A Three-Dimensional Triple-Resonance $^{1}H,^{13}C,^{31}P$ Experiment: Sequential Through-Bond Correlation of Ribose Protons and Intervening Phosphorus along the RNA Oligonucleotide Backbone

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Sequential through-bond assignment of the ribose protons across the phosphate backbone of oligonucleotides has long been a goal in NMR structural studies of RNA and DNA.[1-3] However, sequential assignment via ^{31}P–^{1}H correlations has been limited in the past due to difficulties in resonance assignment (which is especially true of the poorly dispersed RNA ribose ^{1}H resonances) as well as to small J(H5′,P), and J(H5″,P), and J coupling constants. Recently, the development of efficient methods for the synthesis of uniformly $^{13}C,^{15}N$-labeled RNA[4-6] has provided the ability to apply many of the double- and triple-resonance experimental methods previously employed in NMR studies of labeled proteins[7,8] to the unique problems of NMR of RNA oligonucleotides.[9-13] In this communication, we present a three-dimensional triple-resonance proton–carbon–phosphorus (HCP) experiment that allows the unambiguous correlation of ribose H3′/C3′, H4′/C4′ resonances on the 5′ side and H4′/C4′ and H5′,H5″/C5′ resonances on the 3′ side of the intervening phosphorus along the RNA backbone bonding network. The favorable C,P coupling constants and the dispersion of the chemical shifts that is afforded in the three-dimensional correlation make the HCP experiment a powerful assignment tool for RNA.

The HCP pulse sequence (Figure 1) is analogous to a constant time[14,15] version of the HNCO experiment.[16] Two consecutive ^{1}H–^{13}C and ^{13}C–^{31}P INEPT and reverse INEPT transfer steps[17]

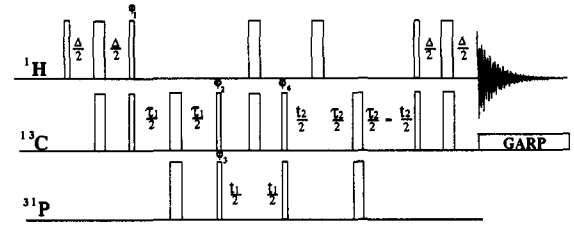

Figure 1. Pulse sequence diagram for 3D HCP experiment. Narrow bars represent $\pi/2$ pulses, and wide bars represent π pulses. The four-step phase cycle is as follows: $\phi_1 = y$, $\phi_2 = y$; $\phi_3 = x, -x$; $\phi_4 = 2(y), 2(-y)$; Acq = $(x, -x, -x, x)$. The delays were $\Delta/2 = 1.5$ ms $(1/4J_{CH}$, where $J_{CH} = 165$ Hz), $\tau_1 = \tau_2 = 26$ ms. A constant time evolution of ^{13}C ($\tau_2 = 1/J_{CC} = 26$ ms) is achieved simultaneously with the reverse INEPT delay. The ^{13}C carrier was centered in the middle of the ribose carbons (~83 ppm), and the ^{31}P carrier was centered in the middle of the phosphorus resonances (–4.10 ppm). GARP decoupling[27] was used to decouple ^{13}C during acquisition. ^{31}P was not decoupled during acquisition. Quadrature in the ω_1 and the ω_2 dimensions were obtained with the TPPI-states method.[28]

are used to transfer magnetization from H3′, H4′, and H5′/H5″ ribose protons to their respective carbons and then to the intervening phosphorus. A constant time evolution of ^{13}C chemical shift is accomplished by the CT delay τ_2 ($\tau_2 = 1/^{1}J_{CC}$), which also serves for the refocusing of the J(C,P) couplings. The correlations observed in the HCP experiment are H3′$_i$–C3′$_i$–P$_i$; H4′$_i$–C4′$_i$–P$_i$; H4′$_{i+1}$–C4′$_{i+1}$–P$_i$; and H5′/H5″$_{i+1}$–C5′$_{i+1}$–P$_i$.[18] The ^{2}J(C3′$_i$,P$_i$) and ^{2}J(C5′$_{i+1}$,P$_i$) coupling constants (^{2}J(C,P) ~ 3–5 Hz, as measured from a PFIDS-CT-HSQC[19] experiment on the RNA stem–loop) and the ^{3}J(C4′$_i$,P$_i$) and ^{3}J(C4′$_{i+1}$,P$_i$) coupling constants (^{3}J(C4′,P) ~ 8–10 Hz, as measured from GMP) allow all these cross peaks to be observed.

The HCP experiment has been applied to the 19mer RNA stem–loop (~1.5 mM), shown schematically in Figure 2A, derived from the RNA I antisense repressor molecule of Col E1 replication control system.[20] A series of expanded H4′/C4′ regions of the $^{1}H,^{13}C$ planes at the ^{31}P chemical shift of the "loop" residues are shown in Figure 2C. The planes are labeled with the sequential assignments that have been made for the H4′$_i$–C4′$_i$–P$_i$ and H4′$_{i+1}$–C4′$_{i+1}$–P$_i$ correlations. The assignment procedure is quite robust against partial overlap of two sequential ^{31}P resonances that correlate with one C4′/H4′ pair, as is the case for the P$_8$–G9(C4′,H4′)–P$_9$ step in Figure 2C. Although the P$_8$ and P$_9$ sequential ^{31}P resonances overlap, the $^{13}C,^{31}P$ planes at the U8 H4′ and G9/G10 H4′ proton resonances (Figure 2D) show that the assignment can be achieved due to the well-resolved resonances of the P$_7$–U8(C4′,H4′)–P$_8$ and P$_9$–G10(C4′,H4′)–P$_{10}$ steps. In addition to the sequential correlation via H4′–C4′–P, the correlations to the H3′ proton in the 5′ direction and H5′ and H5″ protons in the 3′ direction can also be observed in the $^{1}H,^{13}C$ plane of a given ^{31}P resonance. Expansions of the H5′,H5″/C5′ and H3′/C3′ regions of the $^{1}H,^{13}C$ plane at the ^{31}P chemical shift of residue A12 are shown in Figure 2B. In general, these cross peaks together with the carbon-connectivity information obtained from HCCH-TOCSY experiments provide a good internal check to the sequential assignments made using the H4′/C4′ correlations to phosphorus. This is especially true in A-form helical regions that have poorly dispersed C4′ carbon and H4′ proton chemical shifts. In this RNA stem–loop the chemical shift dispersion of the C3′ and C5′ carbons was extremely good and in general much better than that of the C4′ carbons.

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(1) Pardi, A.; Walker, R.; Rappoport, H.; Wider, G.; Wüthrich, K. *J. Am. Chem. Soc.* **1983**, *105*, 1652–1653.
(2) Varani, G.; Cheong, C.; Tinoco, I., Jr. *Biochemistry* **1991**, *30*, 3280–3289.
(3) Sklenar, V.; Miyashiro, H.; Zon, G.; Miles, H. T.; Bax, A. *FEBS* **1986**, *208*, 94–98.
(4) Nickonowicz, E. P.; Sirr, A.; Legault, P.; Jucker, F. M.; Baer, L. M.; Pardi, A. *Nucleic Acids Res.* **1992**, *20*, 4507–4513.
(5) Batey, R. T.; Inada, M.; Kujawinski, E.; Puglisi, J. D.; Williamson, J. R. *Nucleic Acids Res.* **1992**, *20*, 4515–4523.
(6) Michnicka, M. J.; Harper, J. W.; King, G. C. *Biochemistry* **1993**, *32*, 395–400.
(7) Ikura, M.; Kay, L. E.; Bax, A. *Biochemistry* **1990**, *29*, 4659–4667.
(8) Kay, L. E.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1990**, *89*, 496–514.
(9) Nickonowicz, E. P.; Pardi, A. *J. Mol. Biol.* **1993**, *232*, 1141–1156.
(10) Nickonowicz, E. P.; Pardi, A. *Nature* **1992**, *355*, 184–186.
(11) Sklenar, V.; Peterson, R. D.; Rejante, M.; Feigon, J. *J. Biomol. NMR* **1993**, *3*, 721–727.
(12) Sklenar, V.; Peterson, R. D.; Rejante, M. R.; Wang, E.; Feigon, J. *J. Am. Chem. Soc.* **1993**, *115*, 12181–12182.
(13) Farmer, B. T., II; Müller, L.; Nikonowicz, E. P.; Pardi, A. *J. Am. Chem. Soc.* **1993**, *115*, 11040–11041.
(14) Santoro, J.; King, G. *J. Magn. Reson.* **1992**, *97*, 202–207.
(15) Vuister, G.; Bax, A. *J. Magn. Reson.* **1992**, *98*, 428–435.
(16) Kay, L. E.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1990**, *89*, 496–514.
(17) Morris, G. A.; Freeman, R. *J. Am. Chem. Soc.* **1979**, *101*, 760–762.
(18) Note that the ^{31}P resonances are numbered by the preceding 5′ nucleotide to which they are bound, which is consistent with the convention of previous nucleic acid NMR studies, but which does not conform to IUPAC standards.
(19) Schwalbe, H.; Samstag, W.; Engels, J. W.; Bermel, W.; Griesinger, C. *J. Biomol. NMR* **1993**, *3*, 479–486.
(20) Eguchi, Y.; Itoh, T.; Tomizawa, J. *Annu. Rev. Biochem.* **1991**, *60*, 631–652.

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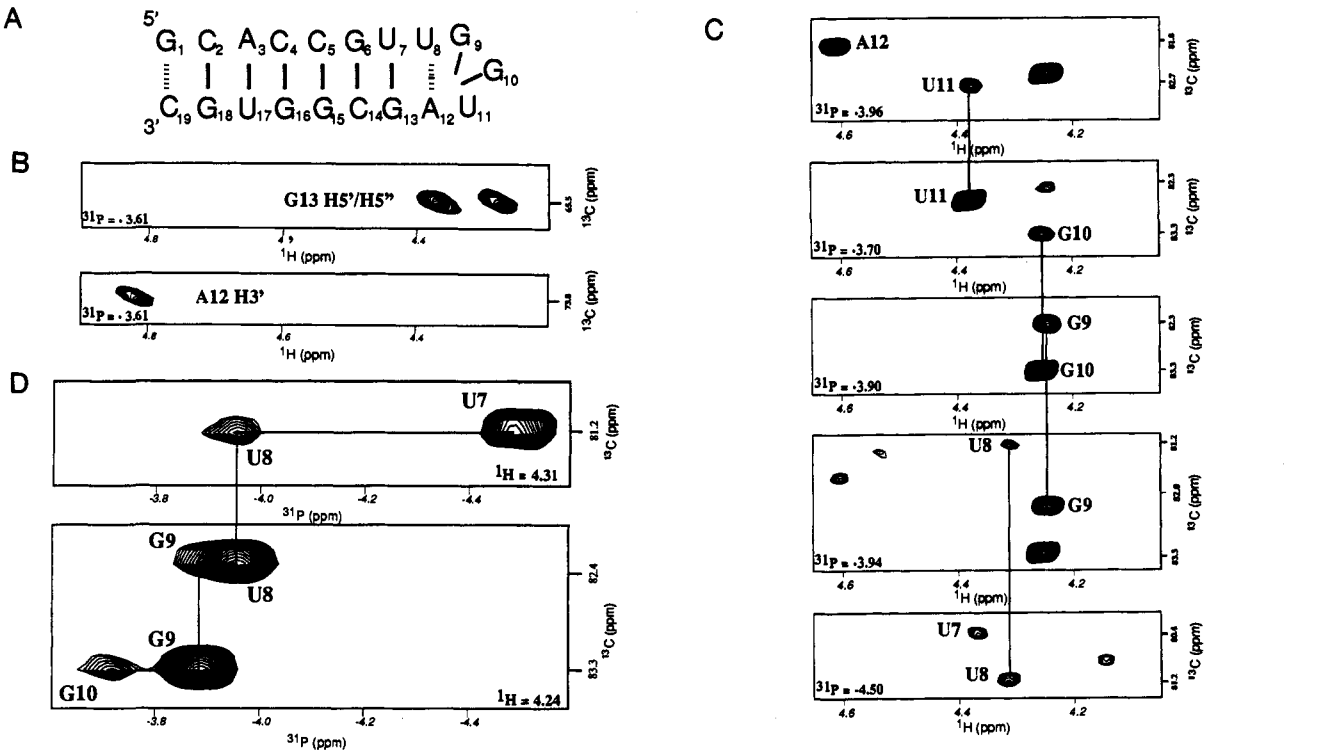

Figure 2. (A) Sequence and secondary structure model of the RNA stem–loop used in this study. The RNA oligonucleotide was enzymatically synthesized using T7 polymerase run off transcription.[29] The ^{13}C,^{15}N-labeled NTPs were prepared from RNA isolated from *Methylophilus methylotrophus* grown in minimal media with [^{13}C]methanol and [^{15}N]NH_4Cl as the sole carbon and nitrogen sources.[5] Solid lines indicate bases for which stable imino protons have been observed. Dashed lines between bases indicate base pairs for which stacking has been established, but for which no stable imino proton is observed. (B) Expansion of the H3'/C3' and H5',H5"/C5' regions of the ^1H,^{13}C plane at the ^{31}P chemical shift of residue A12 in the 3D HCP portion of a pseudo 4D simultaneous HCP,HCN experiment collected on a Bruker four-channel AMX600 spectrometer equipped with a quadruple resonance probe at 25 °C. The experiment was collected with 512, 93, and 34 complex points in t_3, t_2, and t_1, respectively, four scans per t_1, t_2 increment and spectral widths of 6000, 4000, and 400 Hz for each respective dimension. t_{1max} corresponds to $2*T_2$ (^{31}P). Total experiment time = 18 h. The spectra were processed with a 60° shifted sine bell in t_3 (using 256 points), t_2, and t_1 and was zero-filled to a final matrix size of 1024 × 256 × 64. A first-order linear phase correction[30] was applied to the t_2 dimension to unfold the C5' resonances. The observed correlations to the A12 H3' ribose proton in the ribose 3' of the intervening phosphorus and the G13 H5' and H5" protons in the ribose 5' of the phosphorus are indicated. (C) An expansion of the C4'/H4' regions of the ^1H,^{13}C planes at the ^{31}P resonances of the "loop" residues U7 through A12 of the 3D HCP experiment. Each ^1H,^{13}C plane shows two correlations in the H4'/C4' region to the H4' protons of the ribose on both the 5' and 3' sides of the intervening phosphorus. Sequential assignment across the loop region of the stem–loop is shown by the solid lines connecting the labeled H4'/C4' cross peaks from one ^{31}P plane to the next. The additional unlabeled peaks observed in the selected H4'/C4' regions are due to overlap in the ^{31}P dimension. (D) An expansion of the P/C4' regions of the ^{13}C,^{31}P planes at the H4' resonance of the "loop" residues U8 and G9/G10 of the 3D HCP experiment. Note that the ^1H chemical shift of the H4' resonances of G9 and G10 are overlapped so that only one ^1H plane at 4.24 ppm is shown which contains both G9 and G10 C4'–P correlations. Unambiguous sequential ^{13}C–^{31}P correlations are shown with solid lines, and cross peaks are labeled by their ^{31}P assignment.

Using the HCP experiment, together with previous assignments of the ribose spin systems using HCCH experiments[21] and glycosidic anomeric–aromatic correlations by HCN experiments,[11,22] we have been able to connect individually assigned aromatic–ribose spin systems and make a complete through-bond assignment of the backbone protons of this RNA stem–loop. Assignments were confirmed by conventional sequential resonance assignment techniques[23] in helical regions as well as by a ^1H–^{31}P hetero-TOCSY-NOESY experiment.[24]

Although the HCP experiment is quite dependent on the ^{13}C and ^{31}P spectral resolution, it is interesting to note that the most distinctly resolved C5', C4', and C3' carbons[25] and ^{31}P resonances[26] are those in non-helical RNA structure elements which are precisely the areas where there is usually a breakdown in conventional NOE assignment schemes. Thus, the HCP connectivities should complement other conventional assignment methods. The sensitivity of the experiment will depend mostly on the efficiency of the C,P transfer steps and of course will decrease for larger molecules. For RNA molecules with insufficient ^{31}P or ^{13}C resolution, the problem of spectral overlap can be reduced by HCP-C,C-TOCSY experiments which add a C,C-TOCSY transfer step to the HCP experiment.

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(21) Pardi, A.; Nickonowicz, E. P. *J. Am. Chem. Soc.* **1992**, *114*, 9202–9203.
(22) Farmer, B. T., II; Müller, L.; Nikonowicz, E. P.; Pardi, A. *J. Biomol. NMR* **1994**, *4*, 129–133.
(23) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
(24) Kellogg, G. W.; Szewczak, A. A.; Moore, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 2727–2728.
(25) Varani, G.; Tinoco, I., Jr. *J. Am. Chem. Soc.* **1991**, *113*, 9349–9354.
(26) Legault, P.; Pardi, A. *J. Magn. Reson., Ser. B* **1994**, *103*, 82–86.
(27) Shaka, A. J.; Barker, P.; Freeman, R. *J. Magn. Reson.* **1985**, *64*, 547–552.
(28) Marion, D.; Ikura, R.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393.
(29) Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783–8798.
(30) Bax, A.; Ikura, M.; Kay, L. E.; Zhu, G. *J. Magn. Reson.* **1991**, *91*, 174–178.