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Complementary Segmental Labeling of Large RNAs: Economic Preparation and Simplified NMR Spectra for Measurement of More RDCs

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Structure determination of large RNAs at the atomic level is necessary to rationalize their cellular roles. NMR spectroscopy is a powerful method to determine RNA solution structures up to 30 kDa.[1−3] Along with conventional distance and torsion angle restraints, angular restraints derived from residual dipolar couplings (RDCs) help to better define the local and global shape of larger RNA molecules.[4−8] The precision of the overall structure increases with the number of angular restraints, and therefore it is crucial to obtain the maximum number of RDC restraints for a given RNA molecule. However, with large RNA oligonucleotides, increasing resonance overlap in combination with line broadening severely limits the number of obtainable RDC-derived angular restraints. To simplify the NMR spectra of large RNA oligonucleotides to a great extent, thereby allowing the extraction of additional RDC values, we have established a time- and cost-effective methodology for the preparation of segmentally labeled RNA oligonucleotides. The method has been applied to the 25 kDa dendritic targeting element (DTE) of brain cytoplasmic 1 RNA (BC1 RNA), called BC1-DTE hereafter.[9] Two RNA oligonucleotides with complementary ^{13}C-only and ^{15}N-only labeled segments were prepared. The resulting spectral simplification yielded almost double the number of RDCs in comparison to conventional uniform ^{13}C,^{15}N-labeling.

Segmental ^{15}N-labeling has been applied previously to the 5′ half of *Caenorhabditis elegans* spliced leader RNA[10] and the 100 kDa HCV IRES.[11] In the latter study, a 25 kDa subdomain of the entire HCV IRES was ^{15}N-labeled to confirm its independent fold in the context of the entire molecule. That study used RNA fragments with engineered ends to allow for only one combination with T4 RNA ligase, which joins a 3′-hydroxyl with a 5′-phosphate group. Two separate in vitro transcriptions had to be performed to produce individual segments for ligation. For the 5′-fragment, a hammerhead ribozyme generated a free 5′-hydroxyl terminus during in vitro transcription with ^{15}N-labeled nucleotide triphosphates (NTPs) yielding an RNA oligonucleotide with two hydroxyl groups. A second in vitro transcription reaction with unlabeled NTPs primed with GMP used a 3′-hammerhead ribozyme to yield the 3′-fragment with two monophosphates. The resulting fragments were then ligated with T4 RNA ligase to produce an RNA oligonucleotide with an ^{15}N-labeled segment. This approach yielded greatly simplified ^{1}H−^{15}N HMQC spectra compared to the fully ^{15}N-labeled RNA molecule.

Applying the above scheme to generate simplified NMR spectra for the measurement of more RDCs would require the preparation of two individual samples employing complementary labeling schemes in both segments to measure RDCs of the entire RNA molecule. As a consequence, four individual in vitro transcriptions would have to be performed to produce the individual RNA fragments for subsequent complementary ligations. To minimize the cost for sample preparation and purification, we designed a plasmid for run-off transcription, which allows obtaining the four

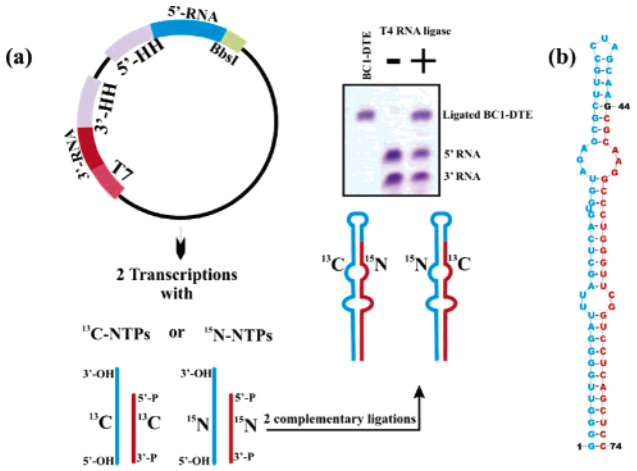

Figure 1. (a) Overview of the methodology for the preparation of ^{13}C and ^{15}N complementary segmentally labeled RNAs. The 5′- and 3′-*cis*-hammerhead ribozymes are denoted as 5′- and 3′-HH, respectively. The denaturing polyacrylamide gel in the upper right analyzes the result of the ligation reaction. (b) Secondary structure of BC1-DTE RNA colored according to the complementary labeling scheme as in (a). Different labeling schemes could also be applied.

desired fragments from only two transcription reactions (Figure 1a). The plasmid contains a T7 promoter sequence followed by a template region for the 3′-fragment and its 3′-hammerhead ribozyme in *cis*. The latter is connected to the hammerhead ribozyme for the 5′-fragment via a flexible linker sequence. In vitro transcription from this plasmid linearized with the restriction enzyme *BbsI* yields a primary transcript from which two RNA fragments are excised by the hammerhead ribozymes yielding the desired 3′-monophosphate for the 3′-fragment and the 5′-hydroxyl group for the 5′-fragment, respectively. Placing the 3′-fragment at the beginning of the transcript also permits priming with GMP to yield a 3′-fragment with two monophosphates, while placing the 5′-fragment at the end of the template region yields the desired 3′-hydroxyl terminus during run-off transcription. Therefore only two transcriptions with complementary labeling schemes are necessary to produce the four fragments for the subsequent complementary ligations.

The complementary segmental labeling scheme was applied to the 74-nucleotide (nt) BC1-DTE RNA (Figure 1b). The following plasmid was constructed to prepare two complementary labeled RNA oligonucleotides: the template region for the 3′-fragment, a 31-nt RNA (nts 43−74 of BC1-DTE) followed by UC to allow cleavage by a 3′-hammerhead ribozyme, a 17-nt UC-rich, flexible linker, a 5′-hammerhead ribozyme, and the 5′-fragment, a 43-nt RNA (nts 1−43 of BC1-DTE).

Two transcription reactions were performed with either ^{13}C-labeled or ^{15}N-labeled NTPs. The resulting 179-nt RNA transcript was cleaved by the two hammerhead ribozymes within 2 h (about 80% efficiency) to yield a 43-nt 5′-fragment, a 33-nt 3′-fragment,

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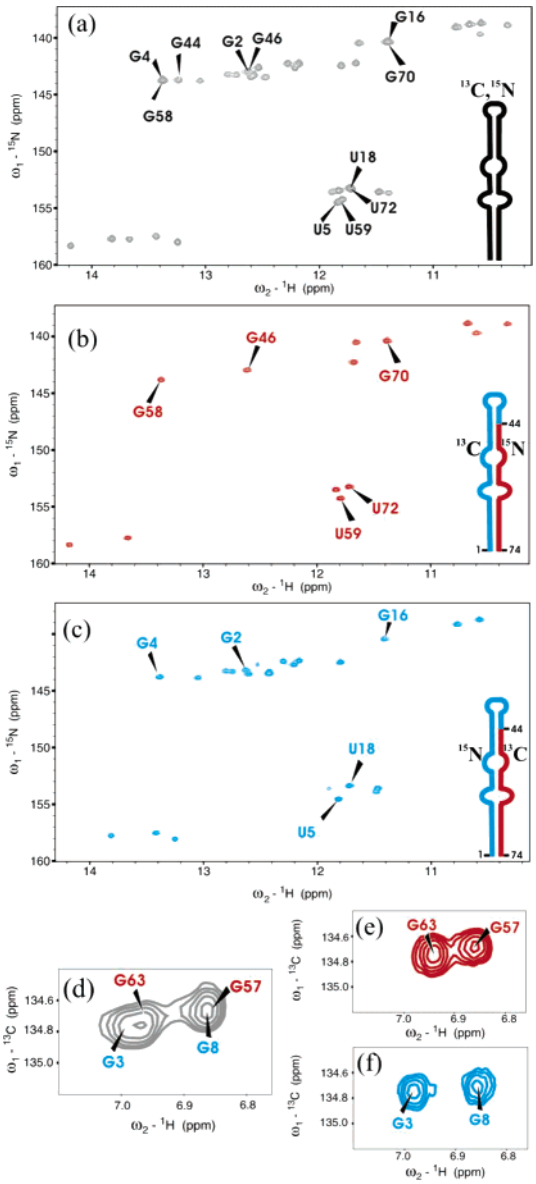

Figure 2. Imino region of ^{1}H−^{15}N TROSY spectra of (a) 1 mM uniformly ^{13}C,^{15}N-labeled BC1-DTE RNA (black), (b) 0.08 mM of 5′ ^{13}C- and 3′ ^{15}N-labeled BC1-DTE RNA (red), (c) 0.07 mM of 5′ ^{15}N- and 3′ ^{13}C-labeled BC1-DTE RNA (blue). (d−f) Representative aromatic regions of the ^{1}H−^{13}C TROSY spectra for the three samples following the same color coding.

and a 103-nt product containing the two cleaved hammerhead ribozymes and the linker region. The RNA oligonucleotides were then separated by denaturing gel electrophoresis and electroeluted into tris-borate-EDTA buffer. Subsequent ligation combining the 5′-RNA "donor" ^{13}C-labeled with the 3′-RNA "acceptor" ^{15}N-labeled and vice versa yielded two ^{13}C and ^{15}N complementary, segmentally labeled RNAs. It should be noted that our methodology is flexible and can be applied to various other complementary labeling schemes, such as the combination of ^{13}C,^{15}N-labeled and unlabeled segments, which would work as efficiently for the extraction of more RDCs.

The ^{1}H−^{15}N TROSY and ^{1}H−^{13}C TROSY spectra of the uniformly ^{13}C,^{15}N-labeled 74-nt RNA showed severe chemical shift degeneracy. Interestingly, most of the observed resonance overlap originated from resonances in the right strand overlapped with resonances in the left strand of the RNA. For example, as shown in Figure 2a, the imino resonances of G4 and G58, G2 and G46, G16 and G70, U18 and U72, and U5 and U59 in the ^{1}H−^{15}N TROSY as well as the aromatic resonances of G3 and G63, G8 and G57 (Figure 2d) in the ^{1}H−^{13}C TROSY were completely overlapped. In total, 10 imino and 50 aromatic resonances were found to suffer from extensive chemical shift degeneracy. This overlap problem reduces the number of RDC values that can be measured from the TROSY spectra and thus limits the resolution of the overall structure that can be obtained for the large RNA.

As shown in Figure 2, chemical shift degeneracy could be completely surmounted using the two segmentally labeled RNA samples. All the imino resonances are observed in the simplified spectra of the segmentally labeled BC1-DTE RNA, except G44, since the transcription reaction was primed with unlabeled GMP in this position. Remarkably, the spectral simplification achieved by this methodology resulted in almost doubling the number of measured RDCs, yielding 34 RDCs for the imino and 95 RDCs for the aromatic spin pairs versus only 24 and 45 RDCs, respectively, in the conventional case of a ^{13}C,^{15}N uniformly labeled sample.

In conclusion, we established a fast and efficient method for complementary segmental labeling of large RNAs. Simplified NMR spectra were recorded for the 25 kDa BC1-DTE RNA, and a considerably larger number of RDCs could be obtained, which will benefit the global and local precision of the BC1-DTE RNA structure. This powerful approach should be generally applicable to the NMR structure determination of large RNAs and open new directions for the study of RNA secondary structure and tertiary interactions through ^{13}C- or ^{15}N-filtered experiments.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Varani, G.; Aboul-ela, F.; Allain, F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *29*, 51−127.
(2) Lantham, M.; Brown, D.; McCallum, S.; Pardi, A. *ChemBioChem* **2005**, *6*, 1492−1505.
(3) Tzakos, A. G.; Grace, C. R.; Lukavsky, P. J.; Riek, R. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 319−342.
(4) Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114.
(5) Hansen, M. R.; Mueller, L.; Pardi, A. *Nat. Struct. Biol.* **1998**, *5*, 1065−1074.
(6) Hansen, M. R.; Hanson, P.; Pardi, A. *Methods Enzymol.* **2000**, *317*, 220−240.
(7) van Buuren, B. N.; Schleucher, J.; Wittmann, V.; Griesinger, C.; Schwalbe, H.; Wijmenga, S. S. *Angew. Chem., Int. Ed.* **2004**, *43*, 187−192.
(8) Lukavsky, P. J.; Puglisi, J. D. *Methods Enzymol.* **2005**, *394*, 399−416.
(9) Tiedge, H.; Zhou, A.; Thorn, N. A.; Brosius, J. *J. Neurosci.* **1993**, *13*, 4214−4219.
(10) Xu, J.; Lapham, J.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 44−48.
(11) Kim, I.; Lukavsky, P. J.; Puglisi, J. D. *J. Am. Chem. Soc.* **2002**, *124*, 9338−9339.

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