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

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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, glmS, 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 structural 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...
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 (Mg$^{2+}$) (Campbell and Legault 2005; Campbell et al. 2006), the IV/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. 2001a, 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 $^1$H–$^{15}$N heteronuclear NMR methods. In the presence of 5 mM MgCl$_2$, the 2D $^1$H–$^{15}$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 $^1$H–$^{15}$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 observed 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 staking 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 $^1$H–$^{15}$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.

**FIGURE 2.** The J236 RNA adopts a stable structure that requires Mg$^{2+}$ ions and an intact bulge in stem II. (A,B) 2D $^1$H–$^{15}$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 $^1$H–$^{15}$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 RNA that result in the J236–ΔA$_6$ and the J236–A$_6$-bp RNAs.
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 Mg\(^{2+}\) 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 Mg\(^{2+}\) ions using heteronuclear NMR spectroscopy of uniformly labeled \(^{15}\text{N}\) or \(^{13}\text{C}/^{15}\text{N}\) and selectively labeled (with \(^{13}\text{C}/^{15}\text{N}\)-labeled A, C or G residues) J236 RNAs. This labeling strategy allowed for almost complete assignments of the observable resonances (\(^1\text{H}, ^{15}\text{N}, \) and \(^{13}\text{C}\) of the bases and the \(^{1}\text{H}, ^{1}\text{H}, ^{1}\text{C} \)–\(^{1}\text{C}, ^{1}\text{C} \)–\(^{1}\text{C}, ^{1}\text{C} \)–\(^{1}\text{C}\) and \(^{1}\text{C} \)–\(^{1}\text{C}, ^{1}\text{C} \)–\(^{1}\text{C}\)–\(^{1}\text{H}\) 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 Mg\(^{2+}\) ions (J236\(^{Mg}\)) by adding metal–RNA restraints derived from Mn\(^{2+}\)-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 (ϕ\(_{\text{II–VI}}\) = 77.5° ± 10.0°), whereas the other two stem pairs define obtuse interhelical angles (ϕ\(_{\text{III–III}}\) = 127.0° ± 7.9° and ϕ\(_{\text{III–VI}}\) = 149.3° ± 9.6°). The orientation of stems II and VI allows for the formation of a remote interaction between the A6 bulge of stem II and the A\(_{32}\)A\(_{33}\) bulge of stem VI.

The J236\(^{Mg}\) structure contains a total of eight Mg\(^{2+}\)-bound sites that were identified based on Mn\(^{2+}\)-induced PRE and modeled as hexahydrated Mg\(^{2+}\) complexes [Mg(H\(_2\)O)\(_6\)]\(^{2+}\) (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 Mg\(^{2+}\) ions at these sites, but simply reveal preferential sites for Mg\(^{2+}\) binding (Bonneau and Legault 2014b). Accordingly, several residues within the J236 core and bulge–bulge interaction display \(^{1}\text{H}–^{13}\text{C}\) and \(^{1}\text{H}–^{15}\text{N}\) correlation signals of either relatively low intensity (C\(_{\text{y}}\), A\(_{\text{y0}}\), A\(_{\text{y10}}\), C\(_{\text{12}}\), U\(_{\text{13}}\), C\(_{\text{30}}\), U\(_{\text{31}}\), C\(_{\text{45}}\), A\(_{\text{51}}\), A\(_{\text{52}}\), and G\(_{\text{53}}\)) or that correspond to two populations in slow exchange (G\(_{\text{7}}\)), suggestive of conformational exchanges associated with Mg\(^{2+}\)-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 A\(_{11}\) (Fig. 4). In contrast, the stacking is disrupted
between stems III and VI, with G_{28} and G_{36} 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 A_{49}, C_{50}, A_{51}, and A_{52} (Fig. 4). Within this ACAA turn, there is continuous stacking between C_{50}, A_{49}, and C_{48} of stem VI, whereas the backbone is reversed after C_{50} to allow for continuous stacking between A_{51}, A_{52}, G_{53}, and C_{54} 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 (C_{50} –OH to A_{51} 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 A_{51} and A_{52} 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 Peña et al. 2009; Bonneau and Legault 2014b; Bouchard and Legault 2014b).

The core of the three-way junction within J236Mg is stabilized by a cis WC/WC G+A base pair and two minor groove base triples (Fig. 5). The (U_{13}–A_{27})•A_{51} base triple (RMSD of 0.51 ± 0.10 Å) is formed by the interaction of the WC edge of A_{51} with the minor groove of the WC U_{13}–A_{27} base pair, whereas the (C_{12}–G_{28})•A_{52} base triple (RMSD of 0.53 ± 0.17 Å) involves the WC edge of A_{52} and the minor groove of the WC C_{12}–G_{28} base pair. The (C_{12}–G_{28})•A_{52} base triple stacks with the (U_{13}–A_{27})•A_{51} base triple on one side and with the well-defined cis WC/WC A_{10}•G_{53} 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 A_{10}•G_{53} base pair. The Mg$^{2+}$ ion at Site 4 is located at the A_{49}G_{50}A_{51}A_{52} turn, making outer-sphere interactions with both A_{49} and G_{53} and stabilized by electrostatic interactions with the 5'-phosphates of A_{49}, G_{53}, and C_{54}. The Mg$^{2+}$ ion at Site 7 is located within the major groove of stem II, making outer-sphere contacts with G_{9}, A_{10}, G_{28}, and G_{53} and stabilized by electrostatic interactions with the 5'-phosphates of G_{9}, A_{10}, and G_{28}. Although the Mg$^{2+}$ ion at Site 7 may play a specific role in stabilizing the sharp backbone turn at the G_{28}–G_{29} 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 J236Mg also reveals a remote tertiary interaction in which the A_{6} bulge from stem II interacts with the A_{32}A_{33} bulge of stem VI (Fig. 6A, left panel). In this bulge–bulge interaction, A_{6}, which protrudes from stem II, forms two hydrogen bonds with A_{32} to adopt a cis Sugar edge/WC A_{6}A_{32} base pair (RMSD of 0.80 ± 0.24 Å) (Fig. 6B). In addition, the base of A_{6} is sandwiched between the bases of residues C_{45} and A_{46} in stem VI (Fig. 6A, right panel). This tertiary interaction may be facilitated by the S-turn motif centered at the A_{32}A_{33} bulge (Fig. 6A, right panel), which involves the backbone reversal and the 2'-endo conformation of the A_{12} ribose and the exclusion of A_{33} 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 A_{6}•A_{32} base pair. Instead, two critical NOE interactions were observed that helped define the geometry of the A_{6}•A_{32} base pair: A_{32} H2 to A_{6} H1' and A_{32} H2 to A_{6} 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$^{2+}$

### Table 2. Structural characteristics of Mg(H$_2$O)$_6^{2+}$-binding sites in J236Mg

| Site   | Outer-sphere ligands | Electrostatic interactions | Cation–π interactions |
|--------|----------------------|---------------------------|-----------------------|
| Site 1 | G_{36} O6 G_{36} N7 | G_{36} 5'-PO$_4$          |                       |
| Site 2 | A_{49} O2' G_{53} 5'-pro-R$_{py}$ OP |                       |                       |
| Site 3 | A_{46} N7 A_{47} O6 | A_{46} 5'-PO$_4$          |                       |
| Site 4 | A_{49} O2' G_{53} 5'-pro-R$_{py}$ OP |                       |                       |
| Site 5 | G_{26} 5'-pro-R$_{py}$ OP G_{26} O6 | G_{26} 5'-PO$_4$         |                       |
| Site 6 | G_{17} O6 G_{21} O6 |                       |                       |
| Site 7 | G_{6} 5'-pro-R$_{py}$ OP | G_{6} 5'-PO$_4$          |                       |
| Site 8 | G_{7} O6 G_{57} O6 | A_{8} 5'-PO$_4$ G_{5} 5'-PO$_4$ |                       |

*Outer-sphere ligands were determined by selecting all hydrogen-bond acceptors within 4.0 Å of a Mg(H$_2$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.

Electrostatic interactions were determined by selecting 5'-PO$_4$ with a distance smaller than 5.2 Å and at an angle smaller than 52° from the ring centroid (McFail-kson et al. 1997).

Cation–π interactions were determined for Mg$^{2+}$ ions within 5.2 Å and at an angle smaller than 52° from the ring centroid (McFail-kson et al. 1997).
ion at Site 8 interacts with stem II near the A6 bulge (Fig. 6A; Table 2). It is likely that these three Mg$^{2+}$ 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 interaction and the presence of Mg$^{2+}$ 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 A49$^*$C50$^*$A51$^*$A52 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 (G29–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
WC edges of all core residues are protected from chemical probing in the presence of Mg\textsuperscript{2+} ions, with the exception of A\textsubscript{11} and C\textsubscript{50}, 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 A\textsubscript{10}, A\textsubscript{11}, and G\textsubscript{29} are protected from ethylnitrosourea (ENU) modification (Sood et al. 1998) and the riboses of A\textsubscript{10}, A\textsubscript{27}, G\textsubscript{28}, and G\textsubscript{29} are protected from hydroxyl radical footprinting (Hiley and Collins 2001), in agreement with backbone distortion near these residues, reduced accessibility of ribose C\textsubscript{4}′ within base triples (for A\textsubscript{27} and G\textsubscript{28}; Fig. 5) and binding of a Mg\textsuperscript{2+} ion at Site 7 (for A\textsubscript{10} and G\textsubscript{29}; 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, A\textsubscript{11} 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 A\textsubscript{11} 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 (A\textsubscript{10}) and VI (A\textsubscript{32}A\textsubscript{33}) 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 A\textsubscript{6}, A\textsubscript{32}, and A\textsubscript{33} do not significantly affect the cleavage activity.

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WC edges of all core residues are protected from chemical probing in the presence of Mg\textsuperscript{2+} ions, with the exception of A\textsubscript{11} and C\textsubscript{50}, 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 A\textsubscript{10}, A\textsubscript{11}, and G\textsubscript{29} are protected from ethylnitrosourea (ENU) modification (Sood et al. 1998) and the riboses of A\textsubscript{10}, A\textsubscript{27}, G\textsubscript{28}, and G\textsubscript{29} are protected from hydroxyl radical footprinting (Hiley and Collins 2001), in agreement with backbone distortion near these residues, reduced accessibility of ribose C\textsubscript{4}′ within base triples (for A\textsubscript{27} and G\textsubscript{28}; Fig. 5) and binding of a Mg\textsuperscript{2+} ion at Site 7 (for A\textsubscript{10} and G\textsubscript{29}; 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, A\textsubscript{11} 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 A\textsubscript{11} 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 (A\textsubscript{10}) and VI (A\textsubscript{32}A\textsubscript{33}) 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 A\textsubscript{6}, A\textsubscript{32}, and A\textsubscript{33} do not significantly affect the cleavage activity.
The overall fold of the II–III–VI junction also 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 (Laing et al. 2011; Lipfert et al. 2008). Similarly, continuous stacking between stems II and III is not compatible with comparative gel electrophoresis activity (Lafrance et al. 2001b; Sood and Collins 2002). These results are in agreement with the bulged out position of A₂₃ and a large subset of isosteric base pairs for the cis sugar edge/WC A₄₇/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 A₄₇/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.
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,
were inserted into the HindIII/EcoRI sites of the pTZ19R-derived pTR-4 vector (Rastogi and Collins 1998) to generate the pl236, pJ236-ΔAα, and pJ236-ΔAα-bp plasmids. These plasmids were fully linearized using EcoRI (New England Biolabs) and used for transcription of the J236, J236-ΔAα, and J236-ΔAα-bp RNAs (Fig. 1B) with a VS ribozyme substrate at their 3’ end (Rastogi and Collins 1998).

RNA synthesis and purification
Unlabeled, 15N-labeled and 13C/15N-labeled RNAs (J236- VS, J236-ΔAα-VS, and J236-ΔAα-bp-VS) were synthesized in vitro with the T7 RNA polymerase, as previously described (Bonneau and Legault 2014b). Nucleotide-specific 13C/15N-labeled J236-VS RNAs were also synthesized using purified 13C/15N ATP, 13C/15N CTP, or 13C/15N GTP for preparation of A15N, J236, C15N, J236, and G15N, 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-Sephrose 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 NaN3 and 90%:10% H2O:DCDO) 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 MgCl2 99.995% [Sigma-Aldrich]). For NMR studies in D2O, the samples were obtained by four cycles of lyophilization and resuspension in 99.996% D2O.

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-HCC-H-COSY (Pardi and Nikonowicz 1992), 3D HCCH-TOCSY (Pardi and Nikonowicz 1992), and 13C-edited HMBC-NOE/SY (Ikura et al. 1990) spectra collected on nucleotide-specific 13C/15N-labeled J236 RNAs (A15N, J236, C15N, J236, and G15N, J236 RNAs) significantly contributed to the unambiguous assignment of NMR signals from the fully 13C/15N-labeled J236. NOE-derived distance restraints were separated in four classes based on NOE crosspeak intensities, and dihedral angle restraints for the sugar puckers (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 J236648 as BMRB accession numbers 25634 and 25655 and PDB ID codes 2N3Q and 2N3R, respectively.

Metal-ion binding studies
Manganese (Mn2+) titrations were performed with two J236 samples (1.5-mM 15N-labeled J236 and 2.0-mM 13C/15N-labeled J236) in NMR buffer A containing 5 mM MgCl2, as previously described (Bonneau and Legault 2014a). RNA–metal–distance restraints were derived from Mn2+-induced paramagnetic relaxation enhancement (PRE) using the ratio of signal intensity (I0/I) determined from spectra collected with (I0) and without (I) 20 µM MnCl2 (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 [Mg(H2O)62+] (Kleywegt and Jones 1998) termed J236Mg, were calculated as described for J236, but using additional metal–RNA restraints (Bonneau and Legault 2014a). For both J236 and J236Mg, the 20 lowest-energy structures that satisfied all the experimental restraints (all distance violations <0.2 Å and all torsion-angle violations <5°) 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 Mg2+ (PDB ID code 1YN1) (Campbell et al. 2006), the IV kissing-loop interaction (PDB ID code 2MI0) (Bouchard et al. 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).
ACKNOWLEDGMENTS

We thank Aurélie Tréfier and Dominique Chaussé for their help with sample preparation, Ryan Richter for computer support, as well as Pierre Dagenais and James G. Omichinski for discussions and critical reading of the manuscript. We also thank Calcul Québec for providing computing facilities. This work was supported by a Canadian Institutes of Health Research (CIHR) grant to P.L. (MOP-86502). E.B. was supported by a Frederick Banting and Charles Best Canada Graduate Scholarship PhD scholarship from CIHR and graduate scholarships from the Université de Montréal. P.L. holds a Canada Research Chair in Structural Biology and Engineering of RNA. Funding for open access charge was provided by CIHR.

Received March 31, 2015; accepted June 8, 2015.

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