Direct Correlation of Exchangeable and Nonexchangeable Protons on Purine Bases in ^{13}C,^{15}N-Labeled RNA Using a HCCNH-TOCSY Experiment

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The assignment of proton resonances is a necessary step in any structural study of RNA by NMR spectroscopy. Traditionally, the assignments rely mainly on through-space interactions due to the nuclear Overhauser effect (NOE). The basic problem with this technique is that it represents a conformation dependent approach. It works well for the stem regions and small loops where the conformation is close to the standard one while it fails in regions where the structure differs significantly from the usual A-form helix. The recent introduction of techniques for the preparation of ^{13}C- and ^{15}N-labeled RNA[1−3] has opened opportunities for utilizing heteronuclear techniques to characterize RNA structure in solution. Triple-resonance experiments have been proposed that approach the nonexchangeable proton assignment problem based on through-bond interactions involving either ^{13}C and ^{15}N for sugar−base correlations[4−8] or ^{13}C and ^{31}P for sequential backbone assignments.[9−14] None of these experiments, however, address the issue of exchangeable proton assignments in nonhelical RNA segments.

Here we present an experiment correlating guanine imino proton resonances with their own guanine H_8 and/or C_8 resonances. A somewhat different experiment has already been proposed[15] on the nucleoside level to achieve the same goal but has not yet been demonstrated on an RNA sample. At the same time, our experiment provides correlations of adenine amino protons with their own H_8/C_8 and H_2/C_2 resonances. The imino and amino proton resonances can be assigned as long as the resonance frequencies of either the corresponding nonexchangeable proton or the attached carbon are resolved and assignable in the spectrum. The pulse sequence we used (Figure 1) is a slightly modified version of the HCCNH-TOCSY pulse sequence proposed for correlating side chain protons with amide protons and nitrogens in proteins.[16] We used carbon rather than nitrogen resonance frequencies as a second indirect dimension since the C_8/C_2 carbons can be independently assigned from the NOESY-HMQC experiment or, more reliably, from the through-bond experiments mentioned above. In the present experiment, the WATERGATE water suppression scheme[17] was incorporated into the refocusing period of the last HSQC-type polarization transfer step.[18] In principle, however, presaturation could also be used in this experiment as all the measured

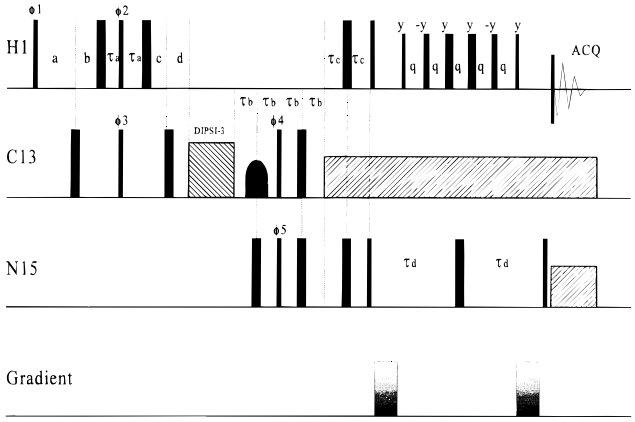

Figure 1. The 3D ^1H, ^{13}C, ^{15}N triple-resonance pulse sequence HC(CN)H-TOCSY used to establish guanine imino-H_8, C_8 and adenine amino-H_8, H_2, C_8, C_2 correlations in ^{13}C,^{15}N uniformly labeled RNA oligonucleotides. Narrow and wide black rectangles represent nonselective 90° and 180° pulses, respectively. The last six proton pulses function as a selective 180° pulse in WATERGATE; their lengths are in the ratio 3:9:19:19:9:3 and were applied with a power of 8.9 kHz. The diagonally striped rectangles stand for DIPSI-3[19] spin lock applied at a power of 3.0 kHz for 55 ms and Garp[20] decoupling with 2.2 kHz power for ^{13}C and 930 Hz for ^{15}N. All ^{13}C pulses were applied at 150 ppm except for the selective refocusing C_6 pulse, represented by the thick top rounded bar, for which a 1.5 ms reburp[21] pulse was frequency shifted by phase modulation[22] to cover the region between 150 and 170 ppm. The ^1H frequency was set to 10.5 ppm and the ^{15}N frequency to 120 ppm. Rectangular gradient pulses of 1.0 ms duration and a strength of 8 G/cm were used. Unless indicated otherwise, 90° pulses were applied with phase x and 180° pulse with phase y. The phase cycle was $\phi_1 = x$ + States-TPPI; $\phi_2 = 4(y), 4(-y)$; $\phi_3 = x, -x$ + States-TPPI; $\phi_4 = 8(x), 8(-x)$; $\phi_5 = x, x, -x, -x$; receiver: $x, -x, -x, x, 2(-x, x, x, -x), x, -x, -x, x$. Delays were $a = \tau_a + t_1/2$, $b = t_1/2$, $c = \tau_a + t_2/2$, $d = t_2/2$; τ_a was set to 1.1 ms, τ_b to 6.5 ms, τ_c to 1.4 ms, and τ_d to 2.7 ms. The value of 150 μs for q was found by optimization for best water suppression.

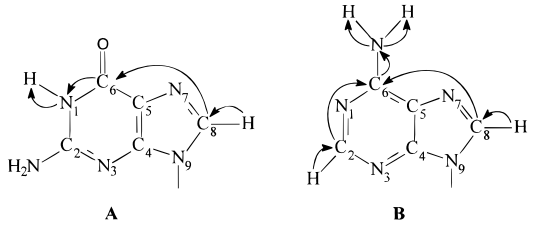

Figure 2. Schematic representation of the coherence transfer pathways in guanine (A) and adenine (B) in the HCCNH-TOCSY experiment.

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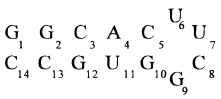
Scheme 1



magnetization comes from nonexchangeable protons. The magnetization is transferred by INEPT-type transfer from the H_8/H_2 proton to its neighboring carbon, and the refocused ^{13}C-magnetization is then transferred by homonuclear TOCSY to the C_6 carbon. From there, in two consecutive INEPT steps, the magnetization is transferred to imino (amino) nitrogens and imino (amino) protons, where it is detected (Figure 2). To prevent the magnetization from going back to C_5 from C_6, a selective refocusing pulse is applied on C_6 in the carbon−nitrogen transfer step. The use of the selective pulse does not improve the polarization transfer from adenine H_2, however, since the chemical shifts of C_2 and C_6 are very close to each other. As the carbon−carbon transfer includes two-bond steps, where the coupling constant is generally about four times smaller than for one-bond coupling, the mixing time has to be significantly longer than that used in this experiment for proteins. As in other experiments of this kind, the choice of polarization transfer parameters represents a compromise between allowing enough time for the polarization transfer to occur and accepting the consequences of the signal loss due to relaxation. In this case, the problem of relaxation is further aggravated by the necessity to work at low temperature, typically between 0 and 10 °C, to slow down the proton exchange rate. We found that the optimal spin-lock time varied (38−75 ms) depending on the size of the molecule, with 55 ms as an almost universally acceptable value.

We tested the experiment on a 4.5 mM solution of a 14-mer RNA hairpin (Scheme 1) in 90% H_2O/10% D_2O buffer. The planes from three-dimensional spectra are shown in Figure 3. We observe all five cross peaks for guanine residues in the molecule. There was only one adenine residue in the molecule. For an adenine residue we typically observe four cross peaks, namely, $H_8-C_8-H_{amino}$ and $H_2-C_2-H_{amino}$ for each of the two amino protons. Not surprisingly, H_8 cross peaks are stronger than H_2 as the experiment was optimized mainly for the guanine H_8-H_{imino} correlation. The signals of the amino proton at 6.2 ppm are weaker than of those at 8.2 ppm, probably due to the influence of water suppression. With different settings for carbon−carbon TOCSY transfer we were able to also obtain cytosine H_5, H_6 to amino correlations and uracil H_5, H_6 to imino correlations (data not shown). However, the cytosine correlations, where the sensitivity was high, provide little new information and the intensity of the uracil correlations was rather low for H_6/C_6. Therefore, we expect the method based on INEPT-type transfer only[15] to prove more useful for uracil correlations. We were able to get a high-quality 3D data set on our test sample in an over-the-weekend experiment. The high sensitivity of the experiment was not surprising considering that the 14-mer RNA hairpin sample we used was rather concentrated and involved a relatively small molecule. It has been observed for other heteronuclear experiments involving multibond polarization transfer that they became significantly less effective for RNAs larger than the ones on which they had been demonstrated because of signal loss resulting from reduced transverse relaxation times. As expected, the sensitivity of the HCCNH-TOCSY experiment does drop dramatically with the size of the molecule. In spite of that, application of a 2D version of the experiment on less concentrated samples of 35- and 40-mer oligonucleotide RNA aptamer complexes proved feasible and provided spectra of very good quality (Figure S1, supporting information). We found the combination of $^1H-^1H$ and $^{13}C-^1H$ 2D correlation experiments to be a viable alternative to complete 3D data collection for these larger molecules.

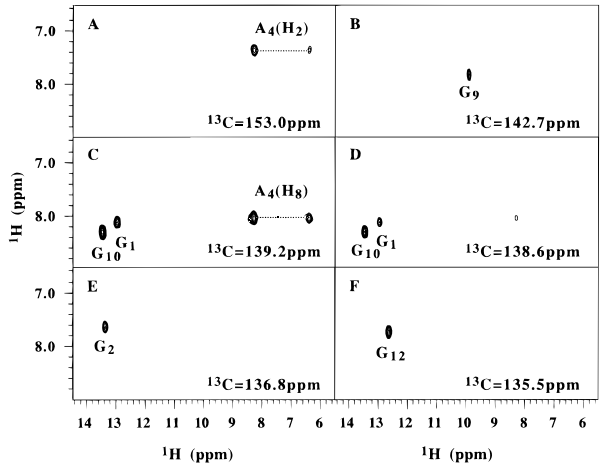


Figure 3. $^1H-^1H$ planes from the HCCNH-TOCSY experiment showing the correlations of exchangeable and nonexchangeable protons in the 14-mer RNA hairpin in Scheme 1. (A) H_2 of A_4 to amino; (B) H_8 of G_9 to imino; (C) H_8 of A_4 to amino; (D) H_8 of G_1 and G_{10} to imino; (E) H_8 of G_2 to imino; (F) H_8 of G_{12} to imino. The vertical scale of A and C was increased 4 times to show much weaker adenine peaks. Therefore, G_1 and G_{10} appear stronger in C than in D. The spectrum was recorded on a Varian UNITY *Plus* 500 spectrometer at 10 °C. Spectral widths were 15 ppm in proton dimensions and 40 ppm in the carbon dimension. We collected 80 × 32 × 256 complex points (t_1, t_2, t_3), 16 scans per increment with relaxation delay of 1.5 s resulting in the total experimental time of 72 h. The data were processed into a 256 × 128 × 512 complex point matrix using Varian VNMR software. The window functions used were nonshifted Gaussian in t_3 and 90°-shifted sine-bell window functions in indirectly detected dimensions. Residual water signal was removed from the spectra through subtraction in the time domain.

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Supporting Information Available: Figure S1 consisting of 2D HCCNH-TOCSY spectra showing correlations of guanine imino protons with H_8 and C_8 for a 40-mer RNA aptamer complex (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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