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^{15}N NMR of RNA Fragments Containing Specifically Labeled GU and GC Pairs

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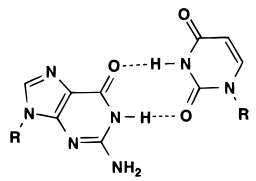
Abstract: ^{15}N and ^{13}C NMR have been used to study three specifically labeled RNA fragments that include two tandem GU wobble pairs of different thermal stability and a tetraloop GU pair. The data are also compared to earlier ^{15}N NMR work on an isolated, intrahelical GU pair. The results provide qualitative information on the relative contributions by stacking and hydrogen bonding to chemical shift changes at specific sites in a variety of GU pairs. Chemical shifts for the unpaired guanine amino groups in the isolated and both tandem wobble pairs are all significantly upfield of corresponding paired GC aminos. The guanine amino of the tetraloop is further downfield, as expected for a base paired hydrogen bond donor. Thus, ^{15}N NMR can be diagnostic for the presence of base−base pairing in RNA. Chemical shifts of the guanine N1 in all four GU pairs show significant shielding effects, particularly those of the more stable tandem GU pair. Similar shielding is also seen in the ^{13}C data for the C2 atom of the GU wobble pairs. Our results demonstrate that these adjacent ring atoms (N1 and C2) are in the shielding regions of neighboring bases, and that specific labeling can provide information on base stacking. Thus, where global structure is known, specific labeling can be invaluable as a complement to probe local interactions. It is likely, however, that the most important use of specific labeling will be with complex systems in which global structural information is incomplete.

^{15}N NMR chemical shift changes of selectively labeled DNA fragments have provided model-independent insight into local hydrogen bonding,[1−5] protonation,[6] hydration,[7] and ligand interactions.[3] We have recently extended our work to an RNA fragment containing isolated, intrahelical guanine−uracil (GU) wobble pairs to probe the unusual stacking present with this geometry.[8] Specific labeling is thus emerging as a powerful tool to probe local interactions in complex systems. We now describe the results of a more extensive ^{15}N NMR study of GU pairs, including two tandem GU pairs with different thermal stabilities and a GU pair within an ultrastable tetraloop.

Wobble GU Pairs. Whereas a guanine−thymine mispair in DNA forms a mutagenic lesion, the guanine−uracil pair in RNA is a common motif that stabilizes loops and branches,[9−11] forms tertiary interactions,[12,13] and binds to proteins.[14,15] In the GU "wobble" geometry first proposed by Crick,[16] the guanine is displaced toward the minor groove, leaving the amino group



G•U Wobble Pair

unpaired. Subsequent X-ray and ^{1}H NMR structural work has shown that the resulting local helical distortion causes the

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guanine to overlap extensively with its 3′ base, but only slightly with its 5′ base.[9,17,18] In addition to this unique stacking associated with wobble pairs, direct interactions with the exposed amino group also play critical roles in biological systems. In the *Tetrahymena* group I intron, the guanine amino of an invariant GU pair at the splice site has been shown to help form the correct tertiary structure by interacting with the ribozyme active site.[12,13] The protruding amino group is also important in the binding of RNA to proteins, as has been shown for accurate and efficient recognition of $tRNA^{ala}$ by alanyl-tRNA synthetase.[14,15] Wobble GU pairs often occur in tandem as well as separately.[11] The thermal stabilities of a series of self-complementary RNA fragments containing tandem GU pairs have been shown to vary significantly, depending on the order in the tandem pair as well as the flanking bases.[19,20] The reasons for these differences are not clear, but undoubtedly involve stacking and hydrogen bonding.

Specific Labeling of an Isolated Wobble GU. We recently described ^{15}N and ^{13}C NMR of an isolated, intrahelical GU wobble pair in the RNA octamer, 5′-G*A*UGCGUCp-3′ (**1**),[8] in which specifically labeled bases are underlined. We reported chemical shifts for the guanine N1 and N2 atoms in the intact GU pair that are 2−3 ppm upfield of those of corresponding labels in the intact GC pair. This shielding reflects the absence of base−base hydrogen bonding of the GU amino as well as the altered stacking known to occur with the wobble geometry.[11,17,18] We now present ^{15}N and ^{13}C NMR results for GU pairing in three other examples.

Specific Labeling of a Tandem Wobble GU. We have prepared two RNA fragments containing tandem GU wobble pairs in which the only difference is the order in the tandem pair: 5′-GGCGU*G*CCp-3′ (**2**) and 5′-GGCUGGCCp-3′ (**3**). The underlined guanines were labeled with ^{15}N at the N1 and N2 positions and differentiated in each case with a ^{13}C "tag" at the C2 position of the GU pair.[21] Fragments with the sequence in **2** (5′-CGUG-3′) have previously been shown to be about 3 kcal/mol less stable than those with the sequence in **3** (5′-CUGG-3′), although 1D ^1H NMR and CD studies did not reveal any obvious reason for this difference.[19,20] In **2** and **3**, the presence of the GU wobble pairs was confirmed by ^1H NMR spectra which showed characteristic exchangeable GU resonances between 10.4 and 12 ppm (data not shown). The $^{15}N2$ chemical shifts of the amino groups in the GC pairs for both **2** and **3** behave as expected, with cooperative upfield changes (Figure 1A: **2** (●) and **3** (▼)). Such 1−4 ppm upfield shifts

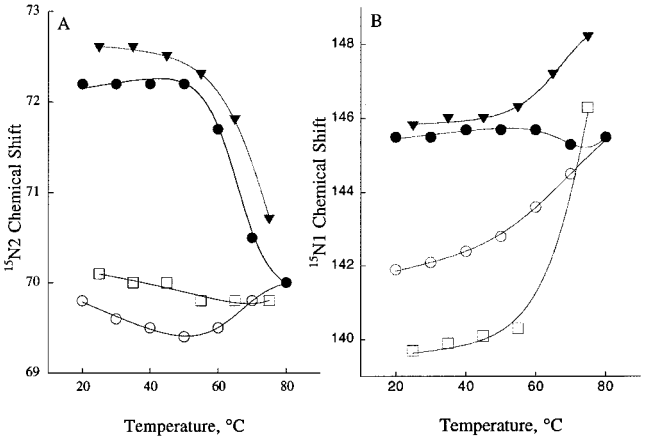

Figure 1. Plots of ^{15}N chemical shifts vs temperature for (A) guanine amino groups in fragments **2** and **3** with tandem GU pairs, where ● represent the GC N2 in **2**, ▼ represents the GC N2 in **3**, ○ represents the GU N2 in **2**, and □ represent the GU N2 in **3**, and (B) guanine N1 atoms in fragments **2** and **3** with tandem GU pairs, where ● represent the GC N1 in **2**, ▼ represents the GC N1 in **3**, ○ represents the GU N1 in **2**, and □ represent the GU N1 in **3**.

are typical for hydrogen bond donor amino groups upon loss of hydrogen bonding during melting, as we have seen before in other Watson−Crick pairs.[1,2,4,8] In contrast, the $^{15}N2$ chemical shifts of the guanine amino groups in the intact GU pairs show much less change, consistent with their lack of base−base hydrogen bonding (Figure 1A: **2** (○) and **3** (□)). The melting transition for the more stable **3** is nearly linear, consistent with loss of hydration being the only significant effect on chemical shift. The chemical shifts for the less stable **2** at low temperatures, however, are about 0.5 ppm further upfield, and during the transition, they change direction to meet the values associated with all melted guanine aminos. This additional shielding in the intact GU pair in **2** is almost identical with what we reported earlier for the isolated wobble pair in **1**,[8] and reflects its distinct altered stacking pattern.

The $^{15}N1$ chemical shifts of the intact GC pair in the less stable **2** are nearly 1 ppm downfield of values extrapolated back from high temperature, and move upfield upon melting (Figure 1B, ●). This upfield shift is again typical of hydrogen bond donors and is virtually identical with that of a labeled GC pair adjacent to the isolated GU wobble pair reported previously in **1**.[8] However, the corresponding GC chemical shifts in the more stable **3** move downfield by about 2 ppm during melting and have not completed the transition at our highest temperature (Figure 1B, ▼). This presumably transient downfield change may be caused by a temporary deshielding associated with the disruption of stacking. Changes in stacking effects on chemical shift depend on the exact position of the observed atom relative to the ring currents of adjacent aromatic bases, and can be in either direction. In this fragment (**3**), the labeled guanine in the GC pair is immediately 3′ of the guanine in the GU pair, whereas in both **1** and **2**, it is 3′ of the smaller uracil. Presumably, at a higher temperature at which all residual structure is disrupted, the guanine N1 chemical shifts of **2** and **3** would merge.

The $^{15}N1$ chemical shifts for the intact GU pairs in both **2** and **3** are significantly more upfield than those for the GC pairs, and move downfield during melting toward the GC values (Figure 1B: **2** (○) and **3** (□)). The chemical shifts for the less stable **2** (○) are again almost identical with those of the isolated wobble pair,[8] while those of **3** (□) are over 2 ppm more shielded. Because the ^{15}N chemical shift behavior of the less stable tandem arrangement in **2** (5′-CGUG-3′) at both the N1 and N2

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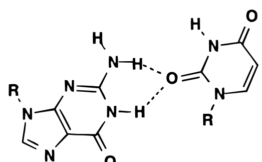
positions is nearly identical with that of the isolated GU wobble,[8] we conclude that this less stable tandem most likely consists of two consecutive wobble pairs, each of which has approximately the same hydrogen bonding and stacking as a single wobble pair. In contrast, the more stable tandem arrangement in **3** (5′-CUGG-3′) results in somewhat different behavior, in particular, the larger shielding at the N1 position. Gautheret *et al.* have noted that in a 5′-UG-3′ tandem pair, the unstacked sides of the guanines are aligned.[11] They propose that this particular arrangement should result in a strong, stabilizing *inter*strand stacking of the two guanines that does not occur in the 5′-GU-3′ tandem. Such interstrand stacking was found by X-ray crystallography in the A-form DNA fragment, dGGGTGCCC, which contains the related tandem, 5′-TG-3′,[22] and recently in r(GUAUGUA)dC.[23] The larger shielding we observe for the GU guanine N1 of **3** relative to that of **2** provides strong support for such interstrand stacking along with intrastrand stacking.

Tetraloop GU Pair. The ultrastable UNCG tetraloops (where N is any base) contain a markedly different GU pairing geometry that contributes significantly to the enhanced stability of the tetraloops.[24,25] This type of tetraloop occurs widely[26] and is thought to create specific protein recognition sites, provide signals for reverse transcription, and initiate and maintain correct folding of complex RNA by serving as nucleation sites.[27] As opposed to the wobble arrangement, in which the guanine is in the *anti* conformation and its amino group is not base paired,[16] in the tetraloop GU, the guanine is in the *syn* conformation[25] and its amino as well as its N1H are thought to be involved in hydrogen bonding to the uracil O2.[18] In spite of these major differences, in both the wobble and the tetraloop GU pairs, the guanine is fully stacked on its 3′ base but not on its 5′ base.[11,18,25]



Tetraloop GU

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Specific Labeling of a Tetraloop GU. We have also prepared a UUCG tetraloop, **4**, in which we have seen a different combination of stacking and hydrogen bonding effects. The two underlined guanines were labeled with ^{15}N at the N1 and N2 positions and differentiated with a ^{13}C "tag" at the C2 position of the GC pair.[21] ^{1}H NMR showed a resonance at 9.9 ppm, which is diagnostic for the loop guanine N1H (data not

5′-GGAC U U
3′-pCCUG G C

shown).[28] The ^{15}N2 chemical shift of the amino group in the GC pair behaves as expected, with a 2 ppm cooperative upfield change upon melting (Figure 2A, ▼). However, because of the high thermal stability, we do not observe the complete transition. The ^{15}N2 chemical shift for the guanine amino group in the intact GU pair at low temperature is only about 1 ppm upfield of that in the GC pair, and moves further upfield upon melting to give the same values associated with the nearly melted GC pair (Figure 2A, □). Again, this behavior is typical of loss of base−base hydrogen bonding by a donor and is consistent with the hydrogen bond proposed between the amino and the uracil O2.[18] This result is strikingly different from the *unpaired* guanine aminos in the three GU wobble pairs described above. While stacking most likely has some effect on the guanine amino chemical shift in this tetraloop GU pair, the hydrogen bonding effect predominates.

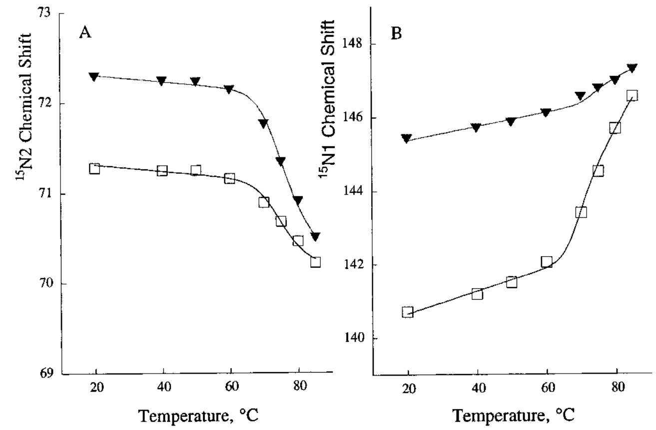

Figure 2. Plots of ^{15}N chemical shifts vs temperature for (A) guanine amino groups in the tetraloop **4**, where ▼ represents the GC N2 and □ represent the GU N2, and (B) guanine N1 atoms in the tetraloop **4**, where ▼ represents the GC N1 and □ represents the GU N1.

The ^{15}N1 chemical shift of the GC pair moves downfield by about 2 ppm during melting, but again, we cannot observe the full transition because of the high T_m (Figure 2B, ▼). This pattern is similar to that of the GC N1 in the more stable tandem GU fragment, **3**. In both cases, guanines in the labeled GC pairs are 3′ to the guanines of the GU pairs, and these GC ^{15}N1 atoms appear to be sensitive indicators of the disruption of this strong stacking. In contrast, the guanine ^{15}N1 chemical shift for the intact tetraloop GU is 5 ppm further upfield of the GC (Figure 2B, □), and moves downfield during melting to give the same values associated with the nearly melted GC pair. Thus, even though the GU N1 is hydrogen bonded, shielding effects from the strong stacking prove to be a greater influence on ^{15}N chemical shift, just as we have seen for this ring nitrogen in all three wobble GU pairs described above.

^{13}C Labeling. Although we used the ^{13}C atoms primarily as tags, their chemical shift changes also provide useful information. In the tetraloop (**4**), the label is in a GC pair, and its chemical shift does not change much with temperature (Figure 3A, ○). In the other three examples, the label is in the GU pairs. The ^{13}C chemical shifts for the isolated GU wobble (**1**)[8] and the thermally less stable tandem GU (**2**) at low temperatures are very similar, with about 2 ppm shielding relative to lines extrapolated back from high temperature (Figure 3A: **1** (●) and **2** (▼)). The ^{13}C chemical shifts for the more stable tandem GU (**3**) at low temperatures are somewhat more shielded (Figure 3A, ■), similar to the behavior of the adjacent ring ^{15}N1 described above. Thus, the ^{13}C data fully support our conclusions from the ^{15}N results.

NMR Exchange Regime and UV Melting Studies. Intermediate exchange, which can complicate the interpretation of

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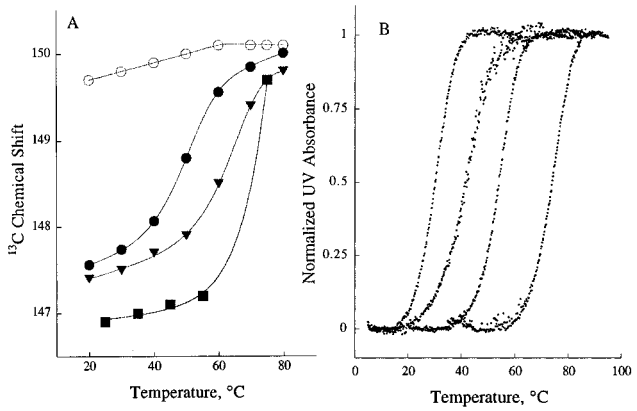

Figure 3. Plots of (A) ^{13}C chemical shifts vs temperature for guanine C2 in all four fragments, where ○ represents a GC pair in the tetraloop **4**, ● represents the GU pair in **1**, ▼ represents the GU pair in **2**, and ■ represents the GU pair in **3**, and (B) representative normalized UV absorbance melting curves at 260 nm for, from left to right, fragments **1** (4.6 μM), **2** (6.2 μM), **3** (9.7 μM), and **4** (6.0 μM) at the indicated concentrations.

Table 1. Comparison of T_m's (deg) from NMR and UV

fragment	T_m(NMR)[a]	T_m(UV)[b]
1	52	52
2	65	60
3	71	70
4	72	74

[a] Average of values for all ^{15}N and ^{13}C atoms, determined from a nonlinear least-squares curve fitting of each melting curve. [b] Determined at an NMR concentration from $1/T_m$ vs ln C plot, constructed by using $4T_m$ values from $1 - \alpha$ vs T plots.

some NMR data,[29] does not appear to be a problem with these examples. None of the resonances described here show evidence of line broadening during melting. The chemical shift differences between the high- and low-temperature forms of the ^{15}N atoms generally are less than 100 Hz. Furthermore, melting temperatures (T_m) calculated from curve fitting of the ^{15}N NMR data agree well (Table 1) with the corresponding values calculated from UV melting studies, examples of which are shown in Figure 3B.

Conclusion. ^{15}N NMR of specifically labeled RNA fragments has provided qualitative information on the relative contributions to chemical shift changes by stacking and hydrogen bonding at specific sites in a variety of GU pairs. Chemical shifts for the unpaired guanine amino groups in the isolated and both tandem wobble pairs are all significantly upfield of corresponding paired GC aminos. The GU guanine amino of the tetraloop is further downfield, as expected for a base paired hydrogen bond donor. Thus, specific labeling can be diagnostic for the presence of base−base pairing in RNA. Chemical shifts of the guanine N1 in all four GU pairs show significant shielding effects, particularly those in the more stable tandem GU, **3**. Similar shielding is also seen in the ^{13}C data for the C2 atom. Our results demonstrate that these adjacent ring atoms (N1 and C2) are in the shielding regions of neighboring bases, and that specific labeling can provide information on base stacking. Thus, where global structure is known, specific labeling can be invaluable as a complement to probe local interactions. It is likely, however, that the most important use of specific labeling will be with complex systems in which global structural information is incomplete.

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Experimental Section

Synthesis. Synthesis was done on solid support with use of the universal and base-stable allyl linker, 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic acid.[30,31] Oligonucleotide syntheses were done on 30 μmol scales with the hydrogen phosphonate method as previously described,[32] except that the 3′ terminal monomer, not the penultimate monomer, was added in the first coupling step. Monomers were protected with 5′-dimethoxytrityl groups and 2′-*tert*-butyldimethylsilyl groups, using the procedures reported elsewhere.[33] The labeled bases [1,NH_2-$^{15}N_2$]-guanosine[21] (X) and [2-^{13}C-1,NH_2-$^{15}N_2$]guanosine[21] (Y) were incorporated into each strand at specific sites, as shown below. Each molecule was deprotected while still attached to the support and then purified by HPLC as previously described.[32] For the experiments reported here, the 3'-phosphates were not removed.

removed.

Isolated GU (**1**)	GAUXCYUCp pCUYCXUAG	11% yield
Less stable tandem GU (**2**)	GGCYUXCCp pCCXUYCGG	10%
More stable tandem GU (**3**)	GGCUYXCCp pCCXYUCGG	3%
Tetraloop (**4**)	5'-GGAC $^{U}_{U}$ 3'-pCCUY$_X$ C	9%

NMR. NMR spectra were acquired at 40.5 MHz on a Varian XL400 by using 1D experiments with a delay of 1 s. ^{15}N chemical shifts are reported relative to NH_3 with use of external 1 M [^{15}N]urea in DMSO at 25 °C at 77.0 ppm as a reference. The total strand concentrations were the following: **1** (10 mM), **2** (11 mM), **3** (3.5 mM), and **4** (11 mM). The solutions were in 100% D_2O, 0.1 M NaCl, 10 mM phosphate, and 0.1 mM EDTA at pH 6.7. A nonlinear least-squares fit[34] for chemical shift as a function of temperature using the equation $\delta_T = \alpha\delta_{ds} + (1 - \alpha)\delta_{ss}$ (α = fraction of total strands in duplex state), where the chemical shifts (δ) of the double strand and single strand species are assumed to be linear functions of temperature, gave the curves shown and assumed the transitions were two-state. T_m's were calculated using $\Delta H°$ and $\Delta S°$ determined as variable parameters from each fit.[35] Equations for a unimolecular process were used for the tetraloop, and for a bimolecular process for the other molecules.[35]

UV. UV spectra were obtained at four different concentrations on an Aviv 14 spectrophotometer at 260 nm with 1, 0.5, 0.2, and 0.1 cm path length cells in the same buffer described above. T_m's were determined by converting each data set to $1 - \alpha$ plots and reading the temperature at $\alpha = 0.5$.[35]

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Supporting Information Available: Tables of ^{15}N and ^{13}C chemical shifts for **1** to **4**, and a representative ^{15}N NMR spectrum (2 pages). See any current masthead page for ordering and Internet access instructions.

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