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Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome

Shyue-Lee Yean, Gerald Wuenschell, John Termini, and Ren-Jang Lin

Department of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010-3011, USA

Abstract

Introns are removed from nuclear messenger RNA precursors through two sequential phosphotransesterification reactions in a dynamic RNA–protein complex called the spliceosome^{1,2}. But whether splicing is catalysed by small nuclear RNAs^{3,4} in the spliceosome is unresolved. As the spliceosome is a metalloenzyme^{5–7}, it is important to determine whether snRNAs coordinate catalytic metals. Here we show that yeast U6 snRNA coordinates a metal ion that is required for the catalytic activity of the spliceosome. With Mg^{2+} , U6 snRNA with a sulphur substitution for the pro-*R*_P or pro-*S*_P non-bridging phosphoryl oxygen of nucleotide U₈₀ reconstitutes a fully assembled yet catalytically inactive spliceosome. Adding a thiophilic ion such as Mn^{2+} allows the first transesterification reaction to occur in the U6/sU₈₀(*S*_P)- but not the U6/sU₈₀(*R*_P)-reconstituted spliceosome. Mg^{2+} competitively inhibits the Mn^{2+} -rescued reaction, indicating that the metal-binding site at U6/U₈₀ exists in the wild-type spliceosome and that the site changes its metal requirement for activity in the *S*_P spliceosome. Thus, U6 snRNA contributes to pre-messenger RNA splicing through metal-ion coordination, which is consistent with RNA catalysis by the spliceosome.

The spliceosome forms by incorporating small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP proteins onto premRNA, and it attains its catalytic conformation by undergoing a series of protein and RNA rearrangements^{1–3,8}. Much evidence indicates that splicing is catalysed by the snRNAs in the spliceosome, although there is no definitive proof^{3,4}. Among the five spliceosomal snRNAs (U1, U2, U4, U5 and U6), U6 is most widely assumed to be involved in catalysis because it is highly conserved through evolution and very sensitive to chemical changes. In addition, U6 forms intramolecular and intermolecular helices that are analogous to autocatalytic group II introns³. If U6 snRNA were directly involved in catalysis, it might coordinate catalytic metals, because the spliceosome, like most RNA enzymes⁹, uses divalent metal ions in catalysis^{6,7}. If a phosphoryl oxygen/ Mg^{2+} interaction is crucial for function, a sulphur substitution for that oxygen would cause a switch in metal specificity from Mg^{2+} to a thiophilic ion such as Mn^{2+} (ref. 10). Several (*R*_P)-phosphorothioate substitutions in yeast or nematode U6 snRNA fail to reconstitute active spliceosomes with Mg^{2+} (refs 11, 12). However, Mn^{2+} does not

rescue these phosphorothioates, and it is unclear whether any of the oxygens coordinate metal^{13,14}.

We tested synthetic U6 snRNA containing a single phosphorothioate (as a mixture of *R_P* and *S_P* diastereomers) in reconstituting Mg²⁺-dependent splicing in a U6-depleted yeast extract^{11,15}. Many phosphorothioate-containing U6 RNAs reconstituted splicing well (our own unpublished data), except U6 with a sulphur substitution at U₈₀ (Fig. 1, lane 5). Pure sU₈₀(*R_P*) diastereomer completely failed to reconstitute splicing (Fig. 1, lane 6)¹¹. Notably, splicing also did not occur in the extract reconstituted with U6/sU₈₀(*S_P*) (Fig. 1, lane 7), suggesting that both 5' non-bridging oxygens of U₈₀ are important for splicing.

We investigated whether U6/sU₈₀ snRNA blocked spliceosome maturation or Mg²⁺-dependent transesterification. The reactions were carried out with or without the yeast PRP2 ATPase, which is required for the final ATP-dependent spliceosomal rearrangements before the first transesterification reaction¹⁶. Reconstituted spliceosomes were isolated from glycerol gradients and analysed on a non-denaturing gel (Fig. 2a). In the absence of PRP2, unmodified U6, U6/sU₈₀(*R_P*) and U6/sU₈₀(*S_P*) reconstituted a pre-catalytic SS2 spliceosome¹⁶ (Fig. 2a, lanes 1, 3 and 5), which was equivalent to mammalian spliceosome B2 (ref. 1). In the presence of PRP2, the post-PRP2 spliceosomes were formed (Fig. 2a, lanes 2, 4 and 6), which were equivalent to mammalian spliceosomes C1 and C2 (ref. 1). These spliceosome complexes were not detected in U6-depleted extracts without reconstitution (data not shown). Thus, both of the U6/sU₈₀ RNAs support spliceosome maturation but not catalysis.

We tested whether phosphorothioate substitutions at U₈₀ switch the metal requirement for catalysis by incubating the purified post-PRP2 spliceosomes with Mg²⁺ or Mn²⁺ (Fig. 2b). Splicing did not occur in the sU₈₀(*R_P*) spliceosome regardless of metal used (data not shown). No splicing occurred when the sU₈₀(*S_P*) spliceosome was incubated with Mg²⁺ as the sole divalent metal ion (Fig. 2b, lanes 5–8). However, the sU₈₀(*S_P*) spliceosome efficiently carried out the first transesterification reaction in the presence of Mn²⁺ (Fig. 2b, lanes 9 and 10). This reaction did not require additional ATP, PRP2 or the HP splicing factor, confirming that this spliceosome had been fully activated¹⁶. Other thiophilic divalent metal ions also rescued the sU₈₀(*S_P*) spliceosome; Cd²⁺ was more effective than Mn²⁺, whereas Ca²⁺ and Co²⁺ were less effective (Fig. 2c). Rescue occurred at submillimolar concentrations, indicating that there was specific metal interaction at the site of sulphur substitution¹⁷. Thus, these results showed that only the sU₈₀(*S_P*) and not sU₈₀(*R_P*) phosphorothioate caused a switch of metal specificity, and that the transesterification reaction could occur in the *S_P* spliceosome once thiophilic metal occupied the sulphur-substituted site.

As purified spliceosomes are missing second-step factors¹⁶, we tested the ability of Mn²⁺ to rescue the second transesterification reaction in extracts (Fig. 3a). Mn²⁺ rescued the first but not the second transesterification reaction in U6/sU₈₀(*S_P*)-reconstituted extracts (Fig. 3a, lanes 14–18), whereas the U6/sU₈₀(*R_P*)-reconstituted extract could not be rescued (lanes 8–12). This specificity of rescue indicated that desulphurization of the phosphorothioate did not occur during the reaction. The result also suggests that the pro-*S_P* oxygen of U₈₀ may

have a different role during the second step. The pro- S_P and pro- R_P oxygens, for example, may switch roles in the two spliceosomal active sites¹⁸.

We further characterized the Mn^{2+} -rescued site by testing whether Mg^{2+} could competitively inhibit the Mn^{2+} -supported reaction in U6/sU₈₀(S_P). Pre-catalytic SS2 spliceosomes were reconstituted with unmodified U6 or U6/sU₈₀(S_P) and, without gradient purification, incubated with PRP2 and Mn^{2+} plus an increasing amount of Mg^{2+} (Fig. 3b). The ratio of splicing efficiency between the U6/sU₈₀(S_P)-reconstituted reaction and the U6-reconstituted reaction decreased as the Mg^{2+} concentration increased (Fig. 3c). Thus, Mg^{2+} could effectively occupy the Mn^{2+} -rescued site in U6/sU₈₀(S_P) but it could not support splicing. This result indicates that sulphur substitution does not create a binding site for the rescuing metal ion that is not present in the wild-type reaction^{19,20}. It also indicates that the metal-binding site at U₈₀ changes its metal requirement for activity in the U6/sU₈₀(S_P)-reconstituted spliceosome.

We have identified, to our knowledge, the first snRNA site that contributes to catalysis in the spliceosome through metal-ion coordination. Ribozymes such as hammerhead^{21,22}, group I intron of *Tetrahymena*²³ and RNase P (ref. 24) have phosphoryl oxygens coordinating metal that contributes to catalysis. It will be of interest to know the exact geometry of the Mg^{2+} coordinated by the pro- S_P oxygen of U₈₀ with respect to the 5' splice site, and whether this metal ion is the one that interacts with the leaving group⁶. U₈₀ of the yeast U6 snRNA is situated at the bulge region of its 3' intramolecular stem loop (3'ISL, Fig. 4). This 3'ISL of U6 and the helix Ib of U2–U6 have been proposed to be functionally analogous to the catalytically important domain 5 of group II introns, partly on the basis of (R_P)-phosphorothioate interference analysis^{12,25}. For U₈₀ to coordinate a Mg^{2+} at or near the 5' splice site, it has to fold over to the scissile phosphate. A tertiary interaction between U2/A₂₅ and U6/G₅₂ identified in yeast²⁶ and in human²⁷ might be involved in this folding. Tertiary interactions (λ – λ') have recently been described in a group II intron between the conserved G at the fifth position of the intron and the two conserved G–C base pairs above the corresponding U₈₀ bulge in domain 5 (ref. 28). If similar interactions also occurred in the spliceosome, they could juxtapose the U₈₀ of U6 and the 5' splice site. Thus, U₈₀ could coordinate the Mg^{2+} that stabilizes the leaving group at the 5' splice junction⁶, the putative Mg^{2+} that activates the nucleophilic hydroxyl at the branchpoint⁵, or an Mg^{2+} nearby (Fig. 4). In conclusion, we have elucidated a catalytic role for the U6 snRNA in pre-mRNA splicing, and provided evidence to support the notion that the spliceosome is fundamentally an RNA enzyme.

Methods

RNA oligonucleotides

We synthesized RNA oligonucleotides using CE (β -cyanoethyl) phosphoramidites with 2'TBDMS (*t*-butyl-dimethylsilyl) protecting groups (Glen Research) on an ABI 394 DNA/RNA synthesizer at the Caltech Biopolymer Center. Sulphurization of a non-bridging oxygen was performed by using 3H-1,2-benzodithiol-3-one-1,1-dioxide. After synthesis, we deprotected the preparation using triethylamine trihydrofluoride, and purified the full-length oligonucleotide from a denaturing polyacrylamide gel or through a diethylaminoethyl

(DEAE) column. The oligomer containing sU₈₀ was resolved by a C18 Beckman Ultrasphere ODS column on a Hewlett-Packard high-performance liquid chromatography (HPLC) into three peaks: first, the unmodified oligonucleotide; second, the *R_p* diastereomer; and third, the *S_p* diastereomer. The exact mass of the oligomers was confirmed by mass spectrometry on a Voyager Elite instrument at the Caltech Protein/Peptide Micro Analytical Laboratory (PPMAL) facility. The *R_p* and *S_p* diastereomers were verified by digesting ³²P-labelled oligomers with snake venom phosphodiesterase, which preferentially cleaves the *R_p* isomer²⁹

Pre-mRNA and U6 snRNA

SP6 RNA polymerase was used to produce ³²P-labelled actin pre-mRNA¹⁶. U6 snRNA was made by using T7 RNA polymerase¹¹ or by ligating four synthetic RNA oligonucleotides^{15,30}. Four RNA oligos, U6/1–37, U6/38–69, U6/70–83, and U6/84–112 were ligated to generate U6/U₈₀ RNA on a DNA template, cdU6/19–100. Before ligation, three RNA oligomers, U6/38–69, U6/70–83, and U6/84–112 were pooled and phosphorylated for 60 min at 37 °C in 10 µl reaction containing 50 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 60 µM ATP, 10 pmol [γ-³²P]ATP, 10 units of RNasin and 10 units of T4 polynucleotide kinase. The phosphorylation reaction was terminated by heating at 65 °C for 20 min. The fourth RNA oligomer, U6/1–37, and the DNA template, cdU6/19–100, were then added and the mixture was heated at 90 °C for 1 min and cooled at room temperature. Ligations were performed at 30 °C for 4 h in 25 µl reaction containing 5,000 units of T4 DNA ligase, 20 units of RNasin, 4% of PEG and 1 × T4 DNA ligase buffer (New England Biolabs). Ligated RNA was isolated from a 7.5% polyacrylamide/urea gel.

Splicing and spliceosome assays

Extract preparation, *in vitro* splicing, gradient and gel analysis of spliceosomes, and spliceosome conversion assays were done as described¹⁶. U6 reconstitution was done by cleaving endogenous U6 snRNA in heat-inactivated *prp2* mutant extracts in the presence of 450nM d1 (cdU6/28–54) oligodeoxyribonucleotide and 2mM ATP¹¹. Pre-mRNA (0.4 nM), U6 RNA (4 nM), divalent metal ions in chloride form, potassium phosphate and PEG (4.8%) were added and incubated at 23 °C for 30 min to allow reconstitution and spliceosome assembly. The PRP2 protein was added and incubated for splicing at 23 °C for 15 min. ³²P-labelled RNA on gels was quantitated with a Phosphorimager.

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Figure 1.

U6 snRNA with a phosphorothioate substitution at U₈₀ fails to reconstitute splicing. U6-depleted *prp2* mutant extracts were reconstituted with T7 polymerase-transcribed, unmodified U6 (lanes 1 and 3) or ligated, synthetic U6 (lanes 4–8) in the absence (lane 1) or presence (lanes 2–8) of wild-type PRP2 protein. No U6 was added in lane 2 (–). Both input pre-mRNA and U6 RNA were labelled with ³²P and were analysed by denaturing gels and autoradiography. Synthetic U6 used: O, no phosphorothioate substitution (lane 4); M, unpurified R_P/S_P phosphorothioate mixture with some unsulphurized RNA (lane 5); and the R_P diastereomer (lane 6), S_P (lane 7) and the unsulphurized RNA (lane 8) purified from the mixture. The intron–exon2 lariat (IVS–E2*) and exon 1 (E1) are products from the first

transesterification reaction, and the intron lariat (IVS*) and ligated exon 1/exon 2 (E1–E2) are from the second reaction.

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Figure 2.

Spliceosome maturation is not affected by phosphorothioate substitution at U₈₀ of U6 but splicing only occurs in the *S_p* diastereomer with a thiophilic metal ion. **a**, U6/sU₈₀ forms mature spliceosome. Extracts were reconstituted with unmodified U6 (lanes 1 and 2) or U6/sU₈₀ (*R_p*, lanes 3–4; *S_p*, lanes 5–6), in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of PRP2. The reaction mixture was sedimented through a glycerol gradient and the fraction containing the spliceosome was analysed on a native gel. The pre-catalytic and post-PRP2 spliceosomal markers (M; lanes 7 and 8) were generated as described¹⁶. **b**, The first transesterification reaction occurs in U6/sU₈₀(*S_p*)-containing post-PRP2 spliceosome in the presence of Mn²⁺. Spliceosomes isolated from glycerol gradients were incubated in

buffer containing combinations of Mg^{2+} , Mn^{2+} , ATP, PRP2 and HP (a protein factor that facilitates splicing in purified spliceosomes¹⁶). c, Other thiophilic divalent metal ions can also rescue the transesterification reaction. The post-PRP2 U6/sU₈₀(Sp) spliceosome was incubated with metal ions without the addition of ATP, PRP2 or HP. Mg^{2+} was added as the sole divalent metal ion in lanes 1 (2.5 mM) and 2 (5 mM). The concentrations (mM) of the thiophilic metal (Mn^{2+} , Ca^{2+} , Cd^{2+} or Co^{2+}) in each set were 0.02, 0.1, 0.5 and 2.5. Mg^{2+} was added to lanes 3–18 to make the total concentration of divalent metal ions 5 mM.

Figure 3.

With U6/sU₈₀(S_P) RNA, only the first of the two transesterification reactions occurs in the presence of Mn^{2+} , which is competitively inhibited by Mg^{2+} . **a**, Mn^{2+} rescues the first but not the second step of splicing in U6/sU₈₀(S_P)-reconstituted reaction. Extract was reconstituted with U6 (lanes 1–6), U6/sU₈₀(R_P) (lanes 7–12), or U6/sU₈₀(S_P) (lanes 13–18) and PRP2 in the presence of Mn^{2+} and Mg^{2+} . In each set of six reactions, the concentrations (mM) of Mn^{2+} / Mg^{2+} were 0/2.5, 2.5/2.5, 1.25/1.25, 1.7/0.8; 2.0/0.5, 2.5/0. **b**, Mg^{2+} competitively inhibits the Mn^{2+} -rescued reaction. Extract was reconstituted in the absence of PRP2 with U6 (lanes 1–9) or U6/sU₈₀(S_P) (lanes 10–18) and with Mg^{2+} (2.5 mM) as the sole divalent metal ion. Splicing was then triggered by the addition of PRP2 with 1mM

Mn^{2+} (lanes 2–9 and 11–18) and an increasing concentration (mM) of Mg^{2+} (2.5, 5.0, 7.5, 10, 15, 20, 25, 30). Reactions in lanes 1 and 2 contained 2.5mM of Mg^{2+} and no Mn^{2+} (asterisk). **c.** The ratio of splicing efficiency between the U6/sU₈₀(S_P)- and U6-reconstituted reactions (S_P/O). Splicing efficiency in each reaction (Fig. 3b) was calculated as the ratio between the products (IVS–E2*, IVS* and E1–E2) and the total (products plus pre-mRNA).

Figure 4.

A representation of RNAs and metal ions in the yeast spliceosome before the first catalytic step. Intermolecular helices between U6 and U2 (Ia and Ib), U2 and pre-mRNA at the branch site (U2/BS), U6 and pre-mRNA at the 5' splice site (U6/5'SS), as well as the 3' intramolecular stem-loop (3'ISL) in U6 are depicted^{1-3,8}. The branchpoint adenine is highlighted with a star. The U₈₀ of U6 is shaded. Three Mg²⁺ ions are shown as circles: one stabilizes the leaving oxygen at the 5' scissile phosphate⁶ (lined); one activates the attacking oxygen⁵ (dotted); and one binds to the pro-Sp oxygen of U₈₀ (filled). The tertiary interaction between G₅₂ of U6 and A₂₅ of U2 (refs 26, 27), a putative interaction between 3'ISL of U6 and the U6/5'SS helix based on the λ - λ' interaction in a group II intron²⁸, and the potential relationship between the metals are linked with a line.