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Multiple-quantum HCN-CCH-TOCSY experiment for $^{13}C/^{15}N$ labeled RNA oligonucleotides

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Abstract

A multiple-quantum 3D HCN-CCH-TOCSY experiment is presented for the assignment of RNA ribose resonances. The experiment makes use of the chemical shift dispersion of N1 of pyrimidine and N9 of purine to distinguish the ribose spin systems. It provides an alternative approach for the assignment of ribose resonances to the currently used COSY- and TOCSY-type experiments in which either ^{13}C or ^{1}H is utilized to distinguish the different spin systems. Compared to the single-quantum version, the sensitivity of the multiple-quantum HCN-CCH-TOCSY experiment is enhanced on average by a factor of 2 for a 23-mer RNA aptamer complexed with neomycin.

Introduction

As the size of RNA oligonucleotides studied by high resolution NMR increases, the complete assignment of ribose resonances using HCCH-COSY (Bax et al., 1990b; Kay et al., 1990; Ikura et al., 1991; Nikonowicz and Pardi, 1993) and HCCH-TOCSY (Bax et al., 1990a; Fesik et al., 1990; Olejniczak et al., 1992; Nikonowicz and Pardi, 1993) type experiments becomes a difficult task due to the poor dispersion in both ^{1}H and ^{13}C chemical shifts. In an effort to address this problem, some extensions of the above two kinds of experiments have been proposed such as the HCCH-RELAY (Nikonowicz and Pardi, 1993), the high resolution constant-time 2D or 3D HCCH-COSY/TOCSY (Kolk et al., 1998a) and the HCCH-COSY-TOCSY experiment (Hu et al., 1998). Recently, a near complete sugar resonance assignment has been achieved for a 44-mer RNA pseudoknot using high resolution HSQC, CT-HCCH-COSY/TOCSY and ^{13}C edited NOESY combined with three different $^{13}C/^{15}N$-labeled samples (Kolk et al., 1998a,b). The general strategy for the sugar resonance assignment has been nicely summarized (Varani et al., 1996; Wijmenga and van Buuren, 1998). Since the assignment methods discussed above are mainly dependent upon the chemical shift dispersion of $^{1}H1'$ and $^{13}C1'$, severe superimposition of $^{1}H1'$ and $^{13}C1'$ chemical shifts will hamper the effort for the sugar resonance assignment. It is thus desirable to have an experiment in which the ribose spin system will be resolved in a dimension other than $^{1}H1'$ or $^{13}C1'$. Here we propose a multiple-quantum HCN-CCH-TOCSY experiment as a complementary approach for ribose resonance assignment.

The single-quantum HC(N,P)-CCH-TOCSY experiment has been previously reported (Ramachandran et al., 1996). The experiment, designed for sequential assignment and base-type determination, labels ^{15}N and ^{31}P simultaneously in the t1 dimension. The magnetization transfer scheme for the HCN-CCH-TOCSY part is

$$\begin{aligned} H1' &\rightarrow C1' \rightarrow N1/N9(t1) \\ &\rightarrow C1' \rightarrow Cn'(t2) \rightarrow Hn'(t3) \end{aligned}$$

where $n = 1, 2, 3, 4, 5$ stands for the carbons and protons in the ribose ring.

The chemical shifts of N1 of pyrimidine C, U and N9 of purine are well separated, and the dispersion within each group is reasonably good, about 4 ppm for U, 6 ppm for C, and 6 ppm for G and A (Wijmenga and van Buuren, 1998). The resonances of ^{1}H and ^{13}C

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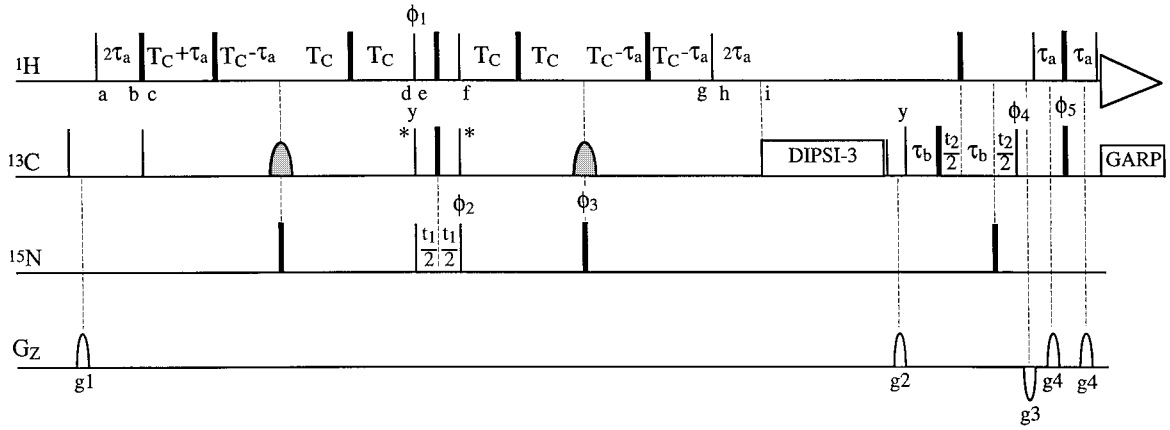

Figure 1. Pulse sequence of the 3D MQ-HCN-CCH-TOCSY experiment for ^{13}C,^{15}N-labeled RNA. Narrow and wide bars represent 90° and 180° pulses, respectively. All pulses are along the x axis unless otherwise indicated. The shaded ones are off-resonance 180° band-selective reburp pulses (Geen and Freeman, 1991) centered at 91 ppm with a duration of 2.4 ms. The phase adjustment is required for the pulses with an asterisk to cancel the shifted phase generated by off-resonance reburp pulses. The ^{1}H, ^{13}C and ^{15}N carrier frequencies were 4.75, 79 and 158 ppm, respectively. Field strengths of the ^{1}H pulse, ^{13}C high power pulse, reburp pulse, DIPSI-3 mixing (Shaka et al., 1988), GARP decoupling on ^{13}C (Shaka et al., 1985) were 32.1, 20, 0.21, 6.0, and 2.5 kHz, respectively. All ^{13}C pulses were applied on resonance with the exception of the reburp pulse which was shifted to 91 ppm through phase modulation. Strengths and durations of gradients were: g1=(18 G/cm, 0.2 ms), g2=(27 G/cm, 0.5 ms), g3=(−27 G/cm, 0.5 ms), g4=(18 G/cm, 0.2 ms). ^{13}C-TOCSY mixing time was 18.2 ms. $\tau_a = 1.4$ ms, $\tau_b = 0.95$ ms and $T_C = 9$ ms. Phase cycling was ϕ_1 = y, −y; ϕ_2 = 4(x), 4(−x); ϕ_3 = x; ϕ_4 = x, x, −x, −x; ϕ_5 = x and Acq. = x, −x, −x, x, −x, x, x, −x. Quadrature detection for t1 was achieved via States-TPPI (Marion et al., 1989) on ϕ_2 and ϕ_3, and for t2 on ϕ_4 and ϕ_5.

in the ribose are thus distinguished by a reasonably resolved nitrogen dimension in the HCN-CCH-TOCSY experiment. Since the J couplings between C1′ and N1/N9 are about 11 Hz (Wijmenga and van Buuren, 1998), it is necessary to develop a sensitivity enhanced version of this experiment in order to obtain the complete chemical shifts of ^{1}H and ^{13}C in the ribose for a larger-sized RNA oligonucleotide. The sensitivity optimization for single-quantum HCN-type experiments has been discussed in great detail in a recent review (Wijmenga and van Buuren, 1998).

Materials and methods

The preparation and conditions of the sample used in this study were reported previously (Jiang et al., 1999). It is a 1.8 mM uniformly ^{13}C,^{15}N-labeled 23-mer RNA aptamer complexed with neomycin B dissolved in D_2O. The sequence of the aptamer is GGACUGGGCGAGAAGUUUAGUCC, which we number as G_4 to C_{26}. The Watson–Crick stem is extended through mismatch formation with chain reversal occurring within the $G_{13}A_{14}G_{15}A_{16}$ segment (Jiang et al., 1999). All the experiments were carried out on a Varian Inova 500-MHz spectrometer equipped with actively shielded performa II Z-gradients at 20 or 25 °C. The data was processed and analyzed using FELIX 97.0 (Molecular Simulations), NMRPipe (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994) software on an SGI O2 workstation.

It is well known that for a two-spin system in a large biomolecule, the multiple-quantum coherence relaxes more slowly than the corresponding single-quantum magnetization state (Grzesiek and Bax, 1995). By applying this technique to a large RNA oligonucleotide, Marino et al. (1997) showed that the sensitivity of the HMQC experiment is two- to threefold higher than that of the HSQC experiment for ribose ^{1}H and ^{13}C correlations. The same group and other authors (Marino et al., 1997; Fiala et al., 1998; Sklenár et al., 1998) further demonstrated that the sensitivity of the HCN and HCNCH experiments for large RNA oligonucleotides can be enhanced significantly by using multiple-quantum coherence in the magnetization transfer step between carbon and nitrogen. In this paper, we present a multiple-quantum version of the HCN-CCH-TOCSY experiment and its utility for the ribose protons and carbons assignment.

The proposed sequence is shown in Figure 1. The coherence transfer pathway up to the point before ^{13}C mixing (i) can be described schematically using

product operators (Sørensen et al., 1983):

$$H_Z \xrightarrow{a} H_Y \xrightarrow{b} H_X C_Z \xrightarrow{c} H_X C_Y \xrightarrow{d} H_X C_C N_Z \xrightarrow{e} H_Z C_Z N_Y \xrightarrow{f} H_Y C_Y N_Z \xrightarrow{g} H_Y C_X \xrightarrow{h} H_Z C_X \xrightarrow{i} C_Y$$

where H and C are the H1′ and C1′ of the ribose, and N stands for the N1 of pyrimidine or N9 of purine. The chemical shifts of H1′ from point a to d, and again from point f to g are refocused by three and two 180° proton pulses, respectively. The chemical shifts of C1′ from point c to d, and from point f to i are refocused by a reburp 180° pulse on C1′. The duration of T_C can be optimized in situ because the scalar coupling interaction between C1′ and C2′ is removed by the selective 180° pulse on C1′. It has been shown that the usage of a selective 180° pulse on C1′ as well as on H1′ can enhance the sensitivity of the HCN experiment dramatically (Fiala et al., 1998). During the long transfer step from C1′ to N1/N9 (point c to d) and back (point f to i), the transverse magnetization of C1′ exists as a mixture of two-spin and three-spin heteronuclear multiple-quantum coherence, with the three-spin coherence antiphase with respect to N1/N9. Slow relaxation of multiple quantum coherence during the long magnetization transfer steps can be used to enhance the signal intensity. The proton homonuclear scalar-coupling interaction during the $4\times T_C$ period attenuates the sensitivity. Since most ribose adapt a C3′-*endo* conformation in RNA oligonucleotides, the loss due to the homonuclear scalar-coupling interaction is small, about 2∼10% estimated from the value of $J_{H1'-H2'}$ (1∼3 HZ). For a ribose with C2′-*endo* conformation, the intensity loss due to a larger proton homonuclear scalar-coupling interaction can be eliminated by using the selective H1′ 180° pulses during the $4\times T_C$ period (Fiala et al., 1998). The trade-off of this approach is that the signals of H1′ protons located in the H2′ region are significantly suppressed. The multiple-quantum coherence pathway is also modulated by the longitudinal relaxation rate of N1/N9. This effect is negligible since N1/N9 relaxation rates are slow compared to the duration of $4\times T_C$.

Results and discussion

To compare the sensitivity of both single-quantum and double-quantum HCN-CCH-TOCSY experiments, we acquired both 1D and 2D spectra from the two 3D sequences. In the SQ-HCN-CCH-TOCSY sequence, slightly modified from the HC(N,P)-CCH-TOCSY experiment (Ramachandran et al., 1996), reburp 180° pulses on ^{13}C1′ are used in the INEPT and reverse INEPT steps for the magnetization transfer between C1′ and N1/N9, and only nitrogen chemical shifts are labeled in the t1 period instead of both nitrogen and phosphorus as in the original HC(N,P)-CCH-TOCSY experiment. Shown in Figure 2A and 2B are the 1D spectra acquired with multiple-quantum and single-quantum HCN-CCH-TOCSY sequences at 20 °C. The duration of T_C is experimentally optimized to 9 and 7.5 ms, respectively, for the multiple and single-quantum HCN-CCH-TOCSY experiments. Both spectra were scaled to the same noise level. The signal intensity of MQ-HCN-CCH-TOCSY is significantly larger when compared to that of the signal-quantum version. A more accurate comparison was obtained from H1′ and ^{15}N correlated 2D spectra acquired from the two 3D sequences under the same conditions as

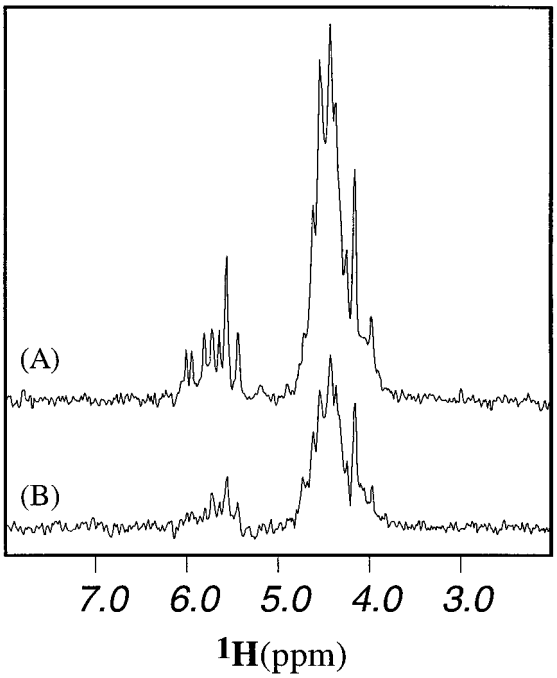

Figure 2. 1D ribose proton spectra acquired using the 3D MQ-HCN-CCH-TOCSY sequence (A) and the single-quantum 3D HCN-CCH-TOCSY sequence (B) from a uniformly ^{13}C/^{15}N-labeled 23-mer RNA aptamer complexed with neomycin. The spectral width (Hz)/complex points was 6000/512 with 320 transients. The delays ($4\times T_C$) for the INEPT transfer step from C1′ to N1/N9 and the back step were optimized experimentally to 36 and 30 ms for (A) and (B), respectively. All other related parameters are the same as in the legend to Figure 1. The data were processed using a sine window function shifted by 75°. Both (A) and (B) are scaled to the same noise level.

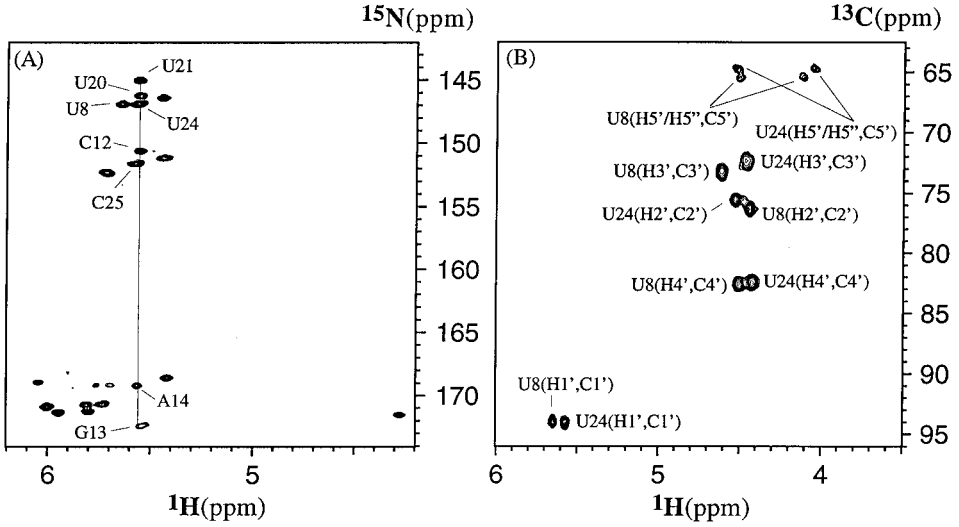

Figure 3. (A) 2D H1′ and N1/N9 correlation spectrum acquired using the 3D HCN sequence (Fiala et al., 1998) with minor modification as discussed in the text. (B) 2D plane from the 3D MQ-HCN-CCH-TOCSY experiment at 147 ppm along the nitrogen dimension for a uniformly $^{13}C/^{15}N$-labeled 23-mer RNA aptamer complexed with neomycin. The experiments for (A) and (B) were carried out at 20 °C and 25 °C, respectively. The spectral widths (Hz)/complex points along ^{1}H and ^{15}N were 6000/512 and 1621/60, respectively with 320 transients per FID for (A). The ^{15}N dimension resolution was enhanced through linear prediction (Delaglio et al., 1995) resulting in a final matrix size of 256×1024. The thin line in (A) highlights the overlapped H1′ chemical shifts of four riboses. The spectral widths (Hz)/complex points along ^{1}H, ^{13}C and ^{15}N were 6000/512, 4274/38 and 1621/38, respectively with 32 transients per FID for (B). Both the ^{13}C and ^{15}N dimension resolution were enhanced by linear prediction (Delaglio et al., 1995) resulting in a final matrix size of 128×128×1024. The 2D plane contains two ribose resonances for U8 and U24. The assignment of the U8 resonance can be easily obtained from the HCCH-COSY-TOCSY experiment, as discussed in the text.

in the 1D experiments described above. The signal-to-noise ratios (S/N) of 17 well-resolved cross peaks with reasonable intensity from both experiments are compared. The S/N ratios of the MQ-HCN-CCH-TOCSY peaks are a factor of 1.2∼3.5 larger than those from SQ-HCN-CCH-TOCSY, with an average value of two-fold. The only exception is the cross peak of A16 which is much stronger in the SQ-HCN-CCH-TOCSY experiment. The possible reasons are first, the $J_{H1'-H2'}$ is large (∼7 Hz) because of the C2′-*endo* conformation of A16 (Jiang et al., 1999). Second, the conformation of A16 may be mobile since this base flips out of the loop and flaps over the bound aminoglycoside in the binding site, and furthermore, the binding of neomycin to the RNA aptamer exhibits moderate affinity (100 nM) (Wallis et al., 1995; Jiang et al., 1999). The multiple-quantum coherence relaxation rate appears not to have advantage over its single-quantum counterpart under these conditions.

Although the RNA aptamer contains only 23 nucleotides, four of the riboses have identical H1′ chemical shifts, and seven H1′ are clustered together within a 0.04 ppm range. Some of these riboses also have overlapped or poorly dispersed $^{13}C1'$ chemical shifts. Two of these riboses could not be fully assigned using ^{13}C-edited NOESY-HMQC and HCCH-COSY-TOCSY experiments (Jiang et al., 1999).

Shown in Figure 3A is the H1′-N1/N9 correlated spectrum acquired using a slightly modified MQ-HCN sequence with hard 180° pulses instead of the selective 180° pulses on the H1′ region as used in the original sequence (Fiala et al., 1998). All of the H1′-N1/N9 cross peaks appear, except for A16, for the reasons outlined above. The cross peaks are located in three distinct regions along the nitrogen dimension: U, C, and A/G. The seven cross peaks whose H1′ chemical shifts are superimposed or poorly dispersed are aligned along the fine line in Figure 3A. Although the H1′ chemical shifts are overlapping or very close, the ribose spin systems can be easily distinguished by their N1/N9 chemical shift values using the MQ-HCN-CCH-TOCSY experiment. Figure 3B shows a ^{1}H-^{13}C 2D plane slice at 147 ppm along the nitrogen dimension from the 3D spectrum acquired using the MQ-HCN-CCH-TOCSY experiment. The U8 and U24 ribose spin systems are located in this plane. The assignment of the U8 ribose is very straightforward using the HCCH-COSY-TOCSY experiment (Hu et al., 1998; Jiang et al., 1999) because its H1′ chemical shift is well separated from other protons. At the same time,

the cross peak of C2′/H2′ of U24 in this plane can also be determined from the HCCH-COSY-TOCSY experiment. Thus, the complete ribose assignment of U24 can be obtained easily from this plane. The assignment of the ribose of C25 is more straightforward from the ^{1}H-^{13}C 2D plane with the ^{15}N chemical shift anchored at 151.6 ppm, since its N1 value is well resolved. By using the MQ-HCN-CCH-TOCSY experiment, 19 out of the 23 ribose spin systems can be fully identified. The three partially identified ones are located either at the end of the sequence (G4, G5) (note that the sequence starts at G4), or in the loop region (G13). Since these riboses are not C2′-*endo* puckered, the possible reason for weak cross peaks might be their dynamic behavior. No cross peaks have been observed for A16 because of the reasons discussed earlier.

Conclusions

In summary, a sensitivity enhanced MQ-HCN-CCH-TOCSY experiment for ribose assignment is presented in this paper. The experiment is a good complement to experiments which rely on protons or carbons of ribose to separate the ribose spin systems, because it resolves the ribose spin systems by reasonably dispersed N1/N9 shifts. The sensitivity of the MQ-HCN-CCH-TOCSY experiment is on average enhanced by a factor of 2 compared to the corresponding single-quantum version, and this significant enhancement makes it useful for larger-sized RNA nucleotides.

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