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^{1}H-NMR OF G-U-C AND G-U-C-C IN D_2O: ASSIGNMENT OF NONEXCHANGEABLE PROTONS AND ANALYSIS OF SOLUTION CONFORMATION

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The ribonucleotide oligomers G-U-C and G-U-C-C have been synthesized enzymatically. These oligomers are cognates of the m^7G^{46}-U^{47}-C^{48}-m^5C^{49} sequence found in the variable loop of t-RNA^{phe}. The ^{1}H-NMR chemical shifts of the base and ribose H1′ protons as well as the couplings, $J_{1'-2'}$, of the ribose protons have been examined as a function of temperature. Assignments for these resonances have been completed, and used in the analysis of solution conformation for these oligomers. The results are consistent with the basic features of the A-RNA structure and suggest the absence of alternative ordered solution structures.

1. Introduction

The crystal structure analysis of the t-RNA^{phe} molecule has been completed by Quigley and Rich [1], Stout et al. [2], Hingerty et al. [3] and Holbrook et al. [4]. Their results show that the TΨC loop and variable loop of this molecule contain somewhat similar oligonucleotide structures designated as an 'arch' conformation [1].

A model of the TΨC loop sequence, A-U-C-C-A, has been synthesized and examined using ^{1}H-NMR spectroscopy. Although the predominant solution conformation of this pentamer appears to be the right-hand stacked A-RNA, the results suggest the presence of an alternative ordered solution structure for AUCCA resembling the arch [5].

In the variable loop portion of t-RNA^{phe}, the sequence m^7G^{46}-U^{47}-C^{48}-m^5C^{49} is found. Here, U^{47} is excluded from the center of the molecule, and projects away from the rest of the molecule. This conformation is stabilized by a hydrogen bond between the 2′-hydroxyl group of ribose 46 and the phosphate of residue 48 [1].

It was thus of interest to examine the ^{1}H-NMR spectra of this oligomer to determine if it also exhibited the presence of alternative ordered conformations. The oligomers G-U-C and G-U-C-C have been synthesized enzymatically in this study, and the solution ^{1}H-NMR of these oligomers has been obtained as a function of temperature.

In this report assignments are made for the G-U-C and G-U-C-C base and ribose H1′ protons using incremental analysis [6]. The chemical shifts are examined as a function of temperature, as are the $J_{1'-2'}$ coupling constants for the ribose H1′ protons. The data suggest the absence of alternative ordered conformations for G-U-C and G-U-C-C in solution. The results do not differ substantially from what would be expected for a standard right-helical structure.

2. Methods

2.1. Synthesis of oligonucleotides

The dimer GpU was obtained as the ammonium salt (Sigma) and used without further purification. Primer-dependent polynucleotide phosphorylase enzyme (PNPase P) [7] (P.L. Biochemicals) was used to achieve chain elongation. In the elongation process, a series of trial reactions was performed to optimize reaction conditions and

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yield. The 50-μl trial reactions were analyzed using HPLC techniques as developed by McFarland and Borer [8]. The optimal trial was then scaled up to a total volume of 25 ml. It contained 30 mM GpU, 60 mM CDP, 100 mM $MgCl_2$, 4 M NaCl, 2 M Hepes, pH 8.2, and 20% (v/v) PNPase P. After incubation for 5 days at 37°C, this mixture was heated to 80°C for 5 min to denature PNPase P and treated with alkaline phosphatase (calf intestine, Boehringer) to degrade any remaining nucleoside diphosphate. GUC* and GUCC were separated from the resulting mixture on a DEAE-Sephadex A-25 column, Cl^- form, pH 8.2, using a 3.010–0.8 M NaCl gradient. Fractions eluting from the column were monitored by the absorbance at 260 nm, and their identity verified by HPLC and paper chromatography (descending mode, 70:30 ethanol/ammonium acetate, v/v). The fractions were desalted by several passes through a BioGel P-2 column, with Millipore-filtered H_2O. Samples were evaporated and quantitated by the absorbance at 260 nm. Extinction coefficients used were 35.5 for GUCC, 28.5 for GUC, and 20.0 for GU (A_{260} units/μmole strand) [9].

2.2. NMR

Each NMR sample contained 1.0 μmole oligomer in 0.5 ml 0.01 M phosphate buffer, pH 6.6, and 10^{-4} M EDTA. Exchangeable protons were replaced by dissolving samples in 99.7% D_2O and evaporation, a procedure which was repeated three times. Finally, the samples were dissolved in 99.98% D_2O (Aldrich) and 5 μl of 4.5 M *t*-butanol in D_2O solution were added as an internal reference; shifts were reported with respect to sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as described by Borer et al. [6].

Spectra were obtained on various spectrometers, operating in the quadrature FT mode. ^{1}H frequencies of 90, 220, 250, 360 and 500 MHz were used; in all cases with a sweep width of 10 ppm. Pulses were accumulated at 3–5 s intervals. At the two lower fields 600–1000 pulses were accumulated, while less than 160 were usually sufficient on the high field instruments. A 16K (8K real) data table was used, except at 500 MHz, where spectra were zero-filled to 32K. A 30–50° flip angle was used in all cases. Temperatures were verified by reference to methanol or ethylene glycol standards [10,11].

* Throughout the rest of this paper we leave out the dashes denoting the phosphodiester linkage; none of the oligomers have terminal phosphates.

3. Results and discussion

3.1. Assignment of resonances

Fig. 1a and b compares the chemical shift versus temperature data for the trimer GUC and the tetramer GUCC base protons. There are two characteristic sets of resonances: the lower field GH8 and pyrimidine H6 protons, and the higher field pyrimidine H5 protons. The G^1H8, U^2H6 and H5, and GUC C^3H6 and H5 can be unequivocally assigned; the tentative assignments for GUCC C^3 and C^4 H6 and H5 are presented along with arguments favoring the present assignment. We note that the latter four resonances cannot be unequivocally assigned by the incremental analysis scheme [6]. Assignments were made on the basis of some well known general features of oligo-RNA NMR spectra. These features include the ap-

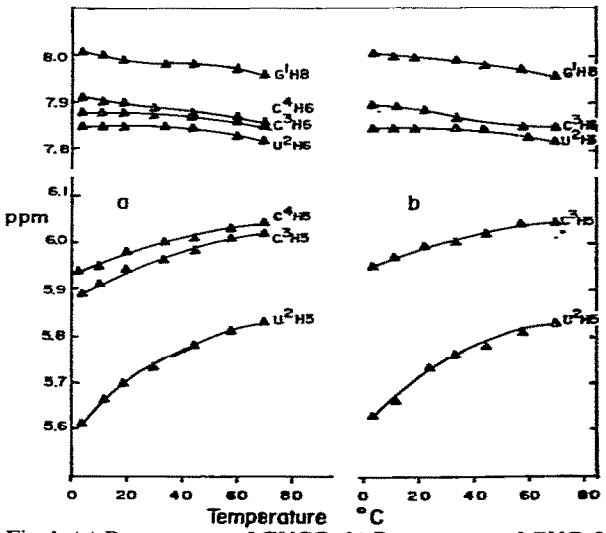

Fig. 1. (a) Base protons of GUCC. (b) Base protons of GUC. δ (ppm from DSS) versus temperature (°C). The GUCC C^3 and C^4 H5 and H6 protons cannot be unequivocally assigned using incremental analysis (see text).

proximate 8 ppm location of the pyrimidine H6 doublets and the H8 guanosine singlet, the approximate 6 ppm chemical shift of the pyrimidine H5 and ribose H1′ doublets, temperature-invariant coupling constants of 7.6 Hz for H5 and H6 of cytosine and 8.1 Hz for uracil, and variable coupling constants in the range 1–6 Hz for ribose H1′ signals which decrease with decreasing temperature.

3.1.1. Aromatic protons

The GH8 proton is least shielded, and it has a δ verus T profile which is quite similar in both GUC and GUCC. This indicates no significant change in environment of the GH8 proton between the trimer and tetramer. This resonance becomes approximately 0.05 ppm less shielded with decreasing temperature.

The CH6 and UH6 doublets are clustered in the 7.8–7.9 ppm region; and often overlap extensively. The UH6 δ versus T profiles of GUC and GUCC are superimposable. The UH6 is deshielded approximately 0.025 ppm over the 70–4°C temperature range. This deshielding of H8 and H6 protons is often observed [12] and is probably due to their close proximity to the oxygen and phosphorus atoms of the backbone chain.

In contrast, the pyrimidine H5 protons show large shielding increases as the temperature is lowered. The shielding is primarily due to ring current effects associated with nearest-neighbor stacking interactions. The most shielded proton is the UH5. The GUC and GUCC UH5 resonances are essentially superimposable over the entire range of temperature values, similar to the UH6 and GH8 behavior. Both the trimer and the tetramer exhibit a shielding effect of approximately 0.25 ppm at lower temperatures. This large shielding arises primarily from the ring current effect of the adjacent guanine ring.

The GUCC C^3 and C^4 H6 and H5 resonances show similar δ versus T profiles. The H5 protons have been linked with their H6 counterparts by homonuclear decoupling experiments. Thus, the less shielded members of both the cytosine H5 and H6 pairs in GUCC are coupled together and hence correspond to the same base. The less shielded of the two CH5 resonances in GUCC is assigned to the C^4H5. The resonance assigned as C^3H5 is consistently 0.05 ppm more shielded. This assignment is supported by the reasoning that addition of a 3′-cytosine unit to GUC should result in an increase in shielding for C^3. GUC CH5 and GUCC C^4H5, both terminal residues, show superimposable δ versus T profiles. However, comparison with the theoretical shielding values calculated for an A-RNA helix [13] leads to the opposite assignment. These calculations show that C^4H5 in GUCC should be more shielded because its 5′-neighbor nucleotide is cytosine, rather than uracil. We note that unequivocal assignments for the CH5 and CH6 protons in GUCC will probably require spectral analysis of oligomers selectively deuterated at these positions.

3.1.2. Ribose H1′ protons

Assignments for the ribose H1′ protons in GUCC are extremely difficult. This is because of the severe overlap of the four H1′ doublets at all temperatures examined. This overlap has also made accurate $J_{1'-2'}$ coupling values difficult to obtain. In a right-hand stacked A-RNA structure, the ribose ring exists in the 3′-*endo* conformation (^{3}E), while in the disordered state, a mixture of ^{2}E and ^{3}E conformers is present. Ribose $J_{1'-2'}$ has values ranging between 0 and 10 Hz, increasing with the proportion of ^{2}E conformers. Thus, base stacking can be followed as a function of temperature by examining the observed ribose coupling constant values. These are shown in tables 1 and 2. The acquisition of 500 MHz ^{1}H-NMR spectra on these oligomers at the Southern California regional facility, combined with spectral simulation using the PANIC simulation and iteration programs (Bruker Instruments, 1980) has allowed accurate coupling constant values to be obtained, as well as tentative assignments. Even at 500 MHz the four H1′ doublets overlap extensively. Calculated spectra were fitted to the experimental data by a least-squares iterative process. The results of such an iteration are shown in fig. 2.

The four H1′ resonances of GUCC are split into two sets of two; one set is significantly more shielded at low temperature (fig. 3a). The following assignments were arrived at by an incremental analysis scheme in which the oligomers GU, GUC

Table 1

GUCC $J_{1'-2'}$ (Hz) versus temperature (°C)

Temperature (°C)	G^1	U^2	C^3	C^4
3	2.0	2.2	2.0	3.0
10	2.0	3.0	3.0	2.5
20	2.0	4.0	4.0	3.0
27	2.8	3.0	4.5	2.8
32	3.0	3.5	3.5	5.0
40	4.0	4.0	4.0	5.0
49	4.0	4.0	5.0	5.0
58	[a]	[a]	4.5	4.5
71	4.5	4.5	5.0	5.0

[a] These values were not determined because of severe overlap with another resonance. The coupling constants are accurate to approximately ±0.5 Hz.

and GUCC were compared. The theoretically calculated chemical shift values for the A-RNA structure [13] were also used as was the PANIC spin simulation software. These assignments are shown in fig. 3a and compared with the trimer GUC in fig. 3b. Note the extensive overlap and frequent crossovers observed in the data.

The GH1′, UH1′ and CH1′ of GUC can be securely assigned using the incremental analysis scheme. The guanosine H1′ chemical shift has a small temperature dependence, which increases in going from GU to GUC to GUCC. This is probably due to an increase in stacking affinities as chain length increases. Ring current calculations [13] indicate that this proton should experience only slight shielding, which agrees with our assignment. The uracil H1′ exhibits a larger increase in shielding as temperature is lowered. This effect

Table 2

GUC $J_{1'-2'}$ (Hz) versus temperature (°C)

Temperature (°C)	G^1	U^2	C^3
4	2.0	3.5	3.5
12	2.5	4.0	4.0
19	3.0	4.5	4.5
34	4.0	5.0	5.0
44	4.0	5.0	5.0
58	4.5	5.5	5.0
70	5.0	5.5	5.5

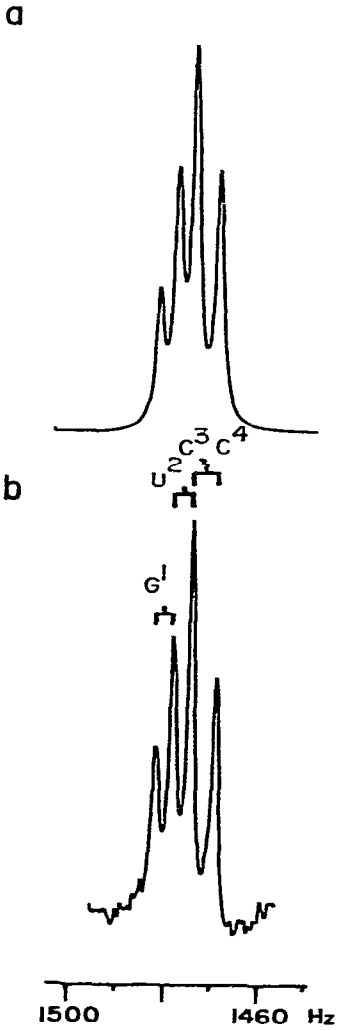

Fig. 2. Comparison of simulated and experimental spectra. The four H1′ protons of GUCC at 53°C. (a) Simulation of the 250 MHz spectrum at 2 Hz linewidth. (b) Experimental 250 MHz spectrum, obtained using a line broadening factor of 0.1 on 128 scans. The four H1′ resonances inferred from the experimental spectrum were used to generate the simulation.

becomes progressively larger in incrementing the chain from GU to GUC to GUCC. Again, this is probably due to increased stacking affinities in the larger oligomers. C^3H1′ in GUC can be assigned

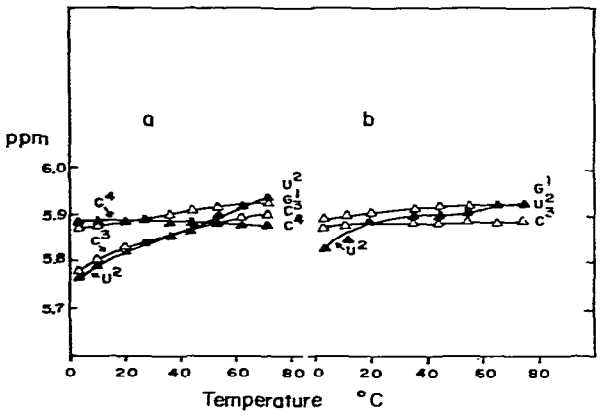

Fig. 3. (a) Ribose H1′ protons of GUCC. (b) Ribose H1′ protons of GUC. δ (ppm) versus temperature (°C). The GUCC C^3 and C^4 H1′ protons cannot be unequivocally assigned using incremental analysis (see text).

by comparison to the dimer GU. This proton is approximately 0.05 ppm more shielded than G or U H1′ at 70°C, and is independent of temperature.

Incrementing the chain from GUC to GUCC adds a fourth H1′ resonance, which is closely parallel to U^2H1′ at low temperature, and to C^3 H1′ at high temperature. Unfortunately, it is not possible to assign unequivocally the C^3 and C^4 H1′ of GUCC at high temperature using the incremental analysis scheme. In the following section we present arguments for both of the possible assignments.

The assignment of C^3 and C^4H1′ (shown in fig. 3a) results in the data being consistent with a stacked A-RNA structure. This gives C^4H1′ a δ versus T transition similar to C^3H1′ of GUC. Since both are 3′-terminal cytosines, one might expect them to have δ versus T transitions which are similar. This assignment also results in C^3H1′ having the smaller $J_{1'-2'}$ value. This would be consistent because C^3H1′ would be expected to be more stacked than C^4H1′, and hence have a higher percent ^{3}E value (smaller $J_{1'-2'}$).

The opposite assignment of C^3 and C^4H1′ makes the more shielded 70°C resonance C^3H1′, and the less shielded resonance C^4H1′. This assignment results in C^4H1′ having a much larger δ versus T transition than does C^3H1′. In addition, it causes C^4H1′ to have a smaller $J_{1'-2'}$ value (2 Hz) than does C^3H1′ (3 Hz) at low temperature. Neither of these observations is consistent with what one would expect for an A-RNA stacked structure. Such a larger $J_{1'-2'}$ value for C^3H1′ could be consistent with an arch model, where C^3 should be 2′-*endo* (^{2}E). However, we prefer the former assignment because the arch model would also predict a ^{2}E conformation for G^1, and a corresponding large $J_{1'-2'}$ value for G^1H1′, which is not seen. The GH1′ coupling constant in GU and GUC is of the same magnitude as in GUCC. No increase of $J_{1'-2'}$ in GUCC as compared to the shorter oligomers is seen.

5. Conclusions

With the possible exception of the C^3H1′ coupling, which awaits an unambiguous assignment of the C^3 and C^4 H1′ protons, the δ versus T and $J_{1'-2'}$ data for these oligomers are substantially consistent with what one would expect to observe for right-hand stacked A-RNA in solution. The difference observed in comparing GUC with GUCC can be attributed to effects associated with addition of a 3′-terminal cytosine.

Stacking and ring current effects in RNA are primarily due to nearest-neighbor interactions, with smaller contributions due to next-nearest-neighbor effects. Thus, chemical shift versus temperature data for G and U in GUC and GUCC show no significant conformational difference in going from the trimer to the tetramer. This is evidenced by the similar δ versus T profiles for the GH8, UH6, and UH5 resonances of GUC and GUCC in figs. 1a and b. The $J_{1'-2'}$ data in table 1 also indicate that the ribose rings of G and U are not changing conformational state in going from GUC to GUCC. We conclude that these protons exist in an environment which is the same in both GUC and GUCC. A similar conclusion was reached in the comparison of AUC with AUCC [5].

The main difference between GUC and GUCC appears in the δ versus T profiles of the cytosine H5 and H6, where C^3 H5 and H6 of GUC become

more shielded upon addition of C^4. This is consistent with a traditional stacked structure for GUCC. An important experiment will be to construct longer oligomers containing the GUCC sequence; such longer sequences may 'lock into' the arch conformation.

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