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Correlation of the guanosine exchangeable and nonexchangeable base protons in ^{13}C-/^{15}N-labeled RNA with an HNC-TOCSY-CH experiment

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Summary

A triple resonance HNC-TOCSY-CH experiment is described for correlating the guanosine imino proton and H8 resonances in ^{13}C-/^{15}N-labeled RNAs. Sequential assignment of the exchangeable imino protons in Watson–Crick base pairs is generally made independently of the assignment of the nonexchangeable base protons. This H(NC)-TOCSY-(C)H experiment makes it possible to unambiguously link the assignment of the guanosine H8 resonances with sequential assignment of the guanosine imino proton resonances. 2D H(NC)-TOCSY-(C)H spectra are presented for two isotopically labeled RNAs, a 30-nucleotide lead-dependent ribozyme known as the leadzyme, and a 48-nucleotide hammerhead ribozyme–RNA substrate complex. The results obtained on these two RNAs demonstrate that this HNC-TOCSY-CH experiment is an important tool for resonance assignment of isotopically labeled RNAs.

The classical sequential resonance assignment procedure for nucleic acids involves a NOE 'walk' between protons on neighboring nucleotides or neighboring base pairs (for a review, see Wijmenga et al., 1993). The assignment of the exchangeable imino protons is made independently of that of the nonexchangeable H8/H6/H5 base protons. The guanosine and uridine imino proton assignments are obtained by observation of NOEs between imino protons on neighboring base pairs, whereas the nonexchangeable base proton resonance assignment involves observation of NOEs between the sugar protons (H1'/H2'/H2") on the 5' nucleotide and base protons (H8/H6) on the 3' nucleotide (Wijmenga et al., 1993). The relatively good resolution for the imino proton spectrum, combined with the fact that there is only one imino proton resonance for each Watson–Crick base pair, means that the sequential assignment of the imino proton resonances is usually much easier than sequential assignment of the nonexchangeable base protons. Thus, it would be extremely useful to have methods for linking the assignments of the imino proton resonances with assignment of the nonexchangeable base proton resonances. Here we report a triple resonance HNC-TOCSY-CH experiment for through-bond correlation of the guanosine imino proton to H8 resonances in isotopically labeled oligonucleotides. This experiment complements recently reported triple resonance experiments for through-bond correlation of uridine, cytidine and adenosine exchangeable and nonexchangeable base protons in ^{13}C-/^{15}N-labeled RNA (Simorre et al., 1995,1996). The 2D H(NC)-TOCSY-(C)H experiment is applied to a 30-nucleotide lead-dependent ribozyme, known as the leadzyme (Pan and Uhlenbeck, 1992) and a 48-nucleotide hammerhead ribozyme–RNA substrate complex (see Fig. 1) (Uhlenbeck, 1987; Heus and Pardi, 1991a; Legault, 1995).

Figure 2 shows the 2D H(NC)-TOCSY-(C)H pulse sequence for correlating guanosine imino proton and H8 resonances in uniformly ^{13}C-/^{15}N-labeled nucleic acids. The experiment starts on the imino protons, which are frequency labeled in t_1 using the time-shared evolution procedure (Logan et al., 1993). This procedure concatenates the t_1 evolution period with the first ^1H-^{15}N INEPT transfer, allowing a reduction of 5 ms for the longest t_1 value. This should help improve sensitivity by reducing the effects of both relaxation and exchange of the imino protons with water. To avoid complete inversion of the

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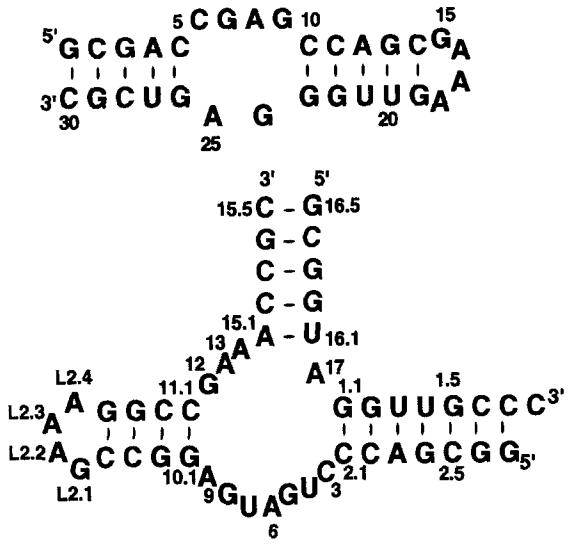

Fig. 1. Sequences and secondary structures of (A) the ^{13}C-/^{15}N-labeled leadzyme (Pan and Uhlenbeck, 1992); and (B) the hammerhead complex, consisting of a ^{13}C-/^{15}N-labeled ribozyme and a ^{15}N-labeled substrate (Uhlenbeck, 1987; Heus and Pardi, 1991a; Legault, 1995).

water signal by the second 90° ^1H pulse, the ^1H carrier frequency is set to the water frequency and the phase of ϕ_1 is shifted by 45°. This allows water magnetization to return to the +z-axis by radiation damping during the subsequent cross-polarization and TOCSY periods. After the N_yH_z magnetization is refocused to N_x, a 44.9 ms DIPSI-3 (Shaka et al., 1988) ^{13}C-^{15}N cross-polarization sequence transfers magnetization from N1 to C6 and C2 (Bertrand et al., 1978; Mueller and Ernst, 1979; Bearden and Brown, 1989; Zuiderweg, 1990). A 37.8 ms FLOPSY-8 (Mohebbi and Shaka, 1991) ^{13}C TOCSY sequence is used to transfer magnetization among all the guanosine base carbons (Braunschweiler and Ernst, 1983). The FLOPSY-8 sequence was employed because it has a broad excitation profile; this is required in order to spin-lock C5, which is over 40 ppm upfield of the base C6 carbons in guanosine. A ^{13}C-^1H reverse INEPT then transfers magnetization from C8 to H8 and a flip-back WATERGATE sequence is incorporated into the final refocusing period to selectively suppress the water signal (Piotto et al., 1992; Grzesiek and Bax, 1993). The water flip-back is achieved with a selective E-BURP pulse, which returns the bulk water magnetization to the +z-axis (Geen and Freeman, 1991; Grzesiek and Bax, 1993). Because the imino protons exchange with water, the water flip-back method provides optimal initial magnetization, even with a relatively short recovery time. The last refocusing period of the INEPT sequence is concatenated with the

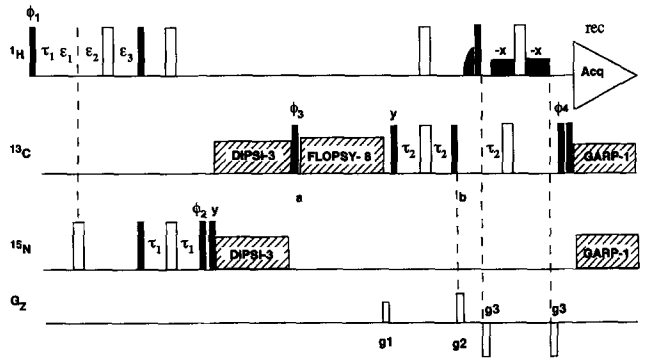

Fig. 2. The 2D H(NC)-TOCSY-(C)H pulse sequence used to connect imino protons to H8 in guanosines. The indirect detection period t_1 was partially concatenated with the first ^1H-^{15}N INEPT using the time-shared evolution procedure, which allows a reduction of 5 ms for the longest t_1 value (Logan et al., 1993). For the first t_1 point, $\varepsilon_1 = \varepsilon_2 = 0.5 * [dw/2 - (4/\pi)PW90(^1H) - PW180(^{15}N)]$ and $\varepsilon_3 = \tau_1$. For subsequent t_1 points, ε_1 was incremented by dw/2, ε_2 was incremented by $dw/2 - \tau_1/(n+1)$ and ε_3 was decremented by $\tau_1/(n+1)$, where dw is the dwell time and n is the total number of complex points in t_1. The τ_1 and τ_2 delays were set to 2.5 and 1.25 ms, respectively. The magnetization was transferred from ^{15}N to ^{13}C by cross polarization using a 44.9 ms DIPSI-3 sequence at an rf strength of 1.9 kHz (Bertrand et al., 1978; Mueller and Ernst, 1979; Shaka et al., 1988). A 37.8 ms FLOPSY-8 sequence (Mohebbi and Shaka, 1991) at an rf strength of 5 kHz was used for the ^{13}C homonuclear TOCSY period (Braunschweiler and Ernst, 1983). Unless otherwise noted, all pulses have phase = x. The phase cycle was $\phi_1 = 2(y+45°), 2(-y+45°)$; $\phi_2 = y,-y$; $\phi_3 = 4(y),4(-y)$; $\phi_4 = 8(x),8(-x)$ and receiver = x,2(−x),x,−x,2(x),−x. Instead of phase cycling ϕ_2, the two 90° ^{15}N pulses before the ^{15}N-^{13}C cross polarization can be eliminated and the phase of the ^{15}N DIPSI-3 can be phase cycled (x,−x) using the same receiver phase cycling as before (Majumdar and Zuiderweg, 1995). Eliminating these two 90° ^{15}N pulses should lead to a slight improvement in sensitivity for probes with low ^{15}N B_1 homogeneity. The ^{15}N frequency was set to 146 ppm during the ^1H-^{15}N and ^{15}N-^{13}C transfers and it was shifted to 195 ppm at point a to decouple N7 and N9 during the acquisition period. The ^{13}C frequency was positioned at 161 ppm for the ^{15}N-^{13}C hetero-TOCSY period and was shifted to 145 ppm at point a for the ^{13}C-TOCSY period and to 142 ppm at point b. To avoid a complete inversion of the water signal by the second ^1H 90° pulse, the phase of ϕ_1 was shifted by 45° and the ^1H carrier was set to the water frequency. Radiation damping returns the water magnetization to the +z-axis during the ^{15}N-^{13}C cross polarization and ^{13}C-TOCSY periods. At point b, water flip-back is achieved with a 2.9 ms selective E-BURP pulse (Geen and Freeman, 1991; Grzesiek and Bax, 1993). During the last INEPT period, a WATERGATE sequence is applied to suppress the residual water signal using two 1.55 ms soft square pulses (Piotto et al., 1992). The phases of the selective E-BURP and soft square pulses were adjusted with a small angle phase shifter for optimal solvent suppression. During the detection period, ^{13}C and ^{15}N GARP1 decoupling was used at rf fields of 1.6 and 1.14 kHz, respectively. All gradients were applied along the z-axis, with g1 = 12 G/cm, g2 = 24 G/cm and g3 = 32 G/cm. The gradient times for g1, g2 and g3 were 300, 300 and 450 μs, respectively. Each gradient was followed by a recovery time of 200 μs.

WATERGATE sequence to reduce ^1H relaxation. Since the time required for the gradients and selective 90° ^1H pulses in the WATERGATE sequence is longer than the last INEPT refocusing period, the ^1H and ^{13}C 180° pulses are not aligned in the final INEPT period. The g1 and g2 z-axis pulsed-field gradients are used to purge transverse magnetization and the g3 gradient selects for refocused magnetization as part of the WATERGATE sequence (Keeler et al., 1994).

Figure 3A shows the 2D H(NC)-TOCSY-(C)H spectrum for the uniformly ^{13}C-/^{15}N-labeled leadzyme (Pan and Uhlenbeck, 1992). This spectrum was acquired in 16 h and has a good signal-to-noise ratio. The magnetization transfer in the HNC-TOCSY-CH experiment is not highly efficient, because the sequence transfers magnetization among all carbons in the guanosine base. Therefore, only a fraction of the starting imino proton magnetization ends up on H8. Nevertheless, except for the terminal base pair, we were able to observe HN to H8 correlations for all guanosines in the stem regions of the leadzyme. The imino proton resonances on terminal base pairs are often difficult to observe, due to fast exchange of these protons with water. A correlation was also observed for G15, which forms a G-A base pair in the GAAA tetraloop (Heus and Pardi, 1991b; Legault, 1995). As previously observed, the imino protons for the guanosines in the internal loop of the leadzyme exchange too fast to be observed in the proton spectrum (Legault, 1995). The correlations observed on the leadzyme confirm previous assignments of the H8 resonances obtained from 3D HMQC-NOESY spectra (Legault, 1995).

There is considerable variation in cross-peak intensity for the different guanosine residues in the leadzyme, even those in base-paired regions. Some of this variation is due to differences in hydrogen exchange rates of the imino protons, but this does not account for all the observed differences. Additional variation of the cross-peak intensity may arise from differences in relaxation properties for specific guanosines.

Since the HN to H8 magnetization transfer occurs via relatively small heteronuclear (^{13}C-^{15}N) and homonuclear (^{13}C-^{13}C) coupling constants, relaxation will be an important factor in the sensitivity of the H(NC)-TOCSY-(C)H for larger molecules. Figure 3B shows the 2D H(NC)-TOCSY-(C)H spectrum for the 48-nucleotide hammerhead ribozyme–substrate complex. This complex consists of a uniformly ^{13}C-/^{15}N-labeled ribozyme combined with a ^{15}N-labeled RNA substrate. Thus, HN to H8 correlations are only observed for guanosines on the ribozyme. Seven HN to H8 cross peaks (six strong, and one weak) are observed for the hammerhead ribozyme. As illustrated in Fig. 3B, four of these correlations could be unambiguously assigned and tentative assignments could be made for two other cross peaks, based on the previous assignments for the imino proton resonances (Heus and Pardi, 1991a; Legault, 1995). No HN to H8 cross peaks were observed for guanosines in the catalytically active core of the hammerhead ribozyme; possibly because these imino protons exchange too rapidly with water to be observed. This 2D H(NC)-TOCSY-(C)H experiment therefore provided the first sequential assignments for the nonexchangeable base protons in the hammerhead ribozyme.

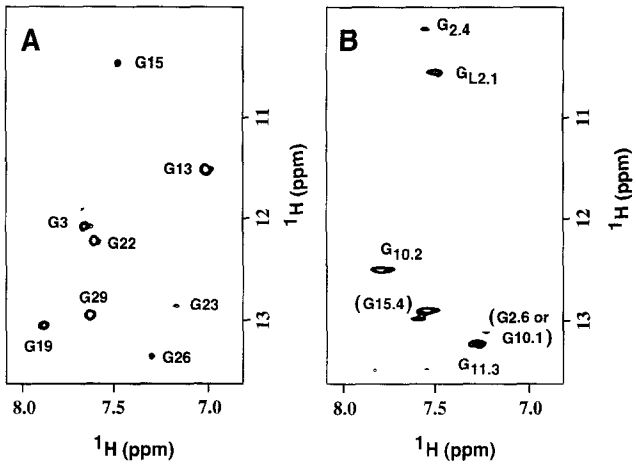

Fig. 3. The 2D H(NC)-TOCSY-(C)H spectra obtained with the pulse sequence given in Fig. 2. The guanosine imino proton frequencies in ω_1 are correlated with their H8 frequencies in the detection dimension, ω_2. The resonance assignments are given in the spectra, with tentative assignments for the hammerhead complex given in parentheses. (A) The H8 to HN region of the spectrum for the 1.8 mM ^{13}C-/^{15}N-labeled leadzyme. Spectral widths in the t_1 and t_2 dimensions were 2750 and 6000 Hz, respectively. This leads to folding of the imino proton resonances in ω_1 and a first order phase correction prior to the Fourier transformation in t_1 was used to shift the imino proton resonances to the center of the spectrum. Quadrature detection in ω_1 was obtained with the hypercomplex method (States et al., 1982). The total experimental time was 16 h, with 80 complex t_1 points, 512 complex t_2 points, 320 scans per FID, and a relaxation delay of 1 s. (B) The H8 to HN region of the spectrum for the 1.5 mM hammerhead ribozyme–substrate complex, recorded with the same parameters as for the leadzyme, except that 1008 transients were acquired for a total experimental time of 50 h. The ribozyme is uniformly ^{13}C/^{15}N labeled and the substrate is uniformly ^{15}N labeled. The isotopically labeled RNAs were synthesized as previously described (Batey et al., 1992; Nikonowicz et al., 1992; Legault, 1995). The buffer for the leadzyme sample was 10 mM sodium phosphate, 0.1 M NaCl, 0.2 mM EDTA, pH 5.5, in 90% H_2O/10% D_2O, and the buffer for the hammerhead sample was 25 mM d_4-succinate, 0.1 M NaCl, 0.1 mM EDTA, pH 5.5, in 90% H_2O/10% D_2O. All spectra were collected at 15 °C on a Varian Unityplus 500 MHz spectrometer and processed on a Silicon Graphics computer with the program FELIX, v. 2.35 (Biosym Technologies Inc., San Diego, CA).

The signal-to-noise ratio of the spectrum for the hammerhead complex is lower than that for the leadzyme, even though acquisition of the spectrum took three times as long. The lower sensitivity is likely due to less favorable transverse relaxation for the larger hammerhead system. However, we were still able to observe HN to H8 correlations for most of the guanosines in the duplex regions of the hammerhead ribozyme. We are currently making ^1H, ^{13}C, and ^{15}N resonance assignments of the

isotopically labeled hammerhead complex by a variety of 2D and 3D heteronuclear experiments, and the guanosine H8 assignments obtained with this 2D H(NC)-TOCSY-(C)H experiment will provide important starting points for further sequential assignment of the nonexchangeable protons in the hammerhead. The 2D version of the H(NC)-TOCSY-(C)H experiment is presented here, but if additional resolution is required, the experiment is readily extended into higher dimensions by frequency labeling of the imino nitrogen or C8 resonances.

After this manuscript was submitted, a communication was published describing an HCCNH-TOCSY experiment for through-bond correlation of exchangeable and nonexchangeable resonances in purine bases (Fiala et al., 1996). This approach is conceptually similar to that employed here, except that INEPT transfers are used for all heteronuclear magnetization transfer steps and the transfer proceeds from the H8 to the imino proton in guanosine. The sensitivity for this HCCNH-TOCSY spectrum is lower than that obtained with the H(NC)-TOCSY-(C)H experiment presented here, which may partially reflect differences between heteronuclear magnetization transfer using INEPT and cross polarization (Majumdar and Zuiderweg, 1995). Given the complex heteronuclear J-coupling network of the guanosine base, the inherent selectivity of the cross polarization leads to more efficient magnetization transfer from N1 to C6 than can be obtained through the use of selective shaped pulses and an INEPT sequence (data not shown).

This guanosine-specific H(NC)-TOCSY-(C)H experiment complements previously reported cytidine-, uridine- and adenosine-specific experiments (Simorre et al., 1995, 1996; Fiala et al., 1996). The guanosine, cytidine and uridine experiments provide unambiguous through-bond correlations between exchangeable (HN or H_2N) and nonexchangeable (H8 or H6) base protons. Because the adenosine amino proton resonances are usually much too broadened by exchange to be observed, the adenosine-specific experiment was designed to correlate the amino nitrogen with the H2 and H8 resonances (Simorre et al., 1996). The ability to assign adenosine and guanosine amino nitrogen resonances is crucial for application of a 2D ^{15}N-correlated NOESY experiment, which has recently been shown to improve structure determinations in isotopically labeled RNAs by detection of NOE contacts to exchange-broadened amino protons (Mueller et al., 1995). In this system, the guanosine amino nitrogen assignments were obtained by observation of strong NOEs between adjacent guanosine imino and amino protons (Mueller et al., 1995). These new techniques for resonance assignment and structure determination, when combined with previously developed techniques (see Pardi (1995) for a review), greatly simplify the assignment and structure determination of isotopically labeled RNAs.

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