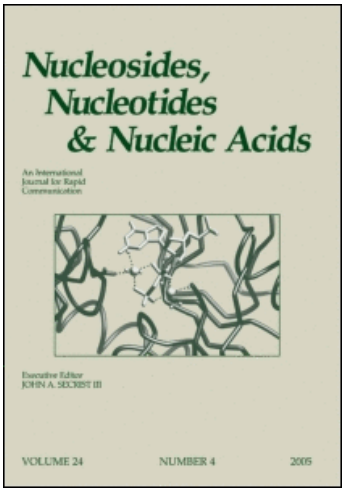

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PREFERENCE FOR RIBOSE OVER DEOXYRIBOSE IN LOOP-CLOSING BASE PAIRS OF EXTRA STABLE NUCLEIC ACID HAIRPINS

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□ *We have investigated the effect of switching ribose to deoxyribose at the closing base-pair of an extra-stable RNA hairpin. Specifically, we studied the sequence 5′-GGAC(UUCG)GUCC, a dodecanucleotide that folds into a well-characterized, "extra stable" RNA hairpin structure. Recently, we showed that hairpins containing a 2′,5′-linked (UUCG) loop instead of the native 3′,5′-linked loop also exhibit extra-stability (Hannoush and Damha, J. Am. Chem. Soc., 2001,* **123***, 12368–12374). In this article, we show that the ribose units located at the loop-closing positions (i.e., rC_4 and rG_9) contribute significantly to the stabilization of RNA hairpins, particularly those containing the 3′,5′-UUCG loop. Interestingly, the requirement of rC4 and rG9 is more relaxed for DNA hairpins containing the 2′,5′-UUCG loop and, in fact, they may be replaced altogether (ribose → deoxyribose) without affecting stability. The results broaden our understanding of the behavior of highly stable (UUCG) hairpin loops and how they respond to structural perturbation of the loop-closing base pairs.*

Keywords RNA structure; 2′,5′-Linked RNA; Hairpin loop-closing base pairs

INTRODUCTION

Hairpin secondary structural motifs are abundant in ribosomal RNA and serve a multitude of functions including nucleation sites for tertiary RNA folding,[1] and recognition signals for interaction with nucleic acids and

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proteins.[2–4] In addition, they stabilize partially double stranded microRNAs (miRNA), whose maturation and mode of action involves pathways overlapping with the RNAi pathway.[5] In rRNA, the most frequently-occurring hairpin motifs contain the four-nucleotide (GNRA) and (UNCG) consensus tetraloops (R = purine, N = any nucleotide), whose topology and contribution to hairpin thermodynamic stability have been studied by Tinoco and coworkers.[6–9] These studies have established that tetraribonucleotide r(UUCG) loop displays extra-stability by virtue of very specific hydrogen bonding and stacking interactions that occur within this structure.[10–12] Furthermore, the identity of the closing base pair (i.e., the identity of the first base pair next to the tetranucleotide loop sequence, Figure 1) has been evaluated in terms of their contribution to overall hairpin stability.[13–15] Hairpins closed by either a C·G, G·C or G·U base pair are the most stable.[16]

Recently our laboratory showed that the sequence 5′-GGAC(UUCG)GUCC (where the underlined residues are linked by 2′,5′-phosphodiester linkages), like its "native" counterpart (only 3′,5′-linkages), exhibits exceptional thermodynamic stability.[17] This is remarkable in view of the structural and conformational differences between 2′,5′-RNA and 3′,5′-RNA,[18] and the fact that 2′,5′-linked nucleic acids are far inferior to the native compounds with respect to pairing strength.[19–22] NMR data has provided a structure-based reasoning for the observed exceptional stability of 2′,5′-linked loops.[23] We also noted that the extra stability imparted by the (UUCG) loop was independent of the composition of the stem.[17] Hairpins containing a (UUCG) loop and either DNA (DD), RNA (RR), or 2′,5′-RNA (RR) stem duplexes all exhibited extra stability. This contrasted the behavior of the native (UUCG) loop, which exerted extra stability only when the stem was duplex RNA (RR). For example, we found that both GGAC(UUCG)GUCC (abbreviated "RRR") and d(GGAC)r(UUCG)d(GTCC) (abbreviated "DRD") are both extra stable. On the other hand, GGAC(UUCG)GUCC ("RRR") is extra-stable,[7] whereas d(GGAC)r(UUCG)d(GTCC) ("DRD") is not.[18] Based on these observations, we reasoned that switching of ribose to 2-deoxyribose at the *loop closing base-pair* residues (Figure 1), i.e., ...C(UUCG)G... to ...**dC**(UUCG)**dG**... was responsible for the lack of extra stability observed in the "DRD" system. Evidently, such a substitution

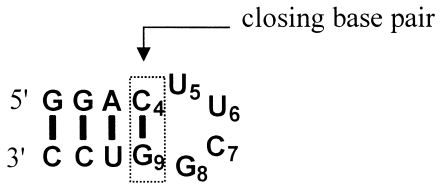

FIGURE 1 Secondary structure of the extra stable hairpin under study showing the $C_4 \cdot G_9$ loop-closing base pair. The loop region comprises U_5-G_8.

has little or no effect in hairpins containing the (UUCG) loop. To test this hypothesis, we describe here the synthesis and base-pairing properties of d(GGA)**C**(UUCG)**G**d(TCC), GGA**dC**(UUCG)**dG**UCC, as well as several other chimeric hairpins containing modifications at the loop, loop-closing base pair, and stem residues. Our aim was to assess the effect of the sugar at the closing base pair on the stability of hairpins containing 2′,5′- and 3′,5′-RNA loops. To our knowledge, there have been no studies examining the influence of sugar/phosphate moieties located at the loop-closing junction, with most studies to date focusing on the uniquely folded tetraloop structure. We feel this is an important endeavor, as it will guide future research in the synthesis and design of chemically modified miRNAs, siRNAs and other biologically relevant RNAs.

MATERIALS AND METHODS

Materials

5′-*O*-Dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilylribonucleoside-3′-*O*-(2-cyanoethyl) N,N'-diisopropyl phosphoramidite monomers for normal RNA synthesis were purchased from Dalton Chemical Laboratories (Ontario, Canada). The corresponding ribonucleoside 2′-*O*-phosphoramidites (2′,5′-RNA synthesis) were obtained from ChemGenes Corp. (Watham, MA). These reagents were stored at −20°C and dried in vacuo (under P_2O_5) for 24 h prior to use.

Oligonucleotide Synthesis and Purification

Oligonucleotides were synthesized using an Applied Biosystems (381A) synthesizer on a 1-μmol scale utilizing LCAA-controlled pore glass (500 Å) as solid support.[24] A detailed description of the synthesis cycle, coupling times and reagents used has been described.[17,24] Following syntheses, the CPG support was treated with aqueous ammonia/ethanol (3:1) for 24–48 h at room temperature or for 16 h at 55°C. After centrifugation, the supernatant was collected and the solid support was washed with 3 × 1000 μL ethanol. The supernatant and ethanol washings were combined and evaporated to dryness under vacuum. The pellet obtained was treated with $NEt_3 \bullet 3HF$ (200 μL) at room temperature for 48 h.[25] The reaction was quenched by addition of deionized double distilled water and the resulting solution was lyophilized to dryness under vacuum. The oligomers were purified either by denaturing polyacrylamide gel electrophoresis (24%, 7 M urea) or by anion-exchange HPLC (Protein Pak DEAE-5PW column; Waters, 7.5 mm × 7.5 cm) using a linear gradient of 0–23% $NaClO_4$ in H_2O with a flow rate 1 mL/min at 55°C. The oligonucleotides were then desalted on a Sephadex G-25 (fine) column. The purity of all sequences was assessed

using 24% analytical gels or HPLC and was determined to be >95%. The structures were confirmed by MALDI-TOF mass spectrometry.

UV Melting and CD Studies

UV thermal denaturation studies were conducted on a Varian CARY 1 UV-VIS spectrophotometer in 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer, pH 7.00 ± 0.02. Absorbance versus temperature spectra were collected at 260 nm over a range from 5°C to 90°C with 0.1°C increments and a heating rate of 0.5°C/min. Samples (~4.5 μM) were annealed by heating rapidly to 95°C for 10–15 min, followed by cooling slowly to room temperature and then refrigerated (5°C) overnight. Single-strand concentration was determined from UV absorbance at high temperature when the hairpin is in the fully dissociated state. Single-strand molar extinction coefficients were calculated from those of mononucleotides and dinucleotides using the nearest-neighbor approximation method.[26] 2′,5′-Linked RNA was assumed to have the same molar extinction coefficient as its 3′,5′ counterpart. Percentage hypochromicity (%H) was calculated from UV absorbances of the hairpin duplex (A_o) and the fully denatured species (A_f) using the following equation: $\%H = (A_f - A_o)/A_f$. CD spectra were measured on a JASCO J710 spectropolarimeter at ambient temperature as previously described.[17] The buffer used was 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.00 ± 0.02. Each spectrum represents the average of five 200–340 nm scans obtained at a rate of 0.5 nm/min (bandwidth: 1 nm; sampling wavelength: 0.2 nm). The data was processed on a PC computer using Windows™ based software supplied by the manufacturer (JASCO, Inc.). The obtained CD spectra were normalized by subtraction of the background scan with buffer. Taking the known oligonucleotide concentration into account, the normalized spectra were converted to molar ellipticities.

Thermodynamic Calculations and Data Analysis

Melting temperatures (T_m) and thermodynamic parameters for hairpin formation were computed using Cary WinUV version 2 software (Varian Ltd.). Melting curves were fit to a two-state (all-or-none) model[27] and the software was adapted for a unimolecular transition. T_m was calculated according to the baseline method[26] by using linear least squares lines for associated and dissociated regions of the melting curve. Thermodynamic parameters were calculated from plots of $\ln K$ versus $1/T$ as previously described.[17] The data shown in Table 1 represent the average of at least five independent measurements for each hairpin. To ensure a unimolecular transition, the samples were melted over at least 30-fold nucleic acid concentration with no detectable change in melting temperature, thus confirming an intramolecular transition. The reported error limits in Table 1

TABLE 1 Thermodynamic Parameters[a] for RNA-Stem Hairpin Formation (Set 1)

Entry	Code	Hairpin[b] 5′ → 2′/3′	T_m (°C)[c]	%H	ΔH° (kcal/mol)	ΔS° (e.u.)	ΔG°_{37} (kcal/mol)
1	RRR	G G A C U | | | | U C C U G C G	71.8 (71.7)[d]	8.5	−53.4 ± 2.3 (−56.5)[d]	−154.8 ± 6.9 (−163.9)[d]	−5.41 ± 0.19 (−5.7)[d]
2	R_cR_gR	G G A **dC** U | | | | U C C U **dG** C G	60.0	6.6	−51.9 ± 1.8	−155.5 ± 5.6	−3.60 ± 0.13
3	R_cRR	G G A **dC** U | | | | U C C U G C G	67.3	9.1	−56.1 ± 1.2	−164.0 ± 3.6	−5.02 ± 0.13
4	RR_gR	G G A C U | | | | U C C U **dG** C G	66.6	8.8	−56.7 ± 1.4	−166.6 ± 4.1	−4.98 ± 0.12
5	R$\underline{\text{R}}$R	G G A C <u>U</u> | | | | <u>U</u> C C U G <u>C</u> <u>G</u>	69.3	9.6	−55.6 ± 1.7	−162.1 ± 5.0	−5.33 ± 0.16
6	$R_c\underline{R}_gR$	G G A **dC** <u>U</u> | | | | <u>U</u> C C U **dG** <u>C</u> <u>G</u>	57.6	6.3	−47.7 ± 1.4	−143.9 ± 4.4	−3.06 ± 0.10

[a]Measurements were made in 0.01 M Na_2HPO_4, and 0.1 mM Na_2EDTA, pH 7.0. Values represent the average of at least five independent measurements. Errors in thermodynamic parameters are standard deviations. For a more accurate calculation, ΔG°_{37} was calculated from ΔH° and ΔS° before rounding off and extra significant figures are given in the values of ΔG°_{37}.

[b]Capital letters represent RNA residues; underlined letters are 2′,5′-RNA residues (e.g., <u>UC</u> = $U_{2'p5'}C_{2'p}$); DNA residues are represented as dG, dA, dU, dT, dC; bold letters are sugar mutations in the loop-closing base pair.

[c]The melting curves show a single cooperative and completely reversible transition that is independent of oligonucleotide concentration over at least a 30-fold range.

[d]Adapted from Antao et al.[8].

for ΔG°_{37}, ΔH°, and ΔS° derived from independent measurements represent standard deviations with all values weighted equally.[28] The variations in T_m values for all hairpin sequences are within ±1°C.

RESULTS AND DISCUSSION

The effect of substituting deoxyribose for ribose within the 3′,5′-UUCG loop (Figure 1) was first investigated by Sakata and coworkers.[29] A significant decline in stability was found upon incorporation of 2′-deoxynucleotides within this tetraloop. Two contiguous 2′-deoxyriboses also revealed a

TABLE 2 Thermodynamic Parameters[a] for DNA-Stem Hairpin Formation (Set 2)

Entry	Code	Hairpin 5'→2'/3'	T_m (°C)	%H	ΔH° (kcal/mol)	ΔS° (e.u.)	ΔG°_{37} (kcal/mol)
7	DRD	dG dG dA dC / dC dC dT dG + U U C G	54.6	11.5	−36.0 ± 2.4	−109.8 ± 7.6	−1.92 ± 0.12
8	D_CR_GD	dG dG dA **C** / dC dC dT **G** + U U C G	59.5	7.5	−45.4 ± 1.0	−136.6 ± 3.1	−3.08 ± 0.09
9	DUD	dG dG dA dC / dC dC dT dG + U U U U	52.8	7.1	−38.7 ± 1.8	−118.8 ± 5.7	−1.87 ± 0.09
10	D_CU_GD	dG dG dA **C** / dC dC dT **G** + U U U U	51.6	9.8	−38.6 ± 1.8	−118.9 ± 5.5	−1.74 ± 0.16
11	D$\underline{R}$D	dG dG dA dC / dC dC dT dG + U $\underline{U}$ $\underline{C}$ G	61.4	12.6	−39.9 ± 2.4	−119.4 ± 7.2	−2.91 ± 0.16
12	$D_C\underline{R}_GD$	dG dG dA **C** / dC dC dT **G** + U $\underline{U}$ $\underline{C}$ G	52.3	7.0	−39.0 ± 1.8	−120.0 ± 5.7	−1.83 ± 0.10
13	$D_{\underline{C}}\underline{R}_GD$	dG dG dA $\underline{C}$ / dC dC dT **G** + U $\underline{U}$ $\underline{C}$ G	57.0	7.0	−41.1 ± 1.5	−124.5 ± 4.8	−2.50 ± 0.05

[a] See footnotes a, b, and c from Table 1.

destabilizing effect.[30] Although the structural basis for destabilization remains unclear, the lack of specific sugar interactions[12,31] and/or the different conformations/dynamics of 2′-deoxyribose versus ribose likely account for these results. Indeed, a d(TTCG) tetraloop displays higher conformational flexibility than the corresponding all-RNA tetraloop.[7,8,32]

To examine the effect of the loop-closing nucleotide residues on hairpin stability, we prepared two different families of hairpins (Tables 1 and 2). The first group (sequences 1–6) was designed to assess the effect of sugar substitutions in RNA-like hairpins. For instance, hairpins RRR (#1) and R_cR_gR (#2) share the same stem and loop sequence, but differ only in the sugar composition at the C·G closing base pair. The second set (sequences 7–13) was designed to probe the effects of deoxyribose to ribose substitution in "DNA-like" hairpin structures. Tables 1 and 2 summarize the

melting temperatures (T_m values) and thermodynamic parameters of these structures. Hairpin formation was also characterized by circular dichroism (CD, Figure 2), whereas unimolecular folding was confirmed by UV melting experiments carried out over at least a 30-fold concentration range (data not shown). Representative melting curves of hairpins are shown in Figure 3.

Effects of Loop-closing Base Pairs on Hairpins Containing RNA Stems (Set 1)

Table 1 shows that the unmodified RNA hairpin [i.e., RRR] exhibits significantly higher thermodynamic stability relative to the hairpin containing deoxyribonucleotides at the loop-closing positions [. . . **dC**(UUCG)**dG**. . . ; R_cR_gR]. A ΔT_m of $-11.8°C$ was observed, with a corresponding increase in $\Delta G°_{37}$ of 1.81 kcal/mol. A similar destabilizing effect was observed for the RNA hairpin sets containing the 2′,5′-tetraloop (compare "R<u>R</u>R" with "$R_c\underline{R}_gR$", Table 1). In this case, $\Delta T_m = -11.7°C$; $\Delta\Delta G°_{37} = +2.27$ kcal/mol. This suggests that ribose → deoxyribose substitutions in the C·G loop-closing base pair destabilize RNA hairpins containing 3′,5′- or 2′,5′-RNA tetraloops; the data shown in Table 1 also suggests that this destabilization is mainly enthalpic in origin.

The destabilizing contribution of each deoxyribose in the C·G base pair was then assessed by comparing the stability of the native hairpin to that of R_cRR or RR_gR. Table 1 shows that each substitution within the native RRR hairpin, whether it be at C or G, contributes equally to the loss in hairpin thermodynamic stability (ΔT_m ~4–5°C drop per deoxy insertion). The effects are nearly additive, suggesting that both riboses of the closing base pair play a role in the stabilization of this sequence. Each single deoxy substitution in the C·G base pair does not significantly affect percentage hypochromicity; however, substitution of both moieties with deoxyriboses seems to perturb base stacking interactions in RRR and R<u>R</u>R hairpins as evidenced by the drop in %H values (compare "RRR" and "R_cR_gR"; "R<u>R</u>R" and "$R_c\underline{R}_gR$"; Table 1).

CD spectral analysis provides further evidence that incorporation of deoxyribose at the loop-closing positions has a significant influence on the stability (conformation) of the hairpin structure. Figures 2A and 2B compare the CD spectra of hairpins with deoxy substitutions, the 'natural' reference hairpin RRR, and the analogous hairpins having the 2′,5′-linked RNA loop. The CD spectra of the RRR and R<u>R</u>R hairpin duplexes (Figure 2, A and B) are characterized by strong positive Cotton effects at ca. 265 nm and a negative Cotton effect at 213 nm.[17] These features are characteristic of A-form helices. After incorporation of two deoxyriboses at the loop-closing positions, the spectral features change significantly. This is seen in the large decrease in the intensity of the negative CD band, and the shift

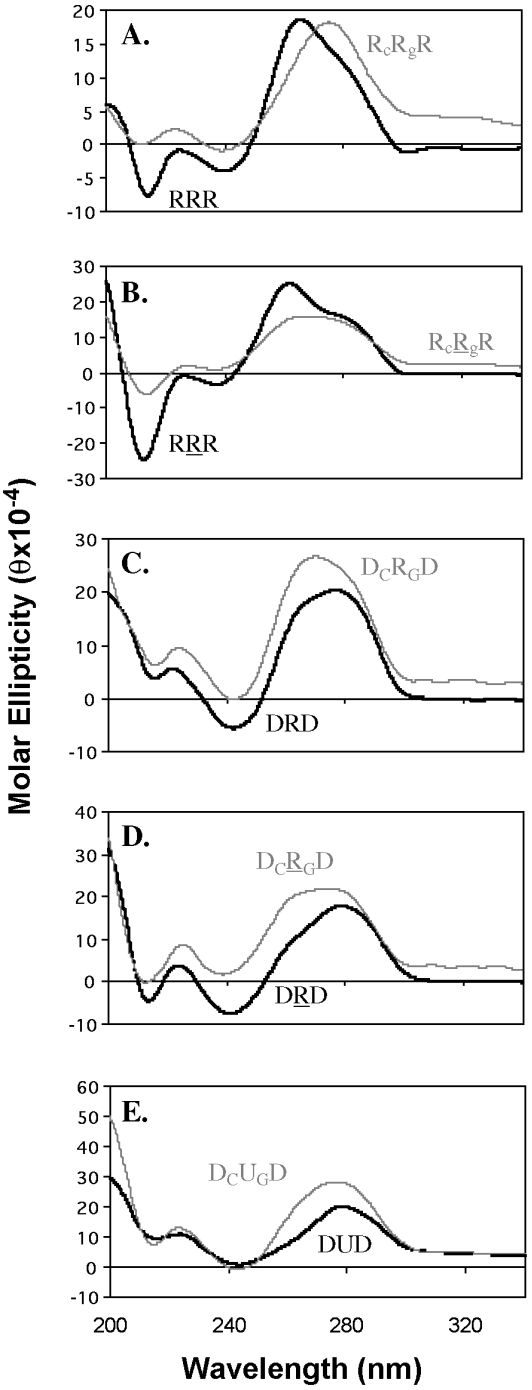

FIGURE 2 Circular dichroic spectra of hairpins differing in the sugar composition of the C·G loop-closing base pair. See Tables 1 and 2 for base sequence. Measurements were carried at 22°C in 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer (pH 7.0). Molar ellipticities were normalized to strand concentration.

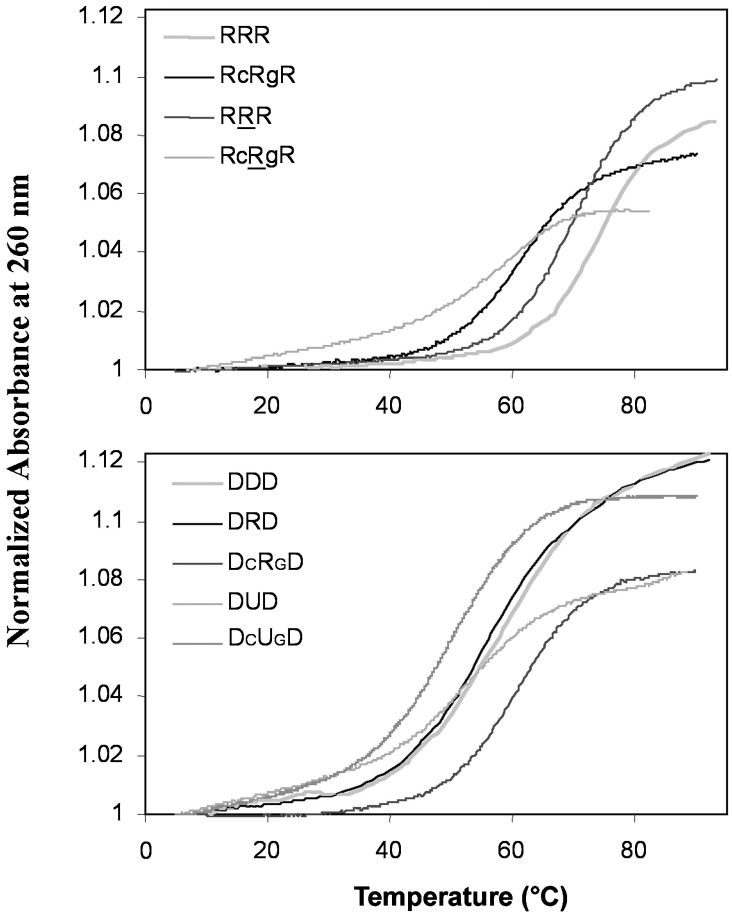

FIGURE 3 Thermal melting curves of representative hairpin duplexes (~4.5 μM). Buffer: 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer, pH 7.0. See Tables 1 and 2 for base sequences.

of the positive CD band toward longer wavelengths. The two deoxyribonucleotide residues incorporated within the RRR and R$\underline{\text{R}}$R hairpins seem to induce a conformational change of the hairpin structure in the direction of 'DNA-like' hairpins. This is demonstrated in the resemblance of the $R_c\underline{R}_gR$ and $D_C\underline{R}_GD$ spectra (Figure 2); the CD profiles of R_cR_gR and D_CR_GD are also very similar.

Effects of Loop-closing Base Pairs on Hairpins Containing DNA Stems (Set 2)

As mentioned earlier, the loop of the all-DNA hairpin d(GGACTTCG GTCC) (or "DDD") is neither uniquely structured nor "extra-stable" with respect to a hairpin of purportedly normal stability, e.g., ggac(tttt)gtcc (or "DTD").[8,39] Thus, d(GGACTTCGGTCC) exhibits the same stability as a

hairpin of purportedly normal stability, i.e., d(GGACTTTTGTCC) (or "DTD"). This contrasts the behavior of the all-RNA hairpin GGAC(UUCG)GUCC whose stability is superior to that of GGAC(UU**UU**)GUCC.[7] Furthermore, incorporation of four riboses in the loop of a DNA hairpin (sequence DRD) is not sufficient to provide extra-stability. As can be seen in Table 2, DRD and DUD exhibit comparable stabilities (entries 7 and 9), which are similar to the DDD stability (data not shown). However, incorporation of two additional ribose moieties at the loop-closing base pair provides a hairpin with extra stability, e.g., d(GGA)**C**(UUCG)**G**d(TCC) or "D_CR_GD" (Table 2). In fact, D_CR_GD is significantly more stable than DRD ($\Delta T_m = +5°C$; $\Delta\Delta G°_{37} = -1.2$ kcal/mol), pointing to the importance of the loop-closing residues. On their own, these residues are not sufficient to confer extrastability since . . . **C**(UUUU)**G**. . . (Table 2; "D_CU_GD") does not show increased thermodynamic preference relative to . . . **dC**(UUUU)**dG**. . . (Table 2; "DUD").[7] These results, altogether, strongly suggest that the sugars of both the loop and the closing base pair (i.e., an "hexaloop") participate in the overall organization and extrastability of D_CR_GD. The observed increase in thermal stability may be ascribed to an organization in the conformation of the DNA stem residues induced upon ribose substitution in the loop-closing base pair.

By contrast, the 2′,5′-tetraloop (<u>UUCG</u>) alone is sufficient to provide extra stability to hairpins with DNA stems. As shown in Table 2, D<u>R</u>D is much more stable than DRD ($\Delta T_m = +7°C$) or ggac(tttt)gtcc ($\Delta T_m = +7°C$). We have also shown earlier that D<u>R</u>D is more stable than the corresponding 2′,5′-homopolymeric loop (d(GGAC)(<u>UUUU</u>)d(GTCC)).[17] Clearly, in this case, UUCG and <u>UUCG</u> respond differently to perturbations made within the sugar of closing base pairs. Finally, we designed the hairpin $D_C\underline{R}_GD$ comprised of 2′,5′-RNA pentaloop, i.e., 5′-d(GGA)**<u>C</u>**(<u>UUCG</u>)Gd(TCC)-3′. The introduction of an additional 2′,5′-linkage near the 5′-end of the loop increases thermodynamic stability by ca. 0.7 kcal/mol compared to 5′-ggaC(<u>UUCG</u>)Gtcc-3′ ($D_C\underline{R}_GD$, Table 2).

The CD profiles of DRD and D_CR_GD, as well as D<u>R</u>D and $D_C\underline{R}_GD$ (Figure 2, C and D) indicate that the perturbation of the overall hairpin conformation in this case is not as dramatic as that observed previously for RNA-stem hairpins. For instance, the CD spectra of DRD and D_CR_GD are similar in shape (Figure 2E), the major difference being the amplitude of the positive peaks.

CONCLUSIONS

The loop-closing base pair works in conjunction with the 3′,5′-r(UUCG) loop to exert extra stability in DNA stems, but it is not required for 2′,5′-RNA loops. On the other hand, deoxyribose substitutions at the closing

base pair within RNA stems cause destabilization in hairpins containing either 2′,5′- or 3′,5′-loops. These results support the existence of differences in stacking interactions and preferred nucleotide conformations in 2′,5′- and 3′,5′-RNA.[23,33] These results also broaden our understanding of the behavior of highly stable (UUCG) hairpin loops and how they respond to various perturbations, particularly to the loop-closing base pairs.

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ABBREVIATIONS

DNA	3′,5′-linked deoxyribonucleic acid (represented as dG, dU, dT, dC, dA)
RNA	3′,5′-linked ribonucleic acid (represented by capital letters)
2′,5′-RNA	2′,5′-linked ribonucleic acid (represented by capital underlined letters), e.g., d(GU) = dG3′p5′ dU3′ p; GU = rG3′p5′ rU3′p; <u>GU</u> = rG2′p5′ rU2′p; <u>UUCG</u> = U2′p5′U2′p5′C2′p5′ G2′p5′
T_m	melting temperature in degrees Celsius
CD	circular dichroism
e.u.	entropy units in cal·K^{-1}·mol^{-1}
%H	percentage hypochromicity