Direct Evidence for Watson−Crick Base Pairs in a Dynamic Region of RNA Structure

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RNA molecules are renown for their exhibition of conformational exchange in regions of structure that are important for function. The crucial role of conformational flexibility in RNA function has been clearly evidenced in structural studies of catalytic RNA ribozymes, RNA−RNA, and protein−RNA interactions.[1−8] The ability to determine and characterize transient base pairing in these regions would afford greater insight into the structure, dynamics, and ultimately, the function of RNA molecules. Recently, direct physical evidence for the existence of hydrogen bonds (H-bonds) across nucleic acid base pairs has been established by the measurement of trans-H-bond scalar $^{2h}J_{NN}$ and $^{2h}J_{HN}$ couplings.[9−13] Trans-H-bond couplings, $^{3h}J_{NiCj}$,[14−17] have also been used to identify and determine the strengths of H-bonds associated with amide protons in proteins. In nucleic acids, the magnitude of $^{2h}J_{NN}$ and $^{2h}J_{HN}$ scalar couplings has been shown to correlate well with H-bond length, $^{1}J_{NH}$ couplings and the chemical shift of the imino proton.[11,18] In addition to providing a physical measure of H-bond strength, these scalar correlations also identify H-bond coupled nuclei. As a result, trans-H-bond couplings provide both unique nuclear magnetic resonance (NMR) restraints for defining nucleic acid structure and novel assignment pathways for defining base pairing and tertiary H-bonded interactions.

In dynamic regions of RNA structure where exchangeable imino and amino protons are unobservable, however, conventional NMR correlation experiments, as well as recently developed methods for measuring trans-H-bond $^{2h}J_{NN}$ scalar couplings,[9−13] fail since these techniques rely on the direct detection of the exchangeable protons. Recently, it has been shown that $^{2h}J_{NN}$ scalar couplings could be measured across a H-bond between two histidine side chains in myoglobin mediated by an exchange-broadened proton.[19] Similarly, it has been shown that H-bonds in DNA base pairs mediated by amino protons, which are exchange broadened due to intermediate rotational exchange about the exocyclic CN bond, can also be measured.[20] Here we report for the first time the measurement of trans-H-bond $^{2h}J_{N1N3}$ scalar couplings for Watson−Crick AU base pairs (Figure 1) in a dynamic region of RNA structure where the H-bond mediating imino proton resonances are exchange broadened beyond detection due to base pair 'breathing.' Through the measurement of $^{2h}J_{N1N3}$ scalar couplings across N1- - -H3−N3 hydrogen bonds (H-bonds), unambiguous evidence for transient Watson−Crick AU base pairs has been established where previously these base pairs had eluded detection using conventional NMR methods.

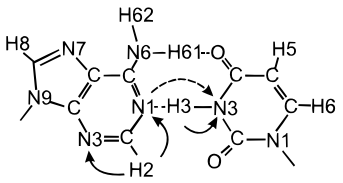

Figure 1. Adenosine-uracil base pair schematic with the trans-H-bond $^{2h}J_{NN}$ correlation between adenosine N1 and uracil N3 nitrogens shown by a dashed arrow. The J_{NN} COSY correlations used to establish AU base pairing in the dynamic upper stem helix of the CopA29 RNA hairpin are shown by solid arrows.

Trans-H-bond $^{2h}J_{N1N3}$ scalar couplings were measured for the RNA hairpin, CopA29, which is derived from the R1 plasmid encoded RNA transcript, CopA,[21] using a dual-detected uracil-H3/adenine-H2 (UH3/AH2) J_{NN} HNN-COSY (see Supporting Information for details), which is an adapted version of the previously described J_{NN} HNN-COSY.[9] The experiment simultaneously correlates both the exchangeable uracil H3 proton to uracil N3 H-bond donor and adenosine N1 H-bond acceptor nitrogens, and the non-exchangeable adenosine H2 proton to adenosine N1 H-bond acceptor and uracil N3 H-bond donor nitrogens in AU base pairs that are $^{2h}J_{N1N3}$ scalar coupled (Figure 1). CopA29 has a predicted secondary structure (Figure 2A) that is comprised of a lower and upper helical stem region separated by a single bulged G nucleotide and a six-nucleotide loop. In disagreement with the predicted secondary structure, AU base pair associated imino proton resonances have been observed for only the lower stem base pairs in this RNA using conventional one- and two-dimensional NMR techniques. Figure 2B shows selected regions of the JNN-COSY experiment applied to a uniformly ^{15}N-labeled 29 nucleotide CopA29 hairpin at 25 °C. In the CopA29 RNA hairpin, five H-bonded AU base pairs are expected based on the predicted secondary structure. Inspection of the adenosine H2 → uracil N3 cross-peak region of the UH3/AH2 J_{NN} HNN-COSY experiment clearly reveals seven, rather than the expected five, correlations. Four H2,N3 correlations are assigned to the lower stem AU base pairs (U4-A27, U5-A26 and U6-A25) and three H2,N3 correlations are assigned to the upper stem base pairs (U21-A10, U12-A19). Note that the additional correlations observed for base pairs U21-A10 and U6-A25 arise from two different conformational states of the bulge that are in slow-exchange on the NMR time scale. In contrast, the H3 detected regions of the experiment show only the four H3,N1 correlations associated with the AU base pairs in the lower stem. Thus, these measurements directly demonstrate AU base pairing

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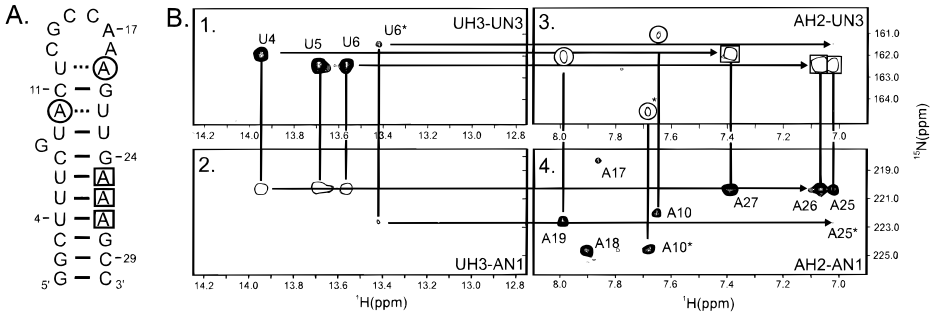

Figure 2. (A) Secondary structural scheme for the CopA 29mer RNA hairpin. H-bonded base pairs for which imino protons are observed are connected by solid lines. Base pairs, that are predicted using RNA structure prediction programs and supported by chemical and enzymatic probing data,[26] but for which no imino proton is observed are indicated by dashed lines. Adenosine bases involved in AU base pairs where both an H3 imino resonance and $^{2h}J_{N1N3}$ coupling are observed are boxed and those where only a $^{2h}J_{N1N3}$ coupling is observed are circled. (B) Selected regions of the UH3/AH2 dual-detected J_{NN} HNN-COSY experiment applied to a 0.75 mM sample of a uniformly ^{15}N-labeled CopA29 hairpin in 90% H_2O/10% D_2O, 1 mM cacodylate [pH = 6.5] and 25 mM NaCl at 25 °C. Region 1 shows the uracil H3 → uracil N3 diagonal peaks and region 2, the uracil H3 → adenosine N1 cross-peaks observed in the experiment. Region 4 shows the adenosine H2 → adenosine N1 diagonal peaks and region 3, the adenosine H2 → uracil N3 cross-peaks that are uniquely observed in the UH3/AH2 dual-detected J_{NN} HNN-COSY experiment. Negative cross-peaks, corresponding to correlations generated by scalar ^{15}N−^{15}N couplings, are displayed with a maximum of three contours. Diagonal peaks are labeled with assignments. ^{1}H,^{15}N diagonal and cross-peaks are connected by solid arrows. In region 3, H2 → N3 cross-peaks arising from adenosines involved in AU base pairs where a uracil H3 proton is observed are boxed and those arising from adenosine involved in AU base pairs where an H3 proton is not observed are circled.

Table 1. $^{2h}J_{NN}$ Scalar Coupling Constants (Hz) Measured for the CopA29 Hairpin (T = 25 °C)[a]

detected proton	U_4-A_{27}	U_5-A_{25}	U_6-A_{24}	U_{10}-A_{16}	U_{10}-A_{16}[c]	U_{12}-A_{19}
uracil H3	6.15 ± 0.5	6.35 ± 0.5	6.45 ± 0.5	*b*	*b*	*b*
adenine H2	6.05 ± 1.0	7.25 ± 1.0	6.95 ± 1.0	5.90 ± 1.0	5.95 ± 1.0	5.7 ± 1.0

[a] Errors are standard deviations estimated from the fitting procedure. [b] Imino proton resonance was not observed due to line broadening. [c] Two sets of correlations were measured for U10-A16 since two states are observed as a result of conformational heterogeneity of the bulge.

in a region of RNA structure that is involved in intermediate conformational exchange.

Table 1 lists the $^{2h}J_{N1N3}$ coupling constants measured for the CopA29 hairpin. The magnitudes of the $^{2h}J_{N1N3}$ couplings were measured and fit as described previously.[9] The $^{2h}J_{N1N3}$ couplings for the three AU base pairs located in the lower stem of CopA29, measured using both adenosine H2 and uracil N3 cross and diagonal peaks, are in good agreement with each other and with previously determined $^{2h}J_{N1N3}$ couplings observed in RNA.[9] These three AU base pairs show the expected H3 imino proton correlations in one-dimensional ^{1}H spectra, as well as in ^{15}N HSQC and ^{1}H NOESY spectra (data not shown), and so the observed magnitude of the trans-H-bond couplings were expected. The average magnitude of the $^{2h}J_{N1N3}$ couplings for the two AU base pairs located in the upper stem of CopA29, measured using solely adenosine H2 cross and diagonal peaks, are only slightly smaller (5.85 Hz ± 1.0 Hz) than for AU base pairs where an H3 imino proton was observed (6.5 Hz ± 0.75 Hz). Imino proton correlations for these AU base pairs were not observed in one-dimensional ^{1}H spectra, nor in either ^{15}N HSQC or ^{1}H NOESY experiments (data not shown), due to exchange broadening of the proton resonances.

Since *J*-couplings are a time averaged measurement, the observation of $^{2h}J_{N1N3}$ scalar couplings that are of the same magnitude for these AU base pairs suggests a 'breathing' model for the exchange broadening of the imino protons, where the dominant state(s) of the AU base pairs are H-bonded. Intermediate fluctuations on the micro- to millisecond time scale in the H-bonded state could explain why the imino protons are not observed; while $^{2h}J_{N1N3}$ couplings, which report on the time average over all states of the system, can be measured. In addition, the observation of relatively sharp adenosine H2 proton and N3 nitrogen resonances in our experiments, even when imino proton resonances are unobservable and adenosine N1 nitrogen resonances are exchange broadened, suggests that the observed "breathing" may be a result of small amplitude variations in the base pairs that perturb the H-bond conformations. Recent ab initio calculations,[11] which predict large changes in the imino proton chemical shift as a function of rather small changes (<0.5 Å) in the r_{N1N3} distance, support such a breathing model for the "selective" broadening of resonances involved in base pair H-bonding. Although other base resonances, such as adenosine H2 and N3, would experience the same conformational exchange due to such base pair dynamics, the effect may not be detected because the difference in chemical shift for these resonances in the different states is not significant. Similar base pair breathing models have been invoked previously to explain the observation of regular A-form helical base stacking within bulges and loops and at the end of helices in RNA where expected imino proton resonances were not detected.[22−24]

In summary, AU base pairs have been defined using $^{2h}J_{N1N3}$ scalar couplings in a region of RNA structure where exchangeable proton resonances were broadened beyond detection. These measurements have provided a new restraint for defining base pairing in dynamic regions of RNA structure and physical insight into base pair dynamics. For the CopA hairpin studied here, the determined dynamics and structure of the upper helix may have a direct implication for how this antisense RNA binds its complementary target RNA with rapid association kinetics.[25]

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Supporting Information Available: Figure and experimental details for the J_{NN} HNN-COSY pulse scheme used to obtain the NMR spectra in Figure 2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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