Improved NOE-Based Sequential Correlation of Base and 1′ Proton Resonances in Labeled Nucleic Acids

Edward P. Nikonowicz,* Malgorzata Michnicka, and Eric DeJong

Department of Biochemistry and Cell Biology Rice University, Houston, Texas 77005

Received November 19, 1997

An efficient procedure is described to facilitate the sequential resonance assignment of nucleic acids using the ^{13}C separated NOESY experiment. A uniformly ^{13}C-enriched RNA oligonucleotide containing the H8 of the purine bases and only the H6 of the pyrimidine bases was prepared by exchanging the protons for deuterons at the C5 positions of uniformly ^{13}C-enriched 5′-CMP and 5′-UMP. Deuteration of C5 eliminates the overlap frequently encountered between H5 and H1′ resonances in the base-1′ region of the NOE spectrum. The procedure is demonstrated for a 23-nucleotide RNA hairpin that contains twelve pyrimidine nucleotides.

The resonance assignment of nucleic acids is primarily accomplished using sequential NOE cross-peaks between adjacent base and sugar protons and has been successfully applied to a variety of oligonucleotide systems.[1−3] This process is now facilitated by ^{13}C and ^{15}N isotopic enrichment of ribonucleic acids which allows the application of multidimensional heteronuclear techniques that dramatically simplify the NOESY spectrum and permit the scalar correlation of intraresidue base-1′ resonances.[4−8] Deuterium labeling of the nonexchangeable sites of oligonucleotides also can reduce cross-peak overlap and improve resolution of the NOESY spectrum.[9−13] We have combined uniform ^{13}C enrichment of ribonucleotides with selective deuteration at the C5 position of uridine and cytidine bases. This labeling strategy eliminates crowding in the base-1′ region of the NOESY spectrum caused by the intense H6−H5 cross-peaks and simplifies the sequential resonance assignment procedure. The constant time variant of the NOESY−HMQC experiment was used to refocus the one-bond C5−C6 coupling and improve the resolution of the pyrimidine C6 resonances.

Deuteration of the pyrimidine nucleotide C5 positions was accomplished using metabisulfite anion[14] as a catalyst in a procedure similar to that employed for hydrogen isotope exchange at C5 and C8 in DNA and RNA oligonucleotides.[9,15] A key step in the procedure for this application is the chromatographic separation of purine from pyrimidine nucleotides in order to preserve the proton at the C8 position of adenine and guanine bases. The 5′-NMPs were eluted sequentially from an anion exchange column using a stepped gradient of formic acid.[16] The exchange reaction (99% ^2H_2O, 10 mM 5′-NMPs, 150 mM $(NH_4)_2SO_3$, 20 mM $Na_2S_2O_5$, pH* 7.7) was incubated at 65 °C for 72 h. The reaction was stopped after ≈90% exchange to facilitate comparison of the deuterated and protonated RNA molecules. An aliquot of the reaction proceeded to >98% completion after incubating for an additional 30 h, and no exchange was detected at other sites. The yield of C5 deuterated 5′-NMPs after separation, exchange, and purification of the protonated 5′-NMPs was ≈93%.

The H6/8-H1′ region of the NOESY spectrum exhibits the greatest dispersion of base-sugar cross-peaks in an oligoribonucleotide and provides the correlations necessary to accomplish the sequence-specific resonance assignment of an RNA molecule. Although the observation of NOE correlations is conformation dependent, each H6/8 and H1′ resonance usually gives rise to a pair of intraresidue and interresidue H6/8-H1′ NOE cross-peaks (Figure 1). The ^{13}C resolved sequential assignment strategy is based on the alternate correlation of these NOE cross-peak pairs to the chemical shifts of the directly attached C1′ and C6/8 nuclei.[17] In general, NOE planes at unique purine C8 frequencies contain an intra- and an interresidue H8−H1′ cross-peak. However, since the pyrimidine H5 resonates in the same region as the 1′ protons, C6 NOE planes generally contain three cross-peaks, two H6−H1′ correlations, and an H6−H5 correlation. The intrabase H6−H5 distance of 2.45 Å results in an intense cross-peak that frequently obscures at least one of the H6−H1′ cross-peaks. Since the C1′ and C6/8 resolved regions of the NOE spectrum are used to obtain sequential correlations, unambiguous identification of H6−H1′ cross-peaks is essential.

Figure 2 compares the NOE-based resonance assignment procedure applied to a C5 protonated (5-^1H) RNA molecule and the corresponding C5 deuterated (5-^2H) molecule. The cross-peaks involving H6 are separated according to the chemical shifts of the directly attached C6 nuclei. In the 5-^2H molecule, the intensities of the residual H6−H5 cross-peaks are significantly weaker than the H6−H1′ correlations (Figure 2b). The near absence of an H6−H5 cross-peak in the spectrum of the 5-^2H molecule permits unambiguous identification of both Cyt-10 H6−H1′ correlations (Figure 2b), whereas in the 5-^1H molecule *both* H6−H1′ correlations are concealed by the intense H6−H5 cross-peak (Figure 2a). The intraresidue H6−H1′ cross-peaks of Uri-12 and Cyt-13 are resolved in the spectra of both RNA molecules, but the interresidue cross-peak of Uri-12 H6 is not observed due to the conformation of the base. The absence of this interaction is immediately clear in the 5-^2H molecule spectrum. In the spectrum of the 5-^1H molecule, it is not possible to distinguish whether the interresidue NOE to Uri-12 H6 is absent or present but simply concealed beneath the H6−H5 cross-peak. The problem of distinguishing between two alternatives arises for the interresidue NOE involving Cyt-13 H6 in the 5-^1H molecule. In this case, the NOE is present but covered by the H6−H5 cross-peak. The sensitivity of HMQC−NOESY−HMQC experiments was insufficient to provide comparable information through 1′−C6/H6 correlations. Thus, substitution of the pyrimidine 5 protons with deuterons eliminates the H6−H5 cross-peaks from the spectrum allowing the H6−H1′ correlations to be readily identified. This permits the sequential resonance assignment to proceed alternately between C6/8 and C1′ NOE planes without interruption.

* Address correspondence to this author. edn@bioc.rice.edu. telephone (713) 527-4912. fax (713) 285-5154.

(1) Hare, D. R.; Wemmer, D. E.; Chou, S.-H.; Drobny, G.; Reid, B. R. *J. Mol. Biol.* **1983**, 171, 319−336.
(2) Scheek, R. M.; Russo, N.; Boelens, R.; Kaptein, R. *J. Am. Chem. Soc.* **1983**, 105, 2914−2916.
(3) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
(4) Farmer II, B. T.; Mueller, L.; Nikonowicz, E. P.; Pardi, A. *J. Am. Chem. Soc.* **1993**, 115, 11040−11041.
(5) Sklenar, V.; Peterson, R. D.; Rejante, M. R.; Feigon, J. *J. Biomol. NMR* **1993**, 3, 721−727.
(6) Pardi, A. *Methods Enzymol.* **1995**, 261, 350−380.
(7) Marino, J. P.; Diener, J. L.; Moore, P. B.; Griesinger, C. *J. Am. Chem. Soc.* **1997**, 119, 7361−7366.
(8) Dieckmann, T.; Feigon, J. *J. Biomol. NMR* **1997**, 9, 259−272.
(9) Brush, C. K.; Stone, M. P.; Harris, T. M. *Biochemistry* **1988**, 127, 115−122.
(10) Agback, P.; Maltseva, T. V.; Yamakage, S.-I.; Nilson, F. P. R.; Foldesi, A.; Chattopadhyaya, J. *Nucleic Acids Res.* **1994**, 22, 1404−1412.
(11) Tolbert, T. J.; Williamson, J. R. *J. Am. Chem. Soc.* **1996**, 118, 7929−7940.
(12) Nikonowicz, E. P.; Michnicka, M.; Kaluarachchi, K.; DeJong, E. *Nucleic Acids Res.* **1997**, 25, 1390−1396.
(13) Huang, X. N..; Yu, P. L.; Leproust, E.; Gao, X. L. *Nucleic Acids Res.* **1997**, 25, 4758−4763.
(14) Hayatsu, H. *Prog. Nucleic Acid Res. Mol. Biol.* **1976**, 16, 75−124.
(15) Nikonowicz, E. P.; Kalurachchi, K.; DeJong, E. *FEBS Lett.* **1997**, 415, 109−113.
(16) Cohn, W. E. *J. Am. Chem. Soc.* **1950**, 72, 1471−1478.
(17) Nikonowicz, E. P.; Pardi, A. *J. Mol. Biol.* **1993**, 232, 1141−1156.

S0002-7863(97)03961-9 CCC: $15.00
Published on Web 04/04/1998

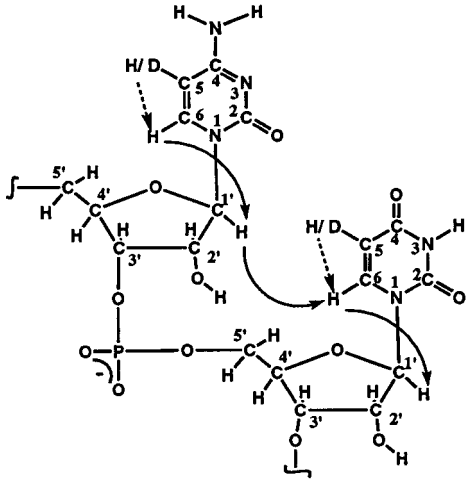

Figure 1. Diagram of a dinucleotide showing the intra- and interresidue base-1′ proton NOEs used for sequential assignments. The intrabase pyrimidine H5−H6 NOE is also shown. Each NOE (represented by an arrow) results in a cross-peak that is correlated with a C6/8 frequency and a C1′ frequency.

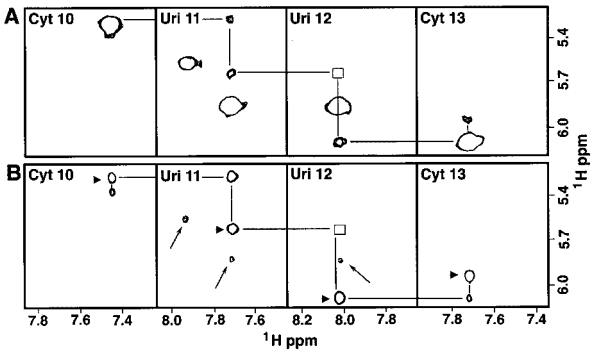

Figure 2. $\omega_1-\omega_3$ planes extracted from {^{1}H, ^{13}C, ^{1}H} 3D NOESY−CT-HMQC spectra showing sequential base-1′ proton correlations in (a) the C5 protonated and (b) C5 deuterated RNA molecules. The primary structure of the RNA is r(GGGAUACUGCUUCGGUAAGUCCC), and the sequential walk is traced from Cyt 10 to Cyt 13. In (b), the H5−H6 cross-peaks are indicated by arrows, and the intraresidue H6−H1′ correlations are marked with a triangle pointed toward the right. The RNA molecules were prepared as reported[20,21,22] using uniformly ^{13}C-enriched and uniformly ^{13}C-enriched/C5 deuterium-labeled 5′ NTPs. Sample concentrations were 2.1 and 1.8 mM for the deuterated and protonated molecules, respectively. The sequence used to acquire the spectra is shown in Figure 3.

The C6 resolved region is further complicated in uniformly enriched molecules as the C6 resonances are split by the C5 nuclei. A constant time (CT) HMQC element (Figure 3) was used to eliminate C6 splitting by refocusing the C6−C5 coupling and provided a significant improvement of sensitivity and resolution. The CT-HMQC takes advantage of the slower relaxation of the multiple quantum coherence relative to single quantum coherence[18] but can result in ^{13}C resonance broadening due to ^{1}H homonuclear coupling. Since there is no H5 in the C5 deuterated RNA molecule, neither the C6 nor H6 resonances suffer broadening originating from the 7−8 Hz H6−H5 coupling.

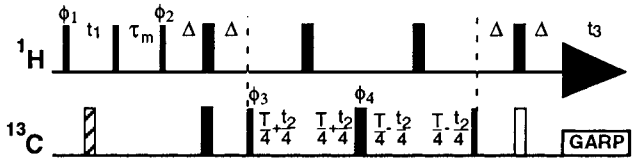

Figure 3. Pulse sequence used to acquire the 3D NOESY−CT-HMQC spectrum. Thin and thick bars represent 90° and 180° pulses; the hatched bar is a composite $90_x240_y90_x$ inversion pulse and the open bar is a composite $90_x180_y90_x$ pulse. The phase cycle is $\phi_1 = x, -x$; $\phi_2 = 4(x)\ 4(-x)$; $\phi_3 = 2(x)\ 2(-x)$; $\phi_4 = x, -x$; rcvr $= x, -x, -x, x, -x, x, x, -x$. The delay $\Delta = 2.6$ ms and the delay $T = 15.2$ ms. Spectra were acquired on a Bruker AMX-500 NMR spectrometer equipped with a ^{1}H-{^{13}C/^{15}N} triple resonance probe. Broadband decoupling of the base carbon resonances was achieved using GARP (γB_2=2314 Hz).[23] Quadrature detection was achieved using the States-TPPI method, and acquisition was delayed by a half-dwell in the indirectly detected ^{1}H dimension.[24] The spectra were acquired with eight scans per FID at 28 °C using a mixing time of 320 ms, and the system was allowed 1.1 s to recover between scans. The spectral widths were $\omega_1 = 3000$ Hz, $\omega_2 = 2083$ Hz, and $\omega_3 = 7000$ Hz with acquisition times $t_1^{max} = 22$ ms, $t_2^{max} = 14.4$ ms, and $t_3^{max} = 146$ ms. The time domain data points were extended in t_1 and t_2 prior to Fourier transformation. The final matrix size was 512 × 256 × 2048 after zero filling.

The removal of the H6−H5 coupling improves sensitivity by an additional ≈35% for this 5-^{2}H RNA molecule relative to the 5-^{1}H molecule.

Deuteration of pyrimidine C5 alleviates ambiguity in the NOESY spectrum resulting from H6−H5 cross-peak overlap and can enhance the sensitivity of cross-peaks involving H6 by eliminating H6−H5 coupling. The success of experiments such as 3D or 4D HMQC−NOESY−HMQC to correlate C1′ to the C6/H6 resonances is limited by the relaxation properties of the molecule of interest. Deuteration of C5 in large molecules and others that exhibit unfavorable relaxation properties facilitates correlation of the H1′ and C6/H6 resonances. We are presently evaluating methods for obtaining scalar correlations within the pyrimidine base, such as the HNCCCH experiment,[19] that can take advantage of the improved relaxation properties of the deuterium decoupled C5.

Acknowledgment. This work was supported by a grant from the Robert A. Welch Foundation (C-1277) to E.P.N. E.D.J. is supported by the National Institutes of Health predoctoral training Grant GM08280.

Supporting Information Available: Experimental details (1 page/PDF). See any current masthead page for ordering and Web access instructions.

JA973961V

(18) Grzesiek, S.; Kuboniwa, H.; Hinck, A. P.; Bax, A. *J. Am. Chem. Soc.* **1995**, 117, 5312−5315.
(19) Simorre, J. P.; Zimmermann, G. R.; Pardi, A.; Farmer II, B. T.; Mueller, L. *J. Biomol. NMR* **1995**, 4, 427−432.
(20) Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, 15, 8783−8789.
(21) Nikonowicz, E. P.; Sirr, A.; Legault, P.; Jucker, F. M.; Baer, L. M.; Pardi, A. *Nucleic Acids Res.* **1992**, 20, 4507−4513.
(22) Batey, R. T.; Inada, M.; Kujawinski, E.; Puglisi, J. D.; Williamson, J. R. *Nucleic Acids Res.* **1992**, 20, 4515−4523.
(23) Shaka, A. J.; Barker, P. B.; Freeman, R. *J. Magn. Reson.* **1985**, 64, 547−552.
(24) Bax, A.; Ikura, M.; Kay, L. E.; Zhu, G. *J. Magn. Reson.* **1991**, 91, 174−178.