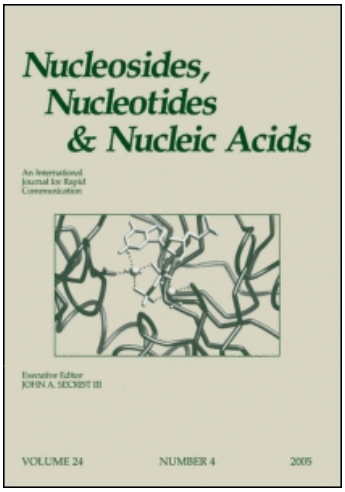

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MODULATION OF RNA METAL BINDING BY FLANKING BASES: ^{15}N NMR EVALUATION OF GC, TANDEM GU, AND TANDEM GA SITES

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□ *^{15}N NMR chemical shift changes in the presence of $Mg(H_2O)_6^{2+}$, Zn^{2+}, Cd^{2+}, and $Co(NH_3)_6^{3+}$ were used to probe the effect of flanking bases on metal binding sites in three different RNA motifs. We found that: for GC pairs, the presence of a flanking purine creates a site for the soft metals Zn^{2+} and Cd^{2+} only; a GG·UU motif selectively binds only $Co(NH_3)_6^{3+}$, while a UG·GU motif binds none of these metals; a 3′ guanosine flanking the adenosine of a sheared GA·AG pair creates an unusually strong binding site that precludes binding to the cross-strand stacked guanosines within the tandem pair.*

Keywords RNA metal binding; ^{15}N NMR; ^{15}N labeling

INTRODUCTION

Metals play a crucial role in forming and maintaining the complex tertiary structure of RNA, as well as in all aspects of its function.[1–6] Although much of the metal binding is nonspecific and primarily serves to neutralize the phosphate negative charge, selective interactions with metals can occur when RNA folds into a precise geometry that provides an array of correctly oriented ligands. While many examples using x-ray crystallography have provided informative, although static, pictures of metals in such high-affinity sites,[7–14] binding in solution is far more difficult to assess.

^{15}N NMR chemical shifts are very sensitive to changes in the local environment around ^{15}N atoms in labeled nucleic acids.[15] As a result, ^{15}N

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Dedicated to Dr. Robins on the occasion of his 70th birthday.
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NMR of RNA containing specifically ^{15}N-labeled nucleosides is a valuable nonperturbing probe for metal binding to nucleic acid nitrogen atoms.[16] In particular, we have used N7 labeled guanosine to show that a sheared tandem GA pair with an adjacent guanosine[17] and a tandem GU pair[18] display strikingly different metal binding properties. The latter selectively binds $Co(NH_3)_6^{3+}$ and K^+ with moderate affinity, but not $Mg(H_2O)_6^{2+}$, Zn^{2+}, or Cd^{2+}, while the former selectively binds $Mg(H_2O)_6^{2+}$, Zn^{2+}, and Cd^{2+} with high affinity, but not $Co(NH_3)_6^{3+}$.

Using ^{15}N NMR, we now describe results that explore the extent to which flanking bases modulate metal binding in three different small RNA motifs: GC pairs, tandem GU pairs, and tandem GA pairs.

MATERIALS AND METHODS

Synthesis of Labeled RNA

[1,7,NH^2-^{15}N$_3$]-Guanosine (**a**), [7-^{15}N]-guanosine (**b**), [8-^{13}C-7-^{15}N]-guanosine (**c**), [2-^{13}C-1,7,NH$_2$-^{15}N$_3$]-guanosine (**d**), [8-^{13}C-1,7,NH$_2$-^{15}N$_3$]-guanosine (**e**), and [8-^{13}C-1,7,NH$_2$-^{15}N$_3$]-adenosine (**f**) were synthesized as previously described.[19–23] Each labeled nucleoside was protected with a phenoxyacetyl group on the amino, a dimethoxytrityl group on the 5′ OH, and a *tert*butyldimethylsilyl group on the 2′ OH.[24,25] After conversion to cyanoethyl phosphoramidites, they were incorporated into RNA on an OligoPilot II synthesizer on 60 μmol scales. Figure 1 indicates the sites at which various labeled nucleosides were incorporated. Duplexes **1–4** are reported here for the first time, while **5**[18] and **6**[17] have previously been described, and are included for comparison. The crude oligonucleotides were deprotected with 40% methylamine, then $Et_3N{\cdot}3HF/Et_3N/N$-methylpyrrolidinone.[26] The excess fluoride was scavenged with isopropyltrimethylsilyl ether.[27] The precipitated RNA was dissolved in 0.1 M triethylammonium acetate at pH 6.8 and purified by reversed phase HPLC, before and again after detritylation. The pure RNA was desalted by reversed phase HPLC using 0.1 M ammonium bicarbonate, and then converted to the sodium form on a cation exchange column.

NMR Sample Preparation

The total strand concentration in each sample was 6.0 mM for duplexes **1** and **3,** 4.3 mM for duplex **2**, and 3.0 mM for duplex **4**. Previously reported **6** was 3.0 mM and **5** was 3.3 mM. They all contained 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 50 mM NaCl in H_2O with 10% D_2O at pH 6.8. Duplexes **1** and **3** are self-complimentary, so were prepared directly from stock solutions, while the two different strands for duplexes **2** and **4** were combined in a 1:1 molar ratio. All samples were

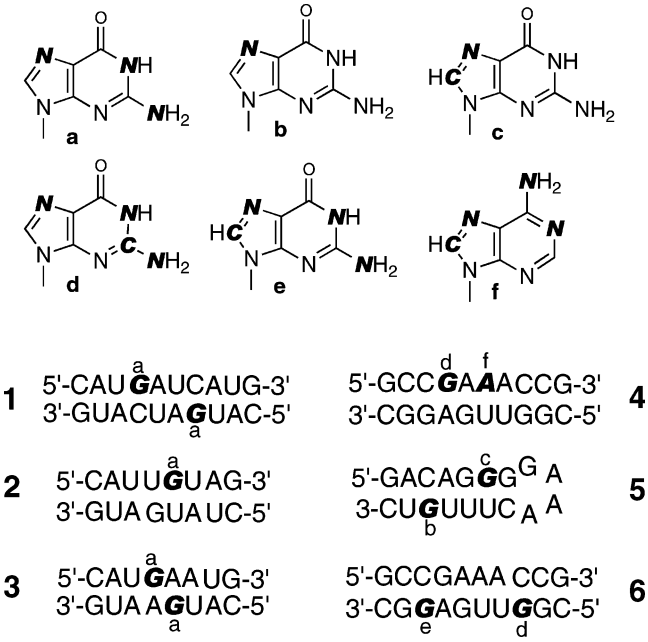

FIGURE 1 Specifically labeled RNA molecules **1–6** synthesized with the designated specifically labeled nucleosides **a–f**. Molecules **5** and **6** have previously been reported, and are included for comparison. The labeled nucleosides are: **a**, [1,7,NH_2-$^{15}N_3$]-guanosine; **b**, [7-^{15}N]-guanosine; **c**, [8-^{13}C-7-^{15}N]-guanosine; **d**, [2-^{13}C-1,7,NH_2-$^{15}N_3$]-guanosine; **e**, [8-^{13}C-1,7,NH_2-$^{15}N_3$]-guanosine; and **f**, [8-^{13}C-1,7,NH_2-$^{15}N_3$]-adenosine.

transferred to 300 mL Shigemi tubes. ^{1}H NMR spectra acquired at 10°C for duplexes **1–3** and 20°C for duplex **4** demonstrated complete duplex formation. In duplex **2**, the presence of a tandem GU wobble pair was indicated by resonances for exchangeable G and U protons between 10 and 11.7 ppm, and in duplex **3**, the presence of a sheared tandem GA pair was indicated by a doublet at 11.7 ppm for the proton on the ^{15}N-labeled GN1.

Addition of Metal to NMR Samples

Solutions of ultrapure $MgCl_2$, $ZnCl_2$, and $Cd(NO_3)_2$ (from Aldrich, St. Louis, MO, USA) and $Co(NH_3)_6Cl_3$ (from Strem Chemicals, Newburyport, MA, USA) were prepared in water and appropriate aliquots were added to the RNA samples in centrifuge tubes. After lyophilization, 0.30 mL 90% H_2O/10% D_2O was added and the pH was adjusted to 6.8 following brief annealing.

NMR Acquisition

^{15}N NMR 1 D spectra were acquired at 30.4 MHz on a Varian Mercury 300 NMR spectrometer with a delay of 1 second for 12–16 hours. Spectra for duplexes **1–3** were acquired at 10°C and duplex **4** at 20°C. Spectra

TABLE 1 ^{15}N NMR chemical shifts in ppm for ^{15}N labeled N7 atoms in molecules 1–6

RNA	no metal	$Mg(H_2O)_6^{2+}$	Zn^{2+}	Cd^{2+}	$Co(NH_3)_6^{3+}$
1	234.87	233.54	231.61	228.12	235.43
2	234.90	235.19	234.78	234.58	235.03
3	234.90	233.86	224.26	222.49	237.89/235.31
$\mathbf{4}^{G}$	233.74	232.94	230.85	230.37	234.14
$\mathbf{4}^{A}$	228.64	227.55	227.84	227.79	228.32

for previously reported **6** were acquired at 20°C and those of **5** at 15°C. Chemical shifts are reported relative to NH_3 using external 1 M [^{15}N]urea in DMSO at 77.0 ppm as a standard.[28] (See Table 1.)

RESULTS

We have synthesized four new duplexes specifically labeled with [1,7,NH_2-$^{15}N_3$]-guanosine (**a**) or [2-^{13}C-1,7,NH_2-$^{15}N_3$]-guanosine (**d**), in one case with [8-^{13}C-1,7,NH_2-$^{15}N_3$]-adenosine (**f**) as well. The sequences of these new duplexes (**1–4**) are shown in Figure 1, along with those of two previously reported molecules, **5**[18] and **6**,[17] that we include for comparison. Since **5** and **6** each have two different ^{15}N-labeled guanosines, ^{13}C labels are selectively included as tags to allow differentiation of the resonances. Although metal interactions are expected to be at guanosine N7 atoms, in most cases we labeled other nitrogens as well for comparison.

^{15}N NMR chemical shift changes for ^{15}N-labeled N7 atoms of GC pairs, tandem GU pairs, and tandem GA pairs upon addition of the four diamagnetic metals $Mg(H_2O)_6^{2+}$, Zn^{2+}, Cd^{2+}, and $Co(NH_3)_6^{3+}$ are shown in Table 2, followed by the number of equivalents of metal added, relative to labeled strand. With Zn^{2+}, Cd^{2+}, and $Co(NH_3)_6^{3+}$, we used stoichiometric amounts of the metal for each potential binding site, normally 1–1.5 equivalents, but when probing a motif in a molecule with a strong binding site elsewhere, we went to 2–3 equivalents. Because of the low affinity of $Mg(H_2O)_6^{2+}$ for nitrogen, we used much larger amounts, ranging from 8–15 equivalents, to probe $Mg(H_2O)_6^{2+}$ binding. Chemical shift changes for the N1 and amino nitrogens were <2 ppm in all cases, and are not shown. Because the RNA sequences and conditions of the six samples are not identical, the comparisons we describe are intended to be qualitative rather than quantitative.

For the GC pairs, we find that with uridines flanking the guanosine (row a), chemical shift changes for its N7 upon addition of all four metals are small (≤1.6 ppm). However, with a flanking guanosine (row b) or adenosine (row c), we observe moderate changes (3–9 ppm) with Zn^{2+} and Cd^{2+}, but small changes with $Mg(H_2O)_6^{2+}$ and $Co(NH_3)_6^{3+}$ (≤1.3 ppm).

TABLE 2 ^{15}N NMR chemical shift changes for the N7 atoms of ^{15}N labeled nucleosides (shown in bold) in individual motifs in RNA **1–6** upon addition of various equivalents (relative to labeled strand) of four different metals. The motifs are shown 5′ to 3′ on top and 3′ to 5′ below. A negative sign for the chemical shift change indicates upfield and a positive sign indicates downfield

			Δδ/equiv metal			
	RNA	Motif	$Mg(H_2O)_6^{2+}$	Zn^{2+}	Cd^{2+}	$Co(NH_3)_6^{3+}$
a	**5**[18]	-U**G**U–ACA-	−0.8/8	−0.6/1	−0.6/1	+1.6/3
b	**6**[17]	-G**G**U–CCA-	−1.2/15	−5.9/2	−9.0/2	+1.1/2
c	**1**	-U**G**A–ACU-	−1.3/10	−3.3/1	−6.8/1	+0.6/1
d	**5**[18]	-AG**G**G–UUUC-	−0.3/8	−1.0/1	−2.0/1	+5.3/1.5
e	**2**	-U**G**U–AGUA-	+0.3/10	−0.1/1	−0.3/1	+0.1/1
f	**6**[17]	-CGAA–**G**AGU-	−6.5/10	−17.5/1	−17.3/1	+0.5/1
g	**4**	-CGA**A**–GAGU-	−1.1/15	−0.8/2	−0.8/2	-0.3/2
h	**4**	-C**G**AA–GAGU-	−0.8/15	−2.9/2	−3.4/2	+0.4/2
i	**3**	-U**G**AA–AAGU-	−1.0/10	−10.64/1	−12.4/1	+3.0/1major +0.4/1minor

For two different arrangements of a tandem GU motif (rows d and e), small changes are found with $Mg(H_2O)_6^{2+}$, Zn^{2+}, and Cd^{2+} (≤2 ppm). In these cases, different flanking bases do not influence the results. With $Co(NH_3)_6^{3+}$, a significant change (5 ppm) occurs for the GG·UU motif (row d), but only a very small change (0.1 ppm) is found for the UG·GU motif (row e).

A 3′ guanosine adjacent to an adenosine of a sheared GA·AG pair (row f) displays strikingly large changes with $Mg(H_2O)_6^{2+}$ (6.5 ppm), Zn^{2+}, and Cd^{2+} (17 ppm), but not with $Co(NH_3)_6^{3+}$ (0.5 ppm).[17] However, a 3′ adenosine in a comparable position (row g) shows only small changes for all metals (≤1.1 ppm). A guanosine within the sheared tandem GA pair (row h) with the nearby strong binding site created by a 3′ flanking guanosine shows modest changes with Zn^{2+} and Cd^{2+} (3–4 ppm), but only small changes with $Mg(H_2O)_6^{2+}$ and $Co(NH_3)_6^{3+}$ (≤0.8 ppm). In contrast, a sheared GA·AG pair with two 3′ flanking adenosines (row i) displays relatively large changes with Zn^{2+} and Cd^{2+} (10–12 ppm) and a moderate change with $Co(NH_3)_6^{3+}$ (3 ppm), but only a small change with $Mg(H_2O)_6^{2+}$ (1 ppm). The spectrum for duplex **3** with $Co(NH_3)_6^{3+}$ displays two N7 resonances, the major one downfield of the minor one.

DISCUSSION

^{15}N NMR is an excellent method for probing interactions at purine sp^2 nitrogens when they are ^{15}N-labeled.[15] It has long been known that addition of excess amounts of soft metals like Zn^{2+} and Hg^{2+} that can accommodate inner-sphere binding to ligands cause ∼20 ppm upfield changes for the guanosine N7.[29] We have demonstrated similar upfield

changes with Zn^{2+} and Cd^{2+} at a labeled guanosine N7 in a RNA duplex decamer that was designed to model a known metal binding site near a sheared tandem GA pair in stem II of a minimal hammerhead ribozyme.[17] We have also reported smaller upfield changes (typically 2–9 ppm) upon formation of hydrogen bonds by purine N1 or N7 atoms in duplexes and triplexes.[30–32] Although the hard metal $Mg(H_2O)_6^{2+}$ with its unusually tight hydration layer normally does not interact selectively with nitrogen atoms, in X ray structures of a minimal hammerhead stem II, a guanosine N7 is precisely positioned near a phosphate from a sheared tandem GA pair and participates in the resulting strong $Mg(H_2O)_6^{2+}$ binding site.[7,12] We observed a selective 6.5 ppm upfield change for the labeled guanosine N7 at this site upon addition of $Mg(H_2O)_6^{2+}$,[17] consistent with unusually strong hydrogen bonding of the N7 with the tight hydration layer of $Mg(H_2O)_6^{2+}$.

In contrast, we have observed small *downfield* chemical shift changes for the purely electrostatic effects of Na^+.[18] We have also reported *downfield* changes for $Co(NH_3)_6^{3+}$: a small nonselective change in the hammerhead model[17] and a larger, selective change at a tandem GU pair.[18] Since the ammine ligands in $Co(NH_3)_6^{3+}$ exchange extremely slowly, the downfield changes we have observed are consistent with a combination of the strong electrostatic effect of trivalent Co^{3+}, and hydrogen bonding to the N7 that is weaker with ammine ligands than with solvent water.[33] Thus, while the direction of ^{15}N chemical shift change upon addition of various metals is a consequence of their individual properties, it is the magnitude of the change that reflects the degree of selectivity. Further, the possible magnitudes for the chemical shift changes vary with the metal, since the direct inner-sphere interactions with Zn^{2+} and Cd^{2+} have a greater effect than the outer-sphere hydrogen bonding with $Mg(H_2O)_6^{2+}$ and the electrostatic effects of $Co(NH_3)_6^{3+}$. Consequently, our discussion below focuses only on the *relative* magnitude of the changes.

Of the four diamagnetic metals used in these studies, only $Mg(H_2O)_6^{2+}$ is biologically abundant. However, a comparison of results from all four can take advantage of their different modes of interaction with RNA metal binding sites, and thus allows a more complete interpretation. This information could prove particularly useful for engineering new artificial RNA systems. Using the framework provided by the key examples of ^{15}N NMR chemical shift changes that we have already reported,[17,18] we are now in a position to extend this approach to the effects of flanking bases on metal binding to GC pairs, tandem GU pairs, and tandem GA pairs.

GC Pairs

We have previously reported that the N7 of a GC pair in hairpin **5** with two uridines flanking the guanosine displays small changes ($\leq$1.6 ppm) upon addition of these four metals (row a).[18] We have also shown that

the GG·UU motif elsewhere in this molecule does not selectively bind $Mg(H_2O)_6^{2+}$, Zn^{2+}, or Cd^{2+}, although it does selectively bind $Co(NH_3)_6^{3+}$. None-the-less, even in the presence of excess $Co(NH_3)_6^{3+}$ (3 equivalents), the GC pair with uridines flanking the guanosine only shows a small change.

We have also previously reported results for a GC pair with a 5′G and a 3′U flanking the labeled guanosine (**G**[**d**]) in duplex **6** that has a strong binding site elsewhere in the molecule at **G**[**e**].[17] Using excess metal, we found that the guanosine of this GC pair (**G**[**d**]) displays significant changes with the soft metals Zn^{2+} (6 ppm) and Cd^{2+} (9 ppm) (row b), but quite small changes with $Mg(H_2O)_6^{2+}$ (1.2 ppm) and $Co(NH_3)_6^{3+}$ (1.1 ppm). Thus, a simple GC pair with a flanking guanosine can form a binding site that attracts soft metals with moderate affinity.

We now present results for a GC pair in **1**, which does not contain another binding site (row c). The labeled guanosine of the GC pair has 5′U and 3′A flanking bases. Although the changes with $Mg(H_2O)_6^{2+}$ and $Co(NH_3)_6^{3+}$ are small ($\leq$1.3 ppm), the changes with Zn^{2+} (3 ppm) and Cd^{2+} (7 ppm) are substantial, indicating that an adjacent adenosine N7 can also help to create a moderately strong binding site for the softer metals, even though it is less basic than guanosine N7. These results demonstrate that two adjacent purines in a region of RNA with standard A form geometry are sufficient to create a binding site for the softer metals that interact through inner-sphere binding to the two closely positioned N7 atoms. However, $Mg(H_2O)_6^{2+}$ and $Co(NH_3)_6^{3+}$ with their very tight solvation layers cannot bind to these sites.

Tandem GU Motifs

Using ^{15}N NMR, we have reported selective K^+ and $Co(NH_3)_6^{3+}$ binding to a GG·UU motif in hairpin **5**[18] to which $Co(NH_3)_6^{3+}$ had already been known to bind.[34] Although $Mg(H_2O)_6^{2+}$ and $Co(NH_3)_6^{3+}$ have been proposed to bind to the same RNA motifs because of their similar size and geometry,[35] we found ^{15}N NMR evidence (row d) for selective binding by $Co(NH_3)_6^{3+}$ (5 ppm), but not for $Mg(H_2O)_6^{2+}$, Zn^{2+}, or Cd^{2+} ($\leq$2.0 ppm). In this GG·UU motif, two guanine N7 atoms and two O6 atoms, but no phosphates, form a broad cavity in the major groove with uniform negative electrostatic potential to which trivalent metal hexamines are attracted by outer-sphere binding and K^+ is attracted because its loose and flexible hydration layer is easily displaced.[18] We now report quite different results for a UG·GU motif in duplex **2** (row e). In this case, the change with $Co(NH_3)_6^{3+}$ is very small, like those with $Mg(H_2O)_6^{2+}$, Zn^{2+}, and Cd^{2+} ($\leq$0.3 ppm).

Using ^1H NMR, Colmenarejo and Tinoco had earlier compared $Co(NH_3)_6^{3+}$ binding to three different tandem GU motifs and found the order of affinity to be GU·UG $\sim$ GG·UU $>$ UG·GU.[36] Their modeling

showed that for the latter, $Co(NH_3)_6^{3+}$ was not accommodated as well into the major groove. Our results definitively support this order of affinity, with the large 5 ppm change for GG·UU demonstrating significant binding, and the very small 0.1 ppm change for UG·GU indicating negligible binding. Thus, the arrangement within the tandem GU pair is critical for metal binding, while we see no evidence that the flanking bases play a significant role.

Duplex **2** is the only example we have seen in which the change for $Mg(H_2O)_6^{2+}$ is downfield. We conclude that in this case, no phosphate is close enough to the guanosine N7 to allow hydrogen bonding with the hydration layer of $Mg(H_2O)_6^{2+}$, and the downfield change is a purely electrostatic effect.

Tandem GA Motifs

We previously used ^{15}N NMR to describe unusually strong, selective binding by $Mg(H_2O)_6^{2+}$, Zn^{2+},and Cd^{2+}, but not $Co(NH_3)_6^{3+}$, to a high-affinity site at a guanosine (**G**[**e**]) adjacent to a sheared tandem GA pair in a duplex model (**6**) of part of stem II of a minimal hammerhead ribozyme.[17] The large chemical shift changes (row f) with $Mg(H_2O)_6^{2+}$ (6.5 ppm), Zn^{2+} (17 ppm), and Cd^{2+} (17 ppm) serve to define very strong binding for these metals as measured by ^{15}N NMR.

We now report results for the same model duplex, but instead with two labeled nucleosides in the strand opposite the binding site, **4**. The labeled adenosine in **4** is in a symmetrical position relative to the tandem GA pair as is the strong binding site at **G**[**e**] in **6**. This adenosine N7 shows limited affinity ($\leq$1 ppm) for all four metals (row g), even with excess metal, contrary to an earlier proposal that it might be a strong binding site.[37] Thus, although the N7 of a flanking adenosine can help create a binding site for soft metals adjacent to a GC pair in standard A form geometry (row c), it does not do so with the altered geometry of a sheared tandem GA pair.

Formation of a sheared GA·AG pair results in characteristic hydrogen bonds between the G amino and the AN7 and between the GN3 and the A amino, as well as cross-strand stacking of G on G and A on A.[38–40] In this unusual geometry, the N7 and O6 atoms of the two cross-strand stacked guanosines are closely aligned, and might form a metal binding site in the absence of a stronger one nearby. Duplex **4** with a labeled internal guanosine has a strong binding site in the opposite strand that involves the 3′ flanking guanosine (**G**[**e**] in **6**). Even with excess metal (row h), we observe only small changes ($\leq$0.8 ppm) for the internal guanosine in **4** with $Mg(H_2O)_6^{2+}$ and $Co(NH_3)_6^{3+}$, and modest changes (3 ppm) with Zn^{2+} and Cd^{2+}.

In contrast, duplex **3** has a sheared GA·AG pair with no additional binding sites because it has 3′ flanking adenosines on both sides (row

i). While the change with $Mg(H_2O)_6^{2+}$ is small (1 ppm), the change for the major resonance with $Co(NH_3)_6^{3+}$ is moderate (3 ppm) and those with Zn^{2+} (10 ppm) and Cd^{2+} (12 ppm) are significant. While not of the 17 ppm magnitude seen at **G**[**e**] in **6** (row f), the results in row i reflect affinities that are somewhat larger than to two adjacent GC pairs (row b) and much larger than to isolated GC pairs (row a). Further, the spectrum for duplex **3** with $Co(NH_3)_6^{3+}$ is unique among all those reported here in displaying an additional minor N7 resonance with only a 0.4 ppm change. These results presumably reflect the presence of two different conformational forms in slow exchange that bind $Co(NH_3)_6^{3+}$ to different extents.

CONCLUSION

The RNA molecules described here contain three common types of small motifs that can bind metals in different ways: GC pairs, tandem GU pairs, and tandem GA pairs. A comparison of their ^{15}N NMR chemical shift changes in the presence of added metal demonstrates the important roles that flanking bases can have, in some cases but not others, on the nature and extent of metal binding to sites containing guanosine N7 atoms. For GC pairs in standard A form geometry, uridines flanking the guanosine have no effect on the limited metal binding, while either a flanking guanosine or adenosine significantly increases binding of the soft metals Zn^{2+} and Cd^{2+}, but not the harder $Mg(H_2O)_6^{2+}$ or $Co(NH_3)_6^{3+}$. Tandem GU and GA pairs have altered geometries that play a significant role in their binding to metals. Of the four metals studied here, the GG·UU motif preferentially binds $Co(NH_3)_6^{3+}$, while the UG·GU motif does not bind any of the metals. Metal binding to sheared GA·AG pairs is profoundly affected by the flanking bases. With a 3′ guanosine flanking one of the adenosines, an unusually strong binding site for $Mg(H_2O)_6^{2+}$, Zn^{2+}, and Cd^{2+}, but not $Co(NH_3)_6^{3+}$, is created that involves that guanosine N7 and a phosphate from the tandem pair. This high-affinity site precludes any significant binding of metals to the cross-strand stacked guanosines within the tandem pair. On the other hand, a 3′ flanking adenosine does not create a comparable high-affinity site. In this case, the cross-strand stacked guanosines within the tandem pair can therefore attract soft metals as well as $Co(NH_3)_6^{3+}$ to their closely aligned N7 and O6 atoms with surprising affinity, while the lack of a close phosphate prevents $Mg(H_2O)_6^{2+}$ from being attracted. For these biologically common motifs,[41,42] a qualitative comparison of ^{15}N NMR chemical shift changes with the four metals described here has provided valuable insight into the differences among their metal binding affinities and the varying effects of flanking bases.

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