RNA Dynamics Hot Paper

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Efficient Detection of Hydrogen Bonds in Dynamic Regions of RNA by Sensitivity-Optimized NMR Pulse Sequences**

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In recent years, RNA has emerged as a central player in the regulation of gene expression in all kingdoms of life, ranging from sRNA in bacteria to miRNAs and long noncoding RNAs in eukaryotes.[1–4] While the sequences and biochemical properties of these RNAs are typically well established, their three-dimensional structures and structure-function relationship are often poorly characterized. Hydrogen bonds (H-bonds) define the secondary and tertiary structures of nucleic acids (Figure 1 a). Thermodynamics-based structure prediction and covariational methods are useful theoretical approaches[5–8] but require experimental validation. Biochemical structure probing methods[7] can provide such information, even for very large RNAs, but the analysis is complicated by alternate structures or by the presence of conformational dynamics. NMR-spectroscopy allows the identification of base pairs in nucleic acids based on the detection of H-bonds involving imino protons, which are shielded from exchange with bulk water. NOE-correlations of imino protons establish base pairing in helical regions of nucleic acids,[10,11] but only provide indirect evidence for H-bonds. Since 1998, the observation of scalar couplings across H-bonds in HNN-COSY experiments[12] has enabled the efficient and direct identification of hydrogen bonding and base pairing in nucleic acids. The size of these cross H-bond scalar couplings ($^{2}J_{NN} \approx 6$–7 Hz) allows efficient magnetization transfer to correlate chemical shifts across base pairs.[12] Various experiments have been designed to measure *J*-couplings across hydrogen bonds in DNA and RNA.[13–21] The sensitivity of HNN-COSY correlations has been optimized by the use of BEST relaxation enhancement and TROSY for large RNAs.[22] However, most of these approaches detect exchangeable protons. Therefore, their applicability is limited to regions of stable secondary structure, as imino protons of weak base pairs are often broadened beyond detection because of chemical exchange with bulk water and conformational dynamics, especially at room temperature or above.

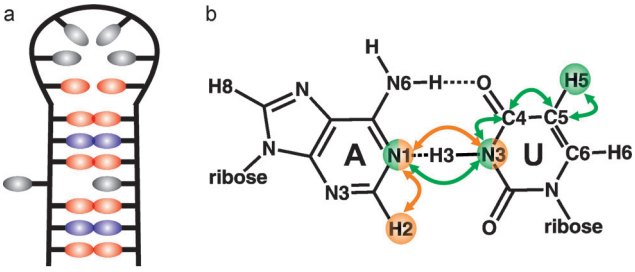

Figure 1. a) A schematic nucleic acid hairpin with an unstructured loop and a dynamic stem region. Bases not involved in base pairing are shown in gray, Watson–Crick base pairs in blue. Hydrogen bonds of weak base pairs (red) are difficult to detect by current methods, as they are next to nonhelical regions and lack stabilizing stacking interactions. b) Magnetization transfer pathways for the detection of cross hydrogen bond *J*-couplings in base pairs.

To overcome these limitations and enable the detection of *J*-couplings across H-bonds in weak base pairs, experiments that detect non-exchangeable, carbon-bound protons have to be used. The long-range (lr) HNN-COSY experiment has been proposed for direct detection of A:U/T base pairs, where the adenosine (A) N1 acceptor nucleus is linked to the H2 proton by the 15 Hz coupling $^{2}J_{H2N1}$,[9,23,24] or where the uracil (U) N3 donor nucleus is linked to the H5 proton via the 5 Hz coupling $^{3}J_{C5N3}$[25] (Figure 1 b). Pulse sequences have been developed for the detection of non-Watson–Crick base pairs involving non-exchangeable protons.[26,27,20,25] However, the poor sensitivity due to alternative magnetization pathways and relaxation during the long transfer periods limits the application of these experiments to small and highly concentrated RNAs. Here, we show that large sensitivity improve-

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ments can be obtained for H-bond correlation experiments by the optimization of magnetization transfers combined with the detection of non-exchangeable protons. The substantially improved sensitivity of these experiments enables the detection of weak and transient base pairs in dynamic regions of large RNA molecules.

We have used optimized band-selective pulses to improve the intrinsically poor sensitivity of the lr HNN-COSY experiment. Because of alternative magnetization transfer pathways 75% of the signal is lost in the original implementation of the experiment (Figure 2a and Supporting Information). This magnetization can be recovered using selective inversion and refocusing ^{15}N pulses during the ^{1}H-^{15}N and ^{15}N-^{15}N transfer steps, respectively (Figure 2, Figure S1). Further sensitivity improvement is achieved when the experiment is recorded in aqueous buffer by using BEST relaxation enhancement with the imino proton[22] (Figure S1). In Figure 2c the original hard pulse experiment, a version employing ^{15}N-selective pulses and the new BEST-selective long-range (BESTsellr) HNN-COSY experiment are compared. The three experiments were recorded for a 24-nt hairpin with a stable GNRA tetraloop derived from the stem region of pri-miRNA 18a (18a stem; nt = nucleotides). As expected, a four-fold sensitivity enhancement is observed for the selective pulse version (without BEST-enhancement). The BESTsellr experiment (combining selective ^{15}N pulses and BEST) shows an additional two-fold enhancement, thus yielding an overall eight-fold improved signal-to-noise compared to the original implementation of the experiment. This substantial sensitivity improvement corresponds to a 64-fold reduction in measurement time and enables the application of this experiment to higher molecular weight RNAs. We thus applied the BESTsellr HNN-COSY experiment to the 76-nt tRNA^{Val} at 318 K. As seen in Figure 3, all of the expected A:U base pairs are observed here, whereas the standard lr HNN-COSY experiment did not yield any signal after two days of experimental time (data not shown). Using an imino-detected BEST-TROSY HNN-COSY experiment[22] only a single A:U correlation can be observed at this temperature (Figure 3).

A drawback of the lr HNN-COSY and similar experiments is that only A:U base pairs are observable.[9,28,25] We therefore developed a novel sensitivity-improved pyrimidine (Py) H(CC)NN-COSY experiment, which enables the simultaneous detection of A:U and G:C base pairs. This is achieved by out-and-back magnetization transfer from the H5 of U/C to C5 to C4 to N3 and across the H-bond (Figure 4a, Suppl. Figure S2). With this approach any base pair involving N–H···N-type H-bonds of pyrimidines can be detected. Since the non-exchangeable H5 protons are observed, the experiment allows the detection of direct H-bond correlations even in dynamic regions and/or at elevated temperature. The magnetization transfer efficiency is optimized by a number of band-selective pulses (Figure S3). The sensitivity of this pulse sequence is comparable to the lr HNN-COSY experiment,[9]

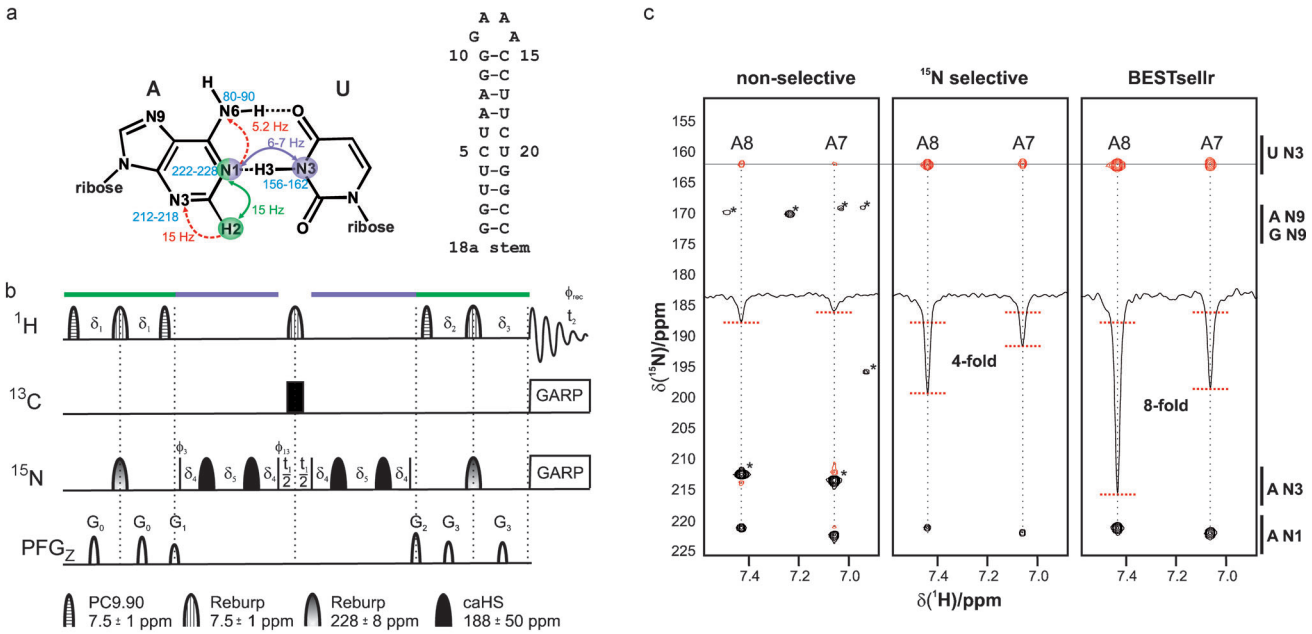

Figure 2. BEST-selective long-range HNN-COSY experiment (BESTsellr HNN-COSY) with selective ^{15}N inversion and refocusing pulses. a) Desired magnetization transfer pathways are indicated by green/purple lines. Undesired pathways, which are suppressed by selective ^{15}N pulses, are indicated by red dotted lines. Chemical shift ranges and *J*-couplings of relevant nuclei are indicated. The secondary structure of the 18a stem RNA is shown on the right. b) Pulse sequence of the BESTsellr HNN-COSY experiment. A detailed description of the parameters can be found in Figure S1. The different magnetization transfer steps are color-coded as in (a). c) Comparison of nonselective, ^{15}N-selective, and BESTsellr HNN-COSY[9] recorded for the 18a stem RNA in H_2O at 298 K. Comparison of the respective 1D slices shows that the selective approach increases sensitivity of the experiment four-fold, whereas introduction of relaxation enhancement by BEST yields an additional factor of two. The use of selective pulses also simplifies interpretation of the spectra, since undesired correlations (signals marked "*" in the nonselective spectrum) are suppressed. Measurement time for each spectrum was 7 h.

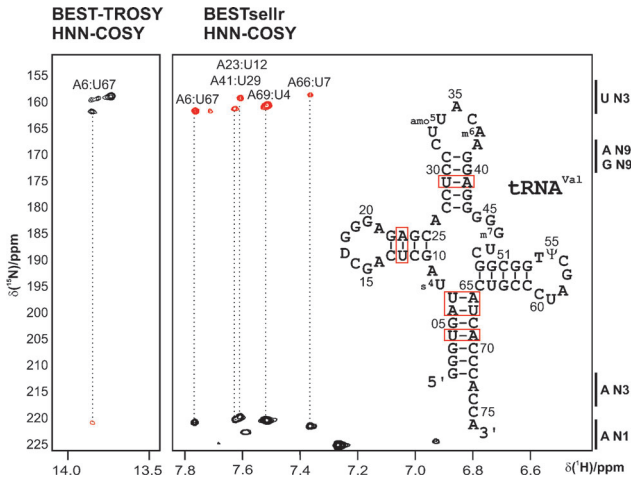

Figure 3. Comparison of an imino-detected BEST-TROSY HNN-COSY experiment (left) with the BESTselIr HNN-COSY, recorded at 318 K on the 24 kDa tRNA^{Val} in H_2O for 12 h. Only one A:U base pair is observed in the HNN-COSY because of fast exchange of the imino protons with bulk water at elevated temperatures, whereas all expected A:U base pair correlations are observed for the long-range experiment.

even though it features two additional transfer steps (Supporting Information). This is achieved by using the large $^1J_{CH}$ and $^1J_{CC}$ coupling constants for the first two transfer steps, where magnetization resides on fast relaxing spins, whereas during the long ^{13}C-^{15}N and ^{15}N-^{15}N transfer steps magnetization resides on the slowly relaxing C4 and N3 nuclei, respectively.

We applied this experiment to three RNAs, which differ in molecular weight and the presence of internal dynamics (Figure 4b; Figure S4). Figure 4b shows NMR spectra for the 18a stem. A tandem U:C mismatch destabilizes the duplex region. As a result, no base pair correlations are observed in the standard imino-detected HNN-COSY experiment at 308 K (data not shown) and only signals for the three nonterminal G:C base pairs are observed at 298 K (Figure 4b). In contrast, the Py H(CC)NN-COSY experiment (Figure 4b) shows all expected G:C and A:U base pair correlations even at 298 K, including the terminal G1:C24 base pair. Importantly, the weak A:U base pair adjacent to the U:C mismatch is only observed in the Py H(CC)NN-COSY and not detected in the standard imino-detected HNN-COSY experiment even at temperatures as low as 278 K (Figure S5). Based on the sensitivity of this experiment one can exclude the possibility of the tandem U:C mismatch forming N–H···N H-bonds, because no corresponding correlations are observed in the Py H(CC)NN-COSY. This is consistent with crystallographic data on tandem U:C mismatches which show the formation of a water-mediated H-bond between the two nitrogen atoms.[29,30] All expected base pairs (except a terminal G:C) were observed in two other RNAs, a 14-nt UUCG tetraloop hairpin, and a Xist A-repeat 14-nt hairpin[31,32] (Figure S4), which further demonstrates the utility of the new experiment. Note, that in case of poor dispersion of the H5 signals, the C5 chemical shifts could be evolved to resolve signal overlap in a 3D version of the experiment.

The set of NMR experiments presented here enables the direct detection of H-bonds even for weak and dynamic base pairs in nucleic acids. Combining BEST relaxation enhancement and band-selective ^{15}N pulses provides an eight-fold improved sensitivity for the lr HNN-COSY experiment. This enabled the detection of all five A:U base pairs in the (24 kDa) 76-nt tRNA^{Val}, whereas only one A:U base pair was observed with the BEST-TROSY HNN-COSY experiment. The novel Py H(CC)NN-COSY experiment enables the simultaneous detection of weak A:U and G:C base pairs in D_2O even at 298 K or above independent of the exchange rate of the imino proton involved in the H-bond. Interestingly, the $^2J_{NN}$-couplings determined here indicate only slightly reduced couplings because of dynamic averaging[33–35] of weak base pairs, even in cases where imino protons exhibit severe line-broadening (Table S1).

In contrast to conventional HNN-COSY experiments, which are typically recorded at 278 K or below, the direct H-bond detection of base pairs is now possible at the same temperature used for measurement of NMR-derived structural data in RNAs (i.e. NOEs, residual dipolar and *J*-couplings). Finally, application of the BESTselIr HNN-COSY experiment at room temperature and above enables the direct detection of cross H-

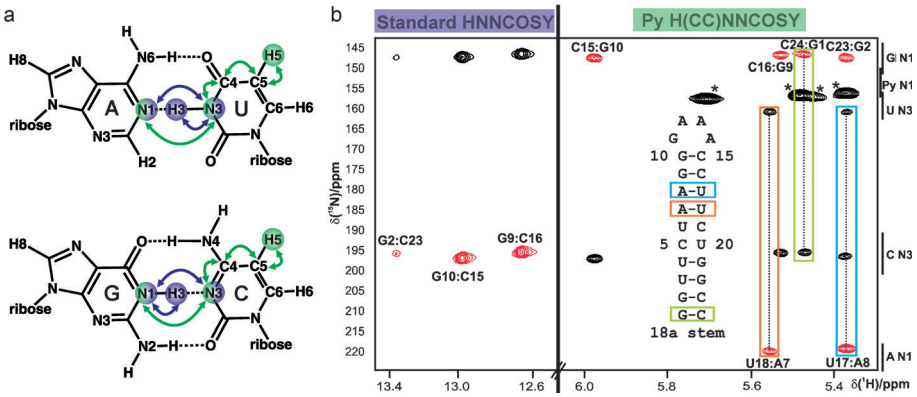

Figure 4. Pyrimidine (Py) H(CC)NN-COSY experiment for the simultaneous detection of A:U and G:C base pairs. a) Out-and-back magnetization transfer steps for the standard (purple) and Py H(CC)NN-COSY (green) experiemnts are illustrated for A:U and G:C base pairs. Details for the pulse sequence and experimental settings are provided in Figure S2. b) Imino region of the standard HNN-COSY recorded for the 18a stem RNA in H_2O and the H5 region of the Py H(CC)NN-COSY experiment recorded in D_2O, each measured for about 3 h at 298 K. Only the three nonterminal G:C base pairs and no A:U base pair are observed in the standard HNN-COSY experiment, while all Watson–Crick (4 G:C, 2 A:U) base pairs are observed with the new Py H(CC)NN-COSY, including the A:U base pair directly adjacent to the U:C mismatch (orange box). The signals at 157 ppm (*) are intraresidual correlations to the N1 atom of pyrimidine. As G:U base pairs lack N–H···N-type H-bonds they are not detectable in the experiment.

bond correlations for secondary structure analysis of larger RNAs, where higher temperature is required for improved sensitivity and thus will be valuable for structural analysis and detection of weak base pairs.

Experimental Section

The following RNA samples were used: A 1.0 mM uniformly ^{13}C,^{15}N-labeled sample of 18a stem was prepared by in vitro transcription. The sample buffer contained 20 mM phosphate buffer, pH 6.5, 50 mM NaCl in 9:1 H_2O:D_2O. A sample of 0.5 mM ^{15}N-labeled native *E.coli* $tRNA^{Val}$ was prepared by overexpression in *E.coli* (BL21(DE3)) from the pVALT7 plasmid in M9 minimal media containing $^{15}NH_4Cl$ as the sole nitrogen source and purified as previously described.[36] The sample buffer contained 10 mM phosphate buffer, pH 7.0, 80 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$ in 9:1 H_2O:D_2O. Uniformly ^{13}C,^{15}N-labeled 14-mer UUCG tetraloop hairpin RNA standard[37] was purchased from Silantes (Munich, Germany) and measured in 20 mM KHPO4, pH 6.4, 0.4 mM EDTA in 9:1 H_2O:D_2O. Finally, a 0.8 mM uniformly ^{13}C,^{15}N-labeled 14-mer AUCG tetraloop hairpin derived from Xist RNA A-repeats r(GGCGCAUCGGCGCC) was prepared as described.[31,32] The sample buffer contained 10 mM phosphate buffer, pH 6.0, 100 mM NaCl, 0.02 mM EDTA, and 0.02 % NaN_3 in D_2O. The sample volume for all samples was 250 µL in susceptibility-matched NMR tubes (Shigemi, Japan). Samples were heated to 95 °C for 5 minutes followed by snap-cooling on ice to trap the kinetically favored intramolecular monomeric hairpin form over possible intermolecular dimers. For more information on data acquisition and processing see Supporting Information.

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