derives, in part, from the low precision of some of the included relaxation rates, the errors are higher than those reported in comparable conformational exchange studies. We attribute this difference to our use of a three-parameter ($R_{1p}^*, \tau_{ex}, \Phi$) fitting procedure as opposed to the widely used two-parameter ($\tau_{ex}$ and $\Phi$) approach. By assuming that the $R_{1p}^*$ parameter can be accurately and independently determined, the two-parameter fit will yield $\tau_{ex}$ and $\Phi$ parameters of seemingly smaller uncertainty. The apparent dramatic improvement in the precision of these fits is a direct result of a high correlation (typically $\geq 0.9$) of the two fitting parameters $\tau_{ex}$ and $R_{1p}^*$ in the expression for the exchange contribution, equation (3). As demonstrated in

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without added MgCl$_2$, relaxation rates were measured for the C6 resonances of the five uridine bases.

The measured relaxation rates determined for the U6 ISL are characteristic for a system with an overall rotational correlation time $\tau_z \approx 4$ ns, thus the contribution from $^{13}$C–$^{13}$C cross-relaxation to the longitudinal relaxation is expected to be small for all sites in the U6 ISL. The application of semi-selective $^{13}$C excitation in the initial INEPT transfer of the $T_1$ experiment serves to further reduce the complicating effects of $^{13}$C–$^{13}$C cross-relaxation. A detailed analysis of the U-C6 $T_1$ data showed that the intensity of the U-C5 carbon signal to be below 10% of the intensity of the corresponding detected U-C6 carbon signal in all cases. This implies an amount of C5 carbon magnetization at the beginning of the relaxation delay that will ensure a minimal contribution from $^{13}$C–$^{13}$C cross-relaxation to the $T_1$ measurements. The relaxation decays were fitted to mono-exponential functions, with none of the fits indicating deviation from the model; we thus conclude that contributions to the extracted longitudinal relaxation rates from cross-relaxation are insignificant (less than 2%).

**Evidence for conformational exchange**

Longitudinal relaxation rates and offset-corrected rotating frame relaxation rates are presented in Table 1. For the U6 ISL at pH 7.0 in the absence of Mg$^{2+}$, the $R_{1p}$ rates from several signals displayed a $B_1$ power dependence indicative of molecular exchange processes in the microsecond to millisecond time range. For 11 of these resonances the exchange parameters were derived from a three-parameter fit of the experimental data to equation (3) (see Materials and Methods). The $R_{1p}$ rates of an additional 11 signals showed visible signs of a spin-lock power dependence, although the data did not allow for simultaneous determination of all three exchange parameters. The results for the fitted resonances are given in Table 2A with examples of the fits shown in Figure 2. The resonances included in Table 2A all display significant exchange contributions, as reported by the relaxation dispersion parameter $\Phi$. We also note that the standard errors of the fitted parameters were sufficiently high in some cases to render the fitted exchange lifetime virtually undetermined. Although this derives, in part, from the low precision of some of the included relaxation rates, the errors are higher than those reported in comparable conformational exchange studies. We attribute this difference to our use of a three-parameter ($R_{1p}^*, \tau_{ex}, \Phi$) fitting procedure as opposed to the widely used two-parameter ($\tau_{ex}$ and $\Phi$) approach. By assuming that the $R_{1p}^*$ parameter can be accurately and independently determined, the two-parameter fit will yield $\tau_{ex}$ and $\Phi$ parameters of seemingly smaller uncertainty. The apparent dramatic improvement in the precision of these fits is a direct result of a high correlation (typically $\geq 0.9$) of the two fitting parameters $\tau_{ex}$ and $R_{1p}^*$ in the expression for the exchange contribution, equation (3). As demonstrated in
the Supplementary Data, the precision of the exchange lifetime parameter $t_{ex}$ for the resonances included in Table 2A will “improve” dramatically by assuming a fixed value of $R_{1r}^{N1}$: a lowering of the standard error by 2–5 times over the three-parameter fit is seen in most cases. However, since our goal was to obtain estimates of the exchange parameters that are both reliable and unbiased, the inherent difficulty of making an independent and accurate determination of the $R_{1r}^{N1}$ parameter warrants the use of the three-parameter fit in the analysis of the $R_{1r}$ data of the U6 ISL. Despite the uncertainty in the fitted $t_{ex}$ parameter, we note that the exchange lifetimes derived for the U6 ISL resonances in Table 2A were the same within the uncertainty for all carbon atoms, and ranged from 60 $\mu$s to 270 $\mu$s. This time-scale is characteristic of motions involving groups of atoms, and since previous structure determinations and RDC analyses indicated that the U6 ISL interconverts between two conformational states, we hypothesize that the $t_{ex}$ parameter reflects concerted motion between the two states.

To further test this hypothesis and confirm its statistical significance, nine resonances of the four nucleotides in the U6 ISL having two or more spin-lock power dependent $^{13}$C $R_{1r}$ rates were subjected to a simultaneous analysis with a single value of $t_{ex}$. This approach allowed a much more precise determination of the exchange parameters, in Table 2 (continued)

C. Conformational exchange parameters derived by one or two-parameter fitting of relaxation rates for individual $^{13}$C resonances

<table>
<thead>
<tr>
<th>$^{13}$C site</th>
<th>$R_{1r}^{N1}$ ($s^{-1}$)</th>
<th>$t_{ex}$ ($\mu s$)</th>
<th>$\Phi$ ($10^3$ s$^{-2}$)</th>
<th>$\Delta\nu_{min}$ (ppm)</th>
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<td>C1'</td>
<td></td>
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<tr>
<td>G62</td>
<td>10.1 ± 0.8</td>
<td>84</td>
<td>0.31 ± 0.18</td>
<td>0.37 ± 0.11</td>
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<tr>
<td>U65</td>
<td>20.2 ± 0.6</td>
<td></td>
<td></td>
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<tr>
<td>C6</td>
<td>24.1 ± 0.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>U74</td>
<td>22.1 ± 0.2</td>
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<tr>
<td>A76</td>
<td>27.1 ± 0.5</td>
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<tr>
<td>G77</td>
<td>24.7 ± 0.07</td>
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<tr>
<td>G78</td>
<td>11 ± 2</td>
<td>84</td>
<td>0.8 ± 0.6</td>
<td>0.6 ± 0.2</td>
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<tr>
<td>A79</td>
<td>8.3 ± 1.3</td>
<td>84</td>
<td>0.8 ± 0.5</td>
<td>0.60 ± 0.17</td>
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<td>C84</td>
<td>20.6 ± 0.4</td>
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<td>C85</td>
<td>13.6 ± 0.4</td>
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<td>C2</td>
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<tr>
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<td>25.4 ± 1.1</td>
<td>84</td>
<td>3.5 ± 0.7</td>
<td>1.24 ± 0.13</td>
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<tr>
<td>A82</td>
<td>27.6 ± 0.3</td>
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<td>C6</td>
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<tr>
<td>U64</td>
<td>33.7 ± 1.0</td>
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<tr>
<td>U65</td>
<td>34.4 ± 1.1</td>
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<tr>
<td>U70</td>
<td>33.2 ± 1.1</td>
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<tr>
<td>C72</td>
<td>17.7 ± 0.7</td>
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<td>C85</td>
<td>27.6 ± 0.6</td>
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<td>C8</td>
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<tr>
<td>G62</td>
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<td>2.4 ± 1.2</td>
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<td>A76</td>
<td>15 ± 4</td>
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<td>G78</td>
<td>36.2 ± 0.8</td>
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<tr>
<td>A79</td>
<td>24.2 ± 0.4</td>
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<td>G81</td>
<td>40.3 ± 1.7</td>
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<tr>
<td>A83</td>
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<tr>
<td>C6 (Mg$^{2+}$)</td>
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<td>U64</td>
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<tr>
<td>U65</td>
<td>33.07 ± 0.05</td>
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<tr>
<td>U70</td>
<td>54.3 ± 1.1</td>
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<td>U74</td>
<td>19.22 ± 0.14</td>
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<tr>
<td>U80</td>
<td>30$^d$</td>
<td>84</td>
<td>6 ± 4</td>
<td>1.5 ± 0.5</td>
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<td>C6 (pH 6.0)</td>
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<tr>
<td>U64</td>
<td>34.2 ± 0.2</td>
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<tr>
<td>U65</td>
<td>33.7 ± 0.4</td>
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<tr>
<td>U70</td>
<td>33.8 ± 0.6</td>
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<tr>
<td>U74</td>
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</tr>
<tr>
<td>U80</td>
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</table>

The observed relaxation rates were obtained at pH 7.0 and in the absence of Mg$^{2+}$ unless otherwise stated. Exchange parameters were extracted from offset corrected $R_{1r}$ rates.

$^a$ $\Phi = p_A p_B (\Delta\nu)^2$.

$^b$ Chemical shift difference between two sites in a two-site-exchange model, with $p_A p_B = 1:1$.

$^c$ Signals that display a significant power dependence in the $R_{1r}$ rates were fitted with the exchange lifetime fixed at 84 $\mu$s (see the text).

$^d$ $R_{1r}^{N1}$ fixed at 30 s$^{-1}$ (see the text).
particular the exchange lifetime. In addition to the nucleotide-specific exchange lifetime $\tau_{ex}$, the atom-specific parameters $\Phi$ and $R^2_{ex}$ were determined by a non-linear, least-squares fitting procedure. The results are presented in Table 2B.

The weighted average of the uniquely determined exchange time constants, $\tau_{ex}$, was $84(\pm 10)$ ms. Notably, none of the individual values of $\tau_{ex}$ differed significantly from the ensemble average. This is consistent with a single motional process being responsible for the conformational exchange-mediated modulation of the chemical shift seen.
in the affected $^{13}$C atoms. The parsimonious explanation for effects occurring in all regions of the molecule (upper and lower stem, both loops) is that they arise from a single global conformational rearrangement within the U6 ISL with a lifetime of approximately 84 μs.

An additional six signals in the U6 ISL showed indications of a significant $B_1$ power dependence in their $R_{1r}$ rates, although the data did not allow for a simultaneous determination of three exchange parameters. However, the power dependence of $R_{1r}$ for these six signals could be analyzed, by assuming that all carbon nuclei in the U6 ISL are affected by a global rearrangement characterized by a single, process-specific exchange lifetime of 84 μs. The fitted results for these six signals are reported in Table 2C.

In summary, 22 resonances were identified as experiencing significant $^{13}$C chemical shift differences between two conformations. They correspond to ten ribose carbon atoms and 12 aromatic carbon atoms. Of the investigated $^{13}$C carbon resonances, the remaining 19 signals showed no significant power dependence; this likely is a consequence of the small changes in carbon chemical shifts observed for these resonances when comparing the stacked and base-flipped conformations. For these signals, the reported estimates of $R_{1r}$ were determined by keeping the exchange term fixed at zero (Table 2C).

**Specific divalent metal ion binding in the U6 ISL**

Previous $^{31}$P NMR measurements of Cd$^{2+}$ binding indicated the existence of one or more metal-binding sites on the U6 ISL. Metal binding to the 5' phosphate of the U80 nucleotide is a critical function of the U6 ISL domain and is required for pre-mRNA splicing. Additionally, the U6 ISL contains a GNRA-type pentaloop fold with a sheared G–A pair analogous to a known metal binding site. The structure of the U6 ISL does not change significantly in the presence of Mg$^{2+}$, however, Mg$^{2+}$ binding to the U6 ISL domain had not been measured directly. Therefore, we performed chemical shift mapping experiments to measure Mg$^{2+}$ binding to the U6 ISL.

In order to detect metal binding to U80, a $^{[13]}$C-uracil-labeled U6 ISL RNA was used. The chemical shift mapping experiments were carried out under conditions, pH 5.7 and pH 7.0, corresponding to predominance of either of the two conformations (Figure 1). Only two out of five uracil bases showed large, magnesium-dependent chemical shift changes: U70 and U80 (Figure 3). Metal binding at U80 correlates well with previous biochemical data and NMR measurements of phosphorothioate-substituted RNA, and U70 is directly adjacent to the sheared G–A pair thought to comprise a metal binding site in the pentaloop.

![Figure 3. Effect of divalent metal on the chemical shift of uridine nucleotides in the U6 ISL RNA. Binding curves of the H6 proton resonances of U80 and U70 versus total metal ion concentration at pH 5.7 (U70 and U80) and pH 7.0 (U80). Experimental data at pH 7.0 for U70 are not available owing to signal overlap. The equilibrium dissociation constants ($K_d$) for Mg$^{2+}$ binding at U70 and U80 were obtained by simultaneous fitting of the data at each pH to a model with two non-interacting sites. For the analysis of the U80 data at pH 7.0 a value of $K_d$ for U70 equal to the experimentally determined $K_d$ at pH 5.7 was assumed. Shown as continuous lines are the calculated metal ion binding curves corresponding to the fitted parameters (Table 3) derived from the expanded model in which metal ion binding at U80 and protonation of A79 N1 are mutually antagonistic. Chemical shift values were obtained from a series of 2D TOCSY experiments recorded at 600 MHz. Sample conditions were 1 mM RNA, 50 mM NaCl, and 30 °C.

Table 3. Mg$^{2+}$ binding in the U6 ISL.

<table>
<thead>
<tr>
<th>pH 5.7</th>
<th>pH 7.0</th>
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<tr>
<td>Two-site model</td>
<td>Two-site model with interference between Mg$^{2+}$ binding at U80 and protonation at A79 N1</td>
</tr>
<tr>
<td>$K_d$ (mM)</td>
<td>$K_d$ (mM)</td>
</tr>
<tr>
<td>U70</td>
<td>U80</td>
</tr>
<tr>
<td>0.90±0.05</td>
<td>1.99±0.16</td>
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<tr>
<td>N/A</td>
<td>0.58±0.04</td>
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</tbody>
</table>

Equilibrium dissociation constants, $K_d$, determined from metal–ion titration of the U6 ISL at pH 5.7 and pH 7.0 and at 30 °C.
A complete binding curve for U70 could not be obtained at pH 7.0 owing to chemical shift overlap. The data were analyzed according to a two-site model with non-equivalent, non-interacting metal binding sites to determine the apparent equilibrium dissociation constants \(K_{d70}\) and \(K_{d80}\) for the RNA–metal ion binding at U70 and U80, both of which are in the low millimolar range (Table 3). The apparent equilibrium dissociation constants for U80 suggest that metal binding is approximately fourfold tighter at high pH (Figure 3 and Table 3). Alternatively, it is possible that the apparent pH dependence of metal binding is actually reflective of the fact that metal binds to U80 only when it is stacked in the helix, which is favored at high pH. In this case, the apparent pH dependence of the observed dissociation constant may be explained by the coupling between pH and the relative populations of the two inter-converting structures, with binding in the stacked-in conformation being essentially independent of pH. To accommodate this view, the two-site model was expanded to reflect interference between metal binding at U80 and protonation of A79 N1. The model assumes that specific metal binding at U80 occurs in the non-protonated, high-pH structure only and that metal bound at U80 inhibits protonation of A79 N1. As such, both \(K_{d70}\) and \(K_{d80}\) can be assumed to be independent of pH, and the intrinsic \(pK_a\) of the protonation of the A79 base is 6.5 and independent of metal ion concentration. The model also assumes that the populations of the high-pH and low-pH conformations of the U64 ISL are determined by the protonation state of the A79 N1 imino group. The results obtained by fitting the data using this model are listed in Table 3. The values of \(K_{d80}\) of 0.28(\(\pm\)0.03) mM and 0.44(\(\pm\)0.06) mM at pH 5.7 and 7.0, respectively, further suggest that \(K_{d80}\) is indeed independent of pH. The weighted average of \(K_{d80}\) of 0.32(\(\pm\)0.03) mM shows that binding at U80 in the high-pH structure is significantly tighter than the apparent binding at U70 (0.90(\(\pm\)0.03) mM at pH 5.7).

To explore the effect of metal ion binding on the observed conformational dynamics, longitudinal relaxation rates and \(R_{1p}\) rates at three spin-lock fields were measured at pH 7.0 in the presence of 3 mM MgCl\(_2\). It is clear from Figure 4 and Table 1 that the rotating frame relaxation rates of none of the uridine bases, with the exception of U80, displayed a spin-lock power dependence. On the other hand, \(R_{1p}\) of U80 contained considerable conformational exchange contributions.

The power dependence of \(R_{1p}\) for U80-C6 was significantly higher in the Mg\(^{2+}\)-containing sample. Although the available data did not support a quantitative determination of the three exchange parameters, a crude estimate of the fitting parameter \(\Phi\) could be obtained from a one-parameter fit of equation (3) to the observed points under the assumption that both the exchange lifetime and the exchange free \(R_{1p}\) are unaffected by the presence of Mg\(^{2+}\). The \(R_{1p}\) data at the highest \(B_1\) fields (Table 1), for U80 with and without Mg\(^{2+}\), provided evidence for the unaltered value of \(R_{1p}\). Since Mg\(^{2+}\) binding to helical nucleic acids takes place on a time-scale considerably faster than the conformational exchange process,\(^{29}\) the lifetime of the bound metal is well separated from the exchange lifetime. An upper limit for \(R_{1p}\) for U80 of 35 s\(^{-1}\) was established from the \(R_{1p}\) observation at the highest applied \(B_1\) field (4 kHz). In the analysis, we fixed \(R_{1p}\) at 30 s\(^{-1}\), as this is similar to the value for U80 in the absence of Mg\(^{2+}\) and is also close to the value of \(R_{1p}\) for the U64 and U65 C6 carbon atoms both with and without added MgCl\(_2\); these presumably represent \(R_{1p}\) values for C6 carbon atoms in the U6 ISL at which no exchange occurs. By selecting a value close to the upper limit for \(R_{1p}\), we may equate the estimate with the lower limit of \(\Phi\) for U80. The resulting \(\Phi\) (Table 2) is about twice the size of the metal-free equivalent, suggesting that metal binding results in a differential change in the chemical shifts of the exchanging conformations and possibly also leads to a shift in the relative populations of the exchanging sites.

Although U74-C6 showed significant power dependence in the absence of Mg\(^{2+}\) (Table 1), it became negligible with Mg\(^{2+}\) present. This suggests that adding Mg\(^{2+}\) lowers the difference in chemical shift of the exchanging sites.

**Effect of pH on the dynamics of uridine nucleotides in the U6 ISL**

The dynamics of the uridine residues in the U6 ISL at pH 6.0 were probed by measuring the longitudinal relaxation rates as well as the rotating frame relaxation rates for the C6 carbon atoms at
two R1 fields (1.4 kHz and 4.0 kHz). For U64, U65, U70, and U74 the observed rates (Table 1) are virtually identical with those at pH 7.0. The similarity of the R1 rates for the C6 of U74 determined at the two pH values suggests that the conformational exchange process that affects the rates at pH 7.0 is active also at pH 6.0. The power dependence of the R1 rates observed for U80 are similar at both pH values. This indicates that the microsecond time-scale motions for this nucleotide are unaffected by pH in this range. Interestingly, the R1 rate of U80 C6 at pH 6.0 is significantly lower than that of U80 C6 at pH 7.0 or those of the other uridine bases at pH 6.0. This suggests that the U80 pyrimidine has increased internal mobility on the picosecond time-scale in the low-pH conformation.

Conclusions

We recently identified the existence of a pH-dependent conformational equilibrium reaction of the U6 ISL that involves a helical movement of approximately 25° (Figure 1) accompanied by base flipping of U80.10 This pH-dependent structural transition is accompanied by numerous changes in 13C chemical shifts of atoms throughout the U6 ISL.10 The 13C R1 relaxation rate analysis described here has revealed the existence of a global conformational exchange process whose underlying mechanism is consistent with the dynamic conversion between the low-pH and the high-pH conformers observed previously. Provided that the time-scale of the transition falls in the microsecond to millisecond time range, in the pH range in which the two states are significantly populated, the structural transition should result in an observable power dependence of the measured R1 rates for carbon atoms that exhibit significant chemical shift differences. Furthermore, the difference between the 13C chemical shifts measured at high pH and at low pH allows us to predict the chemical exchange parameter Φ for resonances displaying a power dependence. To further assess the role of the structural equilibrium as the single, underlying mechanism for the prevalent conformational exchange observed in the U6 ISL, the value of the exchange parameter Φ predicted from chemical shift measurements (Φspec) was compared with the value derived from the R1 relaxation data (Φexch). The observed correlation shown in Figure 5 strongly suggests that the distinct conformations revealed by the analysis of the exchange data are indeed identical with the two pH-dependent conformations identified by structure determination.

Mechanism of the dynamical equilibria in the U6 ISL

The protonation of A79 occurs with a lifetime of approximately 20 μs.10 In the present study, we report on a global conformational exchange that has a lifetime of 84 μs. Structural data indicate that a

![Figure 5](image-url)
rate-limiting step in the exchange. Then we have, $k_{\text{op}} = k_{\text{ex}} = 5 \times 10^4 \text{s}^{-1}$, where $k_{\text{ex}} = 1/\tau_{\text{ex}}$ is the observed proton exchange rate constant derived previously from a line shape analysis of the C2 $^{13}$C resonance of A79.\textsuperscript{10}

The protonated form of A79 may favor additional stacking interactions,\textsuperscript{10} consistent with the evidence derived from the structural study.\textsuperscript{10} In the low-pH form of the U6 ISL, which adopts a straight A-form helix, the A79H$^{-}$–C67 base-pair stacks with a G–C base-pair on both sides (G78–C68 and G81–C66, respectively) (Figure 1(b)). However, in the high-pH structure with a helical bend, the A79–C67 base-pair stacks with a G–C base-pair on one side only (G78–C68), and the intercalation of U80 into the helix leaves a gap in the internal bulge (Figure 1(a)). In the U6 ISL the protonated and unprotonated forms of A79 are in fast intermediate exchange on the $^{13}$C time-scale with a proton exchange rate ($\sim 5 \times 10^4 \text{s}^{-1}$) comparable to the rate of exchange observed for the A25 adenine in the leadzyme.\textsuperscript{31} This rate of exchange is considerably faster than the observed exchange rate of the adenine in the loop A domain of the hairpin ribozyme.\textsuperscript{32} In both these cases the adenine bases are involved in A$^{+}$–C wobble pairs, but in the loop A domain the base-pair has stacking interactions with base-pairs on both sides, whereas in the leadzyme the A$^{+}$–C base-pair stacks with a C–G pair on one side only. By analogy, the base-pair opening in the U6 ISL might be expected to be considerably faster in the high-pH form, which leaves a gap in the helix, than in the low-pH form. Additionally, by analogy to the fully stacked loop A domain, we would expect the rate of A$^{+}$–C base-pair opening in the low-pH form of the U6 ISL to be considerably slower than the rate of conformational exchange determined here from the $T_{1p}$ analysis. Thus, we hypothesize that the transition between the protonated and the unprotonated form is likely to proceed through an intermediate structure that is protonated but has a bent helix with a more accessible adenine (Figure 1(a)). In this scenario, the conformational rearrangement ($\tau_{\text{ex}} = 84(\pm 10) \mu$s) is rate-limiting relative to the proton exchange lifetime (20 $\mu$s).\textsuperscript{10}

**Effects of Mg$^{2+}$ interactions on NMR relaxation rates**

The $^{13}$C relaxation rates of the uridine C6 carbon atoms of the U6 ISL are remarkably susceptible to the presence of Mg$^{2+}$. A comparison of the longitudinal relaxation rates with and without 3 mM MgCl$_2$ is shown in Figure 6. For U64, U65, and U80, the addition of MgCl$_2$ results in significant increases in the longitudinal relaxation rates (25% for U64, 16% for U65, 85% for U80) without any noticeable changes in the transverse rates. For U70, both $R_1$ and $R_2$ increase significantly, $R_1$ by more than 175% and $R_2$ by 64%. No significant change in $R_1$ was observed for U74 upon addition of Mg$^{2+}$. The dramatic site-specific relaxation enhancements of the C6 carbon atoms of nucleotides U70 and U80 upon addition of Mg$^{2+}$ suggest that the metal ions interact directly with these nucleotides, as also indicated by the chemical shift mapping data (Figure 3).

Possible sources of the Mg$^{2+}$-induced enhancement of the longitudinal relaxation rates are direct interaction of the metal ion with the C6 carbon atoms or indirect effects of Mg$^{2+}$ binding in the vicinity of the U-C6 positions. A direct effect due to dipolar coupling between $^{13}$C and $^{25}$Mg nuclei (10% natural abundance) is likely to be negligible, owing to the low magnetogyric ratio of $^{25}$Mg ($\gamma_{^{25}\text{Mg}} = 0.24$). Other direct interactions include scalar relaxation (either of the first or second kind), made possible by scalar coupling between $^{13}$C and $^{25}$Mg directly bound to the uracil base. However, whereas this might contribute significantly to $R_1$, the effect of this relaxation mechanism on $R_2$ would be negligible. As an indirect effect, the coordination of Mg$^{2+}$ to the pyrimidine will likely result in considerable changes in the structure and electron density of the pyrimidine ring system. The interaction between Mg$^{2+}$ and the pyrimidine is likely to increase the CSA of the C6 carbon, thereby amplifying the CSA relaxation contribution. However, a significant change almost exclusively in $R_1$ may not be explained in this way. Alternatively, the observed pattern in the rate changes might come about as a result of a simultaneous increase in the $^{13}$C–$^{1}$H dipolar coupling and an enhanced C6 shielding anisotropy in combination with high internal mobility at the C6 carbon. An increase in the dipolar coupling interaction would be a likely result of Mg$^{2+}$ binding inducing a shortening of the $^{13}$C–$^{1}$H bond distance. However, the 10% decrease in the $^{13}$C–$^{1}$H distance required to explain the observed rate changes seems very unlikely, given the fact that the direct interaction with Mg$^{2+}$ will reduce the overall electron density in the pyrimidine ring. These observations suggest that
static binding of Mg\(^{2+}\) may not explain the observed relaxation rates.

**Modeling of dynamic coordination of Mg\(^{2+}\) in the U6 ISL**

A more plausible explanation for the observed elevated \(R_1\) rates may be found in the dynamics of metal coordination in the U6 ISL. Slow internal molecular motions and exchange processes have been shown to produce large-amplitude distortions in the metal coordination sphere in metal binding enzymes.\(^3\) Similar time-dependent processes might alter the coordination of Mg\(^{2+}\) to the pyrimidine of the uracil nucleotides, and would thus induce considerable fluctuations in the electron density distribution in the base, resulting in librations of the \(^{13}\)C–\(^1\)H bond vector and in modulations of the \(^{13}\)C–\(^1\)H bond distance\(^3\) as well as variations in the CSA interaction. Fluctuations characterized by correlation times in the picosecond to nanosecond time range and faster than the overall rotational correlation of the U6 ISL might affect the observed \(^{13}\)C relaxation rates substantially. Possible coordination distorting processes include slow internal molecular motions involving collective movement of many atoms in proximity of the bound metal, exchange of Mg\(^{2+}\) bound to RNA, which is characterized by mean residence times in the nanosecond range\(^3\) and exchange of water molecules within the inner coordination sphere of bound Mg\(^{2+}\), characterized by mean residence times of 2–10 \(\mu\)s.\(^3\) For coordination distortions characterized by correlation times shorter than the overall rotational correlation time, the effect of the resulting modulations in the Mg\(^{2+}\) coordination sphere may be modeled by the extended Lipari–Szabo formalism.\(^3\) In this description, the influence of the fluctuations on the relaxation rates is treated as a slow internal motion characterized by a correlation time \(\tau_c\) and a separate order parameter \(S^2\). In simulations of U-C6 \(^{13}\)C relaxation rates (Supplementary Data), where a mode of slow internal motion with \(\tau_c=1.5\) ns and \(S^2=0.75\), and an increased chemical shielding anisotropy for the C6 carbon (\(\Delta\sigma_{\text{CS}}=300\) ppm) were included to emulate the effects of specific Mg\(^{2+}\) binding, the predicted \(R_1\) and \(R_2\) rates largely match the observed rates in the U6 ISL in the presence of 3 mM MgCl\(_2\). The values of the motional parameters used in the simulations correspond to previously reported exchange rates for Mg\(^{2+}\) and non-excessive order parameters. The agreement between the simulations and the observed relaxation rates suggests that the dynamics of Mg\(^{2+}\) coordination and a corresponding induced higher shielding anisotropy are largely responsible for the elevated \(R_1\) rates observed for U70 and U80. The smaller, but nevertheless significant, observed increase in the longitudinal relaxation rates of the two nucleotides U64 and U65, suggests that Mg\(^{2+}\) binds to these bases as well. However, the fairly modest increases indicate that Mg\(^{2+}\) binding at these sites is weak and possibly non-specific.

Although the dynamic nature of the Mg\(^{2+}\) binding may by itself explain the observed increase in relaxation rates, it is possible that other relaxation mechanisms, including those discussed above play minor roles as well. However, independent of the exact distribution of the various relaxation mechanisms, the fact that Mg\(^{2+}\) binding has such a profound effect on the C6 relaxation rates of U70 and U80, suggests specific binding of Mg\(^{2+}\) to these pyrimidine bases. Likely coordination sites are the O2 or O4 positions.\(^8\) Both oxygen atoms have the same position relative to the C6 carbon atom, and metal binding to either position is expected to have similar effects on the relaxation rates. The dynamic coordination model may imply exchange between site-bound and diffusely bound states of the ion.\(^4\)

Paramagnetic metal ions are well-known for their ability to significantly affect NMR relaxation rates, even at low concentrations. However, it is unlikely that the observed changes in the relaxation rates of the metal ion-containing U6 ISL can be due to paramagnetic impurities. At realistic levels of paramagnetic contamination (<1 parts per million) the paramagnetic contributions to the observed rates are negligible. Manganese titration experiments indicate that line broadening is not detectable until the addition of parts per thousand, or micromolar amounts (data not shown).

**Differential metal binding in the two conformations at U80**

It is clear from the structures of the U6 ISL at pH 5.7 and pH 7.0 that only in the high-pH structure does the essential U80 nucleotide adopt a well-defined conformation.\(^10\) It has previously been demonstrated that metal binding to U80 \(\text{pro-S}\) oxygen and protonation of the adjacent C67–A79 pair (pK\(_a\) of 6.5) are mutually antagonistic.\(^18\) The present analysis of the \(R_1\) relaxation data suggests that the O2 or O4 oxygen of the base of U80 is also involved in Mg\(^{2+}\) binding at pH 7.0. The relaxation data also showed that adding Mg\(^{2+}\) affects the U80 nucleotide differently in the two conformations (vide supra), but appears to have little or no effect on the rate or equilibrium of the conformational transition. These findings suggest that metal binding at U80 occurs predominantly when it assumes a stacked, well-defined conformation.

**Effects of metal binding at U80 on A79 protonation in the U6 ISL**

It has previously been observed that metal binding in the U6 ISL results in a significant drop in the apparent pK\(_a\) of A79. Under near-saturating conditions, the observed pK\(_a\) is lowered by 0.5 pH unit.\(^8\) A structural change induced by the binding of Mg\(^{2+}\) might alter the apparent pK\(_a\) value;\(^4\) however, this is inconsistent with the structural evidence of identical conformations with and
without Mg$^{2+}$ present. The metal binding results provide evidence for a strong correlation between metal binding at U80 and deprotonation of A79. Mg$^{2+}$ binding inhibits protonation and likely stabilizes the high-pH conformation. We may rationalize the observed changes in $pK_a$ by invoking the model proposed above and by utilizing the results from the determination of the equilibrium dissociation constants for metal binding. In this view, both Mg$^{2+}$ coordination and exchange of the A79H$^+$ proton take place (to a significant degree) in the high-pH conformation only. Upon addition of Mg$^{2+}$, the effective concentration of the U6 ISL molecules available for protonation thus decreases. This effect can explain the lower apparent $pK_a$ value in the presence of 3 mM Mg$^{2+}$ (6.0), versus the apparent $pK_a$ without Mg$^{2+}$ (6.5). As illustrated in Figure 7, the previously determined apparent $pK_a$ values in the presence and absence of Mg$^{2+}$ can be perfectly simulated from first principles, using a $K_{d70}$ of 0.90 mM, a pH-independent $K_{d80}$ of 0.32 mM, an intrinsic $pK_a$ of A79 N1 of 6.5, and a total concentration of RNA of 1 mM. Thus, consistent with structural evidence, the drop in apparent $pK_a$ value upon the addition of Mg$^{2+}$ does not require a model that assumes a Mg$^{2+}$-induced structural change; rather, Mg$^{2+}$ binding simply shifts the conformational equilibrium toward the non-protonated form, either by direct stabilization of the non-protonated conformation, or by indirect means (for example, by providing a steric block towards adenine protonation).

Effects of pH and Mg$^{2+}$ binding on catalysis

A recognition of the relationship between base ionization and RNA structural flexibility is developing. Several examples among ribozymes illustrate the common feature of a pH-controlled conformational switch in regulation of catalysis. This emphasizes the importance of our emerging understanding of the interrelation among conformational dynamics, base protonation, and metal binding in the U6 ISL. It has been shown previously that Mg$^{2+}$ binding at the pro-S phosphate oxygen of U80 is necessary for the first step of splicing. The model proposed above for the interplay between the global conformational transition, protonation of A79, and metal binding at U80 may therefore have significant consequences in spliceosomal catalysis. A potentially important function of the base-flipping motion may be to provide a pH-sensitive conformational switch that effectively controls the affinity for Mg$^{2+}$ binding at the U80 position of the U6 ISL.

Materials and Methods

RNA sample preparation

A uridine (U) (>98%) $^{13}$C, $^{15}$N-labeled RNA sample and an adenosine, guanosine, and cytidine (AGC) (>98%) $^{13}$C, $^{15}$N-labeled RNA sample of the U6 ISL were prepared by in vitro transcription as described. The U6 ISL sequence and structure are shown in Figure 1. The U-labeled U6 ISL NMR samples contained 0.6–0.8 mM RNA, 50 mM NaCl, and were either at pH 6.0 or pH 7.0 (two samples were prepared: with and without 3 mM MgCl$_2$ (Alfa Aesar purotronic)). The sample conditions for the AGC-labeled U6 ISL in all experiments were 0.8 mM RNA, 50 mM NaCl, and pH 7.0.

NMR spectroscopy

All spectra were acquired at the National Magnetic Resonance Facility at Madison (NMRFAM) on a Bruker DMX spectrometer operating at 600 MHz. Two-dimensional $^1$H–$^{13}$C pulse schemes designed for determining $^{13}$C $T_1$ and $T_2$, relaxation times of uniformly $^{13}$C, $^{15}$N labeled proteins were optimized here for use with uniformly labeled RNA. Constant-time evolution was employed in the $^{13}$C indirectly detected dimension to avoid line broadening due to unresolved one-bond $^{13}$C–$^{13}$C couplings. The length of the constant-time period was adjusted to match 1/$T_{2C}$ with $T_{2C}$ assumed to be 39 Hz for C1’–C2’ carbon and 80 Hz for aromatic carbon measurements. During the relaxation delay ($T_1$), $^1$H 180° pulses were applied every 4 ms to minimize the effects of cross-correlation between $^1$H–$^{13}$C dipolar interactions and $^{13}$C CSA and between $^{13}$C–$^{13}$C and $^1$H–$^{13}$C dipolar interactions. The $T_{1p}$ sequence used here employs chemical shift

Figure 7. Simulations of the pH dependence of the chemical shift of the A79-C2 $^{13}$C resonance in the U6 ISL, with and without added MgCl$_2$. The model used assumes that the populations of the high-pH and low-pH conformations of the U6 ISL are determined by the protonation state of the A79 N1 imino group as reported. This emphasizes the importance of our emerging understanding of the interrelation among conformational dynamics, base protonation, and metal binding in the U6 ISL. It has been shown previously that Mg$^{2+}$ binding at the pro-S phosphate oxygen of U80 is necessary for the first step of splicing. The model proposed above for the interplay between the global conformational transition, protonation of A79, and metal binding at U80 may therefore have significant consequences in spliceosomal catalysis. A potentially important function of the base-flipping motion may be to provide a pH-sensitive conformational switch that effectively controls the affinity for Mg$^{2+}$ binding at the U80 position of the U6 ISL.
precession during fixed delays to achieve alignment of the magnetization for individual carbon sites along their effective spin-lock fields. After the precession and the subsequent 90° 13C pulse, the spins are at an angle $\Delta \nu_1$ with the transverse plane. Here $\Delta$ is the offset from the carrier of the jth spin resonance, and $\nu_1$ is the strength ($gB_1/2\pi$) of the spin-lock field. Since the effective field forms an angle $\theta = \arctan(\Delta/\nu_1)$ with the transverse plane, this method assumes $\arctan(0) = 0$, which is true only for small values of $\theta$. Thus as previously recognized, $^{43}$ in the $T_1p$ experiment, the combination of low applied $B_1$ field strengths and large chemical shift dispersions may lead to a significant misalignment for resonances that are not close to the spin-lock carrier frequency. Indeed, a noticeable loss of sensitivity was observed for several resonances in data sets collected at the lowest $B_1$ field strengths. This may potentially result in a substantially reduced precision for the extracted $R_{1p}$ relaxation rates. More importantly, failure to position the magnetization along the effective field may lead to an oscillatory behavior of the signal during the spin-lock period, and, although the oscillations will eventually be destroyed by $B_1$ field inhomogeneity, the use of short relaxation delays may generate biased $R_{1p}$ rotating frame relaxation rates. This has previously been generally recognized to be the case for $T_{1p}$ experiments.$^{34,44}$ The $^1$H $\pi$ pulses applied during the spin-lock period may not completely suppress the cross-correlation between dipole-dipole and chemical shift anisotropy relaxation mechanisms, which may result in an oscillatory behavior of the observed relaxation data.$^{45}$ This in turn may lead to systematic overestimations of the observed $R_{1p}$ rates, particularly for low values of the applied $B_1$ field strengths and for resonances with small chemical shift offsets. Although the deleterious effect decreases with longer inter-$\pi$-pulse delays, and is attenuated by the presence of inhomogeneous $B_1$ fields, specific combinations of spin-lock field strengths and resonance offsets may lead to a significant overestimation of the rotating frame relaxation rate.

To minimize the effect of these experimentally introduced oscillations on the derived $R_{1p}$ relaxation rates, a time-specific weighting function was applied to the data points in the exponential relaxation decays. The observed oscillatory behavior of the intensities is damped by $B_1$ field inhomogeneity, which is one of the issues that affect the observed relaxation rate. $^{46}$ Thus, the effect of the inherent modulation of the intensities may be reduced by applying less weight to the initial points in the exponential decays. A weighting function of the form $1-0.5 \exp(-2mtwo)$, with $\omega = 60$ Hz was found to eliminate essentially the systematic errors in the derived $R_{1p}$ values. With this approach, no detectable systematic deviations were observed in $R_{1p}$ at low $B_1$ fields.

All relaxation experiments were acquired at 600 MHz spectrometer frequency at 30 °C in thin-wall Shigemi microcyls (Shigemi Inc., Alison Park, PA). In the U-labeled U6 ISL RNA sample, the $^{13}$C carrier for C1 and C6 resonances was positioned, respectively, at 92 ppm and 142 ppm. For the AGC-labeled RNA sample, the $^{13}$C carrier was positioned at 92 ppm for C1 and at 146 ppm for C2, C6, and C8 measurements. In each case, ten relaxation delays (4, 40, 96, 152, 220, 296, 388, 500, 644, and 848 ms) were used. In both the $T_1$ and $T_{1p}$ experiments, the delay between consecutive scans was set to 1.5 s, and the $T_1$ and $T_{1p}$ relaxation time-points were acquired in pseudo random order. The proton carrier was set at the residual HDO signal, and $^{13}$C WALTZ decoupling was applied during acquisition ($t_2$) using a 4.72 kHz rf field. In all experiments, $^{15}$N decoupling was employed during the $^{13}$C chemical shift evolution period ($t_1$) as follows: For all $T_1$ and $T_{1p}$ experiments on the U-labeled sample, WALTZ modulated 2.36 kHz fields centered at 92 ppm and 142 ppm were used, respectively, for C1 and C6 measurements. For all other measurements involving aromatic carbon atoms, a GARP modulated field of 2.36 kHz was used for $^{15}$N decoupling, with the field centered at 151, 200, and 217 ppm, respectively, for C-C6, A/G-C8, and A-C2.

In highly enriched uniformly labeled RNA (and DNA) each sugar methine carbon and the purine C5 and C6 carbon is covalently bound to one or two other $^{13}$C carbon atoms. In these spin systems the time evolution of the longitudinal $^{13}$C magnetization is governed by both auto-relaxation and cross-relaxation processes and the measured relaxation decays are generally multi-exponential. If the size of the homonuclear $^{13}$C–$^{13}$C cross-relaxation rate ($\sigma_{CC}$) is small compared to the $^{13}$C auto-relaxation rate ($\rho$), the effect of the cross-relaxation is negligible and it becomes possible to use a mono-exponential decay to model the data and achieve a reliable relaxation rate determination. This is indeed the case for smaller RNA structures such as the U6 ISL ($r_2 = 4$ ns), where $\sigma_{CC}/\rho = 0.05$ (in addition to the proton–carbon dipolar interaction, $\rho$ also contains contributions from all other relaxation mechanisms).$^{15,24}$ To further reduce the effects of carbon–carbon cross-relaxation on the observed relaxation decays, we have used the scheme by Sklenar et al., where $^{13}$C magnetization is positioned along the $z$ and the $–z$ axes in alternate scans,$^{47}$ in combination with minimal excitation of neighboring carbon spins.$^{48}$ Minimal excitation was achieved by the use of semi-selective $^{13}$C pulses throughout the first INEPT transfer of the $T_1$ experiment. In the $R_{1p}$ experiment, $^{13}$C atoms covalently bound to other $^{13}$C atoms may experience appreciable homonuclear Hartmann–Hahn magnetization transfer during the spin-lock period. This transfer of magnetization through the $^{13}$C spin system, mediated by a scalar coupling between $^{13}$C nuclei, may interfere with the interpretation of the relaxation data. The effect is, however, negligible if the magnitude of the scalar coupling is small relative to the difference in effective field strength between the two nuclei:

$$\Delta V_{eff} = |V_{eff} - V_{0,eff}| > 1|\Delta CC|$$

(1)

where $\Delta V_{eff}$ is the difference in effective field strengths for two coupled spins, with:

$$v_{eff} = (\gamma s + \gamma b)^2$$  

$x = a, b$  

(2)

where $\gamma_1$ is the field strength of the $B_1$ field in Hz and $\gamma_b$ is the offset from the spin-lock carrier of nucleus $x$ (in Hz), $x = a, b.$ $^{47,48}$ Of the potentially affected $^{13}$C resonances whose relaxation properties have been measured in the present work, the pyrimidine C6 carbon atoms are in a spectral region remote from that of the adjacent C5 carbon atoms, while the chemical shift of the ribose C1’ carbon atoms differs by ca 15 ppm from the C2’ carbon atoms. In the extreme case of a $B_1$ spin-lock field of 5.6 kHz, and
with the $B_0$ of 14.1 T used here, we find $\Delta \nu_{\text{eff}} \geq 400$ Hz for $\text{C}^1$, and since $J_{\text{C}-\text{C}}$ is ca 40 Hz, we may safely ignore the effect of the Hartmann–Hahn transfer on the measured $R_{1p}$ rates.

The software package Felix 98 (Accelrys) was used to process the NMR relaxation data. All time domain data were apodized in each dimension with a squared sine bell function, zero filled to the final matrix size, Fourier transformed, and phase corrected before being packed into pseudo-3D spectra. The program CHIFT with a pseudo-3D signal model was used to extract signal intensities from the processed spectra by a global fit.49

The $T_1$ and $T_1p$ relaxation times were derived by weighted non-linear, least-squares fitting of two-parameter mono-exponential decays to the measured signal intensities. Uncertainties of relaxation rates were estimated from the covariance matrix of each fit.

**Determination of exchange parameters**

Relaxation measurements in the rotating frame offer a wide window of sensitivity to dynamical processes. For a two-site conformational exchange process in the fast intermediate exchange limit, the $R_{1p}$ relaxation rate is given by:

$$R_{1p} = R_{1p}^e + (p_A p_B (\Delta \omega)^2 \tau_{ex}/(1 + \delta_{0,eff} \tau_{ex}),$$

(3)

The second term on the right-hand side contributes the contribution, $R_{1p}^e$, to the relaxation rate of a possible exchange reaction. Here, $p_A$ and $p_B$ are the fractional populations of each state, $\Delta \omega$ is the chemical shift difference between the two sites, and $\delta_{0,eff}$ is the effective spin-lock field. The process is characterized by the single lifetime for a first-order exchange reaction, $1/\tau_{ex} = 1/\tau_A + 1/\tau_B$, where $\tau_A$, $\tau_B$ are the lifetimes of the individual states. The value of $R_{1p}$ at theoretical infinite spin-lock power, $R_{1p}^e$, is the relaxation rate of nuclei in the rotating frame in the absence of conformational exchange. In the general case of the carbon nuclei being off-resonance from the spin-lock carrier frequency, the effective spin-lock field $\delta_{0,eff}$ is different from the applied spin-lock field $\delta_0 (\frac{\delta_0}{\gamma R_1})$. For each $^{13}C$ resonance, the strength of the effective spin-lock field used in equation (3) is given by:

$$\omega_{0,eff} = \left( \omega_0^2 + \Omega^2 \right)^{1/2}$$

(4)

where $\Omega$ is the offset of the signal from the spin-lock carrier in angular frequency units. For signals that are off-resonance, the effective spin-lock field is tilted out of the perpendicular plane. The proportions of $R_{1p}$ and $R_1$ contributing to the observed relaxation rate are given by:

$$R_{1p}^{\text{obs}} = R_1 \cos^2 \theta + R_{1p} \sin^2 \theta$$

(5)

where $\theta$, the angle between the effective spin-lock field and the static magnetic field, is determined from $\theta = \arctan(\omega_0/\Omega)$. For the U6 ISL, angles $\theta$ of $32^\circ$–$90^\circ$ and $75^\circ$–$90^\circ$ resulted, respectively, from the lowest and highest applied spin-lock powers of 0.97 kHz and 5.6 kHz. The offset-corrected relaxation rates $R_{1p, \omega}$ extracted from equation (5), were used with equation (3) to obtain the exchange parameters. Standard deviations of the offset-corrected relaxation rates were derived by error propagation. Rotating frame relaxation rates were derived for a number of $^{13}C$ resonances at either five or six different spin-lock powers. A three-parameter weighted non-linear, least-squares fit of equation (3) to correspond-

values of offset-corrected relaxation rates and effective spin-lock powers permits the extraction of the exchange parameters $R_{1p}^e$, $\Phi = p_A p_B (\Delta \omega)^2 / \gamma$, and $\tau_{ex}$. An in-house developed software package (LSQFIT) was used in the fitting. Error estimates for the extracted exchange parameters were derived from the covariance matrix.

The low-pH and the high-pH conformers of the U6 ISL have been shown to exist in a dynamic equilibrium with an exchange rate that is fast on the NMR chemical shift scale.10 Thus, the observed $^{13}C$ chemical shift ($\delta_{\text{obs}}$) is given as:

$$\delta_{\text{obs}} = p_A \delta_A + (1 - p_A) \delta_B$$

(6)

where $\delta_A$ and $\delta_B$ are the chemical shift of the low-pH and the high-pH conformer, respectively, and $p_A$ is the mole fraction of the low-pH structure. Assuming that the mole fractions follow the protonation state of the A79 N1 nitrogen,10 $p_A$ is given as:

$$p_A = (1 + 10^{\text{p}\text{Ka} + 1}$$

(7)

where the apparent $p_{\text{Ka}}$ for the protonation of the A79 N1 nitrogen (6.5) has been determined previously.18

**Dissociation constants for specific U6 ISL–Mg$^{2+}$ interactions**

A series of 2D $^1$H–$^1$H TOCSY (40 ms mixing time) experiments were acquired at increasing molar equivalents of Mg$^{2+}$ to the RNA. The chemical shift change upon binding of Mg$^{2+}$ observed for the H6 resonance of nucleotides U70 and U80 was used to characterize metal binding at these sites. For each nucleotide a single H6 resonance was observed, indicating fast exchange between the free and the bound state. This is typically the case when the metal ion binding to RNA is relatively weak ($K_d = 10^{-3}$ M).32 In the fast exchange limit the observed chemical shift is given as:

$$\delta_{\text{obs}} = \delta_\text{f} \times p_\text{f} + \delta_\text{b} \times p_\text{b}$$

(8)

where $\delta_\text{f}$ and $\delta_\text{b}$ are the chemical shift of the free and the bound form, present at mole fractions $p_\text{f}$ and $p_\text{b}$, respectively. The experimental binding curves, describing the $^1$H chemical shift of the H6 resonances of the nucleotides U70 and U80 versus total metal ion concentration, were analyzed by non-linear multiparameter least-squares fitting, applying a model with two non-equivalent, non-interacting metal binding sites. The expressions for the multicomponent binding curves were derived from mass action and mass balance equations. While an analytical solution to the binding isotherm does not exist, the problem could be solved numerically using in-house developed software. In the analysis, the free metal ion concentration was determined from the concentrations of total and bound metal only. Ignoring non-specific binding may result in overestimating the free metal ion concentration and thus the $K_d$ values (representing weaker binding). Therefore, we interpreted the reported $K_d$ values as upper limits. A more accurate determination might be obtained if the concentration of free metal ion were derived by extensive dialysis against a known metal concentration. Nevertheless, the equilibrium constants for Mg$^{2+}$ binding are expected to be only minimally affected by the relatively weaker non-specific binding.
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Supplementary Data

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References


