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New applications of 2D filtered/edited NOESY for assignment and structure elucidation of RNA and RNA-protein complexes

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Abstract

NMR spectra of large RNAs are difficult to assign because of extensive spectral overlap and unfavorable relaxation properties. Here we present a new approach to facilitate assignment of RNA spectra using a suite of four 2D-filtered/edited NOESY experiments in combination with base-type-specific isotopically labeled RNA. The filtering method was developed for use in 3D filtered NOESY experiments (Zwahlen et al., 1997), but the 2D versions are both more sensitive and easier to interpret for larger RNAs than their 3D counterparts. These experiments are also useful for identifying intermolecular NOEs in RNA-protein complexes. Applications to NOE assignment of larger RNAs and an RNA-protein complex are presented.

Introduction

For the last decade techniques for isotopic (^{13}C, ^{15}N and ^{2}H) labeling of RNA have been available (Batey et al., 1992; Nikonowicz et al., 1992). This combined with various heteronuclear experiments has enabled the solution structure of many RNA oligonucleotides of less than about 15 kDa to be solved (Cromsigt et al., 2001; Dieckmann and Feigon, 1997; Wijmenga and van Buuren, 1998). The poor chemical shift dispersion of nucleic acids, particularly RNA, in both the proton and carbon dimensions, and very broad linewidths result in extreme resonance overlap, making the spectra of even moderately sized RNAs quite difficult to assign. Furthermore, for larger RNAs and RNA-protein complexes, 3D NOESY experiments of labeled RNA samples often fail to give rise to a complete set of NOEs due to the short T2 of both protons and carbons. Through bond experiments for sequential assignment involve the phosphate group and are therefore of limited utility for larger RNAs; thus, sequential assignment still relies heavily on NOE-based approaches. Because RNA can adopt a wide range of structural conformations, distinguishing sequential from non-sequential NOEs is problematic, and the assignment process often depends on assumptions about the structure.

Many experiments have been devised to facilitate the sequential assignment of RNA. Standard 2D NOESY and TOCSY methods to assign the H1′-H6/H8 fingerprint region are now supplemented with 3D ^{13}C-edited NOESY and various through bond experiments (reviewed in Wijmenga and van Buuren, 1998) such as HCN-type experiments to correlate intranucleotide H1′-H6/H8 (Sklenář et al., 1993a, b; Yan et al., 2002; Fiala et al., 1998, 2000; Hu et al., 2001, Brutscher and Simorre, 2001), HCCH-COSY and TOCSY to obtain sugar proton assignments (Hu et al., 2000; Simon et al., 2001; Hu et al., 1998; Hu and Jiang, 1999; Pardi and Nikonowicz, 1992) and, for smaller RNAs, HCP-type experiments to correlate sequential residues (Kellogg and Schweitzer, 1993; Heus et al., 1994; Marino et al., 1994, 1995; Luy and Marino, 2001; Kellogg et al., 1992). Even with these experiments unambiguous assignment of larger RNAs usually requires the use of specifically labeled RNAs (Dieckmann and Feigon, 1997). Here we de-

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scribe a strategy based on the use of a suite of four 2D filtered/edited NOESYs with base-type-specific ^{13}C labeled RNA. While this approach requires several isotopically labeled RNA samples, it yields spectra that have very high sensitivity and are easy to interpret.

Since the development of X-filters and X-half-filters (Bax and Weiss, 1987; Otting et al., 1986; Otting and Wüthrich, 1989; Worgotter et al., 1986), various kinds of filtered NOESY experiments have been the primary tool for identifying intermolecular NOEs between isotope labeled and unlabeled molecules in complexes (Breeze, 2000). The most widely used of these experiments generally employ purge filters, which do not require co-addition of spectra and can compensate for variations in J values (Gemmecker et al., 1992; Ikura and Bax, 1992; Kogler et al., 1983), and in our hands give better results. A particularly efficient filtering scheme that takes advantage of an approximately linear correlation between the ^{13}C chemical shift and the ^{1}H-^{13}C J coupling values was developed for use with a 3D ^{13}C F1-filtered NOESY-HSQC (Zwahlen et al., 1997). Iwahara et al. (2001) demonstrated the utility of 2D ^{13}C F1-filtered, F2-filtered and 2D ^{13}C F2-filtered NOESYs in identifying intermolecular NOEs using the same type of purge filter. Here we describe the use of 2D filtered/edited NOESYs in the assignment of RNA and in identifying intermolecular NOEs in molecular complexes. The newly described F1-edited, F2-edited (F1eF2e) and F1-filtered, F2-edited (F1fF2e) NOESY experiments together with the F2-filtered (F2f) and F1-filtered, F2-filtered (F1fF2f) experiments used in conjunction with base-type-specifically labeled RNA provide a uniquely useful tool for assigning larger RNAs and RNA-protein complexes.

Materials and methods

2D filtered/edited NOESY spectra

The 2D-filtered/edited NOESY experiments are performed by applying a purge element just prior to either the t_1 or the t_2 evolution periods of a two-dimensional NOESY. To describe the purge elements, the terminology of Breeze (2000) will be used. 'Labeled' protons are protons that are bound to an NMR-active X nucleus, such as ^{13}C. 'Unlabeled' protons are protons not bound to an X nucleus. A 'filter' is a purge element that removes signal from labeled protons while retaining signal from unlabeled protons. An 'edit' is a purge element that removes signal from unlabeled protons while retaining signal from labeled protons.

The original use of this purge element was as a filter (Zwahlen et al., 1997), but by a simple phase change it can also be used as an edit. The mechanism of the purge element will be described briefly. For ^{13}C-H (labeled protons), the magnetization starts as $-H_y$ at point a (Figure 1A). At point b it has evolved to $-2H_xC_z$. Unlabeled proton magnetization will be in the state H_y at point b. When the purging element is used as a filter, the phase of the next 90° pulse (ϕ_1) is x. The unlabeled proton magnetization is converted to H_z while the labeled proton magnetization stays $-2H_xC_z$. Then a strong pulsed field gradient is applied which purges the labeled signal. At point c the unlabeled signal can be converted back to transverse magnetization. When the purging element is used as an edit, ϕ_1 is applied along the y-axis, and the unlabeled signal remains in the transverse plane while the labeled signal is converted to $2H_zC_z$. The gradient purges the unlabeled signal, but doesn't affect the labeled signal.

Many strategies have been employed to compensate for variations in ^{1}H-^{13}C coupling constants (Breeze, 2000). One of the most efficient uses a special WURST pulse for carbon inversion (Zwahlen et al., 1997). The WURST pulse is designed to sweep the carbon frequencies at a rate such that ^{1}H-^{13}C pairs with different coupling constants become antiphase at the same time (at point b in Figure 1). For optimal suppression, two identical purge elements can be applied sequentially. Note that when the purge is used as an edit, two sequential purges must be done, or else the signal of interest after the purge will be antiphase. If two sequential purge elements are used, they can be applied as a simple double-tuned purge element using a standard WURST pulse for the carbon (Ogura et al., 1996).

In the 2D F1fF2e NOESY, a filter is applied just prior to the f1 evolution period and an edit is applied just prior to the f2 evolution period. In the f1 dimension, the only proton signals are from unlabeled protons, and only unlabeled proton signal is present at the beginning of the NOESY mixing time. Because there is an edit just before f2, the only proton signals that are directly detected are from labeled protons. Thus only signal that originated on an unlabeled proton, and was transferred via NOE to a labeled proton, can be detected in this experiment. In the 2D F1eF2e NOESY, edits are applied just before the f1 and the f2 evolution periods. This ensures that the

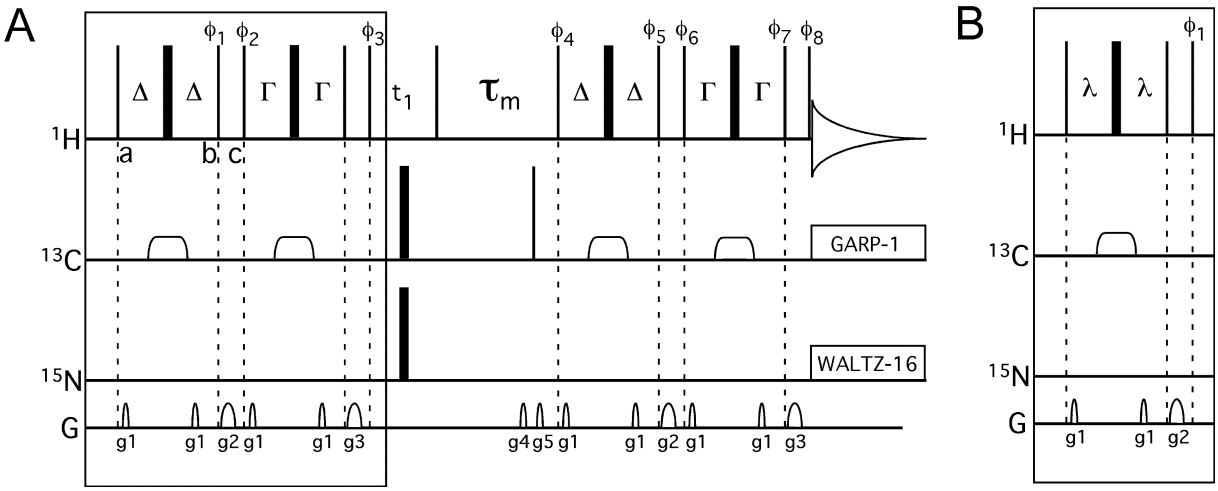

Figure 1. Pulse sequences of the 2D ^{13}C F2fF2e and F1eF2e NOESY experiments. Hard 90° and 180° pulses are represented as narrow and wide lines, respectively. The purge filters were applied in one of two ways: as a doubly tuned purge element, or by the method of Zwahlen et al. (1997). For a doubly tuned purge, the ^{13}C WURST pulses were 500 μs in duration and applied with no frequency offset. The delays, Δ and Γ were set to different values. For example, if RNA were the labeled molecule, Δ would be set to 1.25 ms (1/4J for H6/8-C6/8), and Γ would be set to 1.724 ms (1/4 J for H1′-C1′). If the purge filter was done by the method of Zwahlen et al. (1997), then all parameters of the WURST pulse and the delays Δ and Γ were set as described (Zwahlen et al., 1997). For ^{13}C-labeled RNA the sweep width, pulse length, maximum $\gamma B_1/2\pi$, and center of the WURST pulse were 50 kHz, 2.279 ms, 5 kHz, and 50 ppm, respectively, and the WURST pulse was swept from upfield to downfield. The delays Δ and Γ were each set to 1.8 ms. For ^{13}C-labeled RNA, the doubly tuned purge was usually used because the sensitivity was slightly higher due to the shorter purge delays. Both purge methods seemed about equally effective when two sequential purges were used (as in scheme A). (A) All pulses have phase x unless otherwise indicated. For 2D ^{13}C F1fF2e NOESY, phases are as follows: ϕ_1 = x; ϕ_2 = 4(x), 4(−x); ϕ_3 = 8(x), 8(−x); ϕ_4 = x; ϕ_5 = y; ϕ_6 = 16(y), 16(−y); ϕ_7 = x; ϕ_8 = x, y, −x, −y. For 2D ^{13}C F1eF2e NOESY, phases are as follows: ϕ_1 = y; ϕ_2 = 4(y), 4(−y); ϕ_3 = 8(x), 8(−x); ϕ_4 = x; ϕ_5 = y; ϕ_6 = 16(y), 16(−y); ϕ_7 = x; ϕ_8 = x, y, −x, −y. For both experiments, receiver = (x, y, −x, −y), 2 (−x, −y, x, y), (x, y, −x, −y), (−x, −y, x, y), 2(x, y, −x, −y), (−x, −y, x, y). Sine bell shaped gradients were applied with the following direction, duration, and strength: g1 = (x, 400 μs, 15 G cm^{-1}); g2 = (z, 900 μs, 20 G cm^{-1}); g3 = (z, 1.1 ms, 23 G cm^{-1}); g4 = (z, 3 ms, 20 G cm^{-1}); g5 = (z, 300 μs, 23 G cm^{-1}). (B) Shorter pulse sequence for 2D ^{13}C F1fF2e NOESY. In this scheme, only one purge element is used for the ^{13}C filter for higher sensitivity. If only one purge filter is used, then the method of Zwahlen et al. (1997) must be used for the filter. A doubly tuned purge can still be used for the edit. The phases are as follows: ϕ_1 = 4(x), 4(−x); ϕ_4 = 8(x), 8(−x); ϕ_5 = y; ϕ_6 = 16(y), 16(−y); ϕ_7 = x; ϕ_8 = x, y, −x, −y; receiver = (x, y, −x, −y), 2 (−x, −y, x, y), (x, y, −x, −y), (−x, −y, x, y), 2(x, y, −x, −y), (−x, −y, x, y). Gradients are identical to A. For both experiments quadrature detection in f_1 is achieved by applying States-TPPI phase cycling to the pulse immediately preceding t_1.

only observed signal is from the labeled protons. The 2D F1fF2f NOESY and the F2f NOESY experiments have been described previously (Iwahara et al., 2001). Figure 2 shows schematic versions of all four spectra for a sample that contains both labeled and unlabeled protons.

For RNA assignment these experiments are used with RNAs selectively labeled by nucleotide type. For example, the F1fF2e NOESY of a G-labeled RNA would show only the NOEs from Gs to the unlabeled A, U, and C nucleotides. The F1eF2e NOESY would show only NOEs within the Gs or from one G to another. This is an invaluable tool for assigning larger RNAs because it greatly decreases spectral complexity and allows crosspeaks to be assigned with certainty to base type. These two-dimensional experiments also have higher sensitivity due to the unfavorable relaxation properties of larger RNAs and are easier to interpret than their three-dimensional counterparts. Furthermore, because of the limited base-type specific ^{13}C chemical shift dispersion in RNA, three-dimensional experiments usually do not significantly resolve the resonance overlap problems. For complexes involving nucleic acids and/or proteins, the combination of all four filtered/edited NOESYs provides an efficient means of identifying intermolecular NOEs.

Sample preparation

Unlabeled, uniformly ^{13}C,^{15}N-labeled, and ^{13}C,^{15}N-base-type-specific labeled RNA oligonucleotides were prepared by *in vitro* transcription and purified as previously described (Dieckmann and Feigon, 1997). hTR (human telomerase RNA) p2b hairpin and pseudoknot

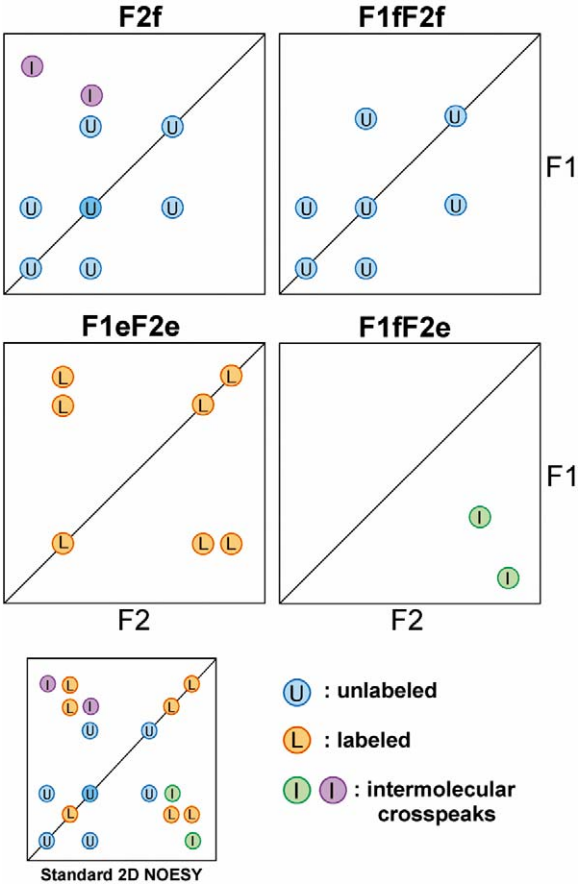

Figure 2. Schematic illustration of 2D filtered/edited NOESY spectra. U and L indicate signals from the unlabeled and labeled protons, respectively. I indicates crosspeaks between labeled and unlabeled protons. A normal proton NOESY spectrum contains all peaks from all four of the filtered/edited NOESY spectra (shown in lower left). The f1 and f2 dimensions are indicated on the figure; all other spectra in this paper are displayed the same way.

RNAs were annealed under dilute conditions (1–10 μM) in NMR buffer (10 mM sodium phosphate, pH 6.3, 200 mM KCl, 50 μM EDTA, 0.2% NaN_3) and concentrated by ultrafiltration to ~1.0 mM. Preparation of the Rnt1p dsRBD (Chanfreau et al., 2000; Nagel and Ares, 2000) will be described elsewhere. The ^{13}C,^{15}N-labeled Rnt1p dsRBD and unlabeled RNA (Wu et al., 2001) complex (Nagel and Ares, 2000) sample was prepared at a 1:1 ratio at 1.2 mM in 50 mM phosphate buffer, pH 6.5, 150 mM NaCl, 0.2% NaN_3 in D_2O.

NMR spectroscopy

All NMR spectra were recorded on Bruker DRX 500, 600 and 750 MHz spectrometers. 2D filtered/edited NOESY experiments were run at 293 K on the RNA samples and 303 K on the RNA-protein complex. Relevant details of experimental set-up are contained in the figure captions. The WURST pulses used in the purge elements were generated using the ShapeTool program (Bruker Biospin). The parameters of these pulses were set according to Zwahlen et al. (1997).

Results and discussion

Application to RNA assignment

The 2D filtered/edited NOESYs have been used in the assignment of several RNA molecules in our laboratory. Two of these will be briefly discussed to illustrate the technique. The first example is the hTR (human telomerase RNA) p2b hairpin (Figure 3A), whose structure we recently reported (Theimer et al., 2003). This 30 nucleotide RNA presented several difficulties for sequential NOE-based assignment. Because

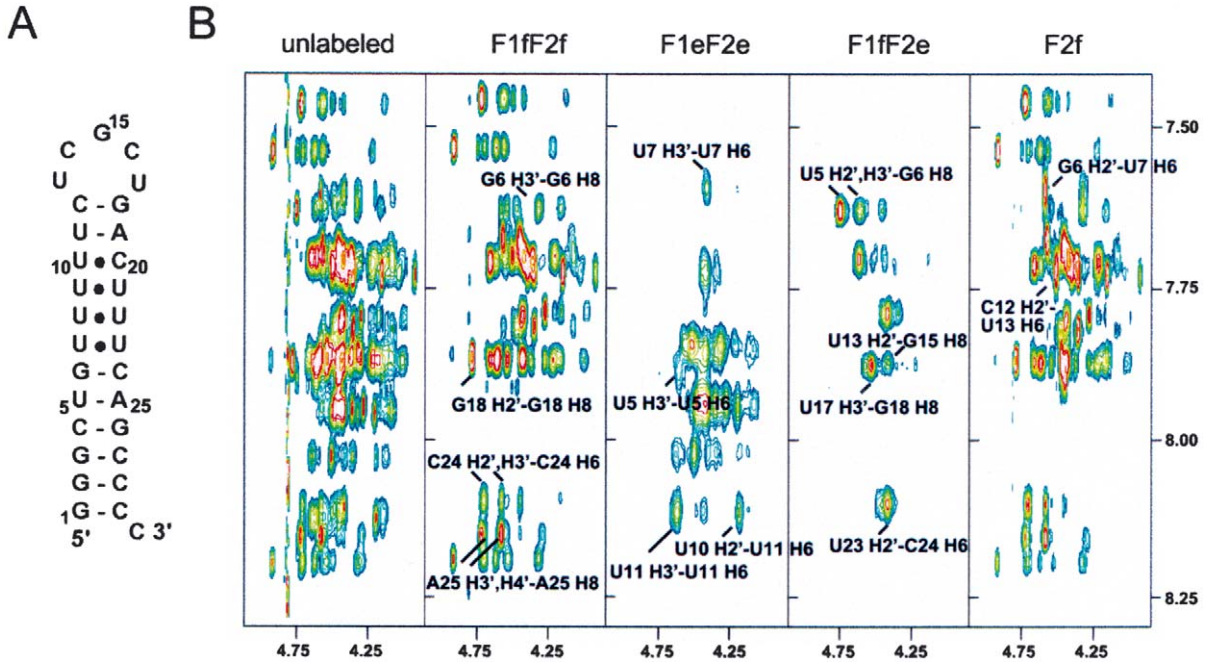

Figure 3. (A) Sequence and secondary structure of the p2b hairpin from human telomerase RNA (nt 95-119) used in these studies (Theimer et al., 2003). Dashes are used to indicate Watson–Crick base pairs and dots to indicate non-Watson–Crick base pairs. (B) The aromatic (H6,H8,H2) to sugar (H2′, 3′, 4′, 5′, 5″) region of 600 MHz NOESY spectra (τ_m = 300 ms) of the p2b hairpin. The spectrum entitled 'unlabeled' is a standard NOESY run on the unlabeled hairpin, and the other four are 2D filtered/edited NOESYs run on a ^{13}C, ^{15}N-U labeled sample of the hairpin. Sample conditions were 1 mM RNA in D_2O, pH 6.3, 200 mM KCl at 20 °C. In t_2 and t_1, 1024 and 512 complex points were acquired, respectively, with 128 scans per t_1 increment. Spectra were processed with a 60° phase shifted squared sinebell in both dimensions and the final data matrix was 2048 × 1024 points. Representative NOEs from the various experiments are indicated.

of the symmetry of the U · U base paired sequence there was a greater problem with overlapping resonances than would normally occur, particularly for the signals from residues U8, U9, U21, U22, and U23 (Figures 3A and B). In addition, due to the high pyrimidine content (21 out of 30 nucleotides), there were several cases of essential base-H1′ cross peaks which overlapped intense H5-H6 cross peaks. Selective labeling of the U nucleotides combined with the 2D filtered/edited NOESY experiments made it possible to remove the 11 uridine H5-H6 crosspeaks, 6 of which were obscuring the most crowded and therefore most critical portion of the aromatic to H1′ region. The overlap problems observed in the aromatic to H1′ region of the spectrum were even more severe in the aromatic to H2′, H3′, H4′, H5′, H5″ (sugar) and H1′ to sugar regions (Figure 3B), making assignment of crosspeaks in these regions particularly difficult for both the U tract and the hairpin loop sequence (U13–U17). Shown in Figure 3B is the aromatic to sugar region with representative peaks annotated in the four different filtered/edited NOESY spectra applied to the ^{13}C-,^{15}N-U labeled RNA hairpin. These experiments clearly differentiate U aromatic to U sugar NOEs (which appear only in the F1eF2e NOESY) from A/C/G aromatic to U sugar (F1fF2e), A/C/G aromatic to A/C/G sugar (F1fF2f), and U aromatic to A/C/G sugar (F2f) NOEs.

Even in regions where the peak overlap is not severe, the combination of filtered experiments makes it possible to more accurately identify NOEs and to determine their intensities qualitatively as strong, medium, or weak. For example, the G6 H8 and the U7 H6 resonances have similar chemical shifts, making it difficult to distinguish their specific NOE crosspeaks. However, as seen in Figure 3B, NOEs to G6 H8 are observed only in the F1fF2f and F1fF2e experiments, from the unlabeled and labeled sugar protons, respectively. NOEs to U7 H6 are observed only in the F1eF2e experiment from labeled sugar protons and in the F2f experiment from unlabeled sugar protons. Thus, NOEs to overlapping resonances can be resolved and easily assigned to the correct nucleotide. Similarly, the 2D filtered/edited NOESYs were useful in separating

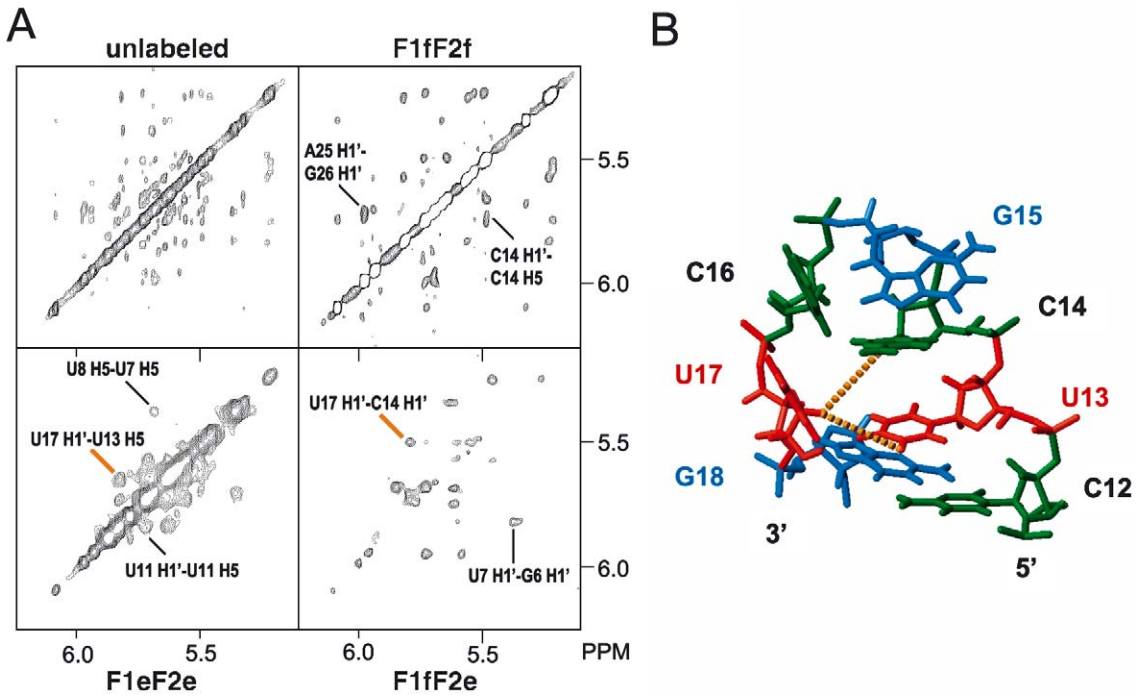

Figure 4. (A) The H1′, H5 region of 600 MHz NOESY (τ_m = 300 ms) spectra of the unlabeled hairpin and F1fF2f, F1eF2e, and F1fF2e experiments on the ^{13}C, ^{15}N-U labeled hairpin. Representative NOEs observed in the various experiments are indicated. Orange lines indicate the two key non-sequential NOEs identified and confirmed in these experiments. (B) Solution structure of the p2b hairpin loop (Theimer et al., 2003). Nucleotides are colored red (U), green (C), and blue (G). The two key non-sequential loop NOEs identified in (A) are indicated by dotted orange lines on the loop structure.

A25 H8 and C24 H6 from the U11 H6 (Figure 3B). Additionally, in the F1fF2f spectrum, both the G15 H8-G15 H1′ and the G15 H8-G15 H2′ were resolved from nearby peaks. This made it possible to determine with confidence that the G15 H8-G15 H1′ NOE is twice as intense as the G15 H8-G15 H2′ NOE, confirming the *syn* conformation predicted for this loop nucleotide (data not shown).

In A-form regions of RNA, there is an expected pattern of sequential NOEs based on helical geometry, making it in general possible to choose between possible NOE identities in overlapped regions. However, in the more interesting regions of RNA such as unusual base pairs or hairpin loops it is extremely difficult to correctly assign NOEs without some idea of the possible structure of the molecule. Use of the filtered/edited NOESYs significantly reduced the complexity of the hTR hairpin spectra and positively eliminated candidate NOEs, making the accurate assignment of critical non-sequential loop NOEs possible. An NOE assignment was confirmed only if it occurred in the correct filtered/edited experiment. The H1′, H5 region of NOESY spectra of the hTR hairpin is shown in Figure 4A. The NOESY spectrum of unlabeled hairpin appears in the upper left. The other three spectra are filtered/edited NOESYs run on the U labeled hairpin. NOEs representative of the type found in particular filtered/edited spectra are annotated. Although 6 loop NOEs were originally assigned as non-sequential based on the NOESY spectrum alone, examination of the filtered/edited NOESY spectra indicated that 4 of these NOEs were actually sequential NOEs. The assignments of the remaining 2 non-sequential loop NOEs were confirmed by their presence in the F1eF2e experiment (U17 H1′–U13 H5) and the F1fF2e experiment (U17 H1′–C14 H1′), respectively. These NOEs, indicated by orange lines in Figure 4A, in conjunction with the rest of the NOE data and dihedral angle restraints, helped to constrain the less ordered 3′ end of the hairpin loop in proximity to the stacked and more structured 5′ side of the hairpin loop (Figure 4B).

The filtered/edited NOESY experiments were also used to assign the larger 47 nucleotide hTR pseudoknot, shown schematically in Figure 5A. Due to the unfavorable relaxation properties of this mo-

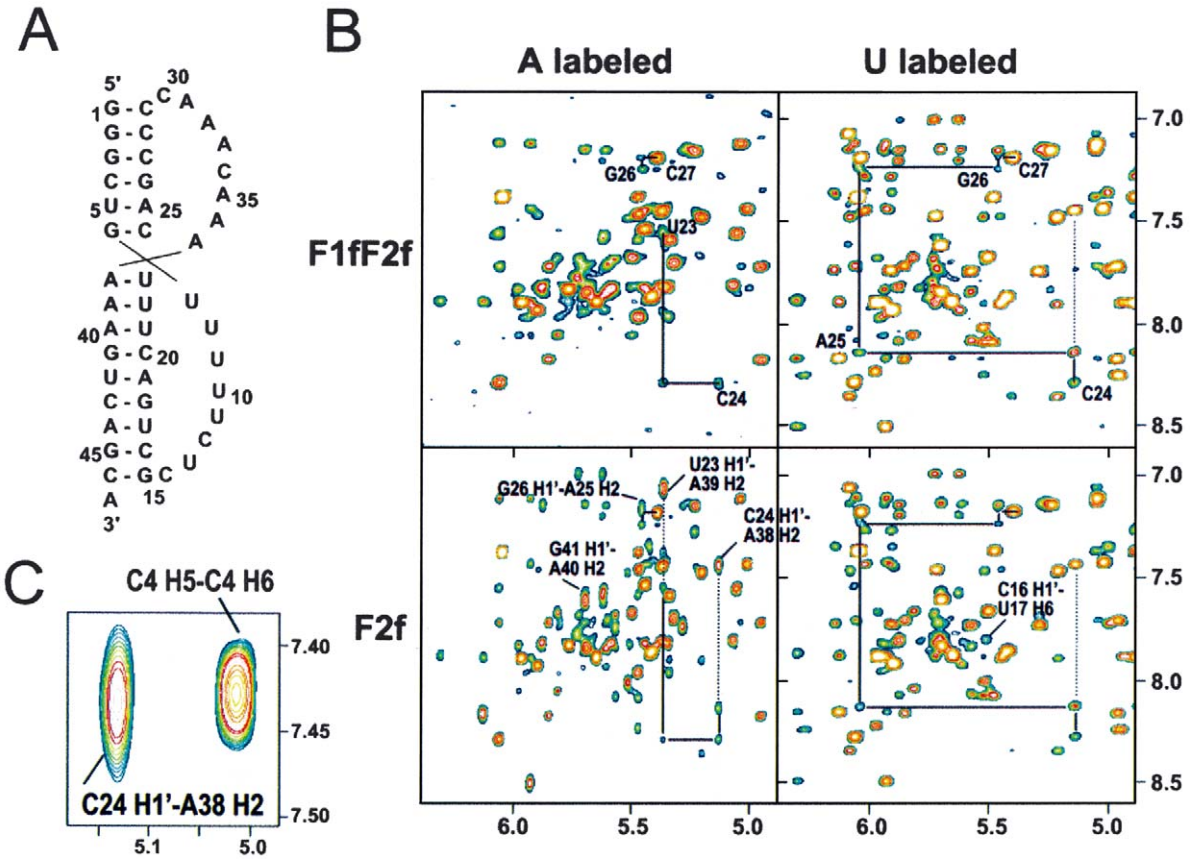

Figure 5. (A) Sequence and secondary structure of the p2b/p3 pseudoknot from human telomerase RNA (nt 95–119 and 168–184). Dashes are used to indicate Watson–Crick base pairs. (B) The aromatic to H1′ region of F1fF2f and F2f NOESY spectra (τ_m = 250 ms) of the ^{13}C, ^{15}N-U and ^{13}C, ^{15}N-A labeled pseudoknots. The NOE sequential walk from U23 H1′-H6 to C27 H1′-H6 is indicated by a solid line with the intranucleotide crosspeaks annotated. Dotted lines are used to indicate the non-sequential adenine H2 to H1′ cross-strand NOEs for these nucleotides. Other representative NOEs that are not observed in the F1fF2f experiments are also annotated in the F2f spectra. Sample conditions were 1 mM RNA in D_2O, pH 6.3, 200 mM KCl at 20 °C. In t_2 and t_1, 1024 and 512 complex points were acquired, respectively, with 128 scans per t_1 increment. Spectra were processed with a Gaussian filter function (LB–18, GB 0.1) in f_2 and a 60° phase shifted squared sinebell in f_1 and the final data matrix was 2048 × 1024 points. (C) Close up of labeled to labeled and labeled to unlabeled peaks from the ^{13}C, ^{15}N-A labeled pseudoknot F2f spectrum showing the different linewidths in the f_1 dimension.

lecule and the high salt (200 mM KCl) concentrations required to maintain the pseudoknot conformation, most through bond experiments usually used for assignment failed. 3D ^{13}C-edited NOESY experiments also had very low S/N, thus limiting their utility for assignments. Because of this, sequential assignment relied almost entirely on 2D NOESY spectra. Complete sequential assignments were obtained by comparing the F1fF2f and F2f NOESY spectra from each of the base-type-specific labeled pseudoknot RNAs. The sequential connectivities from U23 H1′-H6 to C27 H1′-H6 are shown in the F1fF2f NOESY spectra of the A- and U-labeled RNAs (Figure 5B). Comparison of the F1fF2f and F2f NOESY spectra of the U labeled sample also allows identification of U aromatic to A/C/G H1′ peaks. For example, the C16 H1′ to U17 H6 NOE is seen in the F2f spectrum but not in the F1fF2f spectrum. Similarly, comparison of the F1fF2f and F2f spectra of the A labeled sample clearly identifies the non-A H1′ to A H2 cross strand NOEs, which are critically important for structure determination (Figure 5B).

Interestingly, in the F2f experiment, the lineshapes of signals from labeled and unlabeled protons of the pseudoknot RNA are strikingly different in the indirect dimension. This was also observed with the hTR hairpin, although to a lesser extent. Due to faster relaxation, crosspeaks arising from labeled protons have a much broader linewidth in f_1 (Figure 5C). The resulting oval shaped labeled to unlabeled crosspeaks can

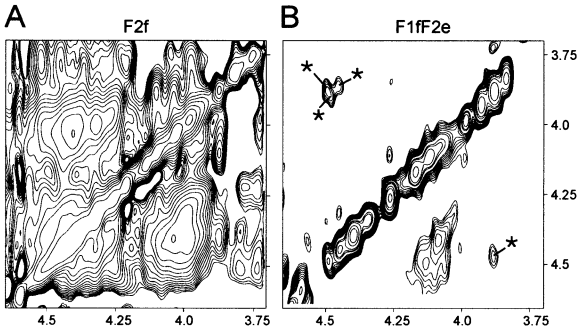

Figure 6. (A) Portion of an F2f NOESY ($\tau_m = 200$ ms) spectrum of $^{13}C,^{15}N$-Rntlp dsRBD/unlabeled RNA complex containing protein $C^{\alpha}H$, and RNA H2′, H3′, H4′, H5′, and H5″ resonances. (B) The same region of an F1fF2e NOESY ($\tau_m = 200$ ms) spectrum of ^{13}C-^{15}N-Rntlp dsRBD/unlabeled RNA complex. Due to overlap in this region in the F2f spectrum, additional intermolecular NOEs can be identified using the F1fF2e NOESY experiment. In both experiments, 2048 and 350 complex pointes were acquired in t_2 and t_1, respectively, with 320 scans per t_1 increment. The final data matrices were 2048 × 2048 points and were processed with a Gaussian filter function (LB −18, GB 0.12 in f_2 and f_1). Newly identified intermolecular NOEs are indicated by * in the spectrum. The appearance of resonance intensity along the diagonal in this experiment is due to magnetization that has leaked through the filters. This breakthrough only occurs in very crowded regions of the spectrum and only along the diagonal, and therefore does not lead to ambiguities in NOE assignment.

easily be distinguished from the round unlabeled to unlabeled crosspeaks in the F2f experiment. In conjunction with the corresponding F1fF2f and F1fF2e experiments, this observation can be a very useful aid in interpreting spectra of very large RNAs.

Application to RNA-protein intermolecular NOEs

The filtered/edited NOESY experiments are also useful in identifying intermolecular NOEs in complexes. The utility of the 2D F1fF2f and F2f NOESYs in identifying intermolecular NOEs has been previously demonstrated for a protein-DNA complex (Iwahara et al., 2001). Using these two experiments, we were able to identify 30 intermolecular NOEs in the 20 kDa $^{13}C,^{15}N$-labeled Rnt1p dsRBD/unlabeled RNA complex. An additional 4 intermolecular NOEs were unambiguously assigned using the F1fF2e NOESY. In the F1fF2e experiment, the only crosspeaks observed are from intermolecular NOEs. While all crosspeaks appearing in the F1fF2e spectra also appear in the F2f spectra, the F2f spectra also contain all the signals from the unlabeled molecule, many of which may overlap with crosspeaks representing intermolecular NOEs. The additional intermolecular NOEs identified in the F1fF2e spectrum could not be resolved in the F2f spectrum because of spectral overlap. Most of these occur between 3.5 and 4.7 ppm in the region where the RNA sugars resonate (see Figure 6).

Summary

This suite of 2D filtered/edited NOESY experiments offers a simple yet very powerful method for sequential assignment of RNA. In our experience these experiments applied to base-type-specific labeled RNA are the single most useful tool for assigning larger RNAs. In addition, the F1fF2e NOESY is a useful addition to the existing arsenal of experiments that can identify intermolecular NOEs in macromolecular complexes. As demonstrated here, it can be used to identify intermolecular NOEs that cannot be resolved using 2D F1fF2f and F2f NOESY experiments alone.

Acknowledgements

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