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Correlation of nucleotide base and sugar protons in a ^{15}N-labeled HIV-1 RNA oligonucleotide by ^{1}H-^{15}N HSQC experiments

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SUMMARY

The advent of methods for preparing ^{15}N- and ^{13}C-labeled RNA oligonucleotides holds promise for extending the size of RNA molecules that can be studied by NMR spectroscopy. A practical limitation is the expense of the ^{13}C label. It may therefore sometimes be desirable to prepare a relatively inexpensive ^{15}N-labeled sample only. Here we show that the two-bond ^{1}H-^{15}N HSQC experiment can be used on ^{15}N-labeled RNA to correlate the intranucleotide H1' and H8,H6,H5 resonances indirectly through the shared glycosidic nitrogen. The nonrefocused version of a standard HSQC experiment for 2D proton-detected ^{1}H-^{15}N chemical-shift correlation is applied in order to minimize the sensitivity loss due to the relatively fast spin–spin relaxation of RNA oligonucleotides. The experiment is applied to the 30-nucleotide RNA RBE3 which contains the high-affinity binding site of the RRE (rev response element) for the Rev protein of HIV. The results indicate that this simple experiment allows a straightforward identification of the base proton resonances CH5, CH6, UH5, UH6, purine H8, and AH2 as well as the intranucleotide H1' and H8,H6,H5 connectivities. When combined with a NOESY experiment, complete sequential assignments can be obtained.

In order to extend the size range of RNA oligonucleotides for which three-dimensional structures can be obtained, methods have been developed for synthesis of uniformly ^{15}N- and/or ^{13}C-labeled ribonucleotides (Batey et al., 1992; Nikonowicz et al., 1992). These methods involve isolation of labeled NMPs from bacteria grown on labeled media, conversion to NTPs (Batey et al., 1992; Nikonowicz et al., 1992; Michnicka et al., 1993), and incorporation of the labeled NTPs into the desired RNA oligonucleotide via enzymatic synthesis on a DNA template with T7 RNA polymerase (Milligan et al., 1987). A practical limitation is the expense of the ^{13}C label. It may

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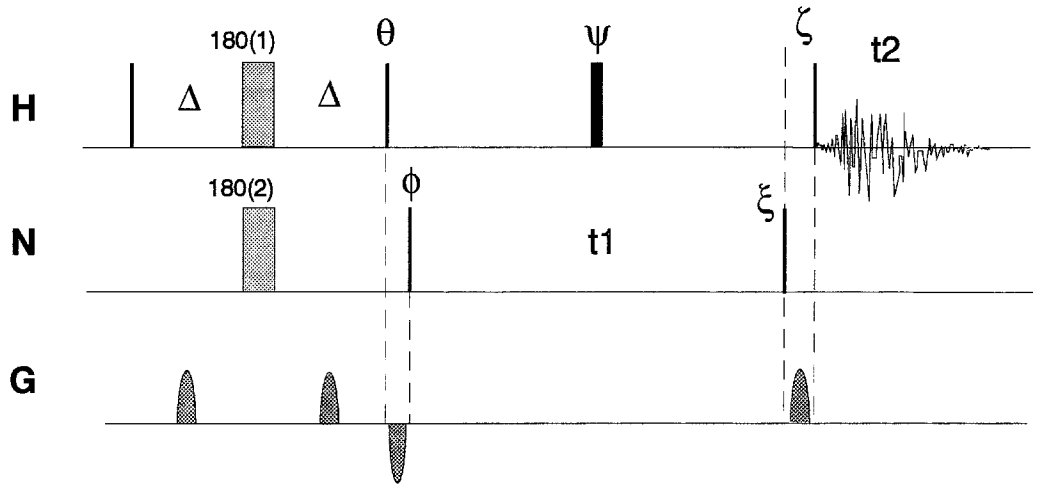


Fig. 1. The pulse scheme of the ^{1}H-^{15}N HSQC experiment. The narrow and broad filled bars represent nonselective 90° and 180° pulses, respectively. The shaded broad bars indicate either a selective or nonselective 180° pulse. The 180(1) pulse is a REBURP refocusing pulse and 180(2) is an IBURP inversion pulse (Geen and Freeman, 1991). The delay $2\Delta \sim 42$ ms optimizes for T_2 and $1/2J_{NH}$. The phase cycling is: θ = y,y,−y,−y; ϕ = x,−x,x,−x; ζ = 4(x), 4(−x); ξ = 8(x),8(−x); receiver = x,−x,−x,x, 2(x,−x,−x,x), x,−x,−x,x. In addition, the ϕ phase is cycled to obtain States-TPPI quadrature detection in t_1 (Marion et al., 1989). The gradient pulses are applied to purge imperfections of the 180° refocusing pulses and to suppress the unwanted coherences during the INEPT transfers when the magnetizations of interest are converted into the zz-orders.

therefore sometimes be desirable to prepare a relatively inexpensive ^{15}N-labeled sample only. The ^{15}N label in RNA oligonucleotides has been used in two- and three-dimensional HMQC experiments to correlate and assign exchangeable imino and amino resonances (Nikonowicz and Pardi, 1992,1993) and to correlate N7,N9 with AH8 and N1,N3 with AH2 resonances (Michnicka et al., 1993). Here we show that the two-bond HSQC (Bodenhausen and Ruben, 1980) experiment can be used on ^{15}N-labeled RNA to correlate the intranucleotide H1' and H8,H6,H5 resonances indirectly through the shared glycosidic nitrogen. The experiment is applied to the 30-nucleotide RNA RBE3 which contains the high-affinity binding site of the RRE (rev response element) for the Rev protein of HIV (Bartel et al., 1991). The results indicate that this simple experiment allows a straightforward identification of the base proton resonances CH5, CH6, UH5, UH6, purine H8, and AH2 as well as the intranucleotide H1' and H8,H6,H5 connectivities. When combined with a NOESY (Kumar et al., 1980) experiment, complete sequential assignments (Wüthrich, 1986) can be obtained.

The pulse sequence is presented in Fig. 1. The nonrefocused version of a standard HSQC experiment for 2D proton-detected ^{1}H-^{15}N chemical-shift correlation is applied (Bodenhausen and Ruben, 1980) in order to minimize the sensitivity loss due to the relatively fast spin–spin relaxation of the large RRE RNA fragment. The ^{1}H-^{15}N HSQC offers several advantages over the ^{1}H-^{15}N HMQC experiment, due to the difference in relaxation properties of single- and multiple-quantum ^{15}N coherences (Norwood et al., 1989,1990; Bax et al., 1990). The INEPT (Morris and Freeman, 1979) delay 2Δ is set to 42 ms to allow sufficient evolution of the small two- and three-bond ^{1}H-^{15}N couplings (Table 1). The pulse sequence can be applied in a nonselective or selective fashion. The nonselective experiment gives the correlation of the purine H8 with N7

TABLE 1
AVERAGE TWO- AND THREE-BOND ^{15}N-^{1}H J-COUPLING VALUES FOR PURINE AND PYRIMIDINE MONOPHOSPHATES[a]

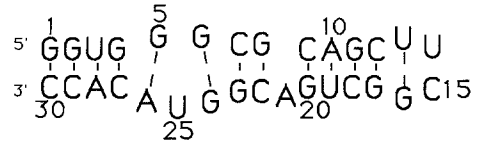
Purines	J_{NH} (Hz)	Pyrimidines	J_{NH} (Hz)
AN1-H2	16	N1-H6	3.5
AN3-H2	16	N1-H5	4–6
N7-H8	10–11	N3-H5	3.6
N9-H8	8	N1-H1'	4
N9-H1'	<3.5		

[a] Coupling constants were measured from splittings observed in one-dimensional spectra of an ^{15}N, ^{13}C-labeled NMR sample in D_2O.

and N9, of H2 with N3 and N1 and of the pyrimidine H6 with N3 and N1, as well as the correlation between N9/N1 and H1' and pyrimidine N1 and H5. However, the efficiency of the desired coherence transfers H8-N9, H6-N1, H5-N1, and H1'-N9/N1 for indirect correlation of H8, H6, and H5 with the intranucleotide H1' through the shared glycosidic nitrogen is decreased by the evolution of competitive H8-N7, H6-H5, and to a lesser extent by H1'-H2' spin–spin interactions. More effective coherence transfers can be obtained using semiselective experiments in which the nonselective INEPT 180° pulses on ^{1}H or on both ^{1}H and ^{15}N are replaced with selective 180° pulses (Bax, 1984; Bax et al., 1984). For correlation of ribose H1' with the glycosidic nitrogen, a selective 180° ^{1}H pulse is centered to refocus the range of H1' and H5 chemical shifts and the 180° ^{15}N pulse is applied nonselectively. As a result, both correlations of H1' and H5 to N9 in purines and to N1 (and N3 for U) are observed at the same time. Because of differences in proton chemical shifts of H6 and H5 and H1' and H2', the selective 180° ^{1}H pulse decouples not only the H5-H6, but the H1'-H2' interactions as well. Although the H1'-H2' coupling is small (1–2 Hz) (Altona, 1982) for the C3'-endo sugar puckers usually found in A-form RNA (Saenger, 1984), the sugar puckers and therefore coupling constants can vary widely in nonstandard RNA structures. Optimal sensitivity for H8-N9 and H6-N1/N3 correlations can be achieved by applying selective INEPT 180° ^{1}H and ^{15}N pulses, covering the range of the proton resonances H8 and H6 (6.8–8.5 ppm) and the nitrogen resonances purine N9 and pyrimidine N1/N3 (140–180 ppm). This effectively decouples N7-H8 and N9-H1' for purines and N1/N3-H5 and N1-H1' for pyrimidines.

These ^{1}H-^{15}N HSQC experiments have been applied to a 0.9-mM labeled sample of a 30-base ribonucleotide RBE3 (see scheme below).

RBE3 was enzymatically synthesized on a DNA template using T7 RNA polymerase to incorporate labeled NTPs, following procedures described by Milligan et al. (1987), and scaled up to yield NMR quantities (Heus and Pardi, 1991; Wyatt et al., 1991). The uniformly ^{13}C- and ^{15}N-labeled NTPs were prepared from RNA isolated from *E. coli* grown in ^{13}C-glucose- and



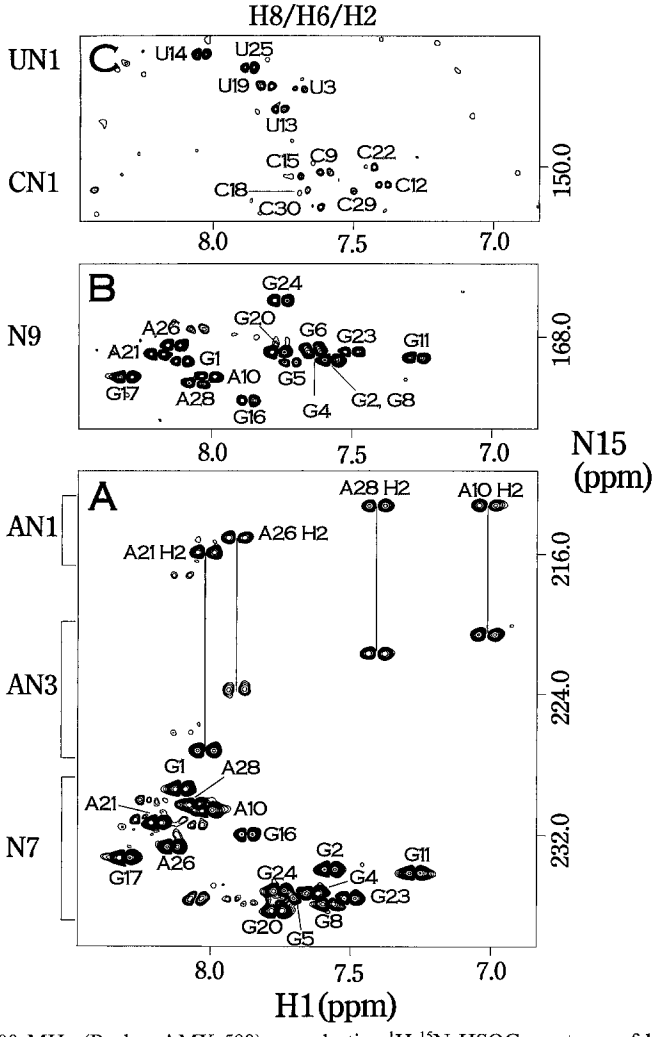


Fig. 2. Portion of the 500 MHz (Bruker AMX 500) nonselective ^{1}H-^{15}N HSQC spectrum of labeled RBE3 at 40 °C, showing cross peaks (A) H8-N7, AH2-N1, and AH2-N3; (B) H8-N9; and (C) CH6-CN1 and UH6-UN1. Assignments are indicated in the spectrum, which was acquired with 512 points in both dimensions, spectral widths of 3012 Hz in f_2 and 5000 Hz in f_1, 208 scans per t_1 increment, and a total measuring time of 42 h. The processed data matrix size was 2K × 2K points. Although only the ^{15}N label was utilized in these experiments, the RNA sample was actually doubly labeled with ^{13}C and ^{15}N. Therefore, the ^{13}C had to be decoupled during the acquisitions. With a ^{15}N-only labeled RNA sample, the sensitivity of the ^{1}H-^{15}N HSQC experiments would be even better than that obtained here.

^{15}N-NH_3-containing media (Nikonowicz et al., 1992), following the procedures of Batey et al. (1992). The RNA was purified by polyacrylamide gel electrophoresis and electrolution, followed by three ethanol precipitation steps. The NMR sample was 0.9 mM RBE3 in 100 mM NaCl, pH 7.0 in 450 µl 99.996% D_2O.

A portion of the spectrum obtained using the nonselective experiment is shown in Fig. 2. The purine N7, N1, N3, N9 (Witanowski, 1973; Uchida et al., 1989) and pyrimidine UN3 (Roberts et al., 1967) and CN3 all resonate in distinct spectral regions. The H8 are all correlated to both N7

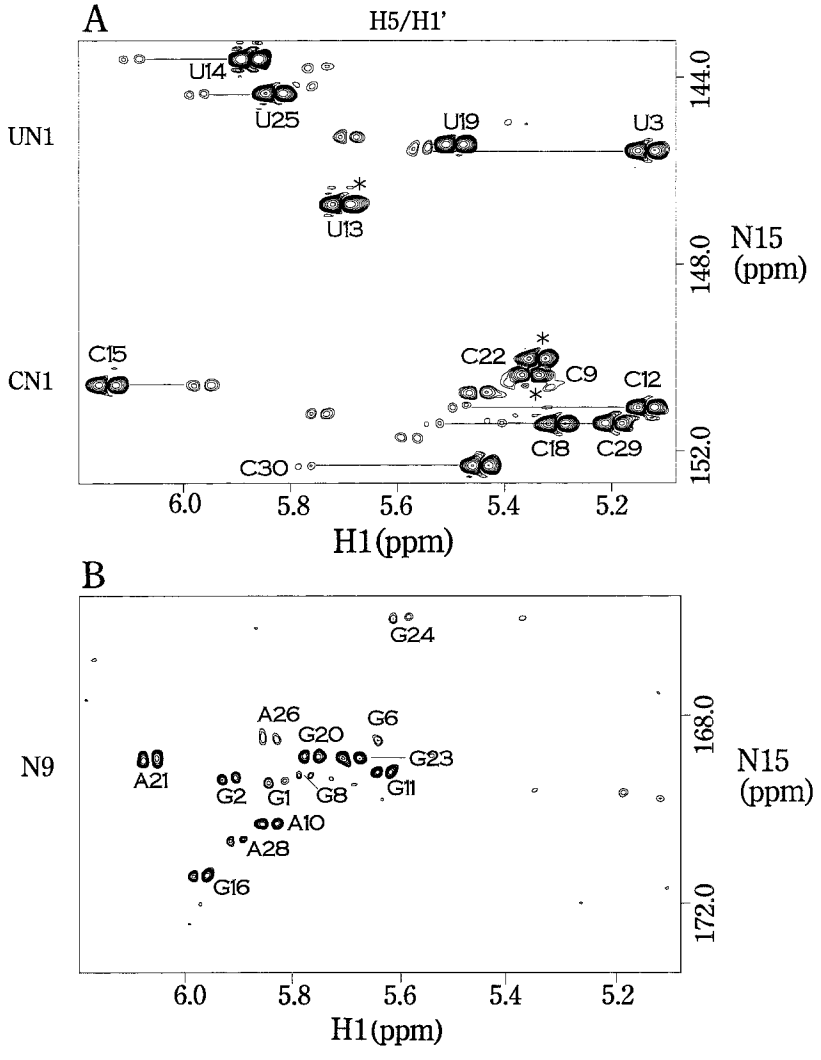


Fig. 3. Portion of the 500 MHz (Bruker AMX 500) H1',H5 selective ^{1}H-^{15}N HSQC spectrum of labeled RBE3 at 40 °C, showing (A) cross peaks UH5-N1, UH1'-N1 CH5-N1, and CH1'-N1. All five UH5-UH1' correlations are seen. The weaker cross peak of each H5-N1, H1'-N1 pair is the H1'-N1 correlation. The U13, C22H5, and C9H5 overlap their respective H1' resonances (indicated by *). (B) H1'-N9 cross peaks. Missing purine N9-H1' correlations (G4, G5) are due to conformational flexibility in the internal loop. G17 H1' resonates out of the plotted range. The selective 180° ^{1}H pulse is 3.3 ms REBURP, centered in the middle of the H1',H5 region at 5.65 ppm. The spectrum was acquired with 256 points in both dimensions, spectral widths of 1501 Hz in f_2 and 1775 Hz in f_1, 368 scans per t_1 increment, and a total measuring time of 37.5 h. The processed data matrix size was 1K × 1K points.

and N9 and the AH2 are correlated to N1 and N3. The UH6 and CH6 are correlated to UN1 and CN1, respectively, and so are UH5 and CH5 (Fig. 3). The UH5 are also correlated to UN3 (not shown), which provides a further distinction between U and C base proton resonances. Thus, the nonselective ^{1}H-^{15}N HSQC experiment provides a straightforward way to identify the base type of all of the nonexchangeable base proton resonances (C, U, purine H8, and AH2).

A portion of the H1',H5 selective ^{1}H-^{15}N HSQC spectrum showing the cross peaks between the pyrimidine H5, H1' and N1 is shown in Fig. 3A. Strong UH5-UN1 and CH5-CN1 cross peaks are observed for all but two CH5 resonances, which are also weak in the proton COSY spectra due to conformational averaging. The H5 resonances are correlated to the intranucleotide H1' via the weaker H1'-N1 cross peaks. Similar results are observed for the purine N9-H1' (Fig. 3B). The information obtained from the ^{1}H-^{15}N HSQC experiment provides a starting point for the sequential assignments (Wüthrich, 1986) in a NOESY spectrum. The intranucleotide base-H1' NOE cross peaks can now be unambiguously distinguished from the internucleotide NOE cross peaks, and any ambiguities in the sequential connectivities are resolvable based on the identification of the type of base proton resonance and comparison to the primary sequence of the RNA.

Alternative approaches employing triple-resonance (H, C, N) experiments for ^{15}N- and ^{13}C-labeled RNA oligonucleotides will be presented elsewhere (Sklenář et al., 1993a,b).

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