Measurement of H,H-Coupling Constants Associated with ν_1, ν_2, and ν_3 in Uniformly ^{13}C-Labeled RNA by HCC-TOCSY-CCH-E.COSY

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Received January 20, 1995

The measurement of homonuclear ^3J(H,H)[1−3] and heteronuclear ^3J(H,C)[4,5] coupling constants is the most direct way to determine the conformation and dynamics of five-membered rings. In RNA, the conformation of the ribose ring not only defines the local geometry but also affects the orientation of the phosphodiester backbone and, as a result, the global conformation of the oligonucleotide chain.[6] Moreover, unusual sugar puckers are often found for nucleotides involved in small loops, junctions, and mismatched base pairs as observed in structures of tetraloops,[7] tRNA,[8] and ribozymes.[9] Precise determination of ribose puckers in regions of noncanonical RNA structure should provide greater detail in these chemically and structurally interesting parts of the oligonucleotide.

To date, the measurement of ^3J(H,H) coupling constants in even moderately sized (6−12 kDa) RNAs has been precluded by the severe chemical shift overlap of the ribose H2′, H3′, H4′ and H5′/H5″ proton resonances. Even application of the 2D or 3D HCCH-E.COSY method[10,11] to uniformly ^{13}C-labeled RNAs[12] has not provided sufficient resolution to measure most ^3J(H2′,H3′) and ^3J(H3′,H4′) coupling constants since they are derived from the C2′,H3′ or C3′,H2′ and C3′,H4′ or C4′,H3′ cross peaks, which usually overlap either mutually or with C2′,H2′, C3′,H3′ or C4′,H4′ peaks. The HCCH-E.COSY did, however, allow complete measurement of all ^3J(H1′,H2′) coupling constants in the RNA[11] under investigation.

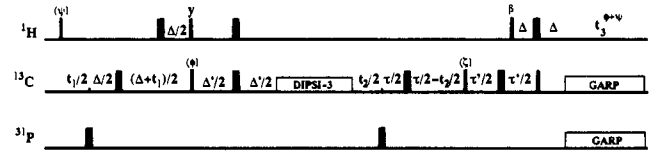

Figure 1. Pulse sequence for the HCC-TOCSY-CCH-E.COSY experiment. Thin bars are 90°, thick bars are 180° pulses. All pulses for which a phase is not indicated are applied along the x-axis. The phase cycle was $\psi = x,-x$, $\phi = 2(x),2(-x)$, $\zeta = 4(x),4(-x)$. $\beta = 45°$. The CC-TOCSY was accomplished with DIPSI-3,[20] with a duration of 9.21 ms and $\gamma B_1/2\pi = 6$ kHz. ^{13}C decoupling was achieved with GARP,[21] using $\gamma B_1/2\pi = 1.8$ kHz. ^{31}P decoupling was not used in the experiment shown in Figure 2. $\Delta = 3.0$ ms, $\Delta' = 1.5$ ms τ_m, and $\tau = 8.3$ ms are matched for the "directed" TOCSY transfer. $\tau' = 6.25$ ms. 80 complex points in ω_1 ($t_1^{max} = 48$ ms), 48 complex points in ω_2 ($t_2^{max} = 8.3$ ms), and 1K complex points in ω_3 ($t_3^{max} = 291$ ms) were recorded. Quadrature detection in ω_1 was obtained by using States-TPPI[22] on ψ and in ω_2 by using States-TPPI on all carbon pulses and the receiver phase after the t_2 evolution. Total measurement time, 51 h.

In this Communication, we introduce the HCC-TOCSY-CCH-E.COSY pulse sequence (Figure 1) that allows both correlation of the HCCH-E.COSY cross peaks with the usually well-resolved H1′ chemical shifts within a given ribose and extremely selective coherence transfer within the ribose spin system. The experiment yields well-resolved C1′,H2′, C2′,H3′, and C3′,H4′ that are normally overlapped in the standard HCCH-E.COSY experiment. The C4′,H5′ and C4′,H5″ cross peaks are weaker, as discussed below. The pulse sequence is a concatenation of a HCC-TOCSY[13] and a constant time CCH-E.COSY sequence.[10,11] In contrast to the usual observation of total correlation in the TOCSY experiment,[14,15] in this experiment, the duration of the CC-TOCSY mixing (9.21 ms) and the ^{13}C constant time evolution delay (8.3 ms) have been exactly matched such that coherence originating from H1′ during t_1 frequency labeling forms exclusively "forward directed" antiphase coherence of the type $2S_{i,y}S_{i+1,z}$ (with $i = 1-4$; 1 = C1′, 2 = C2′, 3 = C3′, 4 = C4′, and 5 = C5′) at the end of the constant evolution time. The delays were optimized for transfer of the C1′ magnetization to C2′ and C3′ (transfer to C4′ is weaker due to the larger numbers of intervening bonds and could be enhanced by delay settings of 13.5 ms for the DIPSI-3 and 7.6 ms for the constant time delay τ in the directed TOCSY). Consequently, strong C1′,H2′, C2′,H3′, and C3′,H4′ cross peaks and weaker C4′,H5′ and C4′,H5″ cross peaks are observed in the ω_1,ω_2 planes taken at each H1′ resonance. In total, only 5 out of 15 potential cross peaks are observed with the autocorrelation peaks (C1′,H1′, C2′,H2′, C3′,H3′, C4′,H4′, C5′,H5′, and C5′,H5″) and the backward-directed cross peaks (C2′,H1′, C3′,H2′, C4′,H3′, and C5′,H4′) effectively removed from the spectrum. Nevertheless, the resolution enhancement is gained without loss of information content since the autocorrelation peaks carry no useful information and the backward-directed peaks contain redundant information.

To demonstrate this resolution enhancement, we applied the pulse sequence to the uniformly ^{13}C/^{15}N-labeled 19mer RNA (GCACCGU***UGGUA***GCGGUGC) stem-loop derived from the

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Table 1. Coupling Constants Determined for the Riboses of the RNA I Stem-Loop Derived from *ColE1* [a]

	G1	C2	A3	C4	C5	G6	U7	U8	G9
^3J(H1′,H2′)	nd	1.5	0.0 ± 0.3	0.8 ± 0.4	0.9	n.d.	1.8	5.6 ± 0.5	5.5 ± 0.2
^3J(H2′,H3′)	nd	<2.5	0.3 ± 0.5	<2.5	4.5	3.9	4.9	6.6 ± 0.5	nd
^3J(H3′,H4′)	nd	11.0	10.3 ± 0.3	12.0 ± 1.0	10.2 ± 0.4	10.6	8.9	< 2.5	3.3 ± 0.3

	G10	U11	A12	G13	C14	G15	G16	U17	G18	C19
^3J(H1′,H2′)	7.0 ± 0.4	7.3 ± 0.4	4.5 ± 0.6	nd	0.7	0.9 ± 0.3	2.8	0.6	0.2	2.0 ± 0.3
^3J(H2′,H3′)	2.0 ± 0.5	4.1 ± 0.5	4.9 ± 0.6	nd	nd	5.2 ± 0.5	0.7	3.1	0.2	3.7 ± 0.4
^3J(H3′,H4′)	3.2 ± 0.5	2.2 ± 0.2	5.1 ± 0.6	nd	10.3	11.3 ± 0.1	10.3	9.6	9.9	3.4 ± 0.5

[a] nd, not determined. All ^3J values in hertz.

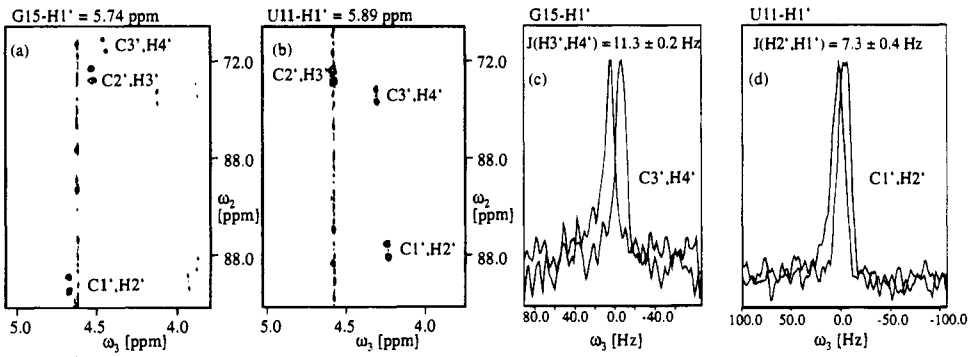

Figure 2. ω_2,ω_3 planes through H1′ of G15 (a) and U11 (b). In these planes, only C1′,H2′, C2′,H3′, and C3′,H4′ are observed. They show no overlap with cross peaks of other nucleotides due to the good resolution of the H1′ region nor with intraresidual peaks due to the "directed" TOCSY transfer. Traces are shown through C3′,H4′ of G15 (c) and C1′,H2′ of U11 (d), together with the coupling constants derived by the fitting procedure described in ref 11. The spectra were recorded on an Bruker AMX 600 spectrometer equipped with four channels and a triple resonance probe with z-gradients (TBI). Transformation with Felix230 (Biosym Inc., San Diego, CA), zero-filling in ω_3 and strip transformed from 6.1 to 3.8 ppm, cosine-squared apodization; zero-filling from 80 complex points to 256 complex points in ω_1, cosine-squared apodization: mirror image linear predicted[23] from 48 to 100 points in ω_2 yields a final matrix size of 1024 × 256 × 256 real points.

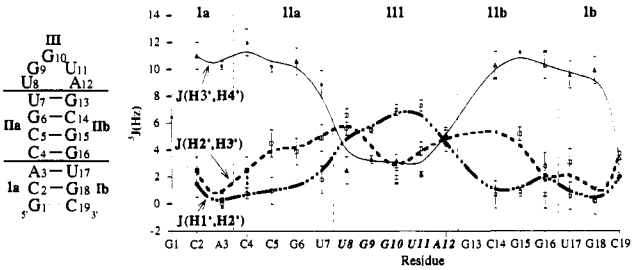

Figure 3. Plot of the ^3J(H1′,H2′), ^3J(H2′,H3′), and ^3J(H3′,H4′) coupling constants given in Table 1, as determined for each residue of the 19mer RNA. The values allow a distinction into five regions, as explained in the text. Bars indicate the error range for each coupling constant.

antisense RNA I molecule found in the *ColE1* plasmid replication control system.[16] The nucleotides in the loop are shown boldface italic. Figure 2 shows the planes taken at the frequencies of the H1′ protons of the residues G15 (in the stem region) and U11 (in the loop region), together with traces through selected cross peaks. The coupling constants extracted according to the procedure presented in ref 11 for all of the nucleotides in the RNA stem-loop, except those associated with G13 and G1, are listed in Table 1.

Presentation of the ^3J(H,H) coupling constants as a function of nucleotide position (Figure 3) shows that the RNA can be characterized by five regions: the stem regions close to the 5′ (**Ia**) and 3′ ends (**Ib**), where the H2′,H3′ coupling constants are smaller than expected for a C2′-endo or C3′-endo conformation; the inner stem regions (**IIa** and **IIb**), where the coupling constants are in agreement with the expected values for pure C3′-endo conformation; and the loop region (**III**), where the coupling constants are indicative of an averaging between C2′-endo and C3′-endo conformations for residues U8, G9, and A12 and a more pure C2′-endo conformation for residues G10 and U11. The coupling constants found here are not all in complete agreement with the values predicted from the Karplus equations.[1] In particular, the ^3J(H1′,H2′) coupling constants are often smaller than the minimum, while the ^3J(H3′,H4′) couplings are often larger than the maximum predicted from the Karplus parametrization.

In summary, we have introduced a method to measure the ^3J(H,H) coupling constants in uniformly ^{13}C-labeled RNAs. The method is especially adjusted to the severely overlapping proton resonances in the ribose rings. In addition, the method is rather insensitive to differential relaxation, as discussed elsewhere,[17] and should therefore be applicable for larger RNAs. The explicit determination of coupling constants and more detailed subsequent conformational analysis of ribose rings by the HCC-TOCSY-CCH-E.COSY method presented here could perhaps contribute to the resolution of controversies surrounding oligonucleotide structures such as RNA/DNA hybrids[18] and the sugar pucker dynamic in oligonucleotides.[19]

Acknowledgment. This work was supported by the Fonds der Chemischen Industrie and the DFG under Gr1211/2-3. H.S. is supported by a stipend of the EU (Human Capital and Mobility). J.P.M. acknowledges support by Prof. Crothers through NIH Grant GM 21966 and by an Alexander von Humboldt postdoctoral fellowship. S.J.G. is supported by the DFG through a Heisenberg Stipendium (Gl 203/2-1). H.S. thanks Dr. C. Dobson for encouragement.

JA950282G

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