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ARTICLE

Enhanced NMR signal detection of imino protons in RNA molecules containing 3′ dangling nucleotides

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Abstract We present a method for improving the quality of nuclear magnetic resonance (NMR) spectra involving exchangeable protons near the base of the stem of RNA hairpin molecules. NMR spectra of five different RNA hairpins were compared. These hairpins consisted of a native RNA structure and four molecules each having different unpaired, or dangling, nucleotides at the 3′ end. NMR experiments were acquired in water for each construct and the quality of the imino proton spectral regions were examined. The imino resonances near the base of the stem of the wild type RNA structure were not observed due to breathing motions. However, a significant increase in spectral quality for molecules with dangling 3′ adenosine or guanosine nucleotides was observed, with imino protons detected in these constructs that were not observed in the wild type construct. A modest improvement in spectral quality was seen for the construct with a 3′ unpaired uridine, whereas no significant improvement was observed for a 3′ unpaired cytidine. This improvement in NMR spectral quality mirrors the increased thermodynamic stability observed for 3′ unpaired nucleotides which is dependant on the stacking interactions of these nucleotides against the base of the stem. The use of a dangling 3′ adenosine nucleotide represents an easy method to significantly improve the quality of NMR spectra of RNA molecules.

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Introduction

The study of RNA structure by nuclear magnetic resonance (NMR) techniques has advanced rapidly since the early 1990s. This progress has been achieved by the development of ^{13}C and ^{15}N isotopic labeling techniques for RNA, improved NMR instrumentation, and the development of a wide variety of new NMR experimental methods (Varani et al. 1996; Puglisi and Puglisi 1998; Mollova and Pardi 2000; Zidek et al. 2001; Furtig et al. 2003; Flinders and Dieckmann 2006). These NMR methods have been used to study a wide range of RNA molecules as well as RNA-small molecule and RNA protein complexes. However, a common problem encountered in NMR based studies of RNA is the significantly reduced signal intensity or even the inability to detect imino and amino protons due to "breathing" motions. These motions result from the transient opening of base pairs, thus allowing the exchange of imino and amino protons with solvent, and the consequent loss in signal intensity. This "breathing" motion is most commonly observed in the terminal base pairs of a stem but can also occur near bulges or internal loops. In many cases, these imino proton signals are seen in one dimensional proton spectra, but are not detected in multidimensional NMR experiments. This loss of signal results in a gap in chemical shift assignments, a lower number of restraints used in structure calculations, and therefore a lower degree of precision in the final structure.

It is known that unpaired, or dangling nucleotides, can stack against the last base pair of a nucleic acid stem structure and stabilize the overall RNA structure

(Romaniuk et al. 1978; Neilson et al. 1980). Thermodynamic data has shown that terminal 3′-dangling nucleotides can stabilize RNA structures. The greatest stabilization, 1.7 kcal mol^{-1}, is achieved with dangling A and G nucleotides stacking against a terminal 5′-GC-3′ base pair (Burkard et al. 1999). In the cases of dangling C and U nucleotides, the stability of the resulting helices are increased by 1.2 kcal $mol^{-1}$and 0.8 kcal mol^{-1}, respectively. This stabilization has been correlated to the degree of screening of the end base pair by the dangling nucleotide (Isaksson and Chattopadhyaya 2005). It has been reported that a dangling adenosine nucleotide has been used to stabilize a terminal base pair and facilitate the detection of the exchangeable NMR signals from the terminal base pair (Huang et al. 2001). Here we further examine the effect of dangling nucleotides on the NMR signals of exchangeable protons in the terminal base pairs of RNA stem-loop structures. This is done by comparing the spectra of an RNA molecule that shows considerable imino signal loss due to breathing motions to the same molecule containing 3′ dangling A, C, G, or U nucleotides.

Materials and methods

RNA sample preparation

Milligram quantities of RNA samples were prepared by in vitro transcription using histidine tagged *E. coli* T7 RNA polymerase and an oligodeoxynucleotide template (ACGT Inc.; Toronto ON) (Milligan and Uhlenbeck 1989). The crude RNA was purified using preparative scale denaturing (8 M urea) 20% polyacrylamide gel electrophoresis, the band of interest was identified using UV backshadowing, excised from the gel, the RNA was then separated from the gel by electroelution (Wyatt et al. 1991). The RNA was extensively exchanged first with 1 M NaCl, then water using YM-3 centricon devises (Amicon). RNA concentrations were determined by absorbance spectroscopy using the extinction coefficients (ε_{260}) of 171.9 mM^{-1} cm^{-1}, wild type; 180.9 mM^{-1} cm^{-1}, dangling-U; 182.8 mM^{-1} cm^{-1}, dangling-G; 178.9 mM^{-1} cm^{-1}, dangling-C; 185.7 mM^{-1} cm^{-1}, dangling-A. Yields of purified RNA were typically 3.3 mg per 15 ml transcription reaction. Prior to the start of NMR analysis, the RNA samples were heated to 95°C for 3 min, and then rapidly cooled in ice water.

Nuclear Magnetic Resonance Spectroscopy

NMR data was acquired on a 600 MHz Bruker Avance spectrometer using a ^{1}H-^{13}C-^{15}N triple resonance probe equipped with triple axis magnetic field gradients. NOESY spectra (Jeener et al. 1979; Macura and Ernst 1980) were obtained using a mixing time (τ_m) of 200 ms as a matrix of 4096 × 760 data points. All NMR experiments were recorded in 90% H_2O/10% $^{2}H_2O$ at 5°C at RNA concentrations of 1.0 mM. Water suppression was achieved through the use of the WATERGATE sequence (Piotto et al. 1992) or the use of the jump and return sequence (Plateau and Maurice 1982). NMR data were processed and analyzed on Linux workstations using the nmrPipe (Delaglio et al. 1995) and NMRView (Johnson and Blevins 1994) software packages, respectively.

Results

The fdnG SECIS RNA is monomeric

The RNA molecules used in this study (Fig. 1) are derived from the selenocysteine incorporation sequence (SECIS) found in the mRNA of the *Escherichia coli* gene *fdnG*. This RNA hairpin directs the incorporation of the rare amino acid selenocysteine into the enzyme formate dehydrogenase N (Kromayer et al. 1996) through its interaction with the special elongation factor B (SelB). The fdnG SECIS RNA is predicted to form a hairpin structure on the basis of a nuclease protection study (Huttenhofer et al. 1996). The structure of related SECIS molecules from the *E. coli fdhF* and *M. thermoacetica fdhA* mRNA were determined by

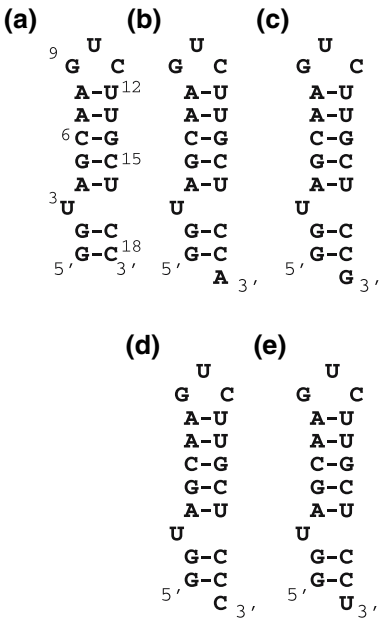

Fig. 1 RNA constructs used in this study. The sequences are based on the selenocysteine incorporation sequence (SECIS) from the mRNA of the *fdnG* gene that encodes for the *Escherichia coli* enzyme formate dehydrogenase N

NMR methods, confirming their hairpin structure (Fourmy et al. 2002; Beribisky et al. 2007).

The monomeric nature of the wild type *fdnG* SECIS RNA used in this study, and those with 3′ unpaired adenosine (fdnG-A), guanosine (fdnG-G), uridine (fdnG-U) and cytidine (fdnG-C) nucleotides was confirmed by native polyacrylamide gel electrophoresis. Samples of the 18 nt wild type fdnG SECIS at 0.5 mM migrated a similar distance as other hairpin RNA molecules of the same size and only very small amounts of non-monomeric *fdnG* SECIS RNA were observed (data not shown) confirming the *fdnG* SECIS RNA as a hairpin in solution. Additionally, the TOCSY spectrum of the wild-type *fdnG* SECIS and fdnG-A SECIS showed the expected ten H6–H5 resonance correlations indicating a single hairpin conformation, and ruling out multiple hairpin conformations being present.

Assignment of the fdnG SECIS imino protons

The NMR assignments of the imino protons in the fdnG SECIS were obtained from a 2 mM sample of wild type fdnG SECIS RNA and a 3 mM fdnG-A SECIS sample (Fig. 2). All imino resonances were observed in the fdnG-A SECIS, with the exception of the U3–H3 proton. This bulged out residue is free to exchange with solvent. In addition to the U3 imino, the signals from G1, G2 and U16 are missing in the spectrum of the wild type hairpin. It is important to note that resonances observed in both the wild type fdnG and the fdnG-A spectra occur at the same chemical shift values indicating that the addition of the unpaired 3′ adenosine stabilizes, but does not significantly perturb the structure of the hairpin.

NMR spectra of the fdnG SECIS RNA with dangling nucleotides

NOESY spectra were acquired at 5°C in H_2O for each of the five fdnG SECIS molecules. The sample volume was adjusted to ensure a constant RNA concentration of 1.0 mM (Fig. 3). The spectrum of the wild type fdnG SECIS shows only the presence of imino resonances at the top of the stem (G9–H1, G10-H1, U12-H3, U13-H3, G14-H1 and G5-H1). The imino protons for G1, G2 and U16 are not observed, most likely due to stem breathing motions. The absence of the U16 imino is unusual as it is the third base pair from the bottom of the stem, relatively far removed from the breathing motion at the base of the stem. However, motions associated with the bulged U3 nucleotide likely increases the effects of breathing motions at this site and the consequent attenuation of the imino signal of U16.

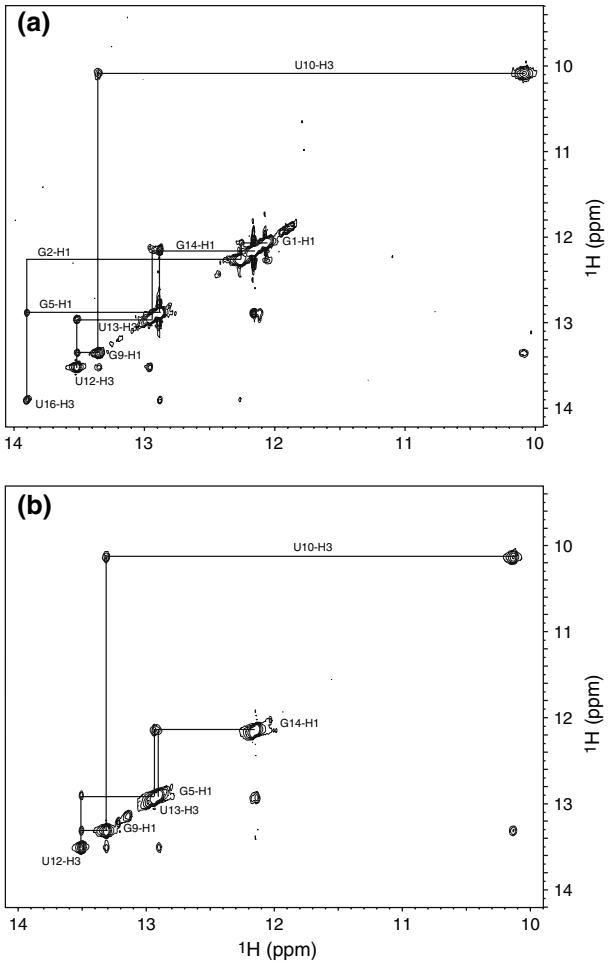

Fig. 2 NOESY spectra of the (**a**) fdnG-A SECIS RNA and the (**b**) wild type fdnG SECIS RNA. Shown in both spectra is the downfield portion of a NOESY experiment for the RNA in 90% H_2O/10% ^2H_2O acquired at 5°C. The assignments of the imino protons in both constructs are traced out. In the molecule containing the dangling-A, imino signals from G1, G2 and U16 are visible that not seen in the wild type construct

A comparison of the imino region of the NOESY spectra of the fdnG SECIS sequences with 3′-dangling nucleotides show a varying degree of improvement compared to wild-type sequence (Fig. 3). The spectra of the dangling-A and dangling-G samples showed the most improvement and gave rise to similar spectra. In both the fdnG-A SECIS and the fdnG-G SECIS samples the previously unobservable imino protons from the G1, G2 and U16 nucleotides are now clearly seen. G1-H1 and G2-H1 are close in chemical shift and their resulting cross peak in the NOESY experiment is near the diagonal and may be difficult to observe (Fig. 2), however the improvement in NOE signals to upfield resonances is much more clear (Fig. 3). The spectrum of the dangling-U construct showed a lower degree of improvement in signal detection compared with the fdnG-A and fdnG-G samples. The G1 and G2 imino protons are

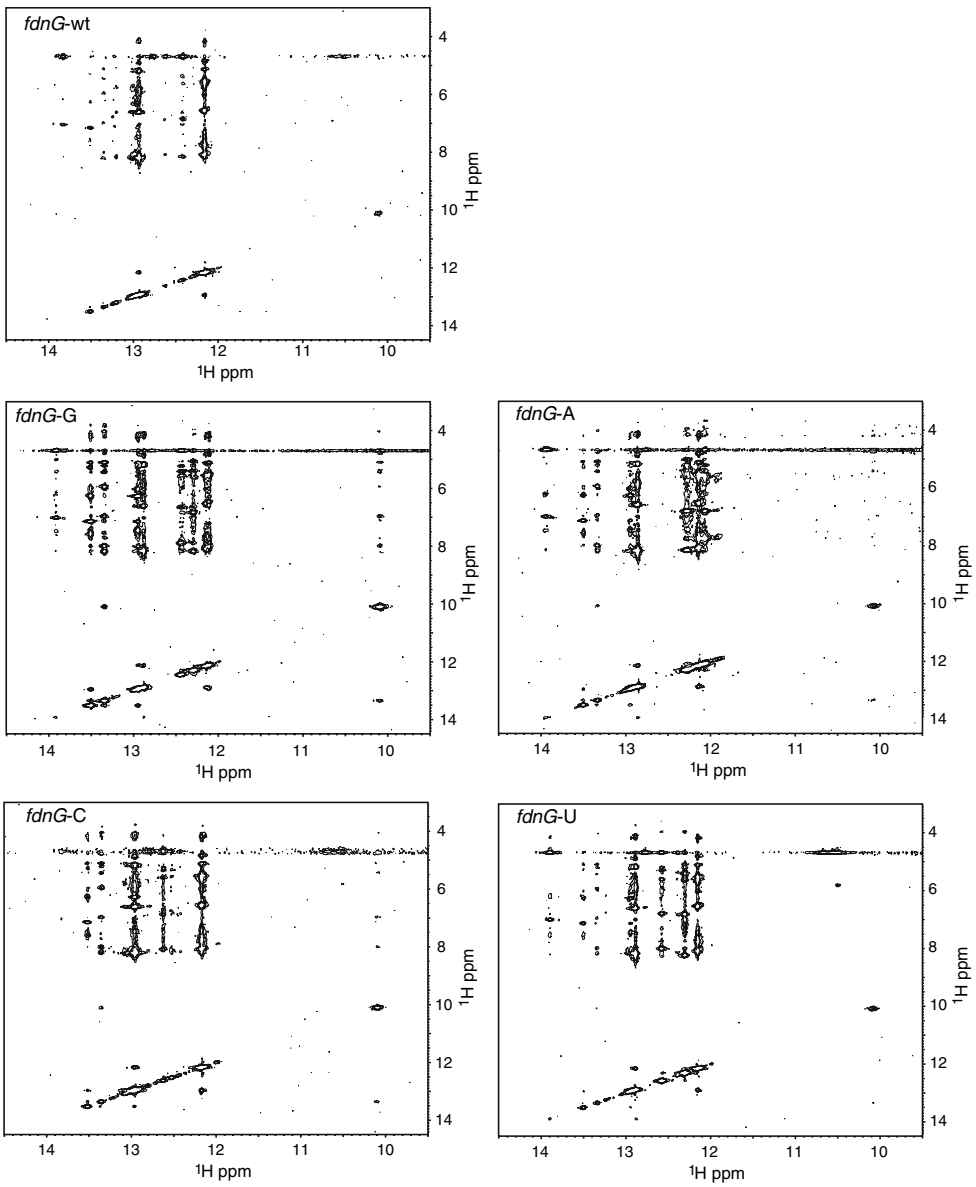

Fig. 3 NOESY spectra of the wild type *fdnG* SECIS RNA and the *fdnG* SECIS RNA with different dangling 3′ nucleotides. Shown is the region where the imino-aromatic/ribose NOEs are observed. Spectra were acquired in 90% H_2O/10% ^2H_2O at 5°C. All data were acquired at 1 mM RNA concentration and all spectra are plotted at the same signal to noise level

observed in the fdnG-U construct, but the U16 imino proton is only weakly observed in this sample. The spectrum of the dangling-C molecule provided a relatively small increase in the detection of the terminal imino protons. NOEs from the imino protons to upfield resonances (Fig. 3) are more intense in the fdnG-C SECIS sample than seen in wild type fdnG SECIS, but no new imino-imino NOEs are observed (Fig. 3). In all samples the imino proton from U3, which is bulged out from the stem, is not observed.

Temperature dependence of imino peaks

As the addition of a dangling 3′ nucleotide is predicted to increase the stability of an RNA helix, we wanted to investigate the effect of temperature on the imino region of the 1D ^1H NMR spectra of both fdnG-wt and fdnG-A. Spectra of these two RNA molecules were recorded using the jump and return sequence at increasing temperatures and are shown in Supplementary Figure 1. As seen with the 2D NOESY the fdnG-A spectrum contains signals from G1 and G2 at the base of the stem, signals not observed in spectrum of the fdnG-wt sequence. With the use of the jump and return sequence, the signal from U16 is now observable in the spectra of both molecules.

As the temperature is increased the signals in both sets of spectra begin to get weaker due to solvent exchange, but clearly in the spectra of the fdnG-A RNA the imino signals are observable at higher temperatures than in fdnG-wt. By 35°C, the imino signals from fdnG-wt have nearly

disappeared, while for the fdnG-A RNA the majority of the imino resonances are still observed at this temperature, and remain observable up to 50°C.

Discussion

These results reveal that the increase in quality of spectra of the fdnG SECIS molecules with dangling 3′ nucleotides is directly related to their predicted increase in thermodynamic stability (Burkard et al. 1999). That is, dangling nucleotides that contribute the most to the increased stability of RNA hairpins produce the best NMR spectra (Figs. 2, 3). Both dangling A and G nucleotides were shown to provide a 1.7 kcal mol^{-1} increase in stability, dangling U provides a 1.2 kcal mol^{-1} increase, and dangling C provides 0.8 kcal mol^{-1} increase. This correlation implies a direct relationship between the stability of the RNA molecule studied and the improved imino proton NMR detection. This correlation is consistent with the addition of a nucleotide that stacks against the terminal base pair and stabilizes its structure, resulting in reduced breathing motions near the base of the stem. A slowing of breathing motions would result in better detection of the exchangeable imino protons as the exchange rate of these protons with the bulk solvent is consequently slowed.

It should be noted that the stabilization of 1.7 kcal mol^{-1} for 3′ dangling A and G nucleotides against a 5′-GC-3′ base pair is the maximum stabilization observed for all the 32 possible different combinations of both 3′ and 5′ dangling nucleotides and four possible terminal base pairs. It is also the easiest combination to incorporate when RNA is produced in vitro using T7 RNA polymerase where it is advantageous to start the sequence with two guanosine nucleotides and therefore there is often an existing terminal 5′-GC-3′ base pair.

The stacking of the dangling nucleotide against the last base pair is consistent with the structural data for the fdnG-A SECIS RNA. The fdnG-A SECIS construct was selected for further structural analysis and its structure has been determined by NMR methods (Amborski and Johnson, unpublished data). Though not well ordered, we have found that the 3′ dangling A nucleotide is stacked upon the terminal base pair. NOE correlations were observed between C18-H6 and A19-H1′, and A19-H8 and A19-H1′, these NOEs are consistent with A19 being part of the A-form helix of the stem.

The use of dangling nucleotides to improve NMR spectral quality as opposed to adding additional base pairs should prove especially useful in cases where it is desirable to keep the NMR spectrum of the RNA molecule as simple as possible, or to keep the molecule studied as small as possible. This could arise when a large RNA is being studied or when the RNA is part of a larger assembly such as a protein-RNA complex. The work presented here was performed exclusively with RNA molecules, but it may be possible to extend the use of dangling nucleotides to improve the NMR spectra of DNA molecules. In the case of DNA the most favorable addition is a 5′ dangling adenosine next to a 5′-CG-3′ base pair which provides a stabilization improvement of 0.96 kcal mol^{-1} (Bommarito et al. 2000). This is 0.16 kcal mol^{-1} more stabilizing than in the case of the dangling C construct used here that led to improvements in the spectrum of the SECIS hairpin.

In conclusion, we presented here a method for improving the NMR signal detection of RNA imino protons using 3′-dangling nucleotides. We recommend the use of either an unpaired adenosine or guanosine as both nucleotides seem to improve the spectra of the fdnG SECIS more significantly than the use of either cytidine or uridine.

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