

## Tandem Electrostatic Effect from the First to the Third Aglycon in the Trimeric RNA Owing to the Nearest-Neighbor Interaction

P. Acharya, S. Acharya, A. Földesi, and J. Chattopadhyaya\*

Contribution from the Department of Bioorganic Chemistry, Box 581, Biomedical Center, Uppsala University, S-751 23 Uppsala, Sweden.

Received August 26, 2002; Revised Manuscript Received December 17, 2002 E-mail: jyoti@boc.uu.se

**Abstract:** We here show an electrostatic polar- $\pi$  interaction from the first to the third aglycon, via the second aglycon, in the ground state in two single stranded trimeric RNAs, 5'-GpA<sup>1</sup>pA<sup>2</sup>-3' (**3**) and 5'-GpApC-3' (**4**), as a result of intramolecular nearest neighbor offset-stacking. The experimental evidence in support of this conclusion has been obtained by comparing the pK<sub>a</sub>s of each aglycone in the two trimers with those of guanosine 3'-ethyl phosphate, GpEt (**1**) and 5'-GpA-3' (**2**): Thus, the pK<sub>a</sub> of N<sup>1</sup>-H of guanin-9-yl of 5'-GpA<sup>1</sup>pA<sup>2</sup>-3' (**3**) could be measured by pH titration (pH 7.3–11.6) of its own  $\delta$ H8G (pK<sub>a</sub> 9.75  $\pm$  0.02) as well as from  $\delta$ H8A<sup>1</sup> (pK<sub>a</sub> 9.72  $\pm$  0.02) and  $\delta$ H2A<sup>1</sup> (pK<sub>a</sub> 9.83  $\pm$  0.04) of the neighboring pA<sup>1</sup>p moiety and the  $\delta$ H8A<sup>2</sup> (pK<sub>a</sub> 9.83  $\pm$  0.02) of the terminal pA<sup>2</sup> moiety. Similarly, the pH titration of GpApC (**4**) shows the pK<sub>a</sub> of N<sup>1</sup>-H of guanin-9-yl from its own  $\delta$ H8G (pK<sub>a</sub> 9.88  $\pm$  0.03) as well as from  $\delta$ H8A (pK<sub>a</sub> 9.87  $\pm$  0.01) of the neighboring pAp moiety, and  $\delta$ H5/H6C (pK<sub>a</sub> 9.88  $\pm$  0.01 and 9.90  $\pm$  0.01 respectively) of the 3'-terminal cytosin-1-yl. This intramolecular nearest neighbor electrostatic interaction in the single-stranded RNA modulates the pseudoaromaticity of the nearest neighbors by almost total transmission of  $\Delta G_{pK_a}^{\circ}$  because they constitute an extended array of offset-stacked coupled aromatic heterocycles within a polyanionic sugar-phosphate backbone at the ground state. The enhanced basicity of Gp residue by ca. 0.6 pK<sub>a</sub> unit in the trimers compared to that of the dimer is a result of the change in the electrostatic microenvironment owing to the nearest neighbors in the former (the nucleobases as well as the phosphates). Thus, the  $\Delta G_{pK_a}^{\circ}$  from the 5'-guanylate ion to the 3'-end aglycon via the central adenin-9-yl is 55 to 56 kJ mol<sup>-1</sup> in each step through a distance spanning  $\sim$ 6.8 Å in an unfolded state. As a result, the pK<sub>a</sub> of guanin-9-yl moiety has become 9.25  $\pm$  0.02 in GpEt (**1**), 9.17  $\pm$  0.02 in GpA (**2**), 9.75  $\pm$  0.02 in GpApA (**3**), and 9.88  $\pm$  0.03 in GpApC (**4**). This means that guanin-9-yl moiety of trimers **3** and **4** is more basic than in the monomer or the dimer. The net outcome of this electrostatic cross-talk between the two neighboring heterocycles is creation of new hybrid aglycones in an oligo or polynucleotide, whose physicochemical property and the pseudoaromatic character are completely dependent both upon the nearest neighbors, and whether they are stacked or unstacked. Thus, this tunable physicochemical property of an aglycon (an array of the extended genetic code) may have considerable implication in our understanding of the specific ligand binding ability of an aptamer, the pK<sub>a</sub> and the hydrogen bonding ability of nucleic acids in a microenvironment, or in the triplet usage by the anticodon-codon interaction in the protein biosynthesis in the ribosome.

### Introduction

The chemical nature of the nucleobase imparts the sequence specificity of nucleic acids, which plays a key role in both hydrogen bonding<sup>1</sup> and stacking,<sup>2</sup> leading to various biological function such as replication, transcription, and translation.<sup>2,3</sup> The nucleobase is also a key element that responds to any micro-environmental changes by protonation, deprotonation, metalation or ligand binding through intra- and intermolecular interaction.<sup>4,5</sup> Although the hydrogen bonding interactions (base-pairing)<sup>1,2,9</sup>

are relatively well studied, the experimental data supporting the nature of stacking interactions<sup>6–10</sup> is very limited. Recent

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studies on the thermodynamic stabilization of the same strand by nearest-neighbor interaction in DNA and RNA<sup>6,7</sup> owing to inter- or/and intrastrand stacking interactions by a dangling base<sup>6</sup> or a 3'- or 5'-tethered chromophore<sup>7</sup> have shed new light on the importance of stacking in the self-assembly process of DNA or RNA. Studies<sup>18</sup> such as temperature and/or concentration dependent intermolecular base-base association constants, as well as substituent effects<sup>11</sup> in various nonbiological aromatic systems have also elucidated the role of aromatic stacking interactions as a major force to the stability of nucleic acids.

We have recently demonstrated that a nucleobase in a dinucleoside (3'→5') monophosphate showed not only its own  $pK_a$  but also the  $pK_a$  of the nearest neighbor, which provided a direct evidence of electrostatic interaction between two nearest neighbor nucleobases in the ground state as a result of intramolecular offset-stacking.<sup>30</sup> This electrostatic interaction leads to almost total modulation of pseudoaromaticity by nearly total transmission of  $\Delta G_{pKa}^\circ$ <sup>14,20</sup> from one nucleobase to the nearest neighbor (16–53 kJ mol<sup>-1</sup>, depending upon the nucleobase and/or cationic or anionic state).<sup>30</sup> This intramolecular electrostatic interaction suggested that the chemical nature of each aglycone in a stacked dinucleotide, unlike simple monomers, constitute a electronically coupled heterocyclic system.

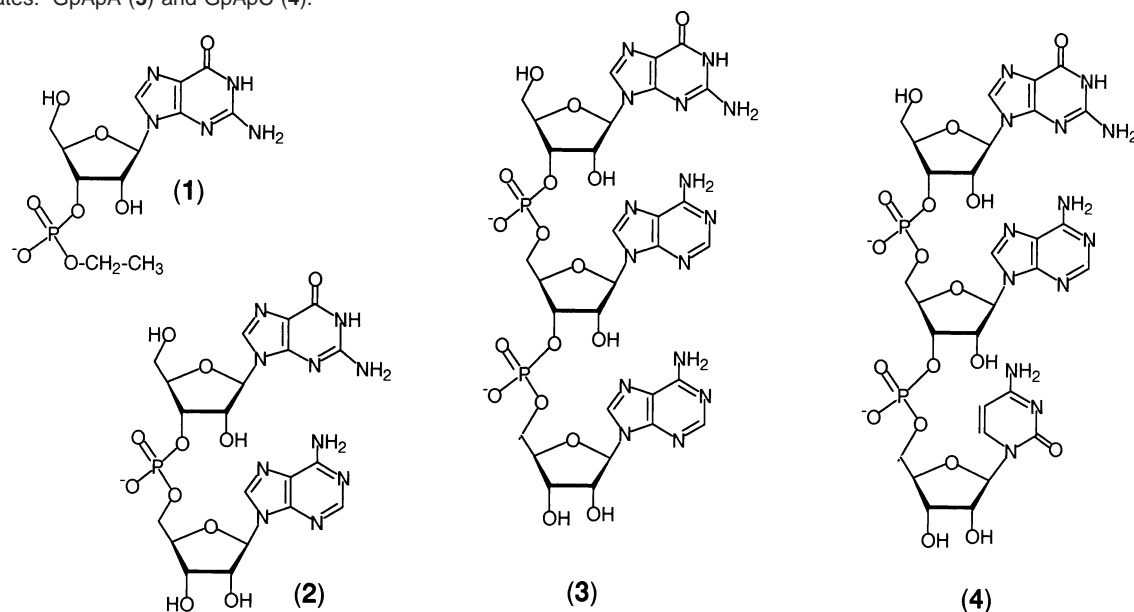
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## Results and Discussion

We here report that this physicochemical modulation of the pseudoaromatic character of aglycones by the nearest-neighbor intramolecular electrostatic interaction can propagate from the first to the third nucleobase, via the second aglycon, in the RNA trimers. This has been evidenced by observing the  $pK_a$  of the

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- (23) The electrostatic interaction between the partial charge distribution of a nucleobase (depending upon its pseudoaromatic character) and the  $\pi$ -electron density corresponding to the next base constitutes atom- $\pi\sigma$  interaction.<sup>8,10</sup> The deprotonation at guanine-9-yl changes partial charge distribution of the aglycone (particularly for heteroatoms), in the ground state, which in turn, causes a differential electrostatic interaction between the neighboring nucleobases in deprotonated state. The pH-dependent chemical shift change of a particular proton (H) between neutral (N) and deprotonated (D) state [ $\Delta \delta_{N-D}^H = \delta_{N-D}^H - \delta_D^H$ , where  $\Delta \delta_{N-D}^H$  corresponds to the relative shielding (upfield shift,  $\Delta \delta_{N-D}^H > 0$ ) or deshielding (downfield shift,  $\Delta \delta_{N-D}^H < 0$ ) as a function of pH] are the basis for this atom- $\pi\sigma$  interaction<sup>8,10</sup> between nearest neighbor nucleobases in **1–4**. The pH-dependent shift of  $\delta_{H8G}$  ( $\Delta \delta_{N-D}^{H8G}$ ) for GpEt (**1**),<sup>30</sup> GpA (**2**),<sup>30</sup> GpApA (**3**), and GpApC (**4**) are as follows: 0.149 for **1**; 0.141 for **2**; 0.152 for **3** and 0.160 for **4**. The pH-dependent shift of  $\delta_{H8A}$  and  $\delta_{H2A}$  ( $\Delta \delta_{N-D}^{H8A}$  and  $\Delta \delta_{N-D}^{H2A}$ , respectively) of terminal pA moieties in GpA (**2**)<sup>30</sup> and GpApA (**3**) are as follows: for **2**,<sup>30</sup>  $\Delta \delta_{N-D}^{H8A}$ : -0.056 and  $\Delta \delta_{N-D}^{H2A}$ : -0.004 and for **3**,  $\Delta \delta_{N-D}^{H8A}$ : -0.052 and  $\Delta \delta_{N-D}^{H2A}$ : -0.002. Similarly,  $\Delta \delta_{N-D}^{H8A}$  and  $\Delta \delta_{N-D}^{H2A}$  of pAp moieties in GpApA (**3**) and GpApC (**4**) are as follows: for **3**,  $\Delta \delta_{N-D}^{H8A}$ : -0.055 and  $\Delta \delta_{N-D}^{H2A}$ : 0.014 and for **4**,  $\Delta \delta_{N-D}^{H8A}$ : -0.117 and  $\Delta \delta_{N-D}^{H2A}$ : 0.003. Moreover, the pH-dependent shift of  $\delta_{H5C}$  and  $\delta_{H6C}$  ( $\Delta \delta_{N-D}^{H5C}$  and  $\Delta \delta_{N-D}^{H6C}$ , respectively) of pC moieties in GpApC (**4**) are as follows:  $\Delta \delta_{N-D}^{H5C}$ : -0.098 and  $\Delta \delta_{N-D}^{H6C}$ : -0.069.
- (24) The difference in the efficiency of the intramolecular electrostatic interaction amongst **2–4** can be quantitatively assessed by comparing the  $\Delta \Delta G_{pKa}^\circ$ , which can be obtained by subtracting  $\Delta G_{pKa}^\circ$  of the GpA (**2**)<sup>30</sup> or GpApA (**3**) or GpApC (**4**) from that of the monomeric GpEt (**1**): Thus, the  $\Delta \Delta G_{pKa}^\circ = 0.4$  kJ mol<sup>-1</sup> (calculated from  $\delta_{H8}$  of guanine-9-yl as well as from other proton markers, see below) for GpA (**2**),<sup>30</sup> 2.8 kJ mol<sup>-1</sup> for GpApA (**3**) and 3.6 kJ mol<sup>-1</sup> for GpApC (**4**) shows that (i) almost total transmission of the free energy of deprotonation (within the experimental error of  $\pm 0.1$  to  $\pm 0.2$  kJ mol<sup>-1</sup>) to the neighboring aglycones of guanylate anion in **2–4** [adenine-9-yl of pA in **2** ( $\delta_{H8A}$ :  $\Delta \Delta G_{pKa}^\circ = 0.5$  kJ mol<sup>-1</sup>); adenine-9-yl of pA<sup>1p</sup> and that of terminal pA<sup>2</sup> in **3** ( $\delta_{H8A}$ :  $\Delta \Delta G_{pKa}^\circ = 2.7$  kJ mol<sup>-1</sup>,  $\delta_{H2A}$ :  $\Delta \Delta G_{pKa}^\circ = 3.3$  kJ mol<sup>-1</sup> and  $\delta_{H8A}$ :  $\Delta \Delta G_{pKa}^\circ = 3.3$  kJ mol<sup>-1</sup>) as well as adenine-9-yl of pAp and further to terminal cytosine-1-yl in **4** ( $\delta_{H8A}$ :  $\Delta \Delta G_{pKa}^\circ = 3.5$  kJ mol<sup>-1</sup>,  $\delta_{H5C}$ :  $\Delta \Delta G_{pKa}^\circ = 3.9$  kJ mol<sup>-1</sup> and  $\delta_{H6C}$ :  $\Delta \Delta G_{pKa}^\circ = 3.6$  kJ mol<sup>-1</sup>]. (ii) the small differences in  $\Delta \Delta G_{pKa}^\circ$  found is owing to slight changes in the microenvironment of the dimer and as well as the electronic properties trimers with respect to the monomer (see also ref 25).

**Scheme 1.** Guanosine 3'-ethyl phosphate: GpEt(1);<sup>30</sup> Di-ribonucleoside (3'→5') Monophosphate: GpA (2);<sup>30</sup> Tri-ribonucleoside (3'→5') Diphosphates: GpApA (3) and GpApC (4).



guanylate anion of the 5'-Gp moiety from the 9-adeninyl moieties of the central pAp as well as from the distant 3'-terminal pA moiety in 5'-GpApA-3' (3), or from the 1-cytosinyl moiety of pC-3' in 5'-GpApC-3' (4). This observation of  $\Delta G_{pK_a}^{\circ}$  (14,20) transmission (55 to 56 kJ mol<sup>-1</sup>) within the aglycons of the RNA trimers is based on the comparison with the  $pK_a$  of the guanylate anion of the monomeric guanosine 3'-ethyl phosphate, GpEt (1), and the dimer, 5'-GpA-3' (2) (Scheme 1). This has led us to observe how the change of the electronic character of guanin-9-yl affect the neighboring aglycones, pAp and pA in 5'-GpApA-3' (3), or pAp and pC in 5'-GpApC-3' (4). Our observation of the tandem intramolecular electrostatic interaction in simple offset-stacked trimeric RNAs shows that the pseudoaromatic characters of the constituent aglycones within the minimal genetic information unit (i.e. the trimer) are

indeed modulated in the ground state by the pseudoaromatic makeup of the nearest neighbor, unlike in the monomers. This may lead us to understand the codon selection or degeneracy in the triplet usage in the anticodon-codon interaction negotiated in the ribosomal machinery.

The pH-dependent titration of aromatic protons of GpA (2) [ $\delta H8G$  ( $pK_a$  9.17  $\pm$  0.02) and  $\delta H8A$  ( $pK_a$  9.16  $\pm$  0.02)] as well as that of the  $\delta H8G$  of GpEt (1) [Panel (A) in Figure 1 and the corresponding Hill plot in Panel (a) in Figure 2]<sup>30</sup> in the pH range 6.9–10.7 are shown for comparison [Panels (B) and (C) for titration plots in Figure 1, and panels (b) and (c) for Hill plots in Figure 2]<sup>30</sup> with the titration profiles of the trimers, 5'-GpApA-3' (3) and 5'-GpApC-3' (4) (see below).

Thus, the pH titration studies (pH 7.3–11.6) of 5'-GpA<sup>1</sup>pA<sup>2</sup>-3' (3) showed the  $pK_a$  of N<sup>1</sup>-H of guanin-9-yl from its own  $\delta H8G$  ( $pK_a$  9.75  $\pm$  0.02) [Panels D–G for titration plots in Figure 1, and Panels d–g for Hill plots in Figure 2], as well as from  $\delta H8A^1$  ( $pK_a$  9.72  $\pm$  0.02) and  $\delta H2A^1$  ( $pK_a$  9.83  $\pm$  0.04) of the neighboring pA<sup>1</sup>p moiety [Panels F and G for titration plots in Figure 1, and Panels f and g for Hill plots in Figure 2] and the  $\delta H8A^2$  ( $pK_a$  9.83  $\pm$  0.02) of the terminal pA<sup>2</sup> moiety [Panel E for titration plot in Figure 1, and panel (e) for Hill plot in Figure 2]. It is noteworthy that  $\delta H2A^2$  of the terminal pA<sup>2</sup> moiety do not respond in the pH titration.<sup>25</sup> This means that both the imidazole and the pyrimidine parts of the middle pA<sup>1</sup>p moiety are experiencing the electrostatic interaction<sup>23</sup> from the neighboring guanylate anion at the 5'-end. The electrostatic interaction from the middle pA<sup>1</sup>p to the imidazole part of 3'-terminal pA<sup>2</sup> moiety is mediated by the offset-stacking, whereas the pyrimidine part of the pA<sup>2</sup> moiety do not participate in any such interaction,<sup>10,26</sup> which is reminiscent of that of GpA (2).<sup>30,31</sup>

Similarly, the pH titration of GpApC (4) [Panels H–K for titration plots in Figure 1, and Panels h–k for Hill plots in

(25) The relative stacking ability as well as  $\Delta pK_a$  (between the trimer and the dimer) show that the strength of the stacking is as follows:  $2 < 3 > 4$ .<sup>22,23</sup> However, in the alkaline pH, 2 becomes slightly destacked, whereas 3 and 4 becomes slightly more stacked vis-à-vis stabilized<sup>22</sup> in the ionic form which may contribute to the relatively high basicity of guanin-9-yl in 3 and 4 compared to either in 2 (ca. 0.6  $pK_a$  unit) or in their monomeric counterpart 1 (ca. 0.5–0.6  $pK_a$  unit). This may also account for relatively higher pH-dependent tunability<sup>23</sup> of  $\delta H8G$  for 3 and 4 compared to that in 1 and 2,<sup>30</sup> which in turn shows the sequence dependent intramolecular modulation of pseudoaromatic character of guanin-9-yl upon the deprotonation. The pH-dependent chemical shift change<sup>23</sup> of  $\delta H2A$  ( $\Delta\delta_{N-D}^{H2A}$ ) in GpA (2) and GpApC (4) are almost invariant, nevertheless in GpA<sup>1</sup>pA<sup>2</sup> (3),  $\Delta\delta_{N-D}^{H2A}$  of pA<sup>1</sup>p shows a detectable upfield shift over the pH range. Thus, the different pH-dependent tunability of  $\delta H8$  and  $\delta H2$  of adenin-9-yl in pA and pAp, indicates the sequence-dependency owing to differential modulation of the pseudoaromatic character of adenine-9-yl in 2–4 by the effect of the nearest-neighbor.

(26) Such pH-dependent atom specific response (evident from specific chemical shift change,  $\Delta\delta_{N-D}^H$ )<sup>23</sup> from the neighbors show the different intramolecular spatial orientation of the nucleobases with respect to each other. Thus, in GpApA (3), the pAp is most probably experiencing a “T-shaped”<sup>21</sup> interaction with the 9-guanylate base, whereas pA is “offset-stacked”<sup>8,10</sup> to pAp to experience the differential electrostatic interaction from the 5'-terminal guanylate to minimize the Coulombic repulsion in GpApA (3). In contrast, the pAp in GpApC (4) is “offset stacked” such that imidazole part of pAp is within the influence of  $\pi$ -electron system of the guanylate anion, whereas the C5–C6 double bond of pC is also “offset stacked” with respect to pAp.

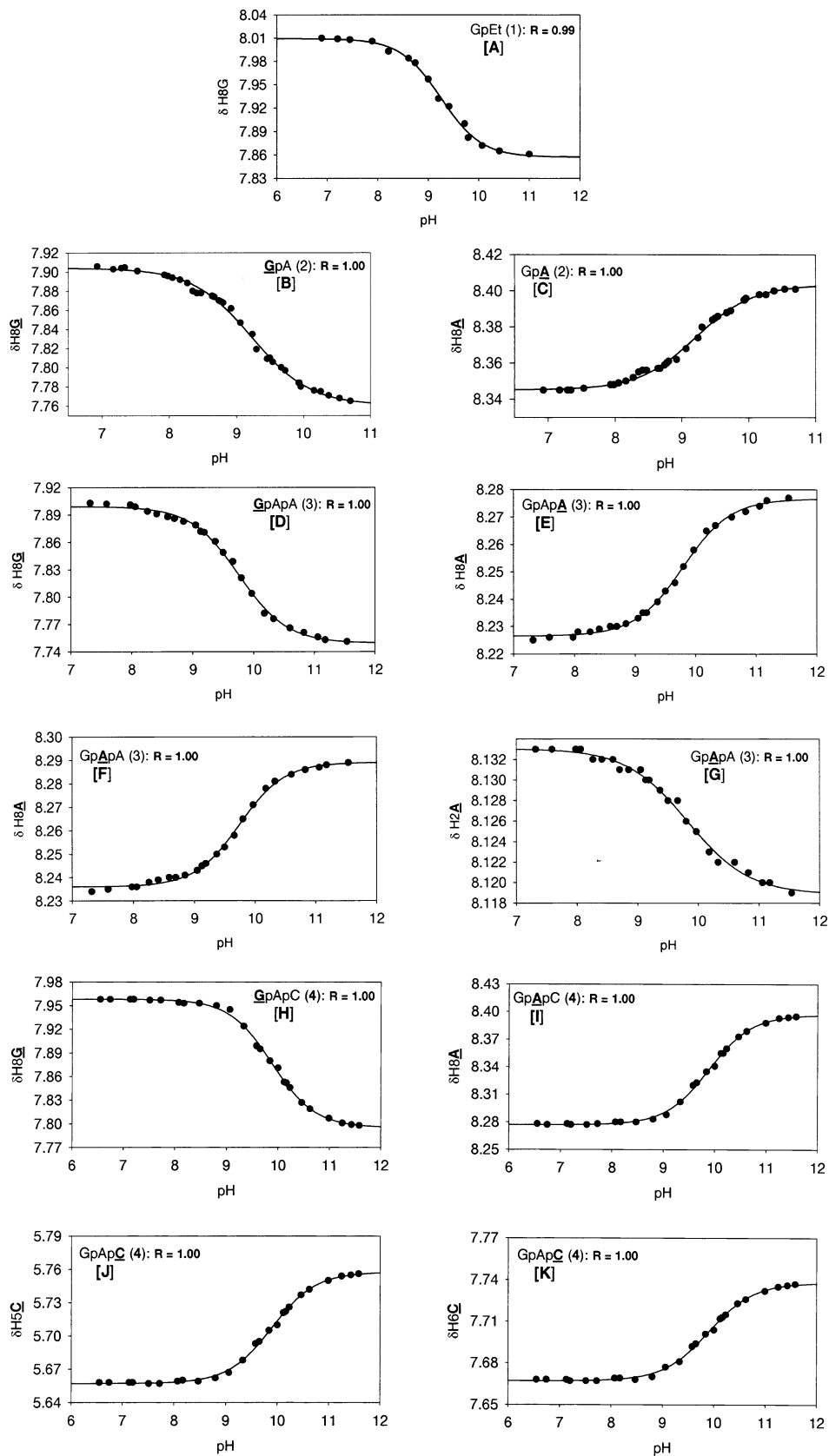
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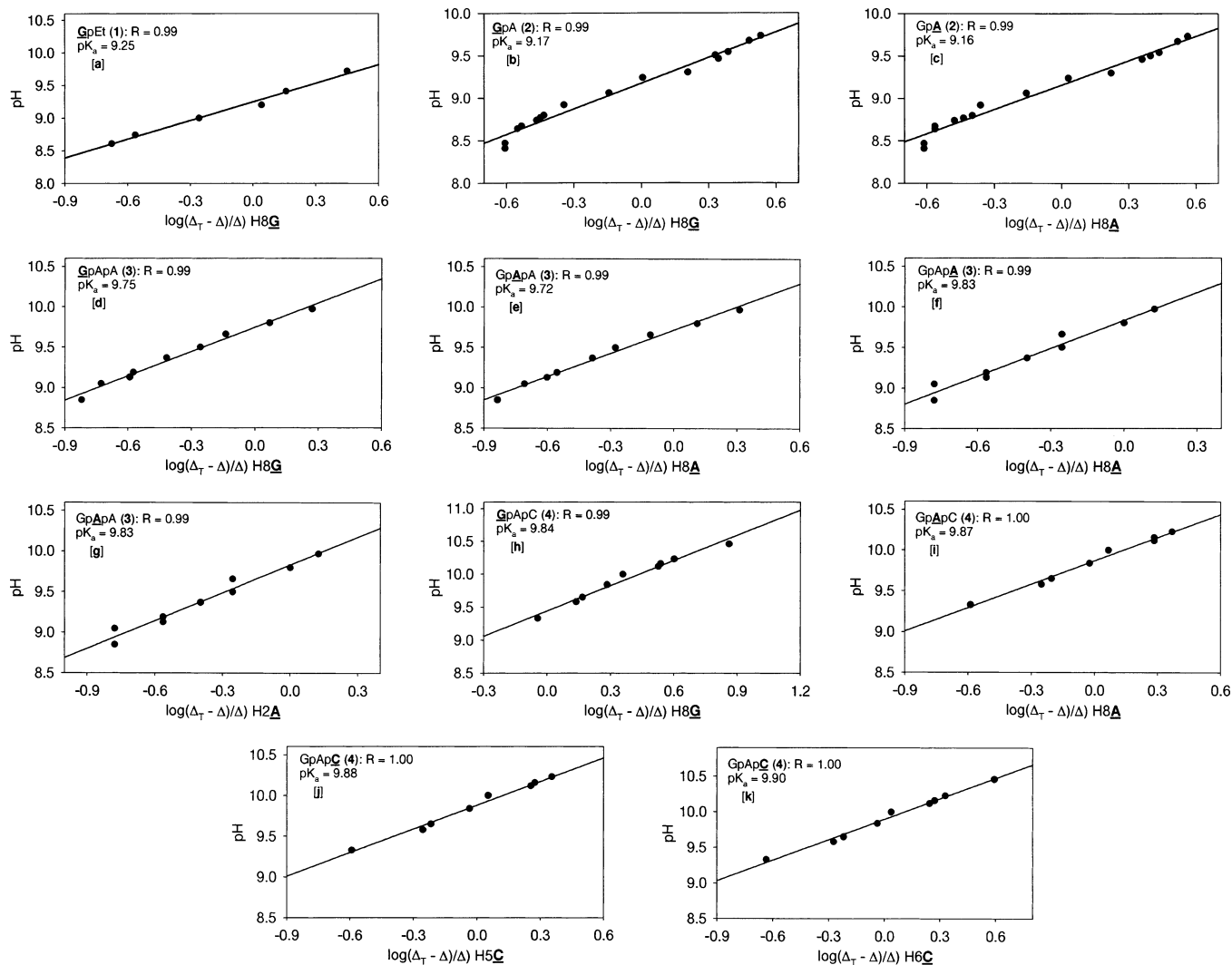
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(31) Since  $\delta H2A$  of neighboring adenine-9-yl in GpA (2)<sup>30</sup> does not respond over the above pH range clearly shows that the transmission of the charge<sup>12,17,23</sup> from the guanylate ion takes place exclusively to the imidazole part of the neighboring adenine-9-yl, in preference to the pyrimidine part.<sup>30</sup> We attribute this electrostatic interaction to offset stacking geometry<sup>10,11</sup> via atom- $\pi$  mechanism,<sup>8,10</sup> which means that the imidazole edge of adenin-9-yl is within the stacking interaction of 9-guanylyl face.



**Figure 1.** Panels A–K show the pH dependent  $^1\text{H}$  chemical shift of aromatic protons:  $\delta\text{H8G}$  of GpEt (1) [Panel A],<sup>30</sup> GpA (2) [Panel B],<sup>30</sup> GpApA (3) [Panel D] and that of GpApC (4) [Panel H];  $\delta\text{H8A}$  of GpA (2) [panel C],<sup>30</sup> pA of GpApA (3) [Panel E], pAp of GpApA (3) [Panel F] and that of GpApC (4) [Panel I];  $\delta\text{H2A}$  of pAp of GpApA (3) [Panel G];  $\delta\text{H5C}$  and  $\delta\text{H6C}$  of GpApC (4) [Panels J and K, respectively] within the pH values of  $6.7 \leq \text{pH} \leq 11.6$ . Chemical shift variations at average 25 different pH values ( $6.7 \leq \text{pH} \leq 11.6$ ) have been measured in an interval of 0.2–0.3 pH units to obtain the sigmoidal curves.  $\text{pK}_a$  values have been calculated [see the Experimental Section for details] from the corresponding Hill plots [see Figure 2].





**Figure 2.** Panel **a** shows the Hill plots for  $\delta\text{H8G}$  of GpEt (1).  $\Delta_T$  ( $7.0 \leq \text{pH} \leq 11.0$ ) = 0.149 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 0.95 ( $\sigma = 0.05$ ) and  $\text{pK}_a = 9.25$  ( $\sigma = 0.02$ ).<sup>30</sup> Panel **b** shows the Hill plots for  $\delta\text{H8G}$  of GpA (2).  $\Delta_T$  ( $6.9 \leq \text{pH} \leq 10.7$ ) = 0.141 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 1.00 ( $\sigma = 0.04$ ). Panel **c** shows the Hill plots for  $\delta\text{H8A}$  of GpA (2).  $\Delta_T$  ( $6.9 \leq \text{pH} \leq 10.7$ ) = 0.056 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 0.96 ( $\sigma = 0.03$ ).<sup>30</sup> Panel **d** shows the Hill plots for  $\delta\text{H8G}$  of GpApA (3).  $\Delta_T$  ( $7.3 \leq \text{pH} \leq 11.6$ ) = 0.152 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 1.00 ( $\sigma = 0.04$ ). Panel **e** shows the Hill plots for  $\delta\text{H8A}$  of pAp in GpApA (4).  $\Delta_T$  ( $7.3 \leq \text{pH} \leq 11.6$ ) = 0.055 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 0.96 ( $\sigma = 0.04$ ). Panel **f** shows the Hill plots for  $\delta\text{H8A}$  of pA in GpApA (3).  $\Delta_T$  ( $7.3 \leq \text{pH} \leq 11.6$ ) = 0.052 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 1.14 ( $\sigma = 0.08$ ). Panel **g** shows the Hill plots for  $\delta\text{H2A}$  of GpApA (3).  $\Delta_T$  ( $7.3 \leq \text{pH} \leq 11.6$ ) = 0.014 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 1.14 ( $\sigma = 0.08$ ). As H2A of pA moiety in **2** and **3** showed negligible change<sup>23</sup> as a function of pH, the Hill plot analyses have not been performed. Panel **h** shows the Hill plots for  $\delta\text{H8G}$  of GpApC (4).  $\Delta_T$  ( $6.7 \leq \text{pH} \leq 11.6$ ) = 0.240 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 1.10 ( $\sigma = 0.07$ ). Panel **i** shows the Hill plots for  $\delta\text{H8A}$  of GpApC (4).  $\Delta_T$  ( $6.7 \leq \text{pH} \leq 11.6$ ) = 0.117 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 0.96 ( $\sigma = 0.03$ ). As H2A showed negligible change<sup>23</sup> as a function of pH, the Hill plot analysis has not been performed. Panels **j** and **k** show the Hill plots for  $\delta\text{H5C}$  and  $\delta\text{H6C}$  of GpApC (3), respectively.  $\Delta_T$  for H5C ( $6.7 \leq \text{pH} \leq 11.6$ ) = 0.059 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 0.97 ( $\sigma = 0.03$ ).  $\Delta_T$  for H6C ( $6.7 \leq \text{pH} \leq 11.6$ ) = 0.098 ppm and the plot of  $\log((\Delta_T - \Delta)/\Delta)$  vs pH gave a straight line with a Hill slope = 0.96 ( $\sigma = 0.04$ ).

Figure 2] in the alkaline range (pH 6.7–11.6) shows the  $\text{pK}_a$  of N<sup>1</sup>-H of guanin-9-yl from its own  $\delta\text{H8G}$  ( $\text{pK}_a$  9.88  $\pm$  0.03) and from  $\delta\text{H8A}$  ( $\text{pK}_a$  9.87  $\pm$  0.01) of the neighboring pAp moiety (but not from its  $\delta\text{H2A}$ ), as well as from  $\delta\text{H5C}$  and  $\delta\text{H6C}$  ( $\text{pK}_a$  9.88  $\pm$  0.01 and 9.90  $\pm$  0.01 respectively) of the 3'-terminal cytosin-1-yl [Panels **J** and **K** for titration plots in Figure 1, and Panels **j** and **k** for Hill plots in Figure 2]. This tandem electrostatic interaction from the 5'-guanylate ion to the 3'-end aglycon through the central 9-adeninyl moiety (which is  $\sim 6.8$  Å between the two terminal aglycons in the unfolded state) in the ground state suggests that the terminal pApC stacking in GpApC (4) is weaker<sup>22,23</sup> compared to pA<sup>1</sup>pA<sup>2</sup> stacking in

GpA<sup>1</sup>pA<sup>2</sup> (3) [compare  $\Delta\delta_{\text{H8G}}^{\text{d/t}}$ ,<sup>22</sup>  $\Delta\delta_{\text{H8A(pAp)}}^{\text{d/t}}$ <sup>22</sup> and  $\Delta\delta_{\text{N-D}}^{\text{H}}$ <sup>23</sup>] because the central pA<sup>1</sup>p moiety is rather poorly offset-stacked<sup>8,25</sup> in the former (because  $\delta\text{H2A}$  does not respond to the deprotonation of neighboring guanin-9-yl) compared to the latter. An estimation of the dimerization and trimerization shift<sup>22</sup> for  $\delta\text{H8G}$  and  $\delta\text{H8A}$  at neutral and deprotonated states allows us to compare the extent of offset-stacking, which suggests that the strength of stacking in neutral<sup>30</sup> versus deprotonated is sequence-dependent.<sup>22,25</sup> Thus, the stacking is comparable in GpApA (3) both in the neutral and deprotonated states, whereas it is slightly favored in the deprotonated state for GpApC (4). This means that the intramolecular electrostatic interaction is

quite universal both in neutral, deprotonated as well as in protonated form<sup>30</sup> to cross-modulate the pseudoaromatic character of nucleobases in nucleic acids by the nearest neighbor interaction.

This study shows that the  $pK_a$ s of guanine-9-yl moiety change owing to the nearest neighbor electrostatic interaction with both the nucleobases as well as with the phosphate(s), which are as follows:  $9.25 \pm 0.02$  in GpEt (**1**),<sup>30</sup>  $9.17 \pm 0.02$  in GpA (**2**),<sup>30</sup>  $9.75 \pm 0.02$  in GpApA (**3**),  $9.88 \pm 0.03$  in GpApC (**4**). Thus, it shows that guanine-9-yl moiety of trimers **3** and **4** are more basic than the monomer or dimer.

Thus, the  $\Delta G_{pK_a}^{\circ}$ <sup>14,20</sup> obtained from H8G for guanine-9-yl is  $55.6 \pm 0.1$  kJ mol<sup>-1</sup> for G<sup>-</sup>pApA (**3**), and  $56.4 \pm 0.2$  kJ mol<sup>-1</sup> for G<sup>-</sup>pApC (**4**). These should be compared with the  $\Delta G_{pK_a}^{\circ}$  of  $52.8 \pm 0.1$  kJ mol<sup>-1</sup> for G<sup>-</sup>pEt (**1**)<sup>30</sup> and  $52.4 \pm 0.1$  kJ mol<sup>-1</sup> for G<sup>-</sup>pA (**2**).<sup>30</sup> Thus  $\Delta \Delta G_{pK_a}^{\circ}$  for the trimers (**3** and **4**) is larger<sup>24,25</sup> than that of dimer (**2**) because of enhanced stacking<sup>25</sup> as well as owing to the influence of phosphate,<sup>32</sup> which effectively increase the intramolecular nearest neighbor electrostatic interactions. It is noteworthy that the  $\Delta G_{pK_a}^{\circ}$  obtained from either H8A/H2A of the middle residue or H8A/H2A or H5/H6C of the 3'-terminal residue within any single RNA trimer is very close to what is obtained from that of H8G of the 5'-terminal residue ( $55$  to  $56$  kJ mol<sup>-1</sup>), which suggests that the intramolecular electrostatic interaction of the 5'-guanylate ion to the aglycon at the 3'-end via the central adenin-9-yl is very close to 100% at the ground state.<sup>24</sup>

The fact that we observe the  $\Delta G_{pK_a}^{\circ}$  both from the guanylate anion as well as from the nearest neighbors in ssRNA, shows that any two nearest neighbors in these trimers are most probably interacting by polar- $\pi$  effect,<sup>21a,b,l-n</sup> where the attractive Coulombic term dominates the electrostatic interaction,<sup>21c,d</sup> in the neutral state either by edge-to-face<sup>11</sup> or center-to-edge (parallel offset)<sup>10,11e,21n</sup> arrangement. However, upon formation of the 9-guanylate anion, the H8, H5, or H6 of both the central pAp and the terminal pAp/pC in GpApA (**3**) and GpApC (**4**) becomes deshielded<sup>23</sup> compared to the neutral state, which again shows that the stabilizing polar- $\pi$  effect in the neutral state becomes destabilized in the deprotonated state because of the Coulombic repulsive anion- $\pi$  interaction (hence destacking). We do not see any charge transfer (CT) band in the UV spectra neither for the neutral or for the deprotonated trimeric RNA, which rules out

any CT effects, and demonstrates the dominance of polar- $\pi$  effect<sup>21a,b,l-n</sup> over the CT effects. The polar- $\pi$  effect by through-space Coulombic interaction<sup>21l</sup> has earlier been invoked, for example, in the molecular complexation of a series of disubstituted naphthalenes<sup>21b</sup> with an electron-rich host, which showed strongest binding with dicyano and weakest with the dimethoxy substituents. The manifestation of polar- $\pi$  effect has also been attributed to explain the interaction between ions and arenes<sup>21e-i</sup> (such as carboxylate ion/arene<sup>21e-g</sup> and trimethylammonium ion/arene interactions<sup>21h,i</sup>) as well as amine/arene interactions<sup>21j</sup> in proteins and hydrogen bonding of water to cyclophanes.<sup>21k</sup> The geometrical requirement for this is that the two nearest neighbors should have an optimal edge-to-face or center-to-edge (parallel offset) arrangement, such that they can have interactions between their respective  $\pi$  and/or  $\sigma$  framework (atom- $\pi\sigma$  interaction).<sup>8,30</sup>

In the classic case of charge-dipole interactions such as an ionic salt, sodium chloride, dissolving in water involves the Coulombic interactions between a positively charged sodium ion and 6 water molecules as well as the corresponding interactions between a negatively charged chloride ion and 6 other water molecules. In the case of the sodium ion, the positive charge attracts the negative end of each water molecule's dipole. The negative charge of the chloride ion attracts the positive end of the O-H bond dipole. In our case, guanylate anion as a charge donor for a potential charge-dipole interactions should interact with the  $\pi$ -deficient pyrimidine system (marker proton H2) of the next 9-adeninyl nucleobase—not with the  $\pi$ -excessive imidazole part (marker proton H8). In fact, what we observe is the pH-dependent sigmoidal behavior causing deshielding of the H8 proton of imidazole part instead of H2 of the pyrimidine part, which rules out the involvement of any charge-dipole interaction, but anion- $\pi$  repulsion.

## Conclusions

Because we can successfully measure the  $pK_a$  of guanine-9-yl from either of the aglycones in the RNA trimers **3** and **4**, it shows that the aglycones in the trimeric RNAs constitute a *coupled heterocyclic system* right across the pH range, 6.7 to 11.5 owing to both 3'→5' and 5'→3' two-way cross-modulation<sup>30</sup> by electrostatic interaction. This may be the reason a trimeric RNA sequence constitutes a single codon signal in recognition and function in the protein synthesis machinery. The magnitude of the chemical shift change in any of the aromatic protons in either of the two coupled aglycones differs in a *variable* manner depending upon the geometry of stacking, partial charge of the heteroatom as well as the sequence (compare GpApA and GpApC), which is evident from relative chemical shift change ( $\Delta\delta_{N-D}^H$ ).<sup>25</sup>

The intramolecular electrostatic polar- $\pi$  interaction ( $\Delta G_{pK_a}^{\circ}$ ) from the 5'-guanine-9-yl (or guanylate ion) to the 3'-end aglycon in GpApA (**3**) or GpApC (**4**), which are 6.8 Å apart, is quite ubiquitous in neutral (protonated or deprotonated) state to cross-modulate the pseudoaromatic character by the nearest neighbor interaction. This is quite similar to the polar- $\pi$  effect found between ions and arene,<sup>21e-i</sup> such as carboxylate-arene interactions<sup>21e-g</sup> and trimethylammonium ion-arene interactions.<sup>21h,i</sup>

The  $\Delta G_{pK_a}^{\circ}$  obtained from either H8A/H2A of the middle residue or H8A/H2A or H5/H6C of the 3'-terminal residue within any single compound (**3** or **4**) is very close to what is

(32) The  $pK_a$  of N1 of guanine-9-yl moiety in various monomer and oligo-RNA are as follows: GpEt (**1**) ( $9.25 \pm 0.02$ ), GpA (**2**) ( $9.17 \pm 0.02$ ), GpApA (**3**) ( $9.75 \pm 0.02$ ), GpApC (**4**) ( $9.88 \pm 0.02$ ), 3'-GpApApC-5' ( $9.76 \pm 0.01$ ), and 3'-GpApApApC-5' ( $9.83 \pm 0.01$ ) (unpublished result). This simply shows that the microenvironment around the Gp residue in the dimer **2** is very comparable to that of the monomer **1**, whereas it is considerably different from the group consisting of trimers **3** and **4**, tetramer and pentamer. We have also compared the  $pK_a$  of N1 of guanine-9-yl moiety in the bis-anionic guanosine-3'-monophosphate ( $9.33 \pm 0.01$ ) with guanosine-3'-ethyl phosphate GpEt (**1**) ( $9.25 \pm 0.02$ ). This shows that the bisanionic 3'-monophosphate in 3'-GMP is slightly more electron-donating (+I effect), hence the constituent guanine-9-yl is slightly more basic than the monoanionic 3'-ethyl phosphate in GpEt (**1**) because of combined electrostatic and +I effects. When the 5'-bis-anionic phosphate and guanine-9-yl residues are at the same side of the pentose ring as in 5'-GMP compared to the 3'-GMP, one notices also the +I effect of 5'-phosphate is causing a more increase of the basicity of the N1 of guanine aglycon by  $\sim 0.15$   $pK_a$  unit (ref 2, pp107–108). This observation is also consistent with the  $pK_a$  comparison of 5'-EtpGpEt-3' ( $9.57 \pm 0.01$ ) and GpEt (**1**) ( $9.25 \pm 0.02$ ). It is thus clear that the position of the phosphate moiety (3' or 5') as well as the number of the phosphate charge (phosphomonoester versus phosphodiester) in the pentose-sugar ring has a distinctive effect on the  $pK_a$  of the constituent aglycon (in the same nucleotide). However, we have seen very little increase of  $pK_a$  (ca 0.1  $pK_a$  unit on the terminal guanine-9-yl in Gp in our case) upon an increase of the total number of phosphates in the molecule as a result of chain elongation.

obtained from that of H8G of the 5'-terminal residue (55 to 56  $\text{kJ mol}^{-1}$ ), which suggests that the modulation of the intramolecular electrostatic interaction ( $\Delta G_{\text{p}K_{\text{a}}}^{\circ}$ ) from the 5'-guanylate ion to the aglycon at the 3'-end (C or A) via the central adenin-9-yl is very close to 100% at the ground state.<sup>24</sup>

A clear outcome of this study is that the  $\text{p}K_{\text{a}}$  of guanine-9-yl moiety changes from monomer/dimer to trimer owing to electrostatic intreraction with the neighbors<sup>8</sup> (nucleobases as well as phosphates), which are as follows:  $9.25 \pm 0.02$  in **GpEt** (**1**),<sup>30</sup>  $9.17 \pm 0.02$  in **GpA** (**2**),<sup>30</sup>  $9.75 \pm 0.02$  in **GpApA** (**3**), and  $9.88 \pm 0.03$  in **GpApC** (**4**). It shows that guanine-9-yl moiety of trimers **3** and **4** are more basic than the monomer or dimer.

$\Delta G_{\text{p}K_{\text{a}}}^{\circ}$  is the free-energy of deprotonation<sup>14</sup> at  $\text{pH} = \text{p}K_{\text{a}}$ . Because the  $\text{p}K_{\text{a}}$  is a measure of the ground-state stability of the anionic or the cationic form of the product, we can estimate the electrostatic free-energy of stabilization<sup>14</sup> at the  $\text{p}K_{\text{a}}$  by the term,  $\Delta G_{\text{p}K_{\text{a}}}^{\circ}$ <sup>14,20</sup>. Thus, the strength of the electrostatic polar- $\pi$  effect via intramolecular offset-stacking interaction in the ground state is here equivalent to  $\Delta G_{\text{p}K_{\text{a}}}^{\circ}$  (**Type 1** effect). When this offset-stacking interactions between two coupled nearest neighbors (as evident by  $\Delta G_{\text{p}K_{\text{a}}}^{\circ}$  transmission, **Type 1** effect) have furthermore contributed to the increase/decrease to their acid/base character ( $\text{p}K_{\text{a}}$ ), we attribute this enhanced stabilization/destabilization to the electrostatic effect, modulated both by the number of phosphate groups<sup>32</sup> as well as by stacked/unstacked nucleobases in an oligomeric nucleic acids (**Type 2** effect). Thus, in the dimer, we see predominantly **Type 1** effect, whereas in the trimers<sup>32</sup> we see **Type 2** effect.<sup>32</sup>

## Experimental Section

**(A) pH-Dependent <sup>1</sup>H NMR Measurement.** All NMR experiments were performed in Bruker DRX-500 and DRX-600 spectrometers. The NMR sample for compounds **3** and **4** (Scheme 1) were prepared in

D<sub>2</sub>O solution (concentration of 1 mM in order to rule out any chemical shift change owing to self-association<sup>18</sup>) with  $\delta_{\text{DSS}} = 0.015$  ppm as internal standard; chemical shifts and their differences are given in  $\delta$  (ppm) and  $\Delta\delta$  (ppm), respectively. All pH-dependent NMR measurements have been performed at 298 K. The pH values [ $\text{pH} = \text{pD} - 0.4$  for the correction of deuterium effect] corresponds to the reading on a pH meter equipped with a calomel microelectrode (to measure the pH inside the NMR tube) calibrated with standard buffer solutions (in H<sub>2</sub>O) of pH 4, 7 and 10. The pD of the sample has been adjusted by simple addition of microliter volumes of D<sub>2</sub>SO<sub>4</sub> and NaOD solutions (0.5M, 0.1M, and 0.01M). The assignments for all compounds have been performed on the basis of selective homo-(<sup>1</sup>H) and heteronuclear (<sup>31</sup>P) decoupling experiments. All spectra have been recorded using 64 K data points and 64 scans for <sup>1</sup>H.

**(B)  $\text{p}K_{\text{a}}$  Determination.** The pH-dependent [over the range of pH 6.7–11.7, with an interval of pH 0.2–0.3] <sup>1</sup>H chemical shift ( $\delta$ ), with error  $\pm 0.001$  ppm) shows a sigmoidal (having average 20 different pH-dependent chemical shifts in each titration profile) behavior [Panels **A–K** in Figure 1]. The  $\text{p}K_{\text{a}}$  determination is based on the Hill plot analysis<sup>4,16</sup> using equation:  $\text{pH} = \log((1 - \alpha)/\alpha) + \text{p}K_{\text{a}}$ , where  $\alpha$  represents fraction of the protonated species. The value of  $\alpha$  is calculated from the change of chemical shift relative to the deprotonated (D) state at a given pH ( $\Delta_{\text{D}} = \delta_{\text{D}} - \delta_{\text{obs}}$  for deprotonation, where  $\delta_{\text{obs}}$  is the experimental chemical shift at a particular pH), divided by the total change in chemical shift between neutral (N) and deprotonated (D) state ( $\Delta_{\text{T}}$ ). So the Henderson–Hasselbalch type equation<sup>20</sup> can then be written as  $\text{pH} = \log((\Delta_{\text{T}} - \Delta_{\text{D}})/\Delta_{\text{D}}) + \text{p}K_{\text{a}}$ . The  $\text{p}K_{\text{a}}$  is calculated from the linear regression analysis of the Hill plot [Panels **a–k** in Figure 2].

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