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Distinguishing between duplex and hairpin forms of RNA by ^{15}N–^{1}H heteronuclear NMR

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Abstract

A general method is described for distinguishing RNA hairpins from RNA duplexes by application of two-dimensional filtered nuclear Overhauser enhancement spectra on a 1:1 mixture of unlabeled and 99% ^{15}N-labeled molecules. The method is applied to the RNA dodecamer rGGCGCUUGCGUC which can form an intramolecular hairpin under low salt conditions and a duplex in high salt. This procedure allows unambiguous identification of RNA hairpins or duplexes under the same conditions that are used in the NMR solution structure determination.

Key words: Heteronuclear NMR; RNA structure; RNA hairpin; RNA duplex; ^{15}N; Isotope

1. Introduction

Most biologically important RNAs, including tRNA and catalytic RNAs, and most RNA structural motifs, such as pseudo-knots and hairpins, fold intramolecularly [1,2]. NMR spectroscopy has been used to generate three-dimensional structures of a variety of RNAs [3] including several hairpins containing frequently occurring and unusually stable tetraloops [4,5] The NMR structures are determined by measurement of proton-proton distance constraints derived from nuclear Overhauser effects (NOEs), and dihedral angle constraints derived from J coupling constants [6]. All molecules that form intramolecular hairpins can also form intermolecular duplexes, and in many cases the duplex represents the stable conformation. Thus for the NMR studies on the RNA tetraloops, optical melting experiments were used to show that the molecules form hairpins in solution [4,5] because standard NMR techniques cannot be used to distinguish a hairpin from a duplex. Since the hairpin consists of a base paired stem and a loop, and the duplex consists of a base paired stem and an unpaired internal loop, the pattern of NOEs are very similar for these two conformations. Thus there generally are no ^{1}H–^{1}H NOEs that can be used to distinguish uniquely the hairpin from the duplex. Other techniques that are used to determine the stoichiometry of nucleic acids include: concentration dependence of melting temperatures, various hydrodynamic techniques, and native gel electrophoresis [7–10]. However these methods can be difficult to carry out under the millimolar concentrations typically used for NMR solution structure determinations. Therefore the goal of this study is to develop a simple technique that can distinguish hairpin from duplex under the experimental NMR conditions.

Isotopic enrichment of RNAs [11,12] provides a means of distinguishing unambiguously between hairpin and duplex formation. The method makes use of the double/half isotope filtered NOESY experiments to distinguish intermolecular and intramolecular NOEs in a 1:1 mixture of unlabeled and labeled molecules [13,14]. In this paper we demonstrate how application of isotope filtered NOESY experiments on a 1:1 mixture of unlabeled and ^{15}N-labeled RNA can be used to distinguish between formation of a single-stranded hairpin and double-stranded duplex. The experiments were performed on the RNA dodecamer, rGGCGCUUGCGUC, which can form either a symmetrical duplex or a hairpin structure (Fig. 1). The distinction between these two forms is determined directly and unambiguously in the NMR experiments through analysis of interstrand interactions.

2. Materials and methods

Unlabeled and 99% ^{15}N-labeled RNA molecules were synthesized using T7 RNA polymerase as previously described [11]. The RNAs were then dialyzed separately against 1 mM EDTA, then 1 M NaCl, and finally five times against 5.0 mM sodium phosphate, pH 6.8, 0.5 mM EDTA. Equal amounts of the labeled and unlabeled RNAs were then mixed, lyophilized to dryness, and resuspended in 550 μl of 90% H_2O/10% D_2O to a total strand concentration of 0.35 mM RNA in 10 mM sodium phosphate, pH 6.8, 1 mM EDTA. This sample represents the low salt conditions. The high salt conditions (200 mM NaCl, 10 mM sodium phosphate, pH 6.8, 1 mM EDTA) were obtained by addition of 23 μl of 5 M NaCl to the low salt sample. Prior to each NMR experiment the sample was heated to 80°C and then cooled on ice.

Fig. 2 shows the isotope filtered NOESY pulse sequence employed here, which is essentially that described by Otting and Wüthrich [15]

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except that water suppression jump and return pulses [16] were substituted for the final 90° and 180° proton acquisition pulses. 1024 complex points were collected in the acquisition time, t_2, and quadrature detection was achieved in the evolution time, t_1, by the hypercomplex method [17] with 50 complex points acquired for the low salt spectrum and 60 complex points for the high salt spectra. A sweep width of 10 kHz was used in both dimensions and 320 scans were collected for each FID. Two separate FIDs were collected for each complex point in t_1, one that employed 180° ^{15}N pulses for both filter elements and the second that employed 0° effective ^{15}N pulses for both filter elements [15]. The two experiments were then summed to yield the double filtered/double selected spectrum and the difference yielded the double half filtered spectrum [15]. These isotope filtered experiments were acquired under low salt conditions at 5 °C, and under high salt conditions at 10°C. The spectra were acquired on a Varian VXR-500S spectrometer and processed on a Sun computer using the program Felix 1.1. A third order polynomial baseline correction was applied in t_2 to remove baseline distortion resulting from the use of the jump and return pulses.

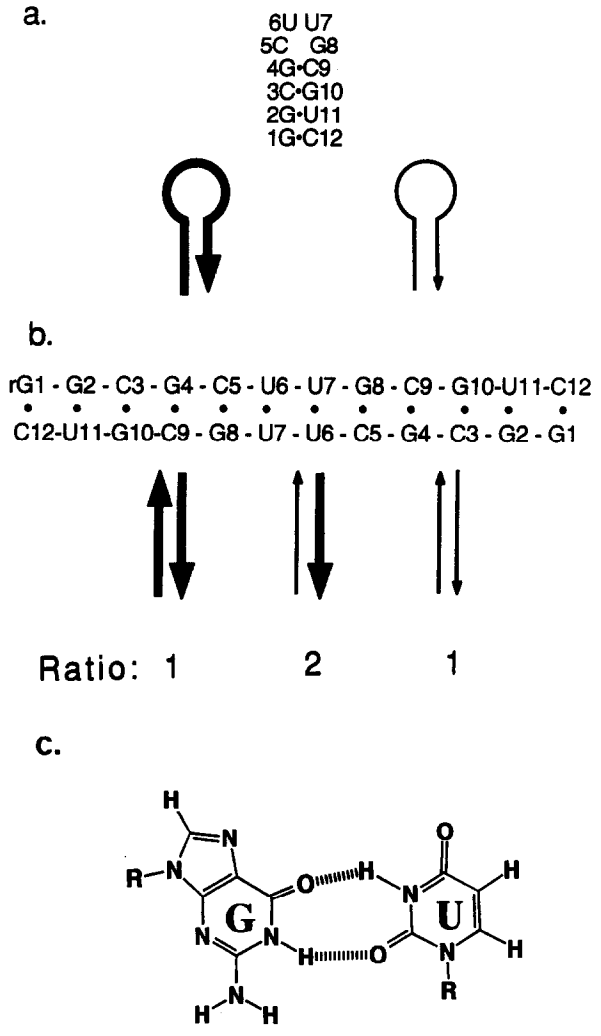

Fig. 1. Schematic illustration of (a) hairpin and (b) duplex forms of the RNA rGGCGCUUGCGUC and the relative populations of ^{15}N-labeled, unlabeled and mixed species for each case. The labeled and unlabeled strands are illustrated by the bold and narrow lines, respectively. A 50:50 mixture of ^{15}N-labeled and unlabeled strands is assumed. Both the duplex and hairpin have the same base pair composition in their helical regions, and therefore have the same number of resonances and many of the same NOEs. (c) A G·U Wobble base pair such as G2·U11 produces a close contact (< 3 Å) between the G and U imino protons.

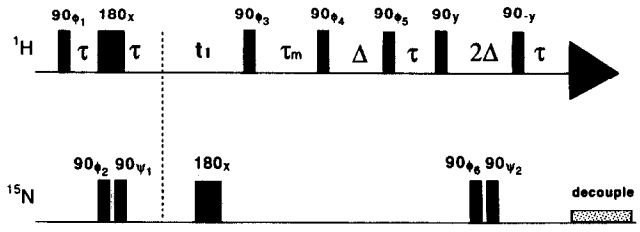

Fig. 2. Pulse sequence for the ^{15}N filtered NOESY experiments in Figs. 3 and 4 [15]. Low power WALTZ ^{15}N decoupling was applied during acquisition. The phase cycling used was: $\phi_1 = x,-x$; $\phi_2 = 8(x), 8(-x)$; $\phi_3 = 2(x),2(-x)$; $\phi_4 = 4(-y), (4y)$; $\phi_5 = -\phi_4$; $\phi_6 = 16(x), 16(-x)$; receiver phase = $x, -x, -x, x, -x, x, x, -x$. Two experiments were acquired and stored separately: one where $\varphi_1 = \phi_2$ and $\varphi_2 = \phi_6$ and a second where $\varphi_1 = -\phi_2$ and $\varphi_2 = -\phi_6$. The sum of these two experiments produced the double filtered/double selected spectra (Figs. 3a and 4a) and the difference produced the double half filtered spectra (Figs. 3b and 4b) [15]. The 180°$_x$ nitrogen pulse in the middle of the t_1 evolution consisted of a composite pulse of the form 90°$_x$180°$_y$90°$_x$. Other experimental parameters were: $\tau = 2.7$ ms, the mixing time, $\tau_m = 250$ ms, the jump and return delay, $\Delta = 69.4$ μs.

3. Results and discussion

The isotope filtered NOESY experiments make it possible to distinguish between intermolecular and intramolecular NOEs in a 1:1 mixture of unlabeled and ^{15}N-labeled RNAs. In the double filtered/double selected experiment, cross peaks are only observed for NOEs where both protons are bound to ^{15}N, or both protons are bound to ^{14}N, whereas in the double half filtered experiment NOEs are only observed when one proton is bound to ^{15}N and the other proton is bound to ^{14}N. For a mixture of labeled and unlabeled RNA, hairpin formation leads to two different species as illustrated in Fig. 1a. Since all the NOEs in a hairpin are intramolecular, all NOEs will be observed in the double filtered/double selected experiment as seen for the RNA in low salt conditions (Fig. 3a). However, since the double half filtered experiment selects only for intermolecular NOEs between labeled and unlabeled molecules, there are no peaks in the double half filtered spectrum of the RNA under low salt conditions as seen in Fig. 3b. The spectrum in Fig. 3a serves as a control for observation of peaks in Fig. 3b since both spectra were produced from the same data set and are plotted at the same contour level. The G2·U11 base pair provides a distinctive marker for identification of intermolecular NOEs, since there is generally a very strong cross peak between the G and U imino protons in a G·U base pair (see Fig. 1c) [6,18]. Thus the standard NOESY spectrum observed in Fig. 3a combined with the absence of peaks in Fig. 3b unambiguously demonstrate that the RNA forms an intramolecular hairpin under the low salt conditions.

For a RNA duplex, a 1:1 mixture of labeled and unlabeled molecules will lead to three different species where half the duplexes are a mixture of labeled and unlabeled

molecules and the rest are either fully labeled or fully unlabeled, as illustrated in Fig. 1b. A double filtered/double selected spectrum of a duplex under these conditions yields a standard NOESY type spectrum, but with reduced intensity since only half the molecules contribute to the spectrum. The double half filtered spectrum of a duplex under these conditions would have standard cross peaks, again at reduced intensity, but no diagonal peaks [15]. This is exactly what is observed in the 1:1 mixture of unlabeled and ^{15}N-labeled RNA under high salt conditions where Fig. 4a and b show the double filtered/double selected spectrum and the double half filtered spectrum, respectively. The G2 imino proton to U11 imino proton cross peak is visible in the double filtered/double selected experiment, though at an intensity somewhat reduced compared to that observed under low salt conditions. However, the same cross peak now appears in the double half filtered experiment in Fig. 4b, demonstrating unambiguously that the NOE in question arises from an intermolecular interaction. The RNA therefore must be forming a duplex under the high salt conditions.

An imino proton to imino proton NOE arising from

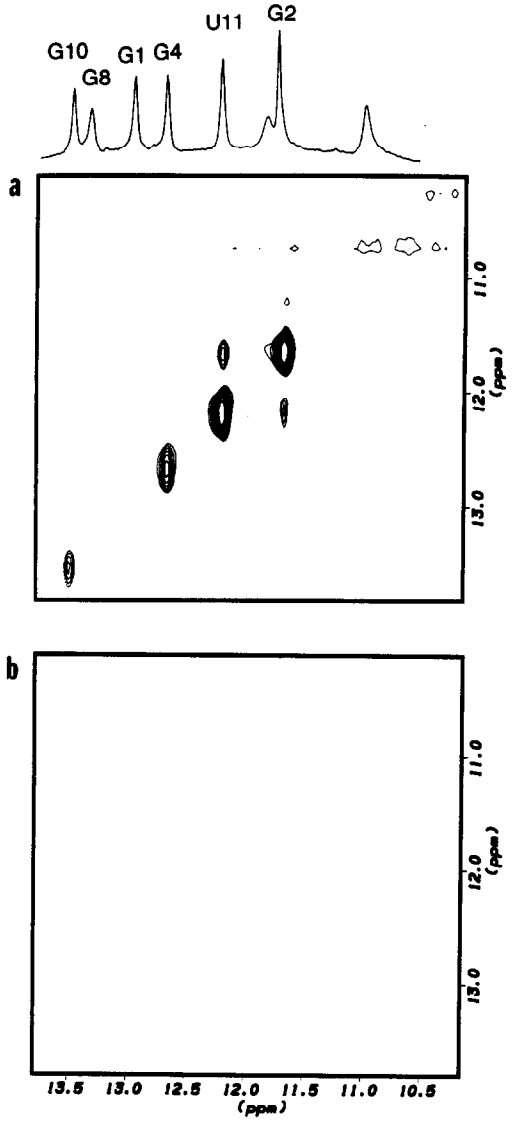

Fig. 3. Imino proton-imino proton region of (a) a double filtered/double selected spectrum and (b) a double half filtered NOESY spectrum of the RNA, rGGCGCUUGCGUC, under low salt conditions. Both spectra are plotted at the same contour level. The 1D imino proton spectrum under the same conditions is plotted above the NOESY spectra. The absence of cross peaks in the double half filtered NOESY spectrum demonstrates that there are no intermolecular NOEs in this molecule [13,14].

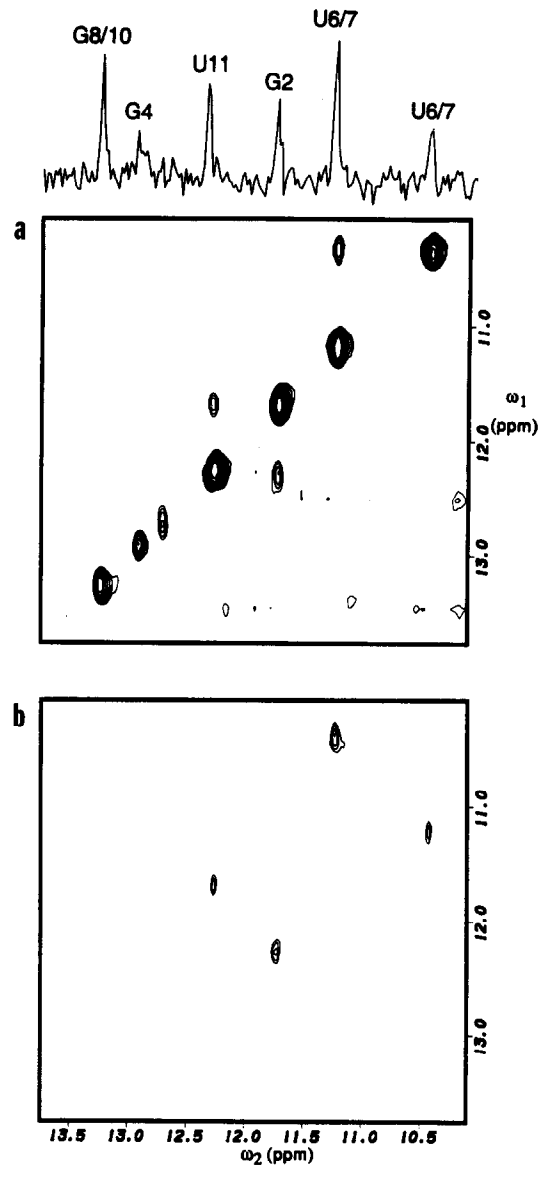

Fig. 4. Imino proton-imino proton region of (a) a double filtered/double selected spectrum and (b) a double half filtered NOESY spectrum of the RNA, rGGCGCUUGCGUC, under high salt conditions Both spectra are plotted at the same contour level. The 1D imino proton spectrum under the same conditions is plotted above the NOESY spectra. The appearance of crosspeaks in the double half filtered NOESY spectrum only occurs for a molecule that contains intermolecular NOEs [13,14].

a G·U Wobble base pair provides the best marker for hairpin vs. duplex formation in a RNA, but for nucleic acids which lack G·U base pairs, the G imino proton to C amino proton NOE provides a convenient marker [6].

4. Conclusions

The experiments reported here demonstrate that a RNA hairpin can be distinguished unambiguously from a symmetric duplex using isotope filtered NOESY experiments on a 1:1 mixture of unlabeled and ^{15}N-labeled RNA. This technique relies upon the ability to distinguish intermolecular NOEs from intramolecular NOEs and is most easily applied to G·U or G·C base pairs. Since it is possible to switch from duplex to hairpin in many RNA oligonucleotides by varying the ionic strength and/or the concentration of oligomers, this procedure allows these two forms to be distinguished directly under a specific set of NMR conditions. The application of filtered NOESY experiments to distinguish hairpin from duplex formation represents another example of the utility of isotopic labeling for the study of RNA structure.

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