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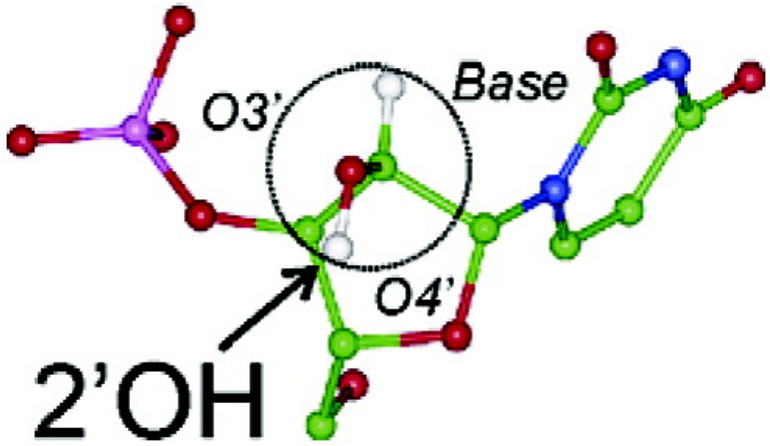


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# Assignment and NOE Analysis of 2′-Hydroxyl Protons in RNA: Implications for Stabilization of RNA A-Form Duplexes

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The ribose 2′-OH hydroxyl group of RNA is the major determinant for differences in conformation, hydration, and thermodynamic stability between RNA and DNA.[1] The 2′-OH hydroxyl group serves as a scaffold for the stabilizing hydration network associated with RNA[2] and is a key player in RNA catalysis.[3,4] NMR assignments for 3 out of 20 2′-OH proton resonances were obtained for a 20mer RNA in aqueous solution from TOCSY and NOESY spectra,[1] while a heteronuclear HMQC[5] correlation was used to assign a 2′-OH resonance of the intensively studied UUCG RNA hairpin loop.[6] Cross hydrogen bond scalar couplings involving two slowly exchanging 2′-OH hydroxyl protons were observed and analyzed in a frame-shifting mRNA pseudoknot.[7] Additional studies have been conducted on single nucleotides in DMSO.[8]

Here we report the assignment and the NOE analysis for the 2′-OH hydroxyl protons of the 30mer HIV-2 transactivation response element (TAR) RNA[9] in aqueous solution (Figure 1A). The resonance assignments were obtained using two-dimensional homonuclear TOCSY[10] and NOESY[11] experiments performed at 600 MHz. All experiments were conducted at 5 °C to slow solvent exchange. The exchange rate constant of the 2′-OH protons with bulk water is approximately 10 Hz under these conditions, as estimated from the intensity of the exchange cross-peaks in the NOESY spectrum. This value is in good agreement with the value reported by Gyi et al.[1] for an RNA and an RNA−DNA duplex. The TOCSY experiment (Figure 1B) shows intense correlations connecting the 2′-OH and the scalar coupled H2′ protons. In qualitative agreement with vicinal $^3J$(2′-OH,H2′) couplings measured for isolated nucleotides in DMSO,[8] the through-bond 2′-OH−H2′ cross-peaks are intense, reflecting $^3J$(2′-OH,H2′) couplings greater than 2 Hz. The NOESY spectrum (Figure 1C) reveals several through-space NOE correlations of medium intensity between the H1′ and the 2′-OH protons. Additional cross-peaks in the region shown stem from NOE correlations between cytidine amino and aromatic H5 protons, as confirmed using a $^{15}$N-labeled RNA sample. NOE correlations between 2′-OH hydroxyl and other ribose protons are difficult to analyze due to extensive resonance overlap (Supporting Information, Figure S1).

We could unambiguously assign 20 out of 30 2′-OH hydroxyl protons of the HIV-2 TAR RNA (Figure 1A and Supporting Information, Table S1). Five of the 10 missing 2′-OH hydroxyl protons belong to the loop nucleotides 30−35, where 2′-OH protons are probably not participating in a stable network of hydrogen bonds. As a consequence, the exchange rate of those 2′-OH protons with water is fast, impeding their detection even at 5 °C. We could not observe the 2′-OH protons of the two nucleotides constituting the terminal, fraying base pair for similar reasons. Three additional 2′-OH hydroxyls in the lower stem could not be identified unambiguously due to resonance overlap (A20 and G43 are tentatively assigned, see Table S1). Our attempts to assign these resonances from a $^{13}$C-edited NOESY acquired using a more dilute, $^{13}$C/$^{15}$N-labeled TAR RNA sample (0.7 mM) failed because of vanishing NOE intensities. To the best of our knowledge, this represents the first nearly complete assignment of 2′-OH proton resonances of an RNA molecule.

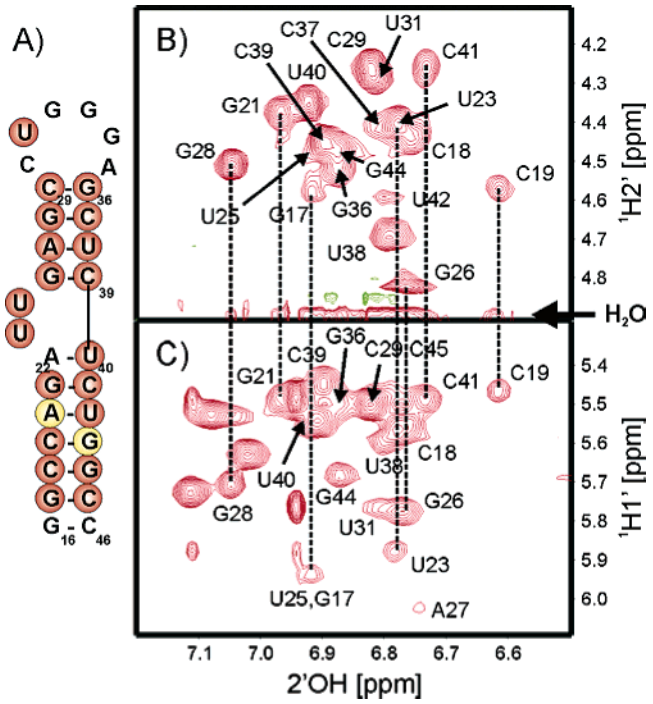


***Figure 1.*** (A) Sequence and secondary structural representation of HIV-2 TAR RNA. Assigned 2′-OH protons are highlighted; tentatively assigned residues A20 and G43 are shown orange. (B) Expansion of the 2′-OH−H2′ region of the TOCSY experiment. Unambiguous assignments are given with one-letter code followed by the residue number. The clean DIPSI-2rc[10] mixing sequence was applied for $\tau_m = 43$ ms, using a $\gamma B_1/2\pi = 10.04$ kHz field strength. (C) Expansion of the 2′-OH−H1′ region of the water flip-back NOESY experiment.[11] The mixing time was $\tau_m = 50$ ms. Water suppression was achieved with excitation sculpting.[12] The $^1$H carrier position was 4.98 ppm. High-power proton pulses were applied with a field strength of 35.5 kHz. Low-power water-selective square $\pi$ pulses of duration 2.2 ms used a $\gamma B_1/2\pi = 250$ Hz field strength. A total of 368 complex points were recorded with an acquisition time of 28.3 ms for $^1$H ($\omega_1$), and 2048 complex points with an acquisition time of 157.5 ms for $^1$H ($\omega_2$). A recycle delay of 1.2 s between transients was used, with 64 scans accumulated per complex increment (total measuring time 17.5 h each). Both spectra were recorded on a four-channel Varian Inova 600 MHz spectrometer equipped with an actively shielded $z$-gradient triple-resonance probe, at a temperature of 278 K. Spectra were recorded on 2 mM unlabeled HIV-2 TAR RNA. The sample buffer contained 10 mM phosphate buffer, pH 6.4, 50 mM sodium chloride, and 0.1 mM EDTA in 500 $\mu$L of 90% $H_2O$/10% $D_2O$.

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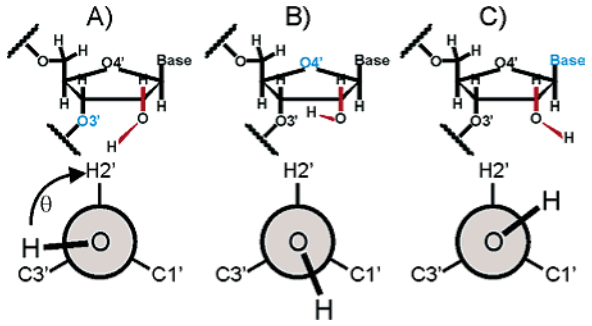


***Figure 2.*** Energetically favored orientations of the 2′-OH hydroxyl group and corresponding Newman projections showing the torsion angle $\theta$ (H2′−C2′−O2′−H) when the ribose is in the C3′-endo conformation: (A) O3′ domain, torsion angle $\theta = 50-140°$, (B) O4′ domain, torsion angle $\theta = 175-230°$, and (C) base domain, torsion angle $\theta = 270-345°$.[13]

With the exception of C19 and G28 (Figure 1), the chemical shift dispersion of the 2′-OH hydroxyl protons is rather limited, with resonance frequencies clustering between 6.75 and 7.00 ppm, which is in good agreement with chemical shift ranges reported previously.[1,5,8,16]

Molecular dynamics (MD) simulations of an RNA hairpin show that the conformation of the 2′-OH group depends on the sugar pucker.[13] Three orientations are accessible to the 2′-OH hydroxyl group when the ribose is in the typical RNA C3′-endo conformation (Figure 2): (A) toward the O3′ stabilized by attractive electrostatic interaction with the phosphate backbone, (B) toward the O4′ stabilized by favorable intra-ribose electrostatic interactions, and (C) toward the base stabilized by electrostatic interaction with the N3 or O2 atom of the attached base. The $^3J$(2′-OH,H2′) coupling can adopt values higher than 4 Hz in all three domains, so that the detection of a 2′-OH−H2′ cross-peak in TOCSY spectra is not indicative of a specific domain.[8]

However, the theoretical through-space NOE correlations differ substantially for the distinct domains. In particular, NOE correlations between the 2′-OH and aromatic H6 or H8 protons of the following nucleotide are only compatible with the 2′-OH proton in either the base or a small region of the O3′ domain (torsion angle $\theta \approx 60°$). Such NOEs can be observed for 13 of the assigned 2′-OH hydroxyl protons (G17, C18, C19, U23, G26, G28, G36, C37, C39, U40, U42, G44, C45). Theoretically, the two orientations with observable 2′-OH($i$)−H6/H8($i$+1) NOEs can be distinguished by inspection of the intraresidual 2′-OH($i$)−H1′($i$) peak, which is predicted to be much weaker in the O3′ domain. A quantitative analysis of the NOE is not possible due to spectral overlap. Our semiquantitative inspection of the cross-peak intensities for C18, C19, U23, G28, U42, G44, and C45 revealed 2′-OH−H1′ NOEs of medium intensity in a short mixing time NOESY ($\tau_m = 50$ ms), which are more intense than the 2′-OH($i$)−H6/H8($i$+1) but less intense than the corresponding intraresidual 2′-OH−H2′ NOEs. These results are in qualitative agreement with the 2′-OH proton in the base domain orientation, where the $\theta$ torsion angle is approximately 300°. The 2′-OH−H1′ NOE intensities for G26, C39, and U40 could not be analyzed because of overlap with interfering cross-peaks stemming from cytidine amino protons, while G17 and C37 show weak 2′-OH−H1′ NOE correlations, suggesting a 2′-OH orientation pointing away from the H1′ in the O3′ domain (torsion angle $\theta \approx 60°$). No 2′-OH($i$)−H6/H8($i$+1) NOE could be observed for the remaining six nucleotides (G21, U25, A27, C29, U31, and C41). Again, the 2′-OH−H1′ are weaker than the corresponding 2′-OH−H2′ NOE correlations, indicative of a populated O3′ domain with a torsion angle $\theta \geq 60°$. Our data do not show evidence for 2′-OH protons in the O4′ domain, where the 2′-OH−H1′ NOE is expected to be more intense than the 2′-OH−H2′ NOE.

It has been commonly accepted that the 2′-OH hydroxyl points toward the phosphate backbone and away from the ribose H1′ proton. This picture has been supported by MD simulation of an RNA hairpin,[14] which provided evidence for a strongly preferred orientation toward the O3′, and by the NMR data available to date,[15] where only very weak 2′-OH−H1′ NOE cross-peaks could be detected. However, more recent MD simulations describe three energetically favored orientations for the 2′-OH hydroxyl proton.[13] The 2′-OH−H1′ NOEs of medium intensity observed for the HIV-2 TAR RNA contradict the paradigm that the 2′-OH hydroxyl typically points away from the ribose H1′ proton. The presence of many detectable NOE correlations involving aromatic H6/H8($i$+1) protons suggest that the orientation toward the base might be more common than currently appreciated. This interpretation is in agreement with the hydration model proposed for an RNA duplex on the basis of a high-resolution crystal structure.[2] The described dense network of hydrogen bonds snakes down the minor groove and involves two water molecules (or the phosphate O2P oxygen), the N3/O2 atoms of purine/pyrimidine bases, and the 2′-OH hydroxyl groups of adjacent nucleotides in base pair steps. This ordered network requires 2′-OH hydroxyl protons to be in the base or O3′ domain, where 2′-OH hydroxyls are able to participate in H-bonding interactions with water. A more quantitative conformational analysis of the 2′-OH hydroxyl group using vicinal homo- and heteronuclear $^3J$-couplings is being currently carried out in our laboratories.

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**Supporting Information Available:** Table of chemical shift assignments; figure showing H6/H8($i$+1) and intraresidual ribose to 2′-OH($i$) NOEs. This material is available free of charge via the Internet at http://pubs.acs.org.

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