Sequential Backbone Assignment in ^{13}C-Labeled RNA via Through-Bond Coherence Transfer Using Three-Dimensional Triple Resonance Spectroscopy (^1H, ^{13}C, ^{31}P) and Two-Dimensional Hetero TOCSY

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The basis for solution structure studies of nucleic acids by NMR[1] is formed by sequential ^1H resonance assignments, which until now have been derived from through-space contacts (NOEs).[2-9] NOE-based assignments have, however, the disadvantage of conformational dependence; e.g., in DNA and RNA hairpins the sequential NOE contacts are disrupted in the loop region.[10-12] The method we propose circumvents this problem since sequential assignment is achieved via through-bond coherence transfer using a combination of two new types of NMR experiments, namely, a triple-resonance experiment (HCP) which correlates ^1H, ^{13}C, and ^{31}P (Figure 1A) and a hetero TOCSY experiment (CCHTOCSY) (Figure 1B). The details of the pulse sequences will be discussed elsewhere; here we demonstrate the assignment procedure on a 1.5 mM sample of a uniformly ^{13}C enriched RNA hairpin, with the sequence 5′(GGGC-CAAA-GCCU)3′, dissolved in D_2O.

The HCP sequence closely resembles the CT-HNCO experiment.[13] It correlates ^1H, ^{13}C, and ^{31}P via through-bond coherence transfer, i.e., via the J-couplings J_{CH} (≈145 Hz) and J_{CP} (with $J_{C4'P5'}$, $J_{C4'P3'} \approx$ 5–11 Hz; $J_{C3'P3'}$, $J_{C5'P5'} \approx$ 6 Hz). The HCP spectrum will therefore show in the $\{^1H,^{31}P\}$ plane at the C4′ frequency of residue i ($C4'_i$ plane) cross peaks at two ^{31}P frequencies, at ($H4'_i$, P_i) (involving P5′ of residue i), and at ($H4'_i$, P_{i+1}) (involving P3′ of residue i), denoted as the P5′ cross peak and the P3′ cross peak, respectively. The $C5'_i$ plane will show cross peaks only at the P5′ resonance position, at ($H5'_i$, P_i (=P5′)), and at ($H5''_i$, P_i (=P5′)). Thus, the $C5'_i$ plane allows one to establish the 5′ → 3′ direction in the $C4'_i$ plane, which is particularly useful when a break in the connectivity pathway would occur. To establish that $C5'_i$ and $C4'_i$ belong to the same ribose residue, we use a CCHTOCSY spectrum (data available as supplementary material). The CCHTOCSY sequence (Figure 1B) efficiently transfers coherence through the system of J-coupled ribose ^{13}C and ^1H spins on account of the large J-couplings involved ($J_{CH} \approx$ 145 Hz, $J_{CC} \approx$ 40 Hz), so that at the ^1Hx_i (x = 1′–5′, 5″) frequency a cross peak appears not only at the frequency of the directly bonded ^{13}C but also at the frequencies of the other ^{13}C nuclei of the ribose ring. Since of all the sugar resonances the H1′ overlap least, we use the TOCSY ladders at the H1′ frequency to unequivocally establish the $C4'_i$ to $C5'_i$ connection. The sequential step in the assignment is subsequently done by finding in the HCP spectrum the $C4'_{i+1}$ plane, i.e., a C4′ plane with a P5′ cross peak at the ^{31}P resonance position of the P3′ cross peak of residue i. Consider as an example the sequential assignment for residues G1 and G2 (Figure 2). The $C4'_{G1}$ plane (Figure 2, first left top panel) shows two cross peaks at the $H4'_{G1}$ resonance frequency. The cross peak connected with the upfield ^{31}P resonance is the P5′ cross peak as follows from the $C5'_{G1}$ plane (Figure 2, second left top panel). The P3′ cross peak is connected with the downfield-shifted ^{31}P resonance. It provides

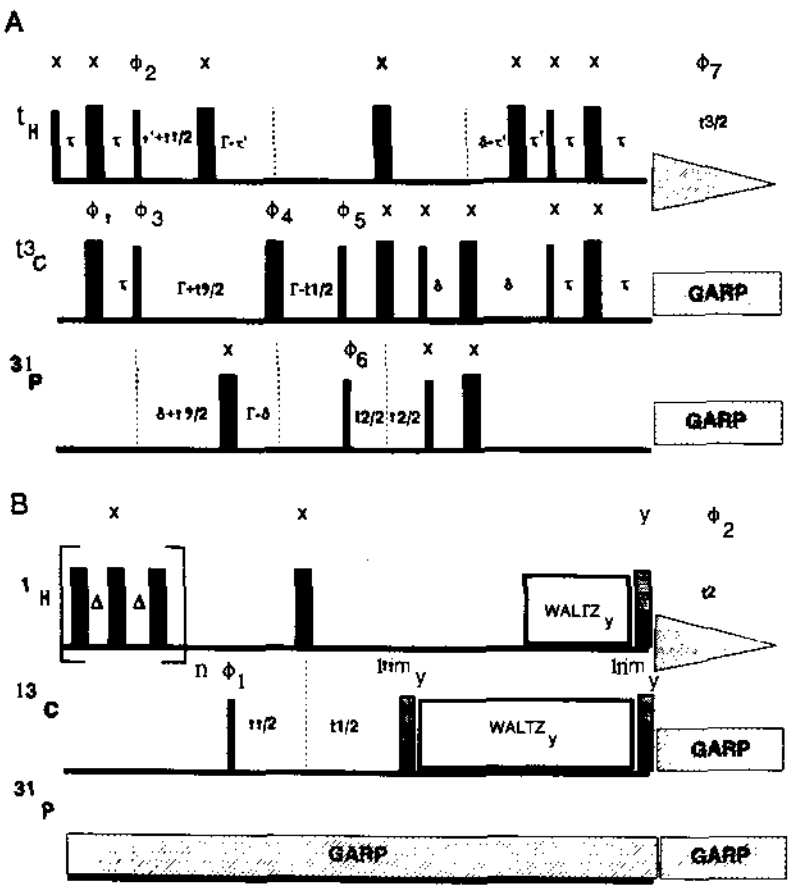

Figure 1. Pulse sequences of (A) the HCP experiment and (B) the CCHTOCSY experiment. The thick and thin bars indicate 180° and 90° pulses, respectively. (A) Constant time ^{13}C evolution applied;[13,14] phase cycling was as in the CT-HNCO:[13] $\phi_1 = x, -x$; $\phi_2 = y, -y$; $\phi_3 = x$(+TPPI[15] (t1)); $\phi_4 = 4x, 4y$; $\phi_5 = x, -x$; $\phi_6 = x, x, -x, -x$ (+TPPI (t2)); $\phi_7 = x, x, -x, -x, -x, -x, x, x$. (B) Phase cycling according to the following: $\phi_1 = x, -x$ (+TPPI (t1)); $\phi_2 = x, -x$. The experiments were performed on a Bruker AM400, equipped with a BB-^{13}C/^1H probe, and modified with home-built electronics for triple resonance and cross-polarization experiments. The HCP spectrum was recorded in approximately 20 h with the following acquisition settings: 16 scans for each FID of 1024 data points (t_3); 128 t_1 values ($t_1^{max}/2 = T = \delta$ = 12.5 ms); 28 t_2 values; $\tau = \tau' = 1.5$ ms (≈$1/(4J_{CH})$); $T - \delta = 5$ μs; 1-s relaxation delay with solvent presaturation; low-power (3 W) GARP decoupling[16] of ^{31}P and ^{13}C; spectral width, 2941, 400, and 2941 Hz; and carrier position 73.6, −2.23, and 4.75 ppm for ^{13}C, ^{31}P, and ^1H, respectively. Typical processing parameters: zero-filling twice in t_1, t_2 (preceded by zero-padding to 32 points), and t_3; applying a $\sin^2$ window function shifted by $\pi/5$, $\pi/3$, and $\pi/2$ in t_1, t_2, and t_3, respectively.

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(1) Abbreviations used: NMR, nulcear magnetic resonance; NOE, nuclear Overhauser enhancement; TOCSY, total correlation spectroscopy; TPPI, time proportional phase incrementation; GARP and WALTZ, decoupling sequences; TSP, (trimethylsilyl)propionic-d_4 acid.

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The 0 ppm value chemical shift calibration relative to TSP is obtained by multiplying the ^1H TSP frequency by 0.251 449 54 for ^{13}C and by 0.404 807 93 for ^{31}P. The latter corresponds to a calibration relative to inorganic phosphate.

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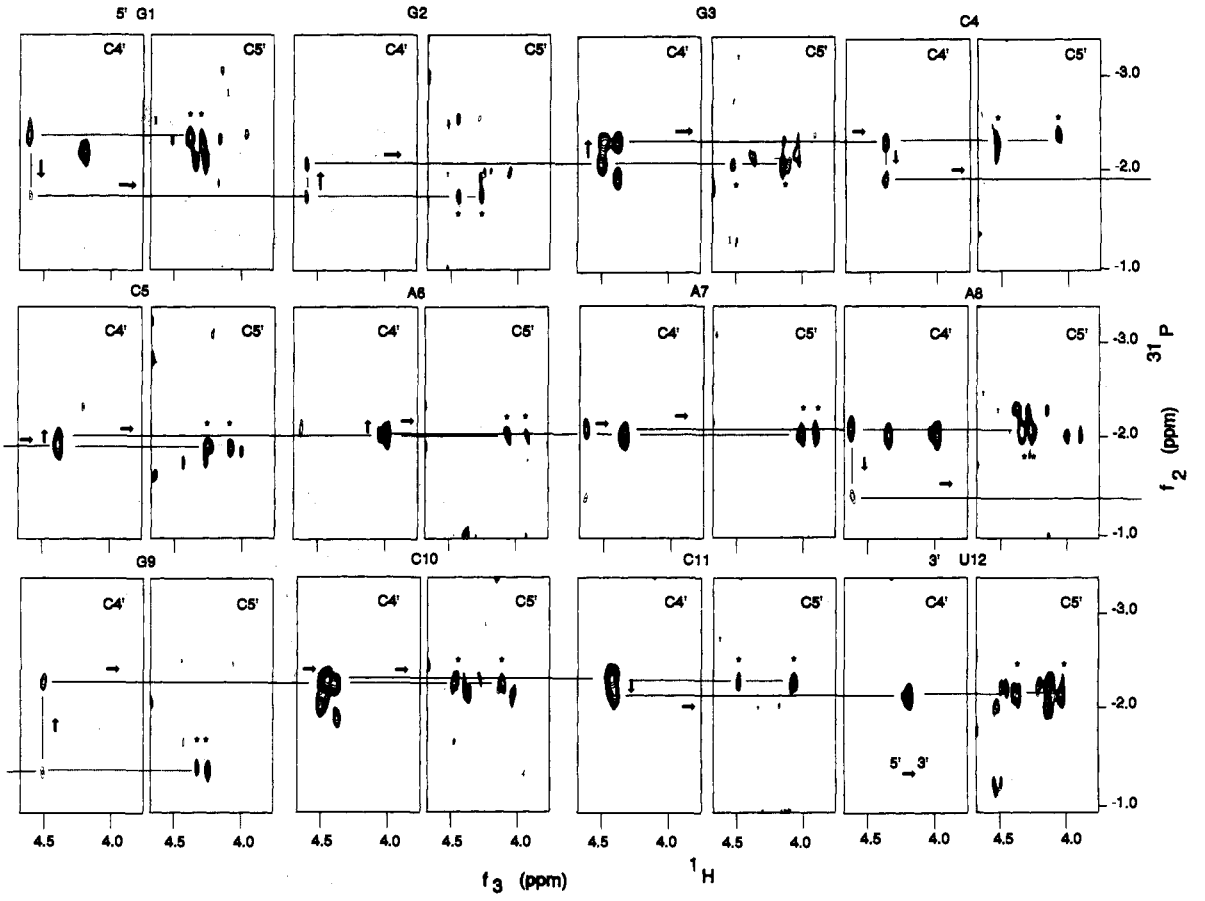

Figure 2. Sequentially arranged C4′ and C5′ planes (see text) from the HCP spectrum. The C4′ planes show the sequential walk through the sugar–phosphate backbone from the first residue G1 (at the 5′-end) to the final residue U12 (at the 3′-end) as indicated by the arrows and the drawn lines; the two cross peaks marked by stars in the C5′$_i$ planes indicate the H5′/5″ resonance positions of residue i; the dotted line connects these H5′/5″ cross peaks with the P5′ cross peak in the C4′$_i$ plane. In some cases, panels (G3; C4′), (C4; C5′), and (G9; C5′), some slight disalignment is observed, due to the presence of an intense nearby cross peak in the same plane (panel (G3; C4′)) or in a nearby plane (cross peak in panel C10; C5′) affects position of cross peak in panel (C4; C5′)) or due to low-frequency noise (number of t2 increments is only 28) of low-intensity cross peaks (panel G9; C5′). The spectrum was calibrated relative to TSP as described by Ikura et al.[17]

the ^{31}P frequency of the P5′ cross peak of G2, visible in the C4′$_{G2}$ plane (Figure 2, third left top panel). Via this procedure a complete sequential walk can be performed (Figure 2). Note that no break in the sequential route occurs in the loop region (residue C4 to G9), as would be encountered if assignment were based on sequential NOE contacts.

The present method provides, apart from the ^{31}P assignments, also the Hx′ and Cx′ ribose sugar assignments. A NOE-based assignment method would most easily provide the H1′ assignments and subsequently via CCHTOCSY the other Hx′ and Cx′ assignments. The two approaches are therefore complementary. Interestingly, the present HCP spectrum contains an example where in fact the ^{31}P nucleus is required to resolve ambiguity. The C5′ resonances of U12 and C10 at 61.4 ppm overlap nearly completely, preventing assignment of their H5′/5″ resonances by the just described NOE-based approach. This overlap is resolved in the C5′$_{C10,U12}$ plane of the HCP spectrum by the ^{31}P frequency (Figure 2, panel C10).

Two-dimensional ^{1}H–^{31}P through-bond correlations provide in principle also a method for sequential assignment[18] not based on NOEs, but this method has met with limited success due to ^{1}H (and also ^{31}P) resonance overlap. The additional ^{13}C frequency axis in the present three-dimensional triple-resonance HCP experiment provides sufficient additional resolution to allow complete sequential assignment.

We have shown that sequential backbone assignments can be obtained in RNAs based solely on through-bond coherence transfer also in those regions where usually sequential NOEs are absent. Whether this approach is indeed applicable for larger systems remains to be demonstrated. An analysis of the transfer efficiencies of C4′ to P3′/5′ and C5′ to P5′, to be presented elsewhere, shows that for larger RNAs (40–50 nucleotides with T2 relaxation of 30 ms) the smaller of the two transfers, i.e., from C5′ to P5′, has still an efficiency of approximately 20%, sufficiently large to lead to a detectable signal ($0.20^2 = 4\%$). For such larger systems the main problem is expected to be overlap, although we find that the C4′, C5′, and phosphorus resonances tend to be more spread out in nonhelical regions then in helical regions, which may make the present approach particularly useful for assigning the nonhelical regions.

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Supplementary Material Available: The H1′ region of the CCHTOCSY spectrum of the uniformly ^{13}C labeled hairpin 5′-(GGGC-CAAA-GCCU)3′ (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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