The NMR structure of the II–III–VI three-way junction from the *Neurospora* VS ribozyme reveals a critical tertiary interaction and provides new insights into the global ribozyme structure

ERIC BONNEAU,¹ NICOLAS GIRARD,¹ SÉBASTIEN LEMIEUX,² and PASCALE LEGAULT¹

¹Département de Biochimie et Médecine Moléculaire, Université de Montréal, Montréal, Quebec H3C 3J7, Canada

²Département d'Informatique et de Recherche Opérationnelle et Institut de Recherche en Immunologie et en Cancérologie, Université de Montréal, Montréal, Quebec H3C 3J7, Canada

ABSTRACT

As part of an effort to structurally characterize the complete *Neurospora* VS ribozyme, NMR solution structures of several subdomains have been previously determined, including the internal loops of domains I and VI, the I/V kissing-loop interaction and the III–IV–V junction. Here, we expand this work by determining the NMR structure of a 62-nucleotide RNA (J236) that encompasses the VS ribozyme II–III–VI three-way junction and its adjoining stems. In addition, we localize Mg²⁺-binding sites within this structure using Mn²⁺-induced paramagnetic relaxation enhancement. The NMR structure of the J236 RNA displays a family C topology with a compact core stabilized by continuous stacking of stems II and III, a *cis* WC/WC G•A base pair, two base triples and two Mg²⁺ ions. Moreover, it reveals a remote tertiary interaction between the adenine bulges of stems II and VI. Additional NMR studies demonstrate that both this bulge–bulge interaction and Mg²⁺ ions are critical for the stable folding of the II–III–VI junction. The NMR structure of the J236 RNA is consistent with biochemical studies on the complete VS ribozyme, but not with biophysical studies performed with a minimal II–III–VI junction that does not contain the II–VI bulge–bulge interaction. Together with previous NMR studies, our findings provide important new insights into the three-dimensional architecture of this unique ribozyme.

Keywords: Varkud satellite ribozyme; NMR structure; RNA three-way junction; remote tertiary interaction; magnesium ions

INTRODUCTION

The Varkud satellite (VS) ribozyme is a member of the family of small nucleolytic ribozymes that includes the hairpin, hammerhead, hepatitis delta virus, *glsms*, and twister ribozymes (Saville and Collins 1990; Collins 2002; Lilley 2004, 2008; Cochrane and Strobel 2008; Wilson and Lilley 2011; Roth et al. 2014). The VS ribozyme is derived from a noncoding satellite RNA found in the mitochondria of certain natural isolates of *Neurospora* filamentous fungi (Saville and Collins 1990). It catalyzes self-cleavage and self-ligation at a specific phosphodiester bond, and both of these transesterification reactions are critical for the replication cycle of the VS RNA (Saville and Collins 1990, 1991). In vitro, these reactions require the presence of metal cations, which are important for both the structure and the chemical reaction (Collins and Olive 1993; Beattie et al. 1995; Beattie and Collins 1997; Murray et al. 1998; Sood et al. 1998; Hiley

and Collins 2001; Maguire and Collins 2001; Sood and Collins 2002; Smith et al. 2008). The minimal contiguous VS ribozyme is composed of six helical domains, numbered I–VI (Beattie et al. 1995). Stem-loop I (SLI) forms the substrate domain, and its internal loop contains the cleavage site. Helical domains II–VI constitute the catalytic domain, also termed the *trans* ribozyme, which is organized around two three-way junctions: the II–III–VI and III–IV–V junctions (Fig. 1A; Beattie et al. 1995). The SLI substrate domain is recognized by stem-loop V (SLV) through the formation of a highly stable kissing-loop interaction, which has been extensively characterized (Rastogi et al. 1996; Andersen and Collins 2000, 2001; Bouchard and Legault 2014a,b). In particular, this kissing-loop interaction is associated with a conformational change in the internal loop of SLI that activates

Corresponding author: pascale.legault@umontreal.ca

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FIGURE 1. Primary and secondary structures of a *cis*-cleaving VS ribozyme (including residues 617–783) (Beattie et al. 1995) (A) and the 62-nt J236 RNA used in this study with a summary of biochemical data (B). In A, the substrate is shown in its active conformation (Andersen and Collins 2000), with the cleavage site indicated by a gray arrowhead. Dashed lines represent tertiary interactions involving the SLI substrate. Gray shading is used to highlight the residues from the II–III–VI junction that are present in the J236 RNA. In B, the filled and open arrowheads represent residues for which site-specific base substitution decreases the activity of the ribozyme by ≥ 20 -fold and < 20 -fold, respectively (Lafontaine et al. 2001a,b; Sood and Collins 2002; McLeod and Lilley 2004). The gray boxes highlight A-rich bulges for which deletion of the bulge and/or strand inversion decreases the activity of the ribozyme by ≥ 20 -fold (Lafontaine et al. 2001b, 2002; Sood and Collins 2002). The black pentagons and circles indicate residues that have their ribose and 5'-phosphate groups protected from hydroxyl radical and ENU modifications, respectively, under native conditions (Sood et al. 1998; Hiley and Collins 2001; Sood and Collins 2002). The W and H letters indicate residues that have their Watson–Crick and Hoogsteen edges accessible to chemical probing under native conditions (Beattie et al. 1995; Beattie and Collins 1997; Maguire and Collins 2001; Sood and Collins 2002). The dashed line represents formation of a UV crosslink between the two residues shaded in light gray (Sood and Collins 2002).

the substrate for catalysis (Andersen and Collins 2000, 2001; Bouchard and Legault 2014b). Similarly to the hairpin ribozyme, the VS ribozyme active site is formed by the interaction of two internal loops: the cleavage site internal loop of SLI and the A730 internal loop of stem-loop VI (SLVI) (Wilson et al. 2007; Lilley 2008; Desjardins et al. 2011; Wilson and Lilley 2011). In addition, the internal loops of SLI and SLVI each contain a key catalytic residue (G₆₃₈ and A₇₅₆) that contribute to the proposed general acid–base

mechanism (Jones and Strobel 2003; McLeod and Lilley 2004; Zhao et al. 2005; Smith and Collins 2007; Wilson et al. 2007, 2010; Jaikaran et al. 2008; Smith et al. 2008).

Although no complete high-resolution structure has been reported for the VS ribozyme, low-resolution models have been derived from biochemical, fluorescence resonance energy transfer (FRET), and small-angle X-ray scattering (SAXS) studies that provide insights into its global structure (Hiley and Collins 2001; Lafontaine et al. 2001a, 2002; Lipfert et al. 2008). Moreover, several high-resolution NMR structures of isolated subdomains of the *Neurospora* VS ribozyme have been determined, including the structures of the SLI substrate in its inactive (Michiels et al. 2000; Flinders and Dieckmann 2001) and active forms (Hoffmann et al. 2003), the terminal loop of SLV in the presence and absence of magnesium ions (Mg²⁺) (Campbell and Legault 2005; Campbell et al. 2006), the I/V kissing-loop interaction (Bouchard and Legault 2014b), the A730 loop of SLVI (Flinders and Dieckmann 2004; Desjardins et al. 2011; Bonneau and Legault 2014a), and the III–IV–V three-way junction (Bonneau and Legault 2014b). Our laboratory has contributed significantly to determining NMR structures of VS ribozyme subdomains with the goal of defining a complete high-resolution solution structure of the *Neurospora* VS ribozyme. The only significant domain that remains to be structurally characterized is the II–III–VI three-way junction.

The II–III–VI junction is a key architectural domain (Fig. 1A) that is essential for the activity of the VS ribozyme; it orients the A730 loop of SLVI in such a way that it can form the active site with SLI (Lafontaine et al. 2001a, 2002; Sood and Collins 2002). Substitutions and chemical modifications of several residues within the junction significantly decrease the catalytic activity of the VS ribozyme, and several of these residues are protected from chemical modifications under native conditions (Fig. 1B). Together, these results indicate that the II–III–VI junction adopts a well-defined compact structure. Interestingly, substitution of the II–III–VI junction by a rRNA junction of similar sequence leads to a 10-fold reduction in the cleavage rate, suggesting that these junctions may share structural characteristics (Lafontaine et al. 2001a). Moreover, bioinformatic (Lescoute and Westhof 2006; Tyagi and Mathews 2007) and biophysical (Lafontaine et al. 2001a) studies of the II–III–VI junction suggest that it adopts a family A topology with coaxial stacking of stems III and VI (Lafontaine et al. 2001a; Lescoute and Westhof 2006; Tyagi and Mathews 2007). In contrast, a more recent bioinformatic study proposed a family C topology with coaxial stacking of stems II and III (Laing et al. 2012). Thus, there is a lack of consensus at this time on the basic topology of the II–III–VI junction within the VS ribozyme. In addition, although the adenine bulges of the adjoining stems have been shown to contribute to the activity and likely to the global structure of the VS ribozyme, it is not clear if they play a role in defining the II–III–VI junction (Lafontaine et al. 2001b, 2002; Sood and Collins 2002; McLeod and Lilley 2004). Similarly,

although divalent metal ions have been shown to stabilize the II–III–VI junction (Beattie et al. 1995; Sood et al. 1998; Hiley and Collins 2001; Lafontaine et al. 2001a; Maguire and Collins 2001; Sood and Collins 2002; Pereira et al. 2008), their precise role remains unknown.

In this study, we investigate the NMR solution structure of a 62-nucleotide (nt) RNA (J236) that encompasses the VS ribozyme II–III–VI junction and its three adjoining stems, each one containing its natural adenine bulge (Fig. 1B). Furthermore, we localize Mg^{2+} -binding sites within the J236 RNA using manganese (Mn^{2+})-induced paramagnetic relaxation enhancement (PRE) (Bonneau and Legault 2014a). The NMR structure of J236 reveals that the II–III–VI junction belongs to the family C of three-way junctions, with a complex network of interactions at the junction and helical stacking of stems II and III. We also identify a remote tertiary interaction between the adenine bulges of stems II and VI that stabilizes the structure of the II–III–VI junction. In combination with the wealth of information available from previous NMR studies of isolated subdomains, the NMR structure of J236 provides important new insights into the global three-dimensional structure of the *Neurospora* VS ribozyme.

RESULTS

The J236 RNA adopts a stable structure in the presence of Mg^{2+} ions

We first investigated the effect of Mg^{2+} ions on the structure of the J236 RNA using 1H – ^{15}N heteronuclear NMR methods. In the presence of 5 mM $MgCl_2$, the 2D 1H – ^{15}N HSQC spectrum (Fig. 2A) is well dispersed with detectable signals for 25 of the 28 imino groups, which is consistent with the formation of a unique stable structure for J236. By comparison, the 2D 1H – ^{15}N HSQC spectrum of J236 collected in the absence of Mg^{2+} ions is considerably different and contains fewer high-intensity signals (Fig. 2B). These results indicate that formation of a stable II–III–VI junction is dependent on Mg^{2+} ions, in agreement with previous biochemical and FRET studies (Beattie et al. 1995; Sood et al. 1998; Hiley and Collins 2001; Lafontaine et al. 2001a; Maguire and Collins 2001; Sood and Collins 2002; Pereira et al. 2008). Interestingly, an imino signal with an unusual ^{15}N chemical shift is ob-

FIGURE 2. The J236 RNA adopts a stable structure that requires Mg^{2+} ions and an intact bulge in stem II. (A,B) 2D 1H – ^{15}N HSQC spectra of the J236 RNA in the presence (A) and absence (B) of 5 mM $MgCl_2$. (Above A,B) Primary and secondary structures of J236 with shading of residues color-coded according to structural elements present in the NMR structure (see Fig. 3). (C,D) 2D 1H – ^{15}N HSQC spectra of (C) the J236- ΔA_6 and (D) the J236- A_6 -bp RNAs, both collected in the presence of 5 mM $MgCl_2$. (Above C,D) Modifications in stem II of the J236- ΔA_6 and the J236- A_6 -bp RNAs.

served for G_{53} only in the presence of Mg^{2+} ions (Fig. 2A), and a 2D HNN-COSY spectrum reveals that this residue forms a WC/WC G•A base pair within the junction (data not shown). Furthermore, a network of NOEs is observed between G_{53} and both G_9 of stem II and G_{28} of stem III (data not shown), suggesting stacking between stems II and III as part of a highly organized junction.

Three-way junctions are generally stabilized by remote tertiary interactions that involve residues from adjacent stems (de la Pena et al. 2009). To test for the presence of a remote tertiary interaction in the II–III–VI junction, we recorded 2D 1H – ^{15}N HSQC spectra of two variants of J236 in which the stem II bulge was removed, either by the deletion of A_6 (J236- ΔA_6 RNA; Fig. 2C) or by the insertion of a U residue

on the opposite strand to form an A–U base pair (J236-A₆-bp RNA; Fig. 2D). The NMR data indicate that these variants do not adopt a stable fold even in the presence of Mg²⁺ ions (Fig. 2C,D). Thus, the A₆ bulge of stem II plays an important role in stabilizing the structure of the II–III–VI junction, possibly by mediating a remote interaction with stem VI.

The overall NMR structure of the J236 RNA

The high-resolution solution structures of the J236 RNA were determined in the presence of Mg²⁺ ions using heteronuclear NMR spectroscopy of uniformly labeled (¹⁵N or ¹³C/¹⁵N) and selectively labeled (with ¹³C/¹⁵N-labeled A, C or G residues) J236 RNAs. This labeling strategy allowed for almost complete assignments of the observable resonances (¹H, ¹⁵N, and ¹³C) of the bases and the C1'–H1', C2'–H2', C3'–H3', and C4'–H4' resonances of the ribose moieties. Experiments on selectively labeled J236 RNAs were particularly valuable for analysis of NMR data given that J236 is larger than 60 nt. In fact, only a few NMR structures are available for such larger RNAs (Lukavsky et al. 2003; D'Souza et al. 2004; Miyazaki et al. 2010; Burke et al. 2012; Miller et al. 2014; Keane et al. 2015).

Three-dimensional structures of J236 were initially determined using NOE-derived distance restraints and dihedral angle restraints. Subsequently, structures of J236 were calculated with bound Mg²⁺ ions (J236^{Mg}) by adding metal–RNA restraints derived from Mn²⁺-induced PRE to the existing set of experimental restraints (Bonneau and Legault 2014a,b). Due to these additional restraints, the J236^{Mg} structure (Fig. 3) is better defined than the original J236 structure with heavy atom RMSD values that are lower for the overall structure (2.95 ± 0.73 Å versus 3.97 ± 1.51 Å) as well as for local structural elements (≤1.4 Å versus ≤1.8 Å; Table 1). Thus, the J236^{Mg} structure represents a high-resolution structure with well-defined local structural elements and is presented in detail, although similar observations were made with the J236 structure.

The J236^{Mg} RNA adopts a Y-shaped fold that belongs to the family C of three-way junctions (Lescoute and Westhof 2006), with a well-defined core domain (RMSD of 0.67 ± 0.10 Å; Table 1; Fig. 3) that orients stems II and VI side-by-side and away from stem III. Stems II and VI define an acute interhelical angle ($\phi_{II-VI} = 77.5^\circ \pm 10.0^\circ$), whereas the other two stem pairs define obtuse interhelical angles ($\phi_{II-III} = 127.0^\circ \pm 7.9^\circ$

TABLE 1. Structural statistics for J236 and J236^{Mg}

	J236	J236 ^{Mg}
Number of experimental restraints		
NOE-derived distance restraints	2341	2341
Internucleotide	1090	1090
Intranucleotide	1244	1244
Ambiguous	7	7
Hydrogen-bond restraints	120	120
Base pair planarity restraints	42	42
Mg ²⁺ –RNA distance restraints	0	95
Dihedral angle restraints	193	193
Total number of restraints	2696	2791
RMSD from experimental restraints		
NOE (Å) (none >0.2)	0.0088 ± 0.0004	0.0117 ± 0.0003
Dihedral (°) (none >5°)	0.06 ± 0.01	0.07 ± 0.01
RMSD from idealized geometry		
Bonds (Å)	0.00375 ± 0.00003	0.00382 ± 0.00002
Angles (°)	1.017 ± 0.001	1.004 ± 0.001
Improper (°)	0.379 ± 0.002	0.386 ± 0.002
Heavy-atom RMSD to the minimized average structure (Å)		
Overall (residues 2–60)	3.97 ± 1.51	2.95 ± 0.73
Stem II (residues 2–9 and 54–60)	1.80 ± 0.72	1.38 ± 0.51
Stem-loop III (residues 13–27)	1.47 ± 0.61	0.97 ± 0.32
Stem-loop VI (residues 30–32, 34–47)	1.50 ± 0.63	1.21 ± 0.35
Core (residues 10–13, 27–29, and 48–53)	0.83 ± 0.14	0.67 ± 0.10
(U ₁₃ –A ₂₇)•A ₅₁ base triple	0.59 ± 0.09	0.51 ± 0.10
(C ₁₂ –G ₂₈)•A ₅₂ base triple	0.69 ± 0.15	0.53 ± 0.17
A ₁₀ •G ₅₃ <i>cis</i> WC/WC base pair	0.58 ± 0.21	0.33 ± 0.15
A ₆ •A ₃₂ <i>cis</i> Sugar edge/WC base pair	1.20 ± 0.25	0.80 ± 0.24

and $\phi_{III-VI} = 149.3^\circ \pm 9.6^\circ$). The orientation of stems II and VI allows for the formation of a remote interaction between the A₆ bulge of stem II and the A₃₂A₃₃ bulge of stem VI.

The J236^{Mg} structure contains a total of eight Mg²⁺-binding sites that were identified based on Mn²⁺-induced PRE and modeled as hexahydrated Mg²⁺ complexes [Mg(H₂O)₆]²⁺ (Fig. 3B; Table 2). As detailed below, several of these sites are associated with structural elements that stabilize the II–III–VI junction. It is important to note that the PRE studies do not provide information about the occupancy of the Mg²⁺ ions at these sites, but simply reveal preferential sites for Mg²⁺ binding (Bonneau and Legault 2014b). Accordingly, several residues within the J236 core and bulge–bulge interaction display ¹H–¹³C and ¹H–¹⁵N correlation signals of either relatively low intensity (C₅, A₆, A₁₀, C₁₂, U₁₃, C₃₀, U₃₁, C₄₅, A₅₁, A₅₂, and G₅₃) or that correspond to two populations in slow exchange (G₇), suggestive of conformational exchanges associated with Mg²⁺-ion binding.

The core of the J236 RNA adopts a compact structure

The core of J236^{Mg} adopts a well-defined structure, in which stems II and III form a continuous helical segment that excludes A₁₁ (Fig. 4). In contrast, the stacking is disrupted

TABLE 2. Structural characteristics of $\text{Mg}(\text{H}_2\text{O})_6^{2+}$ -binding sites in J236^{Mg}

Site	Outer-sphere ligands ^a	Electrostatic interactions ^b	Cation– π interactions ^c
Site 1	G ₃₆ O6 G ₃₆ N7 G ₃₆ 5'- <i>pro</i> -R _p OP	G ₃₆ 5'-PO ₄ [−]	
Site 2	A ₆ O2' G ₃₄ O6 G ₃₄ N7	G ₇ 5'-PO ₄ [−] G ₃₄ 5'-PO ₄ [−]	
Site 3	A ₄₆ N7 C ₄₇ O6 C ₄₇ N7	C ₅ 5'-PO ₄ [−] A ₆ 5'-PO ₄ [−] A ₄₆ 5'-PO ₄ [−] G ₄₇ 5'-PO ₄ [−]	
Site 4	A ₄₉ O2' G ₅₃ 5'- <i>pro</i> -R _p OP	A ₅₂ 5'-PO ₄ [−] G ₅₃ 5'-PO ₄ [−] C ₅₄ 5'-PO ₄ [−]	
Site 5	G ₂₆ 5'- <i>pro</i> -R _p OP G ₂₆ O6 G ₂₆ N7 A ₂₇ N7	A ₂₅ 5'-PO ₄ [−] G ₂₆ 5'-PO ₄ [−]	
Site 6	G ₁₇ O6 G ₂₁ O6 G ₂₁ N7		
Site 7	G ₉ 5'- <i>pro</i> -R _p OP G ₉ N7 A ₁₀ N7 G ₂₈ O2' G ₅₃ O6	G ₉ 5'-PO ₄ [−] A ₁₀ 5'-PO ₄ [−] G ₂₉ 5'-PO ₄ [−]	
Site 8	G ₇ O6 G ₅₇ O6	A ₆ 5'-PO ₄ [−]	C ₅

^aOuter-sphere ligands were determined by selecting all hydrogen-bond acceptors within 4.0 Å of a $\text{Mg}(\text{H}_2\text{O})_6^{2+}$ oxygen and in a favorable orientation for outer-sphere interactions in at least 25% of the lowest-energy structures presenting an occupancy at a site.

^bElectrostatic interactions were determined by selecting 5'-PO₄[−] within 8.0 Å of a Mg^{2+} ion.

^cCation– π interactions were determined for Mg^{2+} ions within 5.2 Å and at an angle smaller than 52° from the ring centroid (McFail-Isom et al. 1998).

between stems III and VI, with G₂₈ and G₂₉ being stacked on their respective stems, but in a splayed conformation relative to each other that creates an abrupt turn in the phosphate backbone (Figs. 3, 4). The longest single-stranded region in the junction connects stems II and VI and contains a backbone turn involving residues A₄₉, C₅₀, A₅₁, and A₅₂ (Fig. 4). Within this ACAA turn, there is continuous stacking between C₅₀, A₄₉, and C₄₈ of stem VI, whereas the backbone is reversed after C₅₀ to allow for continuous stacking between A₅₁, A₅₂, G₅₃, and C₅₄ of stem II. Although the ACAA turn is reminiscent of a U-turn, it does not display as sharp of a backbone reversal as a U-turn and is stabilized by only one hydrogen bond (C₅₀ 2'-OH to A₅₁ N7) that has no equivalent in U-turn structures (Fig. 4; Campbell et al. 2006). Nevertheless, the ACAA turn of J236 positions the bases of A₅₁ and A₅₂ to interact with the minor groove of stem III, and this positioning of bases for tertiary contacts is typical of the U-turn motif (von Ahsen et al. 1997; Lambert et al. 2006; de la Pena

et al. 2009; Bonneau and Legault 2014b; Bouchard and Legault 2014b).

The core of the three-way junction within J236^{Mg} is stabilized by a *cis* WC/WC G•A base pair and two minor groove base triples (Fig. 5). The (U₁₃–A₂₇)•A₅₁ base triple (RMSD of 0.51 ± 0.10 Å) is formed by the interaction of the WC edge of A₅₁ with the minor groove of the WC U₁₃–A₂₇ base pair, whereas the (C₁₂–G₂₈)•A₅₂ base triple (RMSD of 0.53 ± 0.17 Å) involves the WC edge of A₅₂ and the minor groove of the WC C₁₂–G₂₈ base pair. The (C₁₂–G₂₈)•A₅₂ base triple stacks with the (U₁₃–A₂₇)•A₅₁ base triple on one side and with the well-defined *cis* WC/WC A₁₀•G₅₃ base pair (RMSD of 0.33 ± 0.15 Å) on the other side to allow for continuous stacking between stems II and III.

Two Mg^{2+} ions interact with the core domain (Fig. 4; Table 2) that are both associated with the A₁₀•G₅₃ base pair. The Mg^{2+} ion at Site 4 is located at the A₄₉C₅₀A₅₁A₅₂ turn, making outer-sphere interactions with both A₄₉ and G₅₃ and stabilized by electrostatic interactions with the 5'-phosphates of A₅₂, G₅₃, and C₅₄. The Mg^{2+} ion at Site 7 is located within the major groove of stem II, making outer-sphere contacts with G₉, A₁₀, G₂₈, and G₅₃ and stabilized by electrostatic interactions with the 5'-phosphates of G₉, A₁₀, and G₂₉. Although the Mg^{2+} ion at Site 7 may play a specific role in stabilizing the sharp backbone turn at the G₂₈–G₂₉ dinucleotide step, both of these Mg^{2+} ions are likely important for the compact structure at the II–III–VI junction.

A remote tertiary interaction between stems II and VI

The NMR structure of J236^{Mg} also reveals a remote tertiary interaction in which the A₆ bulge from stem II interacts with the A₃₂A₃₃ bulge of stem VI (Fig. 6A, left panel). In this bulge–bulge interaction, A₆, which protrudes from stem II, forms two hydrogen bonds with A₃₂ to adopt a *cis* Sugar edge/WC A₆•A₃₂ base pair (RMSD of 0.80 ± 0.24 Å) (Fig. 6B). In addition, the base of A₆ is sandwiched between the bases of residues C₄₅ and A₄₆ in stem VI (Fig. 6A, right panel). This tertiary interaction may be facilitated by the S-turn motif centered at the A₃₂A₃₃ bulge (Fig. 6A, right panel), which involves the backbone reversal and the 2'-endo conformation of the A₃₂ ribose and the exclusion of A₃₃ from stem VI (Correll et al. 1997; Desjardins et al. 2011). It is important to note that this bulge–bulge interaction is well defined by the NMR data and that we did not include any explicit hydrogen bonding restraint to define the A₆•A₃₂ base pair. Instead, two critical NOE interactions were observed that helped define the geometry of the A₆•A₃₂ base pair: A₃₂ H2 to A₆ H1' and A₃₂ H2 to A₆ H4' (Fig. 6C). Several additional NOEs were observed that allow us to define a total of 19 unique distance restraints between stems II and IV at the bulge–bulge interaction.

Three Mg^{2+} -binding sites were identified near the bulge–bulge interaction. The Mg^{2+} ions at Sites 2 and 3 interact with residues of both stems II and VI, whereas the Mg^{2+}

FIGURE 3. NMR solution structure of the J236^{Mg} RNA. (A) Stereoview of the 10 lowest-energy structures of J236^{Mg}. Only heavy atoms of residues 2–60 are included in the superposition. For clarity, the Mg²⁺ ions were omitted. (B) Stick representations of the lowest-energy structure of J236^{Mg} (residues 1–61), with the Mg²⁺ ions (dark gray spheres) numbered 1–8. (C) Schematic representation of the tertiary structure of J236^{Mg} derived from the NMR structure. Canonical and noncanonical base pairs are represented using the Leontis–Westhof notation (Leontis and Westhof 2001; Abu Almakarem et al. 2012). In A–C, residues are color-coded according to structural elements: the (U₁₃–A₂₇)•A₅₁ base triple in green, the (C₁₂–G₂₈)•A₅₂ base triple in magenta, the A₁₀•G₅₃ base pair in light blue, the unpaired residues of the core (A₁₁, A₄₉, and C₅₀) in orange, the A₆ bulge of stem II in dark blue, and the A₃₂A₃₃ bulge of stem VI in red.

ion at Site 8 interacts with stem II near the A₆ bulge (Fig. 6A; Table 2). It is likely that these three Mg²⁺ ions stabilize the formation of the II–VI bulge–bulge interaction by counteracting the negative charges carried by the backbones of stems II and VI.

DISCUSSION

Our NMR studies of J236 reveal that the II–III–VI junction of the VS ribozyme forms a compact core as well as a bulge–bulge interaction between stems II and VI. The integrity of the structure depends on both the formation of this remote

tertiary interaction and the presence of Mg²⁺ ions. These new findings are discussed below in light of previous biochemical and biophysical studies of the VS ribozyme. Being the final piece of the puzzle in our quest to characterize isolated subdomains of the *Neurospora* VS ribozyme by NMR spectroscopy, the structure of the II–III–VI junction allows us to gain further insights into the overall three-dimensional structure of the complete VS ribozyme.

The NMR structure of the II–III–VI junction core is consistent with biochemical data

The core of the J236 RNA relies on a compact network of interactions (Figs. 3–5) that is compatible with previous mutagenesis studies of the VS ribozyme (Beattie et al. 1995; Lafontaine et al. 2001a; McLeod and Lilley 2004). In agreement with the structural importance of the *cis* WC/WC A₁₀•G₅₃ base pair, single base substitutions of either A₁₀ or G₅₃, but not the A₁₀•G₅₃ base pair inversion, greatly decrease (up to 500-fold) the catalytic activity of the VS ribozyme (for simplicity, J236 numbering is used for VS ribozyme residues) (Lafontaine et al. 2001a; McLeod and Lilley 2004). Similarly, single base substitutions of either A₅₁ or A₅₂ within the A₄₉C₅₀A₅₁A₅₂ turn lead to reduction in cleavage activity (~20-fold), consistent with the participation of both A₅₁ and A₅₂ in base triples (Fig. 5; Lafontaine et al. 2001a). In addition, single inversions of the junction proximal base pairs (G₉–C₅₄ in stem II, C₁₂–G₂₈ and U₁₃–A₂₇ in stem III or G₂₉–C₄₈ in stem VI) did not significantly alter VS ribozyme catalytic

activity (Beattie et al. 1995; Lafontaine et al. 2001a), which is consistent with the lack of tertiary interaction involving the closing base pairs of stems II and VI. Moreover, it suggests that the inversions of the C₁₂–G₂₈ and U₁₃–A₂₇ base pairs in stem III also support the formation of base triples equivalent to the (U₁₃–A₂₇)•A₅₁ and (C₁₂–G₂₈)•A₅₂ base triples. Using WebFR3D, we identified several examples in the PDB of such inverted (A–U)•A and (G–C)•A base triples with the same topology as those observed in J236 (Petrov et al. 2011).

Chemical probing data obtained under native conditions are also consistent with the J236 core structure. Briefly, the

FIGURE 4. NMR structure of the core domain of J236^{Mg}. (A) Superposition of the 10 lowest-energy structures. Only heavy atoms of core residues (10–13, 27–29, 48–53) are included in the superposition. The Mg²⁺ ions associated with each structure are shown as dark gray spheres. (B) Stick representations of the lowest-energy structure of the core domain. The Mg²⁺ ions at Sites 4 and 7 are shown in dark gray, with their bound water molecules in lighter gray. The cyan dashed line represents a hydrogen bond defined on the basis of a short distance (≤ 4.0 Å) between C₅₀ 2'-OH and A₅₁ N7 in the ensemble of structures.

WC edges of all core residues are protected from chemical probing in the presence of Mg²⁺ ions, with the exception of A₁₁ and C₅₀, in agreement with the compact structure of the core (Beattie et al. 1995; Maguire and Collins 2001; Sood and Collins 2002). Similarly, the 5'-phosphates of A₁₀, A₁₁, and G₂₉ are protected from ethylnitrosourea (ENU) modification (Sood et al. 1998) and the riboses of A₁₀, A₂₇, G₂₈, and G₂₉ are protected from hydroxyl radical footprinting (Hiley and Collins 2001), in agreement with backbone distortion near these residues, reduced accessibility of ribose C4' within base triples (for A₂₇ and G₂₈; Fig. 5) and binding of a Mg²⁺ ion at Site 7 (for A₁₀ and G₂₉; Fig. 4). Moreover, the effect of chemical modifications on the VS ribozyme activity is consistent with the impairment of critical interactions observed in the NMR structure (Beattie and Collins 1997; Sood et al. 2002). Thus, the NMR structure of the J236 core is in general agreement with the available biochemical data for the VS ribozyme, indicating that the II–III–VI junction adopts a similar core structure within the active VS ribozyme.

As an exception, A₁₁ adopts a bulged out position in the NMR structure (Fig. 4) that appears incompatible with biochemical data. Substitution of this base by a U leads to a 15-fold reduction in the cleavage activity (Lafontaine et al. 2001a), and this is similar to the effects of carboxyethylation at the N7 position (Beattie and Collins 1997) and base substitution by purine (Jones and Strobel 2003). Taken together, these results suggest that these modifications of A₁₁ prevent the formation of a tertiary interaction that is not present in J236 but may contribute to the cleavage activity of the VS ribozyme.

The II–VI bulge–bulge interaction is supported by biochemical data

The NMR structure of J236 reveals a bulge–bulge interaction between stems II and VI (Fig. 6). Interestingly, such remote tertiary interaction between two stems of a three-way junction is found in almost all junctions with family C topology (Lescoute and Westhof 2006; de la Pena et al. 2009). The one observed in J236 was not previously identified in the VS ribozyme, but is nevertheless consistent with previous mutagenesis data. In particular, it was demonstrated that the adenine bulges in stems II (A₆) and VI (A₃₂A₃₃) play critical roles in the VS ribozyme, since their deletion or strand inversion reduces the cleavage activity by up to 1000-fold (Lafontaine et al. 2001b; Sood and Collins 2002; McLeod and Lilley 2004). Conversely, most single base substitutions of A₆, A₃₂, and A₃₃ do not significantly affect the cleavage

FIGURE 5. Base-pairing within the core domain of J236^{Mg}. The (U₁₃–A₂₇)•A₅₁ minor groove base triple (A), the (C₁₂–G₂₈)•A₅₂ minor groove base triple (B), and the *cis* WC/WC A₁₀•G₅₃ base pair (C) located in the core of J236^{Mg} are shown on the lowest-energy structure (left panels) and on the superposition of the 10 lowest-energy structures (right panels; only the residues shown were used for the superposition). Cyan dashed lines represent hydrogen bonds that are defined on the basis of short distances (≤ 4.0 Å) between heavy atoms in the ensemble of structures.

FIGURE 6. NMR structure of the II–VI bulge–bulge interaction of the J236^{Mg} RNA. (A) Stick representations of one of the lowest-energy structures of the II–VI bulge–bulge interaction showing the A₆•A₃₂ base pair (left panel) and the S-turn at the A₃₂A₃₃ bulge (right panel). Only heavy atoms from the bulges, the closing base pairs of stems II and VI and the S-turn at the A₃₂A₃₃ bulge (residues 5–7, 30–34, 45–47), are shown. The Mg²⁺ ions at Sites 2, 3, and 8 are shown as dark gray spheres, with their bound water molecules in lighter gray. (B) The *cis* Sugar edge/WC A₆•A₃₂ base pair is shown from one of the lowest-energy structures (left panel) and on the superposition of the 10 lowest-energy structures (right panel; only the residues shown were used for the superposition). Cyan dashed lines represent hydrogen bonds that are defined on the basis of short distances (≤ 4.0 Å) between heavy atoms in the ensemble of structures. (C) NMR evidence of the *cis* Sugar edge/WC A₆•A₃₂ base pair provided by NOE signals between A₆ and A₃₂ protons. These NOEs are observed in the 2D ¹H–¹H slice of a 3D ¹³C-edited HMQC-NOESY spectrum ($\tau_m = 100$ msec) taken at the ¹³C chemical shifts of A₃₂ C2 (156.3 ppm). The dotted line is drawn at the ¹H chemical shift of A₃₂ H2 (8.08 ppm).

activity (Lafontaine et al. 2001b; Sood and Collins 2002). These results are in agreement with the bulged out position of A₃₃ and a large subset of isosteric base pairs for the *cis* Sugar edge/WC A₆•A₃₂ base pair (Fig. 6; Leontis et al. 2002).

The II–VI bulge–bulge interaction is also supported by both chemical probing and interference data. In chemical probing experiments performed under native conditions, the WC and Hoogsteen edges of both A₆ and A₃₂ but not A₃₃ are protected from chemical modifications, consistent with the *cis* Sugar edge/WC A₆•A₃₂ base pair being nested between the minor grooves of stems II and VI and the exclusion of A₃₃ within the S-turn (Fig. 6; Beattie et al. 1995; Maguire and Collins 2001; Sood and Collins 2002). In addition, the 5'-phosphates of G₇ and G₃₄ are protected from modification by ENU and phosphorothioate substitution of G₇ interferes with

the cleavage activity, in agreement with the 5'-phosphates of G₇ and G₃₄ contributing to electrostatic stabilization of the Mg²⁺ ion at Site 2 (Fig. 6; Table 2; Sood et al. 1998). Furthermore, a UV-inducible crosslink was reported between A₆ and A₄₆ (Sood and Collins 2002), in accordance with these residues being stacked on each other (Fig. 6A). Hence, the II–VI bulge–bulge interaction revealed by the NMR structure of J236 correlates very well with previously published biochemical data and most likely adopts an equivalent structure in the active VS ribozyme.

The structure of the II–III–VI junction depends on the II–VI bulge–bulge interaction and the binding of Mg²⁺ ions

Although different topologies and helical stacking schemes have been predicted for the VS ribozyme II–III–VI junction (Lescoute and Westhof 2006; Tyagi and Mathews 2007; Laing et al. 2012), the NMR structure of J236 establishes that it adopts a single conformation with a family C topology and continuous helical stacking of stems II and III. Such helical stacking was not observed in a similar three-way junction from rRNA, and this may explain the 10-fold lower activity of a hybrid VS ribozyme in which the core of the II–III–VI junction was replaced by this rRNA three-way junction (Lafontaine et al. 2001a; Lipfert et al. 2008). Similarly, continuous stacking between stems II and III is not compatible with comparative gel electrophoresis

and FRET studies of the II–III–VI junction (Lafontaine et al. 2001a), which rather suggested near-coaxial alignment of stems III and VI. However, in these studies, a minimal II–III–VI junction was used that did not allow for formation of the II–VI bulge–bulge interaction. Correspondingly, we observe that J236 variants in which the II–VI bulge–bulge interaction is impaired do not adopt a stable structure.

The overall fold of the II–III–VI junction also depends on the presence of Mg²⁺ ions. Our NMR data indicate that the junction core and the II–VI bulge–bulge interaction are both destabilized in the absence of Mg²⁺ ions. In addition, they provide evidence for binding of two Mg²⁺ ions in the J236 core and three Mg²⁺ ions within the II–VI bulge–bulge interaction. Notably, the Mg²⁺ ions at Sites 2, 3, and 8 within these two substructures help bridge phosphates that are

remote in the primary structure. In agreement with a Mg^{2+} -dependent structure for the II–III–VI junction, the Hoogsteen and WC edges of several purines, including A_6 and A_{32} of the bulges, are more protected from chemical modification in the presence of Mg^{2+} ions (Beattie et al. 1995; Beattie and Collins 1997). Moreover, the critical role of Mg^{2+} ions in stabilizing the II–III–VI junction is supported by a single-molecule FRET study of the VS ribozyme that revealed important conformational changes induced by Mg^{2+} ions (Pereira et al. 2008).

Novel insights into the global structure of the VS ribozyme

NMR structures of several isolated subdomains of the *Neurospora* VS ribozyme have been previously determined, and with the NMR structure of the II–III–VI junction presented here, all nonhelical domains of the minimal VS ribozyme are now structurally characterized. Based on these NMR structures, we drew a structural schematic and built a three-dimensional model of a *trans* ribozyme/substrate complex that provide significant insights into the global organization of the complete VS ribozyme (Fig. 7). In particular, the two three-way junctions both adopt a family C topology that clearly defines the orientation of their attached stems. In the II–III–VI three-way junction, stem III directly stacks on stem II and an ACAA turn connects stems II and VI.

Similarly, we previously found that in the III–IV–V three-way junction, stem IV directly stacks on stem III and a U-turn connects stems III and V (Bonneau and Legault 2014b). Thus, these two junctions must allow stacking of stems II, III, and IV to create an extended, continuous helical segment from which stems V and VI project alongside stems III and II, respectively. The SLI substrate associates with the *trans* VS ribozyme via the I/V kissing-loop interaction (Lacroix-Labonté et al. 2012), and this interaction creates a more or less continuous helical region encompassing SLV and SLI (Bouchard and Legault 2014b). In the three-dimensional model, the cleavage site internal loop of SLI is not docked with SLVI to create the active site. Instead, it adopts an open state that is compatible with the ground-state conformation characterized by chemical probing and biophysical studies (Hiley et al. 2002; Pereira et al. 2008; Desjardins et al. 2011; Bouchard and Legault 2014a). Future work should focus on refining this NMR-based model of the *Neurospora* VS ribozyme using global structural restraints and characterizing the dynamics that allow formation of the catalytically active docked structure.

MATERIALS AND METHODS

Plasmids

Double-stranded PCR fragments coding for the J236-VS, J236- A_6 -bp-VS, and J236- ΔA_6 -VS RNAs and flanked by a T7 promoter,

FIGURE 7. Overview of the tertiary structure of the complete VS ribozyme based on NMR structures of isolated subdomains. (A) Schematic of the tertiary structure of a *trans* VS ribozyme and bound substrate based on the S_0/R_0 system (Lacroix-Labonté et al. 2012). Canonical and noncanonical base pairs are represented using the Leontis–Westhof notation, except for the $C_{665} \cdot U_{713} \cdot U_{686}$ base triple where intermediate interactions were represented by gray lines (Leontis and Westhof 2001; Abu Almakarem et al. 2012). (B) Stereoview of the NMR-based model of a substrate/ribozyme complex in an open conformation. Due to the modeling approach, this complex differs slightly from the S_0/R_0 complex (see text). The cleavage site (small red sphere) must form an interaction with the A730 loop (dashed line) to form the active site. The color-coding of the helical segments is equivalent in A and B.

were inserted into the HindIII/EcoRI sites of the pTZ19R-derived pTR-4 vector (Rastogi and Collins 1998) to generate the pJ236, pJ236- ΔA_6 , and pJ236- A_6 -bp plasmids. These plasmids were fully linearized using EcoRI (New England Biolabs) and used for transcription of the J236, J236- ΔA_6 , and J236- A_6 -bp RNAs (Fig. 1B) with a VS ribozyme substrate at their 3' end (Rastogi and Collins 1998).

RNA synthesis and purification

Unlabeled, ^{15}N -labeled and $^{13}\text{C}/^{15}\text{N}$ -labeled RNAs (J236-VS, J236- ΔA_6 -VS, and J236- A_6 -bp-VS) were synthesized in vitro with the T7 RNA polymerase, as previously described (Bonneau and Legault 2014b). Nucleotide-specific $^{13}\text{C}/^{15}\text{N}$ -labeled J236-VS RNAs were also synthesized using purified $^{13}\text{C}/^{15}\text{N}$ ATP, $^{13}\text{C}/^{15}\text{N}$ CTP, or $^{13}\text{C}/^{15}\text{N}$ GTP for preparation of A^{CN} -J236, C^{CN} -J236, and G^{CN} -J236 RNAs (Dagenais and Legault 2012). Following RNA synthesis, the VS ribozyme substrate was cleaved using a *trans*-acting VS ribozyme to produce a homogeneous 3' end (Guo and Collins 1995; Rastogi and Collins 1998). The RNAs were then purified by denaturing gel electrophoresis, treated with calf intestinal alkaline phosphatase (CIP, Roche Diagnostics) to remove their 5'-phosphates, and further purified by DEAE-Sepharose chromatography (Delfosse et al. 2010; Bonneau and Legault 2014a). The purified RNAs were concentrated and exchanged in NMR buffer A (10 mM sodium cacodylate [pH 6.5], 50 mM KCl, 0.05 mM NaN_3 and 90%:10% H_2O : D_2O) with Amicon Ultra-4 centrifugation filter devices (Millipore). The RNAs were then heated at 37°C for 2 min and cooled in ice water for 5 min before changing to the final NMR buffer using the same filter device (NMR buffer A with 5 mM MgCl_2 99.995% [Sigma-Aldrich]). For NMR studies in D_2O , the samples were obtained by four cycles of lyophilization and resuspension in 99.996% D_2O .

NMR spectroscopy

All NMR experiments were collected on a Varian UnityINOVA 600 MHz spectrometer. NMR resonance assignment and structural restraints for the J236 RNA were obtained as previously described (Bonneau and Legault 2014b). In addition, an A-specific H(NC)-TOCSY-(C)H spectrum (Simorre et al. 1996) was collected for unambiguous assignment of the adenine protons in the junction. It is important to note that 3D CT-HCCH-COSY (Pardi and Nikonowicz 1992), 3D HCCH-TOCSY (Pardi and Nikonowicz 1992), and ^{13}C -edited HMQC-NOESY (Ikura et al. 1990) spectra collected on nucleotide-specific $^{13}\text{C}/^{15}\text{N}$ -labeled J236 RNAs (A^{CN} -J236, C^{CN} -J236, and G^{CN} -J236 RNAs) significantly contributed to the unambiguous assignment of NMR signals from the fully $^{13}\text{C}/^{15}\text{N}$ -labeled J236. NOE-derived distance restraints were separated in four classes based on NOE crosspeak intensities, and dihedral angle restraints for the sugar puckers (δ) and other backbone dihedral angles (α , γ , χ , and ζ) were defined based on comparative NOE analyses (Wijmenga et al. 1993). Canonical distance restraints and backbone torsion angles were used to define helical regions in agreement with the NMR data. NMR chemical shifts, structural restraints and structural coordinates have been deposited for J236 and J236 $^{\text{Mg}}$ as BMRB accession numbers 25654 and 25655 and PDB ID codes 2N3Q and 2N3R, respectively.

Metal-ion binding studies

Manganese (Mn^{2+}) titrations were performed with two J236 samples (1.5-mM ^{15}N -labeled J236 and 2.0-mM $^{13}\text{C}/^{15}\text{N}$ -labeled J236) in NMR buffer A containing 5 mM MgCl_2 , as previously described (Bonneau and Legault 2014a). RNA-metal distance restraints were derived from Mn^{2+} -induced paramagnetic relaxation enhancement (PRE) using the ratio of signal intensity (I_0/I_{Mn}) determined from spectra collected with (I_{Mn}) and without (I_0) 20 μM MnCl_2 (Bonneau and Legault 2014a).

Structure calculation

Three-dimensional structures of J236 were calculated using restrained molecular dynamics and simulated annealing in X-PLOR-NIH version 2.1.9 (Schwieters et al. 2003) starting from structures with randomized backbone angles, as previously described (Campbell et al. 2006). A force field was used that included bond, angle, improper and repulsive van der Waals energy terms as well as NOE and torsion-angle pseudoenergy terms, but no electrostatic terms. Three-dimensional structures of J236 bound to eight magnesium complexes [$\text{Mg}(\text{H}_2\text{O})_6^{2+}$] (Kleywegt and Jones 1998) termed J236 $^{\text{Mg}}$, were calculated as described for J236, but using additional metal-RNA restraints (Bonneau and Legault 2014a). For both J236 and J236 $^{\text{Mg}}$, the 20 lowest-energy structures that satisfied all the experimental restraints (all distance violations <0.2 Å and all torsion-angle violations $<5^\circ$) were selected for analysis and used to calculate average structures that were minimized against all experimental restraints. Structures were visualized with PyMOL Molecular Graphics System, Version 1.3 Schrödinger, and analyzed with PyMOL and Curves+ (Lavery et al. 2009). Reported values of RMSD, interatomic distances and interhelical angles are given as average values with standard deviations for the 20 lowest-energy structures. To calculate the interhelical angles, the helical segments were defined as residues 7–10 and 53–56 for stem II, residues 12–14 and 26–28 for stem III and residues 29–31 and 46–48 for stem VI.

Three-dimensional modeling of the complete ribozyme

A three-dimensional model of a VS ribozyme substrate/ribozyme complex (Fig. 7A; Lacroix-Labonté et al. 2012) was generated by assembly of NMR structures of individual subdomains in PyMOL based on superposition of overlapping helical segments. The following NMR structures of isolated subdomains were used: the cleavage site internal loop of the SLI substrate in its active form (PDB ID code 1OW9) (Hoffmann et al. 2003), the terminal loop of SLV in the presence of Mg^{2+} (PDB ID code 1YN1) (Campbell et al. 2006), the I/V kissing-loop interaction (PDB ID code 2MI0) (Bouchard and Legault 2014b), the A730 loop of SLVI (PDB ID code 2L5Z) (Desjardins et al. 2011), the III–IV–V junction (PDB ID code 2MTJ) (Bonneau and Legault 2014b) and the II–III–VI junction (PDB ID code 2N3Q). The initial model was then minimized with the molecular dynamics package GROMACS (Pronk et al. 2013) and the Amber99SB force field with the ParmBSC0 nucleic acid parameters and using explicit aqueous solvent (Hornak et al. 2006; Perez et al. 2007; Guy et al. 2012).

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REFERENCES

- Abu Almakarem AS, Petrov AI, Stombaugh J, Zirbel CL, Leontis NB. 2012. Comprehensive survey and geometric classification of base triples in RNA structures. *Nucleic Acids Res* **40**: 1407–1423.
- Andersen AA, Collins RA. 2000. Rearrangement of a stable RNA secondary structure during VS ribozyme catalysis. *Mol Cell* **5**: 469–478.
- Andersen AA, Collins RA. 2001. Intramolecular secondary structure rearrangement by the kissing interaction of the *Neurospora* VS ribozyme. *Proc Natl Acad Sci* **98**: 7730–7735.
- Beattie TL, Collins RA. 1997. Identification of functional domains in the self-cleaving *Neurospora* VS ribozyme using damage selection. *J Mol Biol* **267**: 830–840.
- Beattie TL, Olive JE, Collins RA. 1995. A secondary-structure model for the self-cleaving region of *Neurospora* VS RNA. *Proc Natl Acad Sci* **92**: 4686–4690.
- Bonneau E, Legault P. 2014a. NMR localization of divalent cations at the active site of the *Neurospora* VS ribozyme provides insights into RNA–metal-ion interactions. *Biochemistry* **53**: 579–590.
- Bonneau E, Legault P. 2014b. Nuclear magnetic resonance structure of the III–IV–V three-way junction from the Varkud Satellite ribozyme and identification of magnesium-binding sites using paramagnetic relaxation enhancement. *Biochemistry* **53**: 6264–6275.
- Bouchard P, Legault P. 2014a. A remarkably stable kissing-loop interaction defines substrate recognition by the *Neurospora* VS Ribozyme. *RNA* **20**: 1451–1464.
- Bouchard P, Legault P. 2014b. Structural insights into substrate recognition by the *Neurospora* Varkud satellite ribozyme: importance of U-turns at the kissing-loop junction. *Biochemistry* **53**: 258–269.
- Burke JE, Sashital DG, Zuo X, Wang YX, Butcher SE. 2012. Structure of the yeast U2/U6 snRNA complex. *RNA* **18**: 673–683.
- Campbell DO, Legault P. 2005. NMR structure of the Varkud satellite ribozyme stem-loop V RNA and magnesium-ion binding from chemical-shift mapping. *Biochemistry* **44**: 4157–4170.
- Campbell DO, Bouchard P, Desjardins G, Legault P. 2006. NMR structure of Varkud satellite ribozyme stem-loop V in the presence of magnesium ions and localization of metal-binding sites. *Biochemistry* **45**: 10591–10605.
- Cochrane JC, Strobel SA. 2008. Catalytic strategies of self-cleaving ribozymes. *Acc Chem Res* **41**: 1027–1035.
- Collins RA. 2002. The *Neurospora* Varkud satellite ribozyme. *Biochem Soc Trans Rev* **30**: 1122–1126.
- Collins RA, Olive JE. 1993. Reaction conditions and kinetics of self-cleavage of a ribozyme derived from *Neurospora* VS RNA. *Biochemistry* **32**: 2795–2799.
- Correll CC, Freeborn B, Moore PB, Steitz TA. 1997. Metals, motifs, and recognition in the crystal structure of a 5S rRNA domain. *Cell* **91**: 705–712.
- Dagenais P, Legault P. 2012. Preparative separation of ribonucleoside monophosphates by ion-pair reverse-phase HPLC. *Methods Mol Biol* **941**: 247–256.
- de la Pena M, Dufour D, Gallego J. 2009. Three-way RNA junctions with remote tertiary contacts: a recurrent and highly versatile fold. *RNA* **15**: 1949–1964.
- Delfosse V, Bouchard P, Bonneau E, Dagenais P, Lemay JF, Lafontaine DA, Legault P. 2010. Riboswitch structure: an internal residue mimicking the purine ligand. *Nucleic Acids Res* **38**: 2057–2068.
- Desjardins G, Bonneau E, Girard N, Boissbouvier J, Legault P. 2011. NMR structure of the A730 loop of the *Neurospora* VS ribozyme: insights into the formation of the active site. *Nucleic Acids Res* **39**: 4427–4437.
- D'Souza V, Dey A, Habib D, Summers MF. 2004. NMR structure of the 101-nucleotide core encapsidation signal of the Moloney murine leukemia virus. *J Mol Biol* **337**: 427–442.
- Flinders J, Dieckmann T. 2001. A pH controlled conformational switch in the cleavage site of the VS ribozyme substrate RNA. *J Mol Biol* **308**: 665–679.
- Flinders J, Dieckmann T. 2004. The solution structure of the VS ribozyme active site loop reveals a dynamic “hot-spot”. *J Mol Biol* **341**: 935–949.
- Guo HCT, Collins RA. 1995. Efficient trans-cleavage of a stem-loop RNA substrate by a ribozyme derived from *Neurospora* VS RNA. *EMBO J* **14**: 368–376.
- Guy AT, Piggott TJ, Khalid S. 2012. Single-stranded DNA within nanopores: conformational dynamics and implications for sequencing: a molecular dynamics simulation study. *Biophys J* **103**: 1028–1036.
- Hiley SL, Collins RA. 2001. Rapid formation of a solvent-inaccessible core in the *Neurospora* Varkud satellite ribozyme. *EMBO J* **20**: 5461–5469.
- Hiley SL, Sood VD, Fan J, Collins RA. 2002. 4-Thio-U cross-linking identifies the active site of the VS ribozyme. *EMBO J* **21**: 4691–4698.
- Hoffmann B, Mitchell GT, Gendron P, Major F, Andersen AA, Collins RA, Legault P. 2003. NMR structure of the active conformation of the Varkud satellite ribozyme cleavage site. *Proc Natl Acad Sci* **100**: 7003–7008.
- Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C. 2006. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins* **65**: 712–725.
- Ikura M, Kay LE, Tschudin R, Bax A. 1990. Three-dimensional NOESY-HMQC spectroscopy of a ¹³C-labeled protein. *J Magn Reson* **86**: 204–209.
- Jaikaran D, Smith MD, Mehdizadeh R, Olive J, Collins RA. 2008. An important role of G638 in the cis-cleavage reaction of the *Neurospora* VS ribozyme revealed by a novel nucleotide analog incorporation method. *RNA* **14**: 938–949.
- Jones FD, Strobel SA. 2003. Ionization of a critical adenosine residue in the *Neurospora* Varkud satellite ribozyme active site. *Biochemistry* **42**: 4265–4276.
- Keane SC, Heng X, Lu K, Kharytonchyk S, Ramakrishnan V, Carter G, Barton S, Hosc A, Florwick A, Santos J, et al. 2015. RNA structure. Structure of the HIV-1 RNA packaging signal. *Science* **348**: 917–921.
- Kleywegt GT, Jones TA. 1998. Databases in protein crystallography. *Acta Crystallogr D Biol Crystallogr* **54**: 1119–1131.
- Lacroix-Labonté J, Girard N, Lemieux S, Legault P. 2012. Helix-length compensation studies reveal the adaptability of the VS ribozyme architecture. *Nucleic Acids Res* **40**: 2284–2293.
- Lafontaine DA, Norman DG, Lilley DM. 2001a. Structure, folding and activity of the VS ribozyme: importance of the 2-3-6 helical junction. *EMBO J* **20**: 1415–1424.
- Lafontaine DA, Wilson TJ, Norman DG, Lilley DM. 2001b. The A730 loop is an important component of the active site of the VS ribozyme. *J Mol Biol* **312**: 663–674.
- Lafontaine DA, Norman DG, Lilley DM. 2002. The global structure of the VS ribozyme. *EMBO J* **21**: 2461–2471.
- Laing C, Wen D, Wang JT, Schlick T. 2012. Predicting coaxial helical stacking in RNA junctions. *Nucleic Acids Res* **40**: 487–498.

- Lambert D, Heckman JE, Burke JM. 2006. Three conserved guanines approach the reaction site in native and minimal hammerhead ribozymes. *Biochemistry* **45**: 7140–7147.
- Lavery R, Moakher M, Maddocks JH, Petkeviciute D, Zakrzewska K. 2009. Conformational analysis of nucleic acids revisited: curves+. *Nucleic Acids Res* **37**: 5917–5929.
- Leontis NB, Westhof E. 2001. Geometric nomenclature and classification of RNA base pairs. *RNA* **7**: 499–512.
- Leontis NB, Stombaugh J, Westhof E. 2002. The non-Watson-Crick base pairs and their associated isostericity matrices. *Nucleic Acids Res* **30**: 3497–3531.
- Lescoute A, Westhof E. 2006. Topology of three-way junctions in folded RNAs. *RNA* **12**: 83–93.
- Lilley DM. 2004. The Varkud satellite ribozyme. *RNA* **10**: 151–158.
- Lilley DMJ. 2008. The hairpin and Varkud satellite ribozymes. In *Ribozymes and RNA catalysis* (ed. Lilley DMJ, Eckstein F), pp. 66–91. Royal Society of Chemistry, Cambridge.
- Lipfert J, Ouellet J, Norman DG, Doniach S, Lilley DM. 2008. The complete VS ribozyme in solution studied by small-angle X-ray scattering. *Structure* **16**: 1357–1367.
- Lukavsky PJ, Kim I, Otto GA, Puglisi JD. 2003. Structure of HCV IRES domain II determined by NMR. *Nat Struct Biol* **10**: 1033–1038.
- Maguire JL, Collins RA. 2001. Effects of cobalt hexamine on folding and self-cleavage of the *Neurospora* VS ribozyme. *J Mol Biol* **309**: 45–56.
- McFail-Isom L, Shui X, Williams LD. 1998. Divalent cations stabilize unstacked conformations of DNA and RNA by interacting with base pi systems. *Biochemistry* **37**: 17105–17111.
- McLeod AC, Lilley DM. 2004. Efficient, pH-dependent RNA ligation by the VS ribozyme in trans. *Biochemistry* **43**: 1118–1125.
- Michiels PJ, Schouten CHJ, Hilbers CW, Heus HA. 2000. Structure of the ribozyme substrate hairpin of *Neurospora* VS RNA: a close look at the cleavage site. *RNA* **6**: 1821–1832.
- Miller SB, Yildiz FZ, Lo JA, Wang B, D'Souza VM. 2014. A structure-based mechanism for tRNA and retroviral RNA remodelling during primer annealing. *Nature* **528**: 591–595.
- Miyazaki Y, Irobalieva RN, Tolbert BS, Smalls-Mantey A, Iyalla K, Loeliger K, D'Souza V, Khant H, Schmid MF, Garcia EL, et al. 2010. Structure of a conserved retroviral RNA packaging element by NMR spectroscopy and cryo-electron tomography. *J Mol Biol* **404**: 751–772.
- Murray JB, Seyhan AA, Walter NG, Burke JM, Scott WG. 1998. The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone. *Chem Biol* **5**: 587–595.
- Pardi A, Nikonowicz EP. 1992. Simple procedure for resonance assignment of the sugar protons in ^{13}C -labeled RNAs. *J Am Chem Soc* **114**: 9202–9203.
- Pereira MJ, Nikolova EN, Hiley SL, Jaikaran D, Collins RA, Walter NG. 2008. Single VS ribozyme molecules reveal dynamic and hierarchical folding toward catalysis. *J Mol Biol* **382**: 496–509.
- Perez A, Marchan I, Svozil D, Sponer J, Cheatham TE III, Laughton CA, Orozco M. 2007. Refinement of the AMBER force field for nucleic acids: improving the description of α/γ conformers. *Biophys J* **92**: 3817–3829.
- Petrov AI, Zirbel CL, Leontis NB. 2011. WebFR3D—a server for finding, aligning and analyzing recurrent RNA 3D motifs. *Nucleic Acids Res* **39**(Web Server issue): W50–W55.
- Pronk S, Pall S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, Shirts MR, Smith JC, Kasson PM, van der Spoel D, et al. 2013. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics* **29**: 845–854.
- Rastogi T, Collins RA. 1998. Smaller, faster ribozymes reveal the catalytic core of *Neurospora* VS RNA. *J Mol Biol* **277**: 215–224.
- Rastogi T, Beattie TL, Olive JE, Collins RA. 1996. A long-range pseudoknot is required for activity of the *Neurospora* VS ribozyme. *EMBO J* **15**: 2820–2825.
- Roth A, Weinberg Z, Chen AG, Kim PB, Ames TD, Breaker RR. 2014. A widespread self-cleaving ribozyme class is revealed by bioinformatics. *Nat Chem Biol* **10**: 56–60.
- Saville BJ, Collins RA. 1990. A site-specific self-cleavage reaction performed by a novel RNA in *Neurospora* mitochondria. *Cell* **61**: 685–696.
- Saville BL, Collins RA. 1991. RNA-mediated ligation of self-cleavage products of a *Neurospora* mitochondrial plasmid transcript. *Proc Natl Acad Sci* **88**: 8826–8830.
- Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM. 2003. The Xplor-NIH NMR molecular structure determination package. *J Magn Reson* **160**: 66–74.
- Simorre JP, Zimmermann GR, Mueller L, Pardi A. 1996. Triple-resonance experiments for assignment of adenine base resonances in $^{13}\text{C}/^{15}\text{N}$ -labeled RNA. *J Am Chem Soc* **118**: 5316–5317.
- Smith MD, Collins RA. 2007. Evidence for proton transfer in the rate-limiting step of a fast-cleaving Varkud satellite ribozyme. *Proc Natl Acad Sci* **104**: 5818–5823.
- Smith MD, Mehdizadeh R, Olive JE, Collins RA. 2008. The ionic environment determines ribozyme cleavage rate by modulation of nucleobase pKa. *RNA* **14**: 1942–1949.
- Sood VD, Collins RA. 2002. Identification of the catalytic subdomain of the VS ribozyme and evidence for remarkable sequence tolerance in the active site loop. *J Mol Biol* **320**: 443–454.
- Sood VD, Beattie TL, Collins RA. 1998. Identification of phosphate groups involved in metal binding and tertiary interactions in the core of the *Neurospora* VS ribozyme. *J Mol Biol* **282**: 741–750.
- Sood VD, Yekta S, Collins RA. 2002. The contribution of 2'-hydroxyls to the cleavage activity of the *Neurospora* VS ribozyme. *Nucleic Acids Res* **30**: 1132–1138.
- Tyagi R, Mathews DH. 2007. Predicting helical coaxial stacking in RNA multibranch loops. *RNA* **13**: 939–951.
- von Ahsen U, Green R, Schroeder R, Noller HF. 1997. Identification of 2'-hydroxyl groups required for interaction of a tRNA anticodon stem-loop region with the ribosome. *RNA* **3**: 49–56.
- Wijmenga SS, Mooren MMW, Hilbers CW. 1993. NMR of nucleic acids; from spectrum to structure. In *NMR of macromolecules: a practical approach* (ed. Roberts GCK), vol. 134, pp. 217–288. Oxford University Press, New York.
- Wilson TJ, Lilley DM. 2011. Do the hairpin and VS ribozymes share a common catalytic mechanism based on general acid-base catalysis? A critical assessment of available experimental data. *RNA* **17**: 213–221.
- Wilson TJ, McLeod AC, Lilley DM. 2007. A guanine nucleobase important for catalysis by the VS ribozyme. *EMBO J* **26**: 2489–2500.
- Wilson TJ, Li NS, Lu J, Frederiksen JK, Piccirilli JA, Lilley DM. 2010. Nucleobase-mediated general acid-base catalysis in the Varkud satellite ribozyme. *Proc Natl Acad Sci* **107**: 11751–11756.
- Zhao ZY, McLeod A, Harusawa S, Araki L, Yamaguchi M, Kurihara T, Lilley DM. 2005. Nucleobase participation in ribozyme catalysis. *J Am Chem Soc* **127**: 5026–5027.

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Eric Bonneau, Nicolas Girard, Sébastien Lemieux, et al.

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