Journal of Biomolecular NMR, 5 (1995) 315–320
ESCOM

J-Bio NMR 278

Novel three-dimensional ^{1}H-^{13}C-^{31}P triple resonance experiments for sequential backbone correlations in nucleic acids

Gabriele Varani*, Fareed Aboul-ela, Frederic Allain and Charles C. Gubser

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

Received 11 January 1995
Accepted 24 March 1995

Keywords: ^{1}H-^{13}C-^{31}P correlation; RNA structure determination; Dihedral angle constraints; Spectral assignments

Summary

Backbone-driven assignment methods that utilize covalent connectivities have greatly facilitated spectral assignments of proteins. In nucleic acids, ^{1}H-^{13}C-^{31}P correlations could play a similar role, and several related experiments (HCP) have recently been presented for backbone-driven sequential assignments in RNA. The three-dimensional extension of ^{1}H-^{31}P Het-Cor (P,H-COSY–H,C-HMQC) and Het-TOCSY (P,H-TOCSY–H,C-HMQC) experiments presented here complements HCP experiments as tools for spectral assignments and extraction of dihedral angle constraints. By relying on ^{1}H-^{31}P rather than ^{13}C-^{31}P couplings to generate cross peaks, the strongest connectivities are observed in different spectral regions, increasing the likelihood of resolving spectral overlap. In addition, semiquantitative estimates of ^{1}H-^{31}P and ^{13}C-^{31}P couplings provide dihedral angle constraints for RNA structure determination.

The possibility of using phosphorus correlations for spectral assignments has long been recognized, and succesfully implemented for small oligonucleotides (Varani et al., 1991; Kellogg, 1992; Kellogg et al., 1992; Kellogg and Schweitzer, 1993; Szewczak et al., 1993). Although NOE-based sequential assignment procedures are sufficient to assign regular helical regions (Varani and Tinoco, 1991b; Nikonowicz and Pardi, 1993), these procedures rely on conformational assumptions which are generally not valid in unusual novel structures. These assumptions have led to misassignments in single-stranded regions, and through-bond assignment techniques have allowed correction and verification of assignments based on NOE connections (Varani et al., 1991; Gehring et al., 1993). Through-bond connectivities complement NOE-based procedures in NMR spectral assignments of nucleic acids.

The availability of ^{13}C-labelled RNA has made it possible to use triple resonance ^{1}H-^{31}P-^{13}C experiments to overcome the poor spectral dispersion of both the ^{31}P and ^{1}H dimensions. In the recently introduced HCP experiments (Heus et al., 1994; Marino et al., 1994), magnetization is transferred between neighboring sugar resonances via the intervening phosphorus resonances, thereby sequentially correlating neighboring nucleotides (in the following discussion, each ^{31}P resonance is taken to be part of the nucleotide at its 3' side, in accord with standard IUPAC nomenclature). The cross peaks generated by the C4'(i–1)-P(i) and P(i)-C4'(i) couplings (J ≈ 6–11 Hz) are usually the most intense peaks in HCP spectra, whereas C3'(i–1)-P(i) and P(i)-C5'(i) couplings (J ≈ 5 Hz) generally lead to weaker cross peaks and less reliable spectral identifications at increasing molecular weight. The C4'-H4' region is the least disperse region of the RNA spectrum, and we have found it impossible to obtain spectral assignments for oligonucleotides of 20 or more residues from HCP experiments alone. The scope of HCP experiments has been extended by relaying the ^{31}P-labelled magnetization to other sugar resonances via HCCH-TOCSY (Marino et al., 1995; Wijmenga et al., 1995) or HCCH-COSY steps (Tate et al., 1995). Although these experiments are clearly valuable, our experience with HCCH-TOCSY and HCP-CCH-TOCSY experiments (Marino et al., 1995) suggests that relaxation will severely limit the scope of these experiments at molecular weights above ≈ 10 kDa (30 nucleotides).

One alternative and complement to HCP experiments is utilization of sequential H3'(i–1)-P(i) connectivities (J ≈ 5–15 Hz), that produce especially strong cross peaks in

*To whom correspondence should be addressed.

0925-2738/$ 6.00 + 1.00 © 1995 ESCOM Science Publishers B.V.

two-dimensional ^{31}P-^{1}H Het-Cor (Sklenář et al., 1986) and hetero-TOCSY (Kellogg, 1992) experiments. Although the overlap of two-dimensional ^{31}P-^{1}H Het-Cor and hetero-TOCSY experiments makes it very difficult to use these experiments to supplement the HCP-based information, their extension to three dimensions solves the overlap problems in many cases.

In this communication, we present three-dimensional ^{13}C-edited Het-Cor (P,H-COSY–H,C-HMQC) and hetero-TOCSY (P,H-TOCSY–H,C-HMQC) experiments. These experiments provide information that is generally complementary to the HCP experiment. Although sequential connectivities are difficult to establish because of the generally small intraresidue P(i)-H4'(i) and P(i)-H5'/H5"(i) couplings, these experiments ideally complement the HCP experiments by relying on different coupling constants for magnetization transfer to generate intense cross peaks in different regions of the RNA spectrum. Using a combination of three-dimensional HCP and P,H-COSY–H,C-HMQC experiments, we have completely assigned several RNA oligonucleotides of up to 30 residues and obtained at the same time semiquantitative estimates of scalar coupling constants that are valuable in defining the RNA backbone conformation.

The 3D ^{13}C-edited Het-Cor and Het-TOCSY experiments are shown in the upper and lower parts of Fig. 1, respectively. We have chosen Het-Cor and Het-TOCSY ^{31}P-^{1}H transfer based on the performance of 2D Het-COR and Het-TOCSY which is superior over ^{31}P-^{1}H HSQC experiments at molecular weights of 7 kDa or more (G. Varani et al., unpublished results). In Fig. 1, the ^{1}H-^{31}P cross peaks observed in two-dimensional Het-Cor or Het-TOCSY experiments are edited by an additional ^{13}C dimension via a ^{1}H-^{13}C HMQC period. In two-dimensional experiments acquired on a 500 MHz spectrometer equipped with an inverse probe, Het-TOCSY experiments generally outperformed Het-COR experiments. However, the quality of the three-dimensional P,H-TOCSY–H,C-HMQC was inferior to that of the corresponding P,H-COSY–H,C-HMQC spectrum. We have no explanation for this unexpected result.

The experiments presented in this communication were conducted on a 30-mer oligonucleotide (Fig. 2), isotopically ^{13}C-^{15}N-labelled at a level of >98%. Comparable results were obtained for two other RNA oligonucleotides of similar size and for their complexes with small ligands, but sensitivity was clearly diminished when the molecular weight approached 15 kDa in an RNA–polypeptide complex (F. Aboul-ela and G. Varani, manuscript in preparation). This limitation may partially be overcome by performing these experiments on lower field spectrometers (300 or 400 MHz) to reduce the chemical shift anisotropy contribution to ^{31}P relaxation. However, we have found that the superior resolution of 600 MHz spectra (compared to 300 MHz) more than compensated for the losses due to faster ^{31}P relaxation for oligonucleotides of 10 kDa or less.

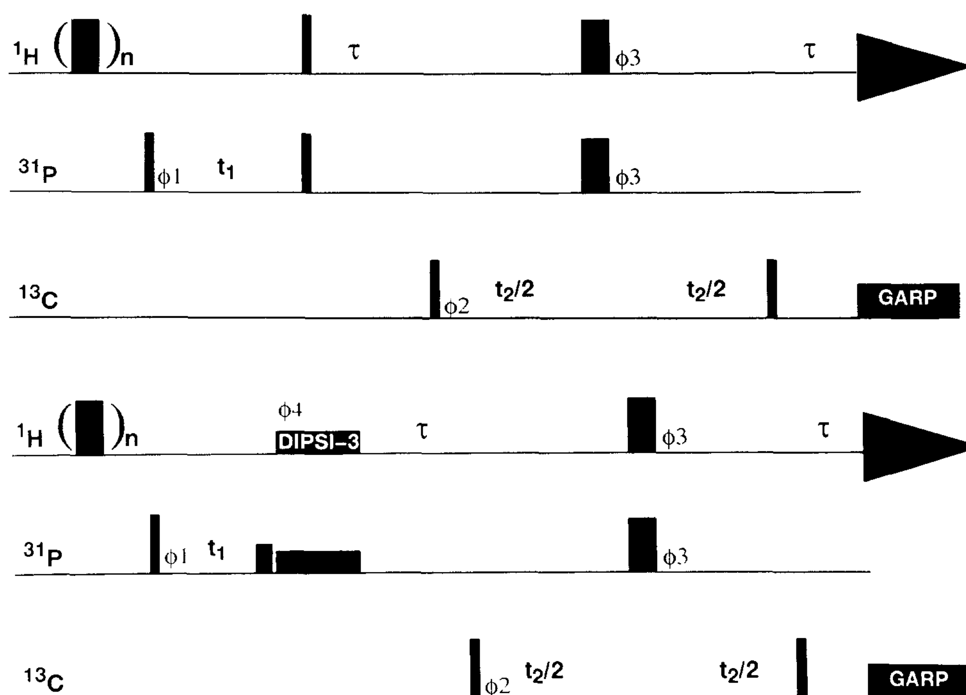

Fig. 1. Three-dimensional P,H-COSY–H,C-HMQC (top) and P,H-TOCSY–H,C-HMQC (bottom) experiments. Thin bars represent 90° pulses; thick bars represent 180° pulses. Heteronuclear cross-polarization was applied using the DIPSI-3 mixing sequence at reduced power (τ_{90} = 42 μs) and was preceded by a 1.5 ms trim pulse applied to ^{31}P; τ = 3 ms. ^{13}C decoupling during acquisition was done with low-power GARP decoupling (Shaka et al., 1985). A train of 25–30 180° pulses (separated by 45 ms delays) was used to saturate the ^{1}H magnetization during the relaxation delay. Phases are along the x-axis unless indicated otherwise; ϕ_1 = x,x,–x,–x; ϕ_2 = x,–x; ϕ_3 = x,x,x,x,–x,–x,–x,–x; ϕ_4 = x,–x; Rec. = +,–,–,+.

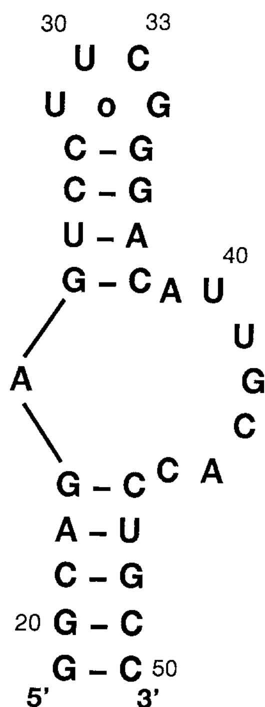

Fig. 2. Sequence and secondary structure of the 30-mer RNA oligonucleotide used in the experiments presented in this communication. This RNA oligonucleotide represents the binding site for the human U1A protein within the 3'-untranslated region of the U1A pre-mRNA; this interaction is involved in the regulation of polyadenylation of the U1A pre-mRNA (Van Gelder et al., 1993). Isotopically labelled RNA was prepared by standard methods using biosynthetically prepared nucleotide triphosphates and the T7-RNA polymerase in vitro transcription system.

The results are illustrated in Fig. 3, where a two-dimensional Het-COR experiment is compared with several planes from the P,H-COSY–H,C-HMQC spectrum. A direct comparison of the 3D Het-Cor and HCP experiments is shown in Fig. 4. A considerable degree of spectral overlap is seen for the ^{31}P resonances in the two-dimensional spectrum (Fig. 3a), particularly those corresponding to the double helical regions of the structure. Even for the well-isolated resonances from single-stranded regions of this oligonucleotide, it would be imprudent to assume that the strongest peaks always correspond to sequential H3'(i–1)-P(i) connectivities. If the β (P-O5'-C5'-C4') torsion angle is not *trans* (as normally observed in regular double helices), but in either of the *gauche* conformers, the intranucleotide P(i)-H5'(i) or P(i)-H5"(i) scalar couplings are as large as the sequential H3'(i–1)-P(i) couplings (Altona, 1982). The ^{13}C sugar chemical shifts (Varani and Tinoco, 1991a) easily allow to distinguish C3' from C5' resonances. The planes of the P,H-COSY–H,C-HMQC experiments illustrate the editing power of the additional ^{13}C dimension (Figs. 3b–d). We found it much easier to analyze spectra that were phased in the directly detected dimension (ω_3), with an additional 90° correction to 'hide' the antiphase character with respect to the H-P coupling.

Restraining the backbone conformation by means of scalar coupling constant measurements improves the accuracy and precision of NMR structures of RNA

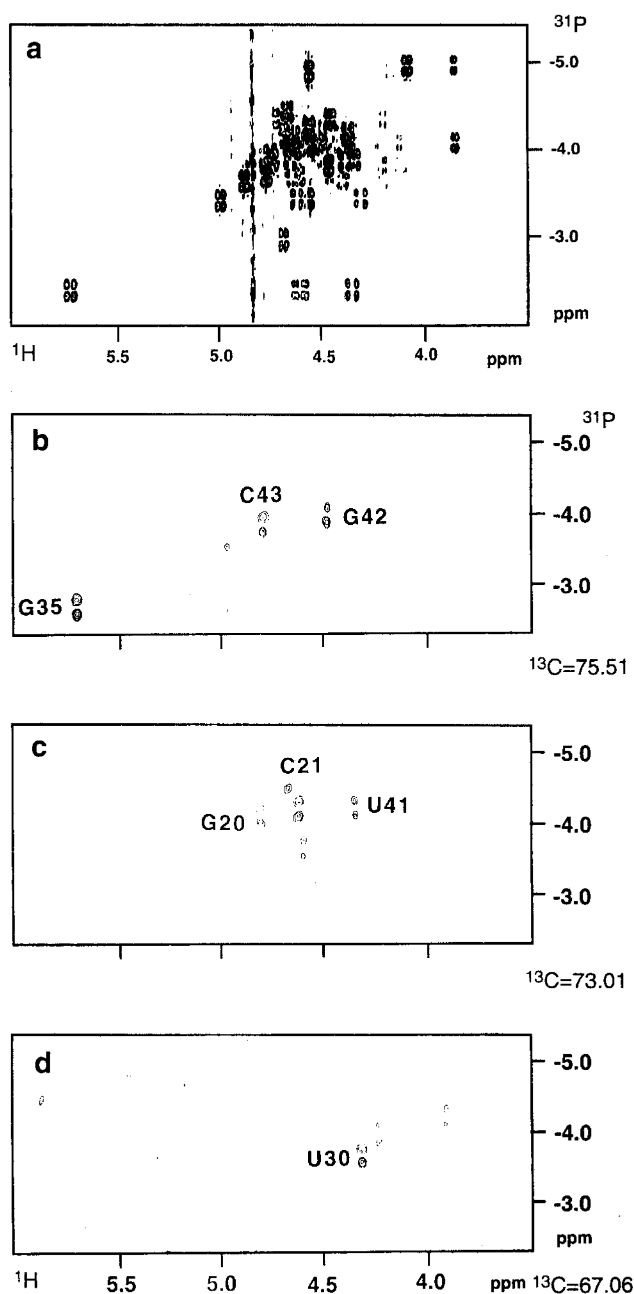

Fig. 3. (a) Two-dimensional ^{1}H-^{31}P Het-Cor spectrum (Sklenář et al., 1986) of the oligonucleotide of Fig. 2, acquired in ≈ 22 h at 500 MHz on a 2 mM RNA sample isotopically ^{13}C-^{15}N enriched (> 98%). (b–d) ^{13}C slices through the three-dimensional Het-Cor spectrum corresponding to C3' frequencies (b,c) or C5' frequencies (d). Assignments are indicated for several cross peaks. This 3D spectrum and the HCP experiment shown in Fig. 4 were acquired in ≈ 22 h each on a 600 MHz Bruker DMX spectrometer equipped with a triple resonance gradient ^{1}H-^{13}C-X probe. Sixty-four transients of 256 complex points were acquired in each of the indirectly detected dimensions, with spectral widths of 3004 Hz (^{1}H, t_3^{max} = 85 ms), 4000 Hz (^{13}C, t_2^{max} = 8 ms) and 2000 Hz (^{31}P, t_1^{max} = 16 ms). The data were apodized with 40°–60° shifted sine bells and zero-filled to a final size of 64 (^{31}P) × 128 (^{13}C) × 512 (^{1}H).

(Varani et al., 1991; Wimberly, 1992). Several experimental strategies have been proposed for measuring homo- and heteronuclear couplings in RNA oligonucleotides (Schmieder et al., 1992; Hines et al., 1993,1994; Schwalbe et al., 1994), including C-P and H-P couplings (Schwalbe et al., 1993). However, it is far from clear how effective those strategies are for molecules larger than 10–15 nucleotides. In addition to providing a means of assigning the RNA spectrum, the combination of HCP and HetCOR 3D experiments provides the opportunity to constrain the nucleic acid backbone conformation.

To a first approximation, cross-peak intensities in correlated spectra depend on the magnitude of the active coupling. Of course other factors (nuclear relaxation and the pattern of passive couplings) contribute to the cross-peak intensity, and it would be naive to quantitate precisely the magnitude of scalar coupling constants from cross-peak intensities. Similar problems are encountered when relating cross-peak intensities in NOESY spectra with interproton distances, and we propose to use a similar semiquantitative approach to obtain backbone dihedral angle constraints in RNA (and DNA) oligonucleotides. The intensities of cross peaks corresponding to known coupling constants can be used for internal control and 'calibration'. In many circumstances, the sequential two-bond C3'(i–1)-P(i) couplings can be employed for this purpose, since these couplings (4–6 Hz) are not strongly sensitive to conformation. By comparison with cross peaks corresponding to couplings of known magnitude, we have estimated a lower limit of approximately 5 Hz for the active coupling generating intense cross peaks in HCP (generally sequential or intraresidual C4'-P couplings) or P,H-COSY–H,C-HMQC (generally sequential H3'-P couplings) spectra. Analysis of the generalized Karplus correlations for ^{31}P-^{1}H and ^{31}P-^{13}C scalar couplings (Altona, 1982; Mooren et al., 1994) reveals that upper or lower limits of 5 Hz can significantly restrain the conformational space if (and only if) several couplings are estimated at the same time.

Additional care should be employed when using the absence or weakness of cross peaks to obtain upper limits on scalar couplings. It must be verified that fast relaxation is not responsible for the absence or weakness of the cross peak. Controls on ^{1}H and ^{13}C line widths can be obtained by analyzing other three-dimensional spectra (such as NOESY-HMQC, HCCH-TOCSY or HCCH-COSY). Controls on ^{31}P line widths can be obtained either from the intensity of the sequential H3'(i–1)-P(i) cross peaks in P,H-COSY–H,C-HMQC spectra, or from sequential and intraresidual H4'-P cross peaks in HCP spectra. A careful analysis of the entire pattern of cross peaks observed for a given ^{31}P resonance is necessary to identify broadened resonances that generate cross peaks of reduced intensity. In our experience, broader resonances are generally found within conformationally flexible regions, where the pattern of scalar couplings indicates significant conformational averaging.

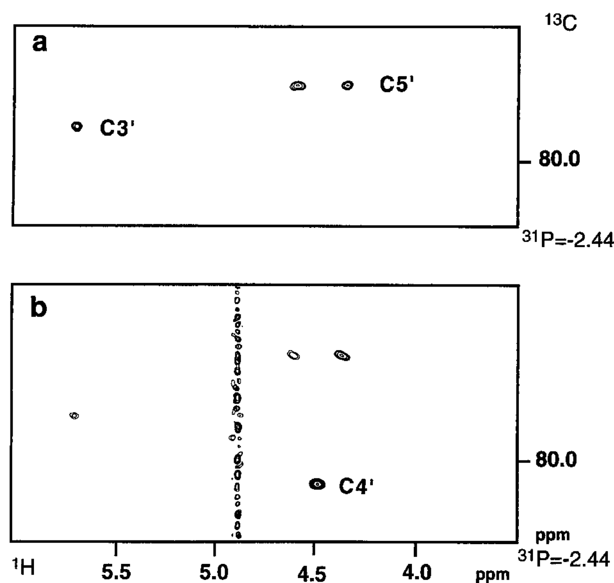

Fig. 4. Comparison of (a) P,H-COSY–H,C-HMQC and (b) HCP spectra, plotted at similar noise levels; ^{1}H-^{13}C planes corresponding to the well-isolated G34 ^{31}P resonance have been chosen for ease of comparison. Notice the appearance of strong connectivities in the C4' region of the HCP spectrum (corresponding to two overlapped resonances), and the decreased intensity of the signal corresponding to the sequential C3'-P connectivity (at ≈ 5.7 ppm).

A graphic demonstration of the procedure used to constrain the ε dihedral angle is provided in Fig. 5. Strong sequential H3'(i–1)-P(i) cross peaks in the P,H-COSY–H,C-HMQC spectrum (implying $^{3}J_{H3'-P} > 5$ Hz) are consistent with ε (C4'-C3'-O3'-P) *trans*, g^{+} or g^{-}. The three possibilities can be distinguished from the pattern of sequential couplings observed in HCP experiments. Large sequential C4'(i–1)-P(i) cross peaks ($^{3}J_{C4'-P} > 5$ Hz), together with weak or absent sequential C2'(i–1)-P(i) cross peaks ($^{3}J_{C2'-P} < 5$ Hz) demonstrate that ε is *trans* (Figs. 5a–c). The opposite situation ($^{3}J_{C4'-P} < 5$ Hz, $^{3}J_{C2'-P} > 5$ Hz) indicates that ε is g^{-} (Figs. 5d–f). As predicted from steric considerations (Saenger, 1984), the g^{-} conformation was found at two nucleotides (U30 and C33) in the single-stranded apical loop characterized by C2'-*endo* sugar conformation. The observation of relatively strong sequential correlations to both the C2' and the C4' resonances (as seen in the internal loop region, U40–C45) indicates the presence of a flexible region in the structure. The observation of several broadened resonances and very large chemical shift changes as a function of temperature for some loop resonances is consistent with this interpretation.

The intensities of the intraresidual P(i)-H5'/H5''(i) cross peaks can be used to constrain the β dihedral angles, after it has been verified that the absence of a cross peak is not due to broadening of the resonance. When β is *trans*, as

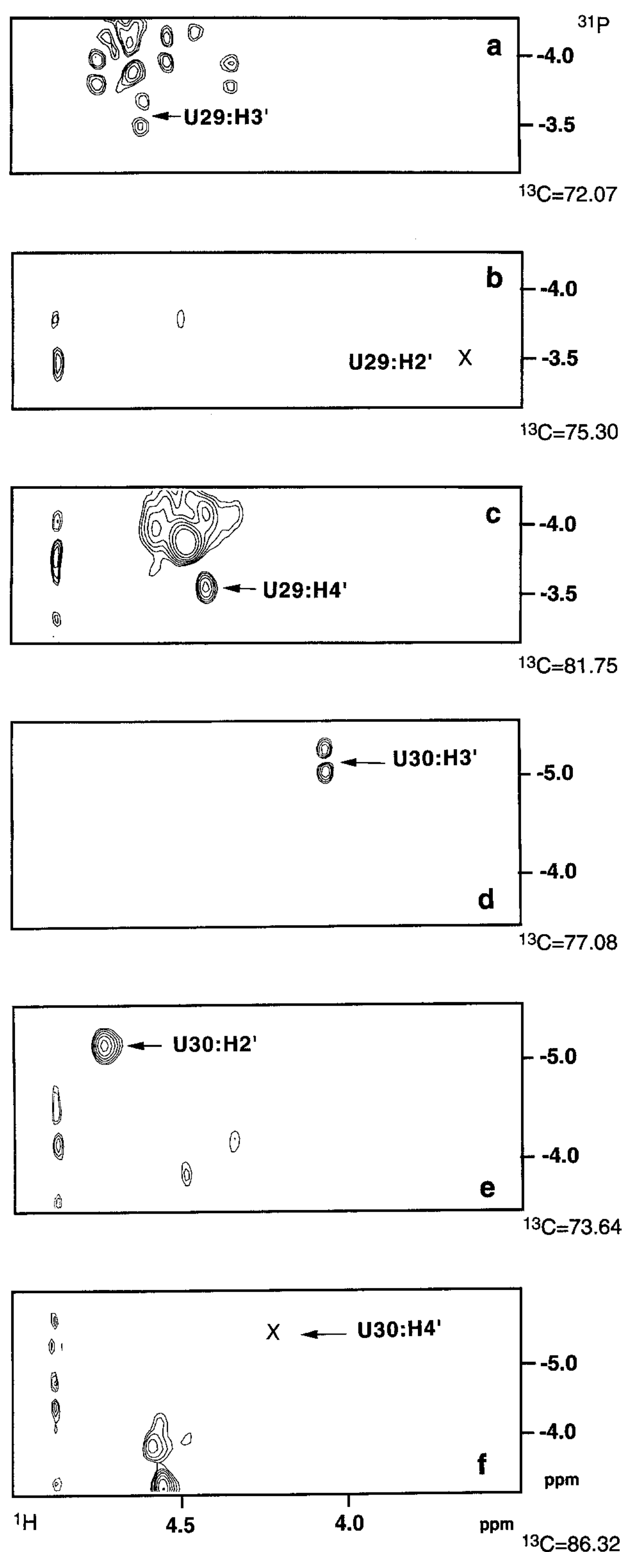

Fig. 5. The combination of Het-Cor and HCP spectra can be used to unambiguously identify the conformation of the ε (C4'-C3'-O3'-P) dihedral angle. If ε is *trans*, as normally observed in regular RNA and DNA double helices, the H3'(i–1)-P(i) coupling is large (a), the C2'-(i–1)-P(i) coupling is small (b; the x indicates the position where the peak should be observed) and the C4'(i–1)-P(i) coupling is large (c). When ε is g^-, the H3'(i–1)-P(i) coupling is large (d), the C2'(i–1)-P(i) coupling is also large (e), but the C4'(i–1)-P(i) coupling is now unresolved (f; the x indicates the position of the missing cross peak).

normally observed in regular double helices, both $^3J_{P\text{-}H5'} < 5$ Hz and $^3J_{P\text{-}H5''} < 5$ Hz, whereas either $^3J_{P\text{-}H5'}$ or $^3J_{P\text{-}H5''}$ are large when β is in the g^+ or g^- range. For all nucleotides with relatively small ^{31}P line widths, the observation of weak or absent intraresidue P(i)-H5'(i) and P(i)-H5"(i) cross peaks in P,H-COSY–H,C-HMQC spectra indicates that β is *trans*. Important controls are provided by the intensity of the intraresidue P(i)-C4'(i) cross peaks in the HCP experiment and, in some cases, by the observation of a resolved four-bond intraresidual P(i)-H4'(i) coupling (Altona, 1982). Since $^3J_{P\text{-}C4'}$ is relatively large for β^t, the observation of a strong intraresidue P(i)-C4(i) cross peak validates the conclusion based on the absence or weakness of intraresidue P(i)-H5'(i) and P(i)-H5"(i) cross peaks. Because of the spectral overlap in the two-dimensional Het-Cor and Het-TOCSY experiments (Fig. 3a), the increased resolution provided by the additional ^{13}C dimension is essential. Relatively strong P(i)-H5'(i) and P(i)-H5"(i) couplings were found in the internal loop region (U40–C45), presumably as a result of conformational averaging.

The semiquantitative analysis of scalar coupling patterns, based on the comparison of cross- peak intensities in 3D ^{13}C-edited correlated spectra, can provide structural information on two of the six torsion angles that define the conformation of the nucleic acid backbone. Examination of the Karplus equations (Mooren et al., 1994) reveals that upper and lower limits of 5 Hz on the scalar couplings are sufficient to constrain torsion angles β and ε to within ±30°–40°, if several coupling constants are obtained for each residue. The sugar conformation can be obtained in many different ways from ^1H-^1H (Altona, 1982) and ^1H-^{13}C scalar couplings (Hines et al., 1993, 1994), and γ (O5'-C5'-C4'-C3') can be estimated from the magnitude of H4'-H5' and H4'-H5" scalar couplings, in addition to $^4J_{P\text{-}H4'}$ (Altona, 1982). Only the two phosphodiester angles α (O3'-P-O5'-C5') and ζ (C3'-O3'-P-O5') cannot be constrained by means of scalar coupling measurements.

In conclusion, the extension to three dimensions of indirectly detected ^1H-^{31}P correlation experiments by means of ^{13}C editing provides a powerful complement to the recently proposed HCP (Heus et al., 1994; Marino et al., 1994) and HCP-CCH-TOCSY (Marino et al., 1995; Tate et al., 1995; Wijmenga et al., 1995) experiments for backbone-driven assignments in isotopically labelled nucleic acids. Furthermore, these experiments can be used to constrain the conformation of the nucleic acid backbone. Work under way in our laboratory indicates that the procedure illustrated here remains effective for oligonucleotides of up to about 40 residues. The favorable spectral dispersion generally observed in the more structurally interesting single-stranded regions may allow the extraction of relevant information for at least part of even larger molecules.

References

Altona, C. (1982) *Recl. Trav. Chim. Pays-Bas*, **101**, 413–433.

Gehring, K., Leroy, J.-L. and Gueron, M. (1993) *Nature*, **363**, 561–565.

Heus, H.A., Wijmenga, S.S., Van de Ven, F.J.M. and Hilbers, C.W. (1994) *J. Am. Chem. Soc.*, **116**, 4983–4984.

Hines, J.V., Landry, S.M., Varani, G. and Tinoco Jr., I. (1994) *J. Am. Chem. Soc.*, **116**, 5823–5831.

Hines, J.V., Varani, G., Landry, S.M. and Tinoco Jr., I. (1993) *J. Am. Chem. Soc.*, **115**, 11002–11003.

Kellogg, G.W. (1992) *J. Magn. Reson.*, **98**, 176–182.

Kellogg, G.W. and Schweitzer, B.I. (1993) *J. Biomol. NMR*, **3**, 577–595.

Kellogg, G.W., Szewczak, A.A. and Moore, P.B. (1992) *J. Am. Chem. Soc.*, **114**, 2727–2728.

Marino, J.P., Schwalbe, H., Anklin, C., Bermel, W., Crothers, D.M. and Griesinger, C. (1994) *J. Am. Chem. Soc.*, **116**, 6472–6473.

Marino, J.P., Schwalbe, H., Anklin, C., Bermel, W., Crothers, D.M. and Griesinger, C. (1995) *J. Biomol. NMR*, **5**, 87–92.

Mooren, M.M.W., Wijmenga, S.S., Van der Marel, G.A., Van Boom, J.H. and Hilbers, C.W. (1994) *Nucleic Acids Res.*, **22**, 2658–2666.

Nikonowicz, E.P. and Pardi, A. (1993) *J. Mol. Biol.*, **232**, 1141–1156.

Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer Verlag, New York, NY.

Schmieder, P., Ippel, J.H., Van den Elst, H., Van der Marel, G.A., Van Boom, J.H., Altona, C. and Kessler, H. (1992) *Nucleic Acids Res.*, **20**, 4747–4751.

Schwalbe, H., Marino, J.P., King, G.C., Wechselberger, R., Bermel, W. and Griesinger, C. (1994) *J. Biomol. NMR*, **4**, 631–644.

Schwalbe, H., Samstag, W., Engels, J.W., Bermel, W. and Griesinger, C. (1993) *J. Biomol. NMR*, **3**, 479–486.

Shaka, A.J., Barker, P. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.

Sklenář, V., Miyashiro, H., Zon, G. and Bax, A. (1986) *FEBS Lett.*, **208**, 94–98.

Szewczak, A.A., Moore, P.B., Chan, Y.-L. and Wool, I.G. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 9581–9585.

Tate, S.-I., Ono, A. and Kainosho, M. (1995) *J. Magn. Reson. Ser. B*, **106**, 89–91.

Van Gelder, C.W.G., Gunderson, S.I., Jansen, E.J.R., Boelens, W.C., Polycarpou-Schwartz, M., Mattaj, I.W. and Van Venrooij, W.J. (1993) *EMBO J.*, **12**, 5191–5200.

Varani, G., Cheong, C. and Tinoco Jr., I. (1991) *Biochemistry*, **30**, 3280–3289.

Varani, G. and Tinoco Jr., I. (1991a) *J. Am. Chem. Soc.*, **113**, 9349–9354.

Varani, G. and Tinoco Jr., I. (1991b) *Q. Rev. Biophys.*, **24**, 479–532.

Wijmenga, S.S., Heus, H.A., Leeuw, H.A.E., Hoppe, H., Van der Graaf, M. and Hilbers, C.W. (1995) *J. Biomol. NMR*, **5**, 82–86.

Wimberly, B.T. (1992) Ph.D. Thesis, University of California, Berkeley, CA.