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# New RNA Labeling Methods Offer Dramatic Sensitivity Enhancements in $^2$H NMR Relaxation Spectra

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Many RNA molecules are inherently plastic and undergo conformational changes that are required for function.[1,2] Despite the importance of dynamics in this class of molecule, in general, detailed site-specific motional information is lacking. For example, although both pico- (ps) to nanosecond (ns) and micro- to millisecond time scale dynamics in proteins have been studied extensively by NMR spectroscopy, the motional properties of relatively few RNA molecules have been characterized using this technique.[3−10] Part of the problem stems from the fact that it is more difficult to prepare RNA molecules with a large number of isolated $^1$H−X two-spin system probes of dynamics than for proteins (where every backbone amide site is potentially available for study in $^{15}$N-labeled proteins). In addition, accurate characterization of dynamics by NMR is predicated on knowledge of the strengths of relaxation interactions; in dynamical regions of RNA and/or regions of irregular structure, chemical shift anisotropies that contribute to relaxation are often variable,[11] and contributions from chemical exchange can complicate analysis. With this in mind, our laboratory has recently developed an alternative approach for the study of ps−ns time scale dynamics in RNA that makes use of ~50% $^2$H, 100% $^{13}$C enrichment[12] and uses the deuteron as a probe.[8] Here, magnetization is transferred from $^1H_i$ on a sugar or base (C,U) to the vicinal hydrogen, $^2H_k$, via the scalar coupled pathway $^1H_i \rightarrow {}^{13}C_i \rightarrow {}^{13}C_k \rightarrow {}^2H_k$, the relaxation properties of $^2H_k$ are measured, and then the magnetization is returned to $^1H_i$ for detection. However, this approach selects only approximately 25% of the molecules that have the appropriate ($^1H_i$,$^{13}C_i$,$^{13}C_k$,$^2H_k$) labeling, and thus the sensitivity of experiments is compromised. Here we report a new labeling approach in which RNA sugars are synthesized enzymatically starting from U-$^2$H/$^{13}$C glucose, with substitution of $^1$H for $^2$H at the 2′ position achieved by carrying out the enzymology in $H_2O$[13] (Supporting Information). As described below, dynamics at the 1′, 3′, and 4′ sugar positions can be probed. The methodology is applied to a 1 mM sample of HIV-2 TAR RNA[14] with very significant gains in sensitivity, on the order of 5-fold, relative to our previous labeling scheme.

The pulse sequences for measurement of $^2$H1′ $R_1$ and $R_2$ relaxation rates are shown in Figure 1A, with the corresponding experiments for measuring $^2$H3′ and $^2$H4′ presented in the Supporting Information. The transfer makes use of $^1J_{HC}$,$^1J_{CC}$, and $^1J_{DC}$ scalar couplings,[8,15] with $^1H_i = {}^1$H2′ and $^2H_k = {}^2$H1′ (see transfer scheme above). A gain in sensitivity of approximately 4-fold is realized relative to random 50% $^2$H labeling since the occupancy of $^2$H and $^1$H at positions 1′ and 2′ is 100%. However, the labeling scheme produces additional benefits. Because the $^1$H2′−$^{13}$C2′ spin system is well isolated from additional $^1$H scalar coupling effects and from dipolar interactions involving sugar spins (but see below), sensitivity advantages are anticipated using a $^1$H−$^{13}$C multiple-quantum element between points *a* and *b* (and *c* and *d*) in the scheme of Figure 1A, since relaxation from the $^1$H2′−$^{13}$C2′ dipolar interaction is very much reduced.[16] Indeed, further gains in sensitivity of a factor of 2.2 ± 0.3 are realized over previously published sequences, where $^{13}$C single-quantum magnetization evolves during these periods,[8] while TROSY-based approaches are 0.95 ± 0.2 as sensitive as the single-quantum variant.

There is, however, a decided disadvantage in preparing a "near"-isolated spin system: although transverse relaxation properties are much improved (a positive feature), the increased longitudinal relaxation times relative to those in samples with more abundant levels of protonation lead to decreases in sensitivity. As a result, a labeling strategy was employed where the sugars are highly deuterated but the bases are not. In the case of A and G there is no $^2$H incorporation in base positions, while for C and U the 6 position is protonated (see Supporting Information). In a regular A-form RNA helix, the H2′ and the H6/H8 protons of the next base are proximal (2.0−2.4 Å). The present labeling scheme thus preserves a pathway for the relaxation of the H2′ proton while ensuring that deleterious homonuclear scalar couplings and relaxation pathways within the sugar are suppressed. $^1$H2′ $T_1$ values have been measured in the HIV-2 TAR RNA sample studied here (30 nucleotides, 25 °C, 600 MHz spectrometer field), and an average value of 3.3 s ($1/T_1 \approx 0.3$ s$^{-1}$) was obtained, with a calculated decrease in $1/T_1$ of approximately 0.05 s$^{-1}$ in cases where all base positions are deuterated. This is to be contrasted with the average value of 1.8 s, measured for a 50% $^2$H-, 100% $^{13}$C-labeled RNA sample containing the U1A binding site,[3] at 25 °C, 600 MHz, that is similar in size to HIV-2 TAR (30 nucleotides, with a correlation time within 12% of HIV-2 TAR RNA). The elevated $T_1$ values in the present labeling scheme translate into decreases in sensitivity of approximately 30% (for a relaxation delay of 2 s), so that an overall gain in sensitivity of $4 \times 2.2 \times 0.7 = 6$ in the experiment of Figure 1 is realized relative to that achieved using a sample prepared with random 50% $^2$H labeling.

Multiple-quantum enhanced $^2$H relaxation experiments have been used to measure relaxation rates at the 1′, 3′, and 4′ positions of HIV-2 TAR RNA at 25 °C. The $R_1$ and $R_2$ rates vary significantly in the RNA, with elevated $R_1$ and reduced $R_2$ values measured for the loop and bulge nucleotides at the D1′ (Figure 1E,F), D3′, and D4′ positions, indicating that they are more mobile than stem residues on the ps−ns time scale. The $^2$H relaxation rates can be used to estimate order parameters ($S$) that provide a measure of the degree of spatial restriction of the C−D bond vectors on a time scale faster than overall tumbling. Values of $S^2$ in the helical region are ~0.85, similar to those obtained on a small 14-mer containing a UUCG tetraloop.[8] In contrast, the bulge and the loop have lower $S^2$ values (as low as 0.4 for U23D4′, see Supporting Information)

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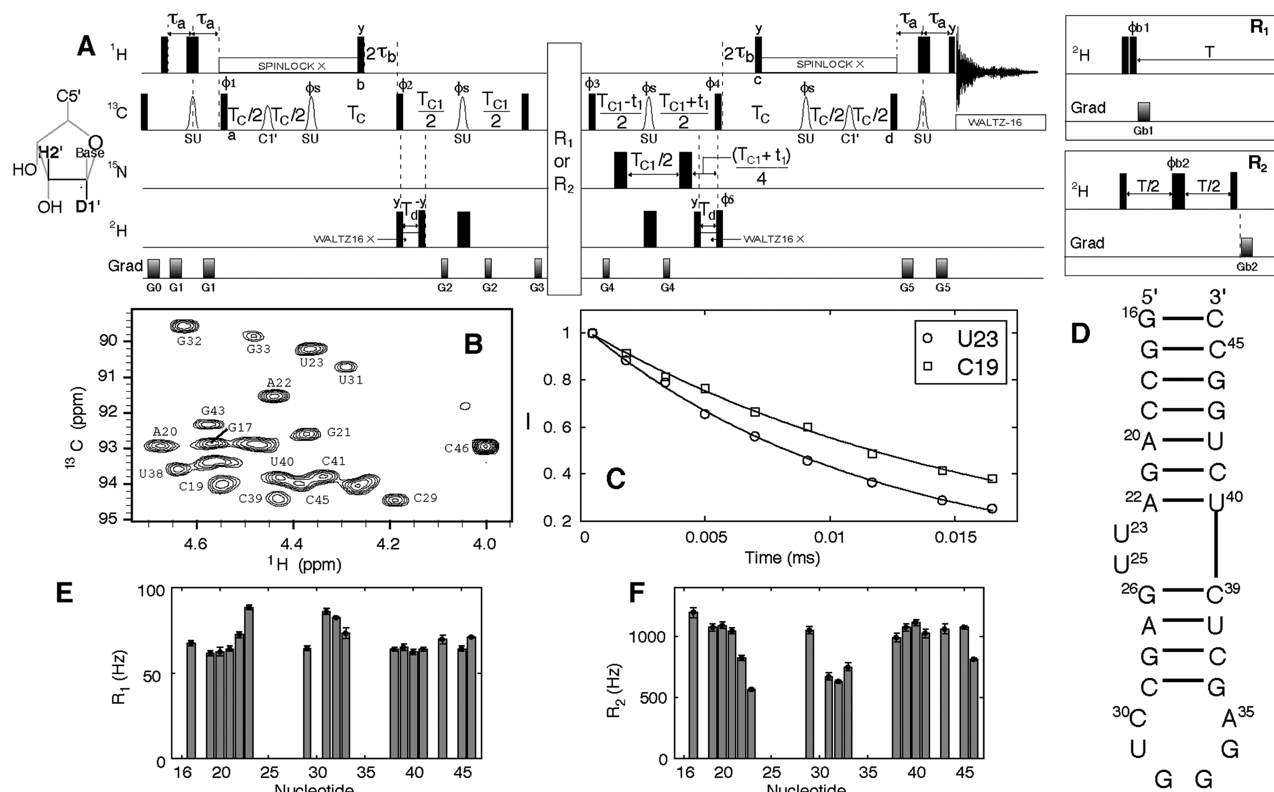


***Figure 1.*** $^2$H relaxation rates at the ribose 1′ position in HIV-2 TAR RNA. (A) The pulse sequence used to record $^2$H1′ relaxation rates. Box $R_1$ or $R_2$ is inserted to record $R_1$ or $R_2$ rates, respectively. Details are given in Supporting Information. $R_1$ and $R_2$ values are measured from a series of 2D spectra with different relaxation delays $T$. (B) Spectrum of the HIV-2 TAR RNA, recorded with the sequence in A, $T = 0.5$ ms, with H2′ shifts correlated to C1′. (C) Representative $R_1$ $^2$H1′ decays for U23 and C19. $^2$H1′, $^2$H3′, and $^2$H4′ rates were measured in 20, 10, and 17.5 h on a 600 MHz spectrometer with a cryogenically cooled probehead; average errors between 2 and 4%. (D) The 2° structure of the HIV-2 TAR RNA construct used here. (E,F) $^2$H1′ $R_1$ and $R_2$ rates for different positions in the molecule.

relative to those of the UUCG tetraloop (lowest $S^2$ value of 0.67 for D5″ of the second U of the tetraloop). It is well known that UNCG sequences are more rigid than other loops, such as GNRA,[17] and that the rigidity likely accounts for a decrease in interactions of such sequences with other RNA and proteins.[17] By contrast, TAR binds to a diverse range of targets,[6] and the range of conformations that the loop and bulge regions sample most likely are important for this activity.

The development of labeling methodology has had a profound effect on NMR studies of biomolecular structure and dynamics. Here we have presented a new labeling scheme with markedly improved sensitivity for the study of RNA dynamics that targets those classes of experiments that were sensitivity limited using previous labeling strategies. Measures of dynamics have been obtained at sugar positions in TAR that provide a nice complement to previous $^{13}$C- and $^{15}$N-based measures of motion that focused on aromatic positions in the molecule.[6] Additionally, samples prepared with this labeling scheme can be used to measure long-range NOEs and dipolar couplings.[18]

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**Supporting Information Available:** Tables of rates, pulse sequences, details of the experiments, dynamics parameters and scheme for the synthesis of the labeled nucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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