^{1}H−^{31}P CPMG-Correlated Experiments for the Assignment of Nucleic Acids

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Phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectroscopy can provide important information about the structure and dynamics of nucleic acids in solution.[1−4] Phosphorus-associated heteronuclear scalar[5] and residual dipolar couplings,[6,7] phosphorus chemical shifts[8] and chemical shift anisotropy (CSA),[9] and cross-correlated relaxation involving phosphorus-CSA[10] can all be used to extract restraints that define the phosphodiester backbone conformation and dynamics. The effective application of ^{31}P NMR, however, requires sensitive methods to first obtain sequence-specific assignment of individual phosphorus resonances. To date, in the absence of ^{13}C-labeling, ^{1}H−^{31}P correlation and phosphorus assignment in nucleic acids has been most effectively accomplished using phosphorus-excited heteronuclear correlation spectroscopy (heteroCOSY)[11] and heteronuclear total correlation spectroscopy (heteroTOCSY)[12,13] experiments. Although inversely detected methods, like heteronuclear single quantum correlation spectroscopy (HSQC), could theoretically yield proton−phosphorus correlations with an enhanced sensitivity, proportional to the gyromagnetic ratios of the proton and phosphorus nuclei ($\gamma_H/\gamma_P \approx 2.5$), previous applications of such methods have not realized such uniform sensitivity gains.[11,14−16] The reason for this is primarily two-fold: first, multiple homonuclear proton−proton couplings, which are of the same magnitude as the heteronuclear ^{1}H−^{31}P couplings of interest, induce complex phase twists in the line shape; second, significantly broad phosphorus resonance line widths can cause losses due to nonideal refocusing.

In this communication, a ^{1}H−^{31}P heteronuclear single quantum correlation (HSQC) experiment (Figure 1A), with an enhanced sensitivity of ∼2.5-fold with respect to phosphorus excited methods, is described for the assignment of phosphorus resonances in nucleic acids. The effective application of HSQC correlation of phosphorus and protons is achieved by the application of a train of closely spaced 180° pulses, a so-called Carr−Purcell−Meiboom−Gill (CPMG)[17] pulse train, during the periods of magnetization transfer between phosphorus and scalar coupled proton nuclei. It is well-known that CPMG-pulse trains can be used to preserve spin coherence in the presence of conformational exchange[18,19] and cross-correlated relaxation[20] so that an optimized refocusing behavior can be achieved. In the present application, the CPMG pulse train provides for optimal refocusing between

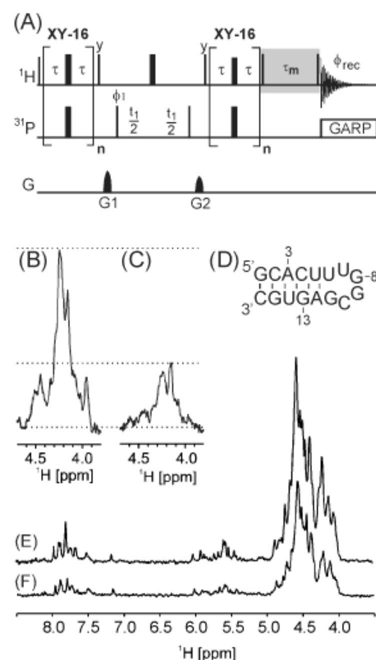

Figure 1. (A) Pulse sequence scheme for the 2D HP-CPMG−HSQC experiment. During proton−phosphorus polarization transfer intervals synchronous proton/phosphorus 180° refocusing pulses with surrounding delays of $\tau \approx 100$ μs expanded in n (XY-16) supercycles[23] are applied. Narrow and wide vertical lines indicate 90° and 180° flip angle pulses, respectively. All pulses are applied along x unless otherwise indicated. Phase cycle: $\phi_1 = x, -x$; $\phi_{rec} = (x, -x)$. Quadrature detection is obtained in ω_1 by incrementing ϕ_1, according to States-TPPI. All experiments were collected using a Bruker DMX500 spectrometer equipped with a triple resonance HCP probe head. For the HP-CPMG−HSQC−NOESY experiment a homonuclear NOESY step (τ_m = 500 ms) was added [shaded box]. 1D spectra of the HP-CPMG−HSQC (B), HP-heteroTOCSY (C), HP-CPMG−HSQC−NOESY (E), and HP-heteroTOCSY−NOESY (F) experiments applied to a 2.5 mM sample of a 16mer CopT16 RNA hairpin (D) in 1 mM cacodylate [pH = 6.5], 25 mM NaCl and 99.96% D_2O are shown. Spectra in (B) and (E) were acquired with 8 (B) and 12 (E) XY-16 supercycles with τ = 100 μs, a 180° ^{31}P pulse of 38 μs and 180° ^{1}H pulse of 20 μs, resulting in a delay of 30.5 ms (B) and 45.7 ms (F) for each CPMG−INEPT period. For the spectra in (C) and (F) planar mixing was achieved with 86.4 ms simultaneous irradiation of the DIPSI-2 multiple pulse sequence with a rf-field of 1 kHz on ^{1}H and ^{31}P, respectively. In all experiments, the proton carrier frequency was set to 4.5 ppm, and the phosphorus carrier frequency was set to 0.5 ppm, corresponding to the center of the ^{1}H ribose and phosphorus spectral ranges, respectively. The comparison of the 32 scan experiments (B) and (C) shows the expected increase in signal-to-noise of about 2.5-fold. With a 500 ms NOESY period added, the average increase in signal-to-noise is still about 2-fold [(E) and (F), 256 scans each].

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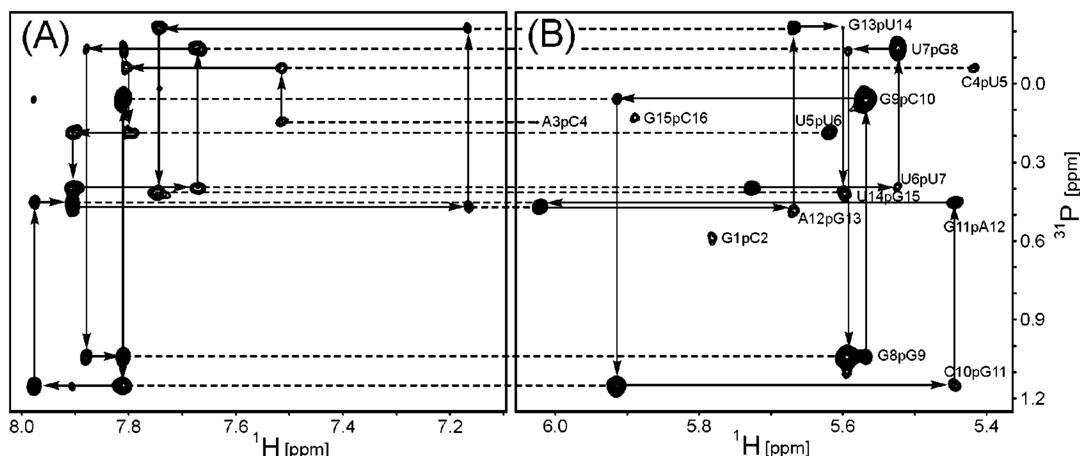

Figure 2. The phosphorus to H8/H6 (A) and phosphorus to H1′ (B) regions of the HP-CPMG−HSQC−NOESY applied to the 16mer RNA shown in Figure 1D. Sequential assignment "walks" are indicated by arrows for residues U6 through G11 in the phosphorus-H1′ region and for residues A3 through G15 in the phosphorus-H6/H8 region of the experiment, while dashed lines are used to annotate the phosphorus frequencies. The spectrum was acquired with the parameters as given for the 1D HP-CPMG−HSQC−NOESY shown in Figure 1 with 2K complex points in the ^{1}H t_2 (acq = 205 ms) and 40 complex points in ^{31}P t_1 (t_{1max} = 263 ms) dimensions. Total experiment time = 15 h.

nuclei correlated along the phosphodiester backbone, which may experience significant conformational exchange.[2,21,22] In addition, the 180° pulse train in a XY-16 expansion scheme[23] results in homonuclear isotropic mixing conditions,[24] leading to in-phase transfer in the ^{1}H−^{1}H-coupling network so that phase twists due to evolution of ^{1}H−^{1}H anti-phase magnetization is avoided. In DNA, HSQC correlation of H3′ protons and phosphorus could previously be obtained through application of selective H3′ proton pulses since these protons resonate in a resolved chemical shift region of the proton spectrum.[9] However, for RNA in general and H5′ correlations in DNA, such an approach is not possible due to complete overlap of the proton chemical shifts. The HP-CPMG−HSQC experiment presented here overcomes the main problems associated with standard HP-HSQC correlation and uses only nonselective pulses. It therefore provides a general method for the detection of proton-phosphorus correlations in nucleic acids with a sensitivity that approaches the theoretical 2.5-fold enhancement over methods that utilize phosphorus excitation.

The HP-CPMG-HSQC experiment is demonstrated using an unlabeled 16mer RNA hairpin, CopT16 (Figure 1D). Figure 1, B and C, shows for comparison the 1D spectra obtained using the HP-CPMG−HSQC pulse sequence and the previously published heteroTOCSY pulse sequence,[12,13] respectively, with comparable acquisition conditions. From these 1D proton spectra, the expected sensitivity gain of approximately 2.5 for the HP-CPMG−HSQC experiment is clearly evident. The HP-CPMG−HSQC pulse sequence element can be combined with an additional relayed magnetization transfer step to correlate the phosphorus nuclei with additional ribose and aromatic base protons that are more well-resolved than H5′ and H3′ protons directly coupled to phosphorus. In this regard, the application of ^{31}P−^{1}H transfer in combination with an NOE mixing period has proven to be an effective method for resolving sequence specific assignment of phosphorus in unlabeled nucleic acids.[12,13] Applied in combination with a nuclear Overhauser effect (NOE) mixing period (HP-CPMG−HSQC−NOESY), the HP-CPMG−HSQC pulse sequence element provides a similar improvement in sensitivity as observed for the HP-CPMG−HSQC correlation. Figure 1, E and F, shows for comparison 1D spectra for the CopT16 hairpin obtained using comparable acquisition conditions with the HP-CPMG−HSQC−NOESY pulse sequence and the previously published phosphorus-excited heteroTOCSY−NOESY[12,13] pulse sequence, respectively. Again, an overall increase in sensitivity of approximately 2-fold is observed for the HP-CPMG−HSQC−NOESY with respect to the phosphorus-excited experiment.

Figure 2 shows expansions of the ^{1}H aromatic (Figure 2A) and anomeric regions (Figure 2B) of a 2D version of the HP-CPMG−HSQC−NOESY experiment. Phosphorus correlations are labeled by dinucleotide steps in the 5′ to 3′ direction of the RNA sequence (i.e., G1pC2). In this experiment, each intervening phosphorus nucleus correlates with two aromatic base proton resonances, as is observed in the heteroTOCSY−NOESY experiment. Due to the increased signal-to-noise ratio and different transfer properties of the HP-CPMG−HSQC sequence, with respect to heteroTOCSY, correlations to H1′ protons in both the 5′ and 3′ direction are also observed in the HP-CPMG−HSQC−NOESY experiment, resulting in a second phosphorus driven pathway for sequential assignment of RNA oligonucleotides. To demonstrate the sequential assignment pathways, cross-peak connectivities are traced in Figure 2 using arrows.

In summary, methods for proton−phosphorus correlation in nucleic acids are presented with significantly increased sensitivity and which provide a new phosphorus-driven sequential assignment pathway. Although demonstrated on a relatively small RNA, these methods should achieve similar sensitivity gains when applied to larger RNAs since efficacy is derived from ^{1}H excitation and attenuation of homonuclear proton couplings and conformational exchange during magnetization transfer, which are factors independent of molecular weight.

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