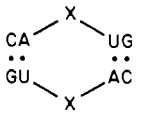
Effects of Internal Nonbonded Bases and a G·U Base Pair on the Stability of a Short Ribonucleic Acid Helix†

Paul J. Romaniuk,‡ Donald W. Hughes, Renê J. Grêgoire, Russell A. Bell,* and Thomas Neilson*

ABSTRACT: Proton nuclear magnetic resonance has been used to examine the effect of both noncomplementary and G·U oppositions in the duplexes formed by the synthetic pentaribonucleotides CpApApUpG, CpApUpUpG, CpApGpUpG, and CpApCpUpG. The lack of any sigmoidal behavior in the chemical shift vs. temperature plots of the base protons in the individual pentaribonucleotides indicates that duplexes with noncomplementary base oppositions of the type



(where X = A, U, G, or C) do not form. Variable temperature spectra of the mixture of CpApGpUpG and CpApUpUpG were recorded over the range of 70–10 °C. The chemical shift vs. temperature plot of the purine aromatic protons displayed sigmoidal curves. This demonstrated both duplex formation and the presence of a G·U base pair. The average T_m of the duplex was found to be 23.4 ± 2.0 °C. This is similar to that of the duplex formed by CpApUpG (24.0 ± 1.0 °C) but less than the T_m of the following duplexes: CpApApUpG:CpApUpUpG (T_m = 28.5 ± 2.1 °C), CpApGpUpG:CpApCpUpG (T_m = 38.4 ± 0.6 °C) and CpApUpApUpG (T_m = 41.5 ± 1.1 °C). The G·U base pair has a T_m (20.0 °C) significantly lower than the rest of the duplex (24 ± 1 °C) and is a region of local instability within the double helix. This ^{1}H NMR study is the first to investigate both the formation and relative stability of an internal G·U base pair neighboring regular Watson–Crick base pairs.

Crick first proposed the G·U base pair as part of the wobble hypothesis (Crick, 1966), which allowed for the formation of non Watson–Crick base pairs at the terminus of a codon–anticodon double helix, thereby explaining the degeneracy of the genetic code. This base pair has since been included *within* double-helical regions of the secondary structures of several tRNAs (Rich, 1977; Rordorf et al., 1976; Robillard et al., 1976), as well as the proposed secondary structures of other native RNAs (Gross et al., 1978; Fiers et al., 1975, 1976).

Evidence for the formation of G·U base pairs at nonwobble positions in RNA double helices has been conflicting to date. Although physical evidence for the formation of G·U wobble base pairs has been implied from certain resonances in the NMR spectra of several tRNAs (Rordorf et al., 1976; Robillard et al., 1976), such assignments have been disputed (Kearns, 1976). Attempts to demonstrate G·U base pair formation using model compounds have been inconclusive for the most part. Studies on poly[r(GU)] using CD measurements found no evidence of duplex formation (Gray et al., 1972). Krugh has studied the deoxynucleotide d(pGpT) by NMR and found that there was no base pairing under conditions where other self-complementary dinucleotides duplex (Krugh & Young, 1975). Two independent CD studies of poly[d(GT)] demonstrated that the polymer forms an intermolecular structure, although there was insufficient proof for a base-paired double helix (Lezius & Domin, 1973; Gray & Ratliff, 1977). More recently, poly[d(GT)] has been the subject of both NMR and ethidium bromide binding studies, which demonstrated that a wobble G·T base-paired duplex is formed (Early et al., 1978).

The relative stability of a G·U wobble base pair within a Watson–Crick hydrogen-bonded double helix is not completely understood. It is known that a terminal G·U base pair in a codon–anticodon interaction can be as stable as an A·U base pair in the same position (Uhlenbeck et al., 1970). Uhlenbeck and co-workers also showed, by optical methods, that the addition of an internal G·U base pair does not increase the stability of an A_nU_n self-complementary duplex (Uhlenbeck et al., 1971). However, there was no evidence from the experiments which would explain the process by which a G·U base pair could form without contributing to the overall duplex stability.

We have taken a more direct approach to studying the formation and stability of a G·U base pair within a Watson–Crick hydrogen-bonded RNA double helix using the self-complementary tetraribonucleotide CAUG (Romaniuk et al., 1978a,b) as a reference duplex. ^{1}H NMR spectroscopy was used to monitor duplex formation in order to gain information about the conformational environment of each constituent nucleotide. The four pentaribonucleotides CAAUG, CAUUG, CACUG, and CAGUG were used to study the effect of a single, additional A·U (CAAUG:CAUUG), G·C (CACUG:CAGUG), and G·U (CAGUG:CAUUG) base pair on the stability of the duplex formed relative to CAUG.[1] By using

† From the Department of Biochemistry, McMaster University, Hamilton, Ontario, L8S 4J9, Canada (P.J.R., R.J.G., and T.N.), and the Department of Chemistry, McMaster University, Hamilton, Ontario, L8S 4M1, Canada (D.W.H. and R.A.B.). *Received April 25, 1979.* This work was supported by the NSERC of Canada. This paper is part 4 in a series entitled Proton Magnetic Resonance Studies of Short Duplexes.

‡ Recipient of an Ontario Graduate Scholarship.

[1] Abbreviations used: CAUG, tetranucleotide triphosphate CpApUpG. In addition to the abbreviations recommended by IUPAC–IUB Commission (1970), the following are also used: trac, triphenylmethoxyacetyl; t, tetrahydropyranyl; pO^-, 3′-*O*-(2,2,2-trichloroethyl) phosphate; *p* between two characters, 3′,5′-(2,2,2-trichloroethyl) phosphotriester; MST, mesitylenesulfonyl 1,2,4-triazolide; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; NMR, nuclear magnetic resonance; T_m, melting temperature (°C). All oligoribonucleotides are written in the 5′ → 3′ sequence, and the bases are numbered from the 5′ end. For double helices, the base pairs are numbered from left to right:

0006-2960/79/0418-5109$01.00/0

Table I: Summary of Preparation of Protected Oligoribonucleotides[a]

reactants						products		
	quantity			quantity			quantity	
compd	mg	mmol	compd	mg	mmol	compd	mg	%
C	1350	1.85	A	842	1.85	CA	1460	78
U	1000	1.58	G	745	1.58	UG	1263	62
CA	377	0.27	A	124	0.27	CAA	328	59
CAA	218	0.11	UG	106	0.11	CAAUG	95	28
CA	377	0.27	U	107	0.33	CAU	304	59
CAU	202	0.11	UG	105	0.11	CAUUG	135	41
CA	730	0.53	C	228	0.53	CAC	910	84
CAC	475	0.24	U	102	0.31	CACU	375	62
CACU	175	0.07	G	32	0.07	CACUG	135	62
CA	730	0.53	G	250	0.53	CAG	820	76
CAG	415	0.20	U	85	0.26	CAGU	300	58
CAGU	150	0.06	G	27	0.06	CAGUG	110	59

[a] Column 1 contains the 5'-trityloxacetyl reactants and C stands for tracBz-CtOH; column 4 contains the incoming nucleosides and A stands for HOBz-AtOH; column 7 contains the trityloxyacetyl product and CA stands for tracBz-Ct-*p*-Bz-AtOH. Pyridinium mono-2,2,2-trichloroethyl phosphate (2 equiv) activated by 4 equiv of MST in anhydrous pyridine is used in each phosphorylation step. The coupling step to the incoming nucleoside is driven by 1.2 equiv of MST.

^{1}H NMR, we were also able to measure the stability of the G·U base pairs in the CAGUG:CAUUG duplex relative to the stability of the Watson–Crick base pairs of the *same* duplex. As well, each single pentaribonucleotide was used to study the effect of nonbinding base oppositions on the formation of neighboring base pairs in very short duplexes. The oligoribonucleotides used in this study were chemically synthesized by the phosphotriester method (Neilson & Werstiuk, 1974) since this is currently the only available synthesis capable of preparing the variety of ribonucleotide sequences required in the amounts necessary for NMR studies.

Materials and Methods

(*A*) *Chemical Synthesis of Oligonucleotides.* The pentamers and hexamer used in this study were synthesized by the phosphotriester method developed in this lab and fully described elsewhere (Neilson & Werstiuk, 1974; England & Neilson, 1976; Werstiuk & Neilson, 1976). The full synthetic details of the preparation of the hexaribonucleotide CAUAUG have been previously published (Ganoza et al., 1978). The fully deblocked sequences were characterized by ^{1}H NMR. The base ratios of the oligomers were confirmed from the distinct aromatic proton resonances of the spectra.

Materials. Nucleosides were purchased from Terochem Labs Limited, Edmonton, Alberta, Canada; mesitylenesulfonyl chloride, 1,2,4-triazole, and 2,2,2-trichloroethanol were from Aldrich, Milwaukee, WI; methylene chloride and silica gel (40–140 mesh) were from J. T. Baker, Philipsburg, NJ; silica gel G (250 μm) thin-layer chromatography plates were from Analtech Inc., Newark, DE.

(*B*) *NMR Studies of Duplex Formation.* The 90-MHz ^{1}H NMR spectra were obtained in the Fourier transform mode of a Bruker WH-90 spectrometer equipped with quadrature detection. Probe temperatures were maintained to within ±1 °C by a Bruker B-ST 100/700 variable temperature unit and were calibrated by thermocouple measurements. The samples were lyophilized twice from D_2O and then dissolved in 100% D_2O (Aldrich) which contained 0.01 M sodium phosphate buffer (pD 7.0) and 1.0 M sodium chloride. The sample concentrations were as follows: CAAUG, 1.1×10^{-2} M; CAUUG, 1.1×10^{-2} M; CAGUG, 3.2×10^{-3} M; CACUG, 5.3×10^{-3} M; CAUAUG, 9.9×10^{-3} M. Total strand concentrations for the duplex samples are listed in Table V. *tert*-Butyl alcohol-*d* was used as an internal reference, and the chemical shifts are reported in parts per million relative to DSS. The field-frequency lock was provided by the deuterium signal of D_2O. Spectra were recorded over a 1200-Hz sweep width in 8K data points (3.411-s acquisition time). The pulse width was 3 μs (67.5° pulse angle). High-temperature spectra were obtained in 200–300 scans while those at lower temperatures required 1000 scans.

Results

Chemical Synthesis and Characterization of Oligoribonucleotides. In order to study the effect one additional internal base pair would have on the stability of the CAUG duplex (Romaniuk et al., 1978a,b), it was necessary to synthesize a series of pentaribonucleotides, CAXUG, where X is A, G, C, or U. Three complementary duplex sets can be prepared by appropriate mixing experiments. All of the oligoribonucleotides used in this work were prepared by the general phosphotriester synthesis developed in the Neilson laboratory (Neilson & Werstiuk, 1974; England & Neilson, 1976; Werstiuk & Neilson, 1976). This method is capable of providing large-scale preparations of the variety of sequences used in this NMR study.

An outline of the synthetic scheme is presented in Figure 1. Stepwise synthesis of a protected oligoribonucleotide starts at the 5′ terminus and builds toward the 3′ terminus of the sequence. The versatility of the synthesis is illustrated by the fact that two of the pentanucleotides were prepared in a stepwise fashion and the other pentamers were synthesized by using a block scheme similar to that shown in Figure 1.

Table I contains the preparative data for the protected oligoribonucleotides. The 5′-specific deblocking of tracUt*p*Bz-Gt (1.0 g, 0.77 mmol) was carried out in methanolic ammonia (1%, 99 mL) to yield HOUt*p*Bz-Gt (830 mg, 63%) for use in the block synthesis of CAAUG and CAUUG. Full deblocking of the sequences was carried out by using a three-step procedure (England & Neilson, 1976). Deblocked oligoribonucleotides were isolated by using paper chromatography (Table II). Sequence integrity was checked by ^{1}H NMR analysis of the products after each synthetic step. The results of these analyses are presented in Table III and have been reported elsewhere (Romaniuk et al., 1978b).

Chemical Shift Assignments of the Aromatic Resonances. The low-field nonexchangeable proton chemical shift assignments were determined by comparison to the data on CAUG (Romaniuk et al., 1978b). In CAAUG the presence of A(3) was indicated by two singlets at 8.255 (H-8) and 8.079 ppm (H-2). The fact that adenine has a strong ring-current shielding effect (Giessner-Prettre et al., 1976) would account

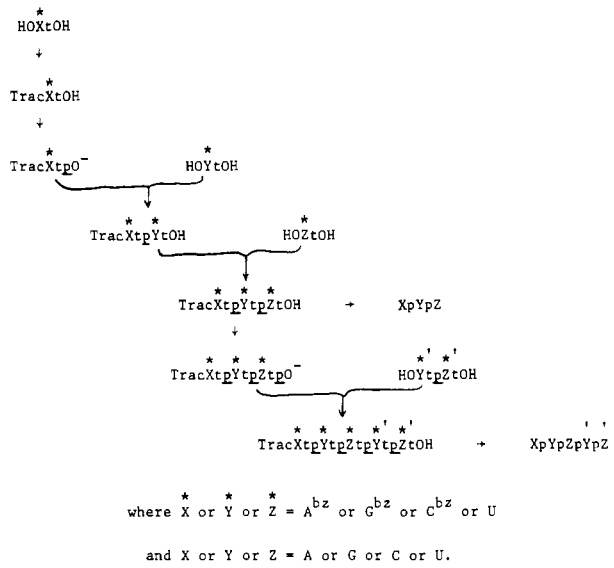


FIGURE 1: Chemical synthesis of oligonucleotides of defined sequence. Abbreviations and details of synthesis are described in the text.

Table II: Experimental Data of Free Oligoribonucleotides

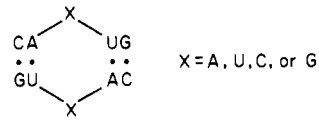
sequence	R_f[a]	yield (%) for deprotection[b]
CAC	0.41	34
CACU	0.29	27
CACUG	0.15	22
CAAUG	0.16	55
CAGUG	0.16	26
CAUUG	0.21	54

[a] Chromatography system: 1 M ammonium acetate–ethanol (50:50) on Whatman 40. [b] Calculated from UV spectrophotometric data, assuming a 90% hypochromicity factor.

for the upfield shifts experienced by the neighboring aromatic protons, when compared to CAUG. The two G H-8 resonances in CAGUG were readily distinguished since the G(5) H-8 resonance at 7.958 ppm shows very little change from that in CAUG. The appearance of a new doublet in the spectrum of CAUUG is attributed to the U(3) H-6 resonance at 7.716 ppm ($J_{5,6}$ = 8.2 Hz). In CACUG the U(4) and C(3) H-6 doublets are overlapped, and confirmation of the chemical shift assignments was provided by incremental analysis (Table IV). The conversion of CAC into CACU causes a shielding of the C(3) H-6 resonance to 7.711 ppm. This proton remains unaffected when G is added to CACU, although the U H-6 is shifted upfield by 0.060 ppm.

The chemical shifts of the C H-6 and G H-8 resonances in CAUAUG were assigned by comparison to those of CAUG (Table III). The U(3) and U(5) H-6 doublets were equivalent at 70 °C. Differentiation of the A(2) and A(4) H-2 signals was achieved by comparison with the data on the trinucleotides CAU and AUG (Romaniuk et al., 1978b). In CAU the A H-2 signal is at lower field (8.250 ppm) than that in AUG (8.198 ppm), and this trend is retained in the hexanucleotide (Table III). Although the same chemical shift trend is observed for the H-8 signals, further support for assigning the lower field H-8 resonance to A(2) was provided by its variable temperature behavior, which was similar to the A H-8 in CAUG (Figure 4b).

Effect of a Nonbonding Base Opposition on the Stability of a Short Duplex. Each of the CAXUG pentaribonucleotides can theoretically form a duplex containing a looped-out region:



Such a duplex would be an ideal model to probe the effect small nonbonding regions have on the formation of adjacent base pairs.

The results of the full variable temperature experiments on each pentaribonucleotide are shown in Figures 2 and 3. For most resonances, the chemical shift vs. temperature plot is characterized by a linear region at high temperatures and a gentle upfield curve at temperatures below 30 °C. These plots do not display the distinct sigmoidal shape characteristic of duplex formation (e.g., Figure 4). In fact, these oligonucleotide sequences do not form a stable duplex under the conditions in which CAUG formed a duplex with a T_m of 24 ± 1 °C (Romaniuk et al., 1978a,b).

Studies on Duplex Formation in Complementary Sets. Before studying the formation and stability of a G·U base pair, we calibrated our model system by studying the effect an additional internal Watson–Crick base pair has on the T_m of CAUG. Thus, the effect of an additional A·U base pair was illustrated by the duplex formed by CAAUG:CAUUG. The results of the full variable temperature experiments are shown in Figure 4a. The low-temperature chemical shift values were determined at 270 MHz since excessive broadening of the 90-MHz spectra at these temperatures made is difficult to assign individual resonances. The average T_m for the duplex, as determined from the eight purine aromatic resonances, is 28.5 ± 2.1 °C which represents an increase of almost 5 °C over the T_m of CAUG (Romaniuk et al., 1978b).

Table III: Chemical Shifts[a] of the Oligoribonucleotides in D_2O[b] at 70 °C

proton	CAUG	CAAUG	CAGUG	CAUUG	CACUG	CAUAUG
C(1) H-6	7.662	7.623	7.641	7.659	7.672	7.643
A(2) H-8	8.346	8.268	8.294	8.359	8.352	8.326
A(2) H-2	8.196	8.134	8.160	8.206	8.183	8.154
A(3) H-8		8.255				
A(3) H-2		8.079				
G(3) H-8			7.906			
U(3) H-6				7.731		7.685
C(3) H-6					7.710	
U(4) H-6	7.692	7.654	7.703	7.716	7.710	
G(5) H-8	7.962	7.942	7.958	7.978	7.978	
A(4) H-8						8.310
A(4) H-2						8.131
U(5) H-6						7.685
G(6) H-8						7.945

[a] Chemical shifts are in parts per million relative to DSS, using *tert*-butyl alcohol-*d* as an internal reference, and are accurate to ±0.005 ppm. [b] pD 7.0. Concentrations: CAUG, 9.2 mM; CAAUG, 11 mM; CAGUG, 3.2 mM; CAUUG, 11 mM; CACUG, 5.3 mM; CAUAUG, 9.9 mM.

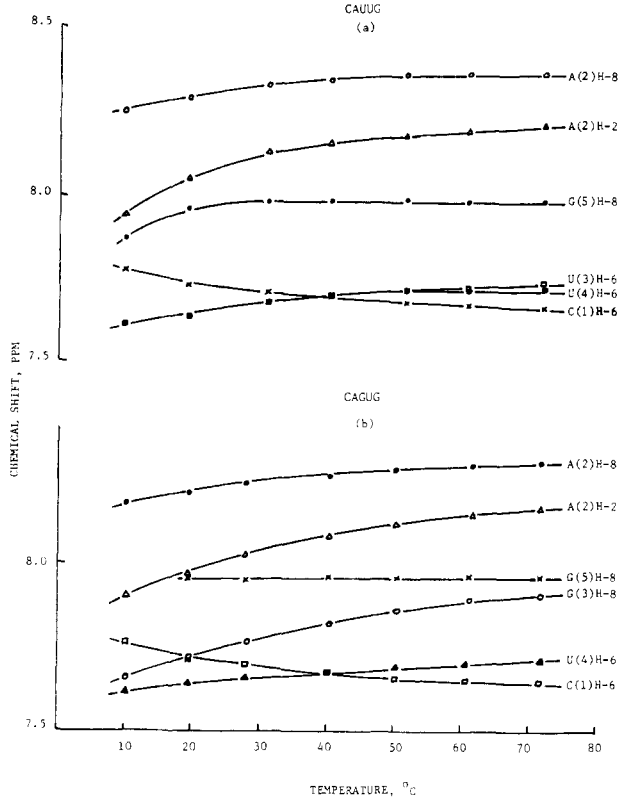


FIGURE 2: Chemical shift vs. temperature plots for the aromatic base protons of (a) CAUUG (11 mM) and (b) CAGUG (3.2 mM).

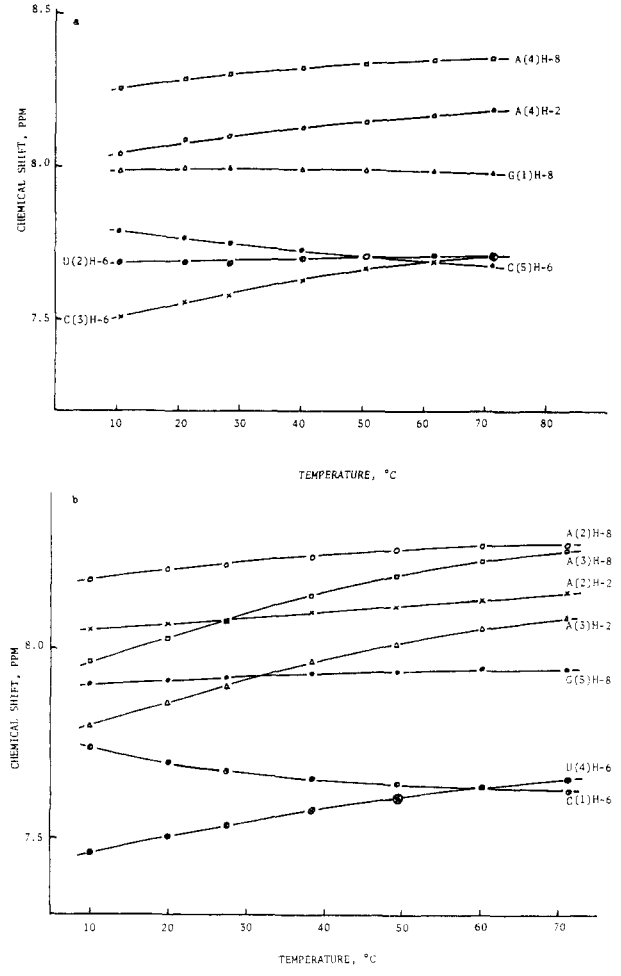


FIGURE 3: Chemical shift vs. temperature plots for the aromatic base protons of (a) CACUG (5.3 mM) and (b) CAAUG (11 mM).

Table IV: Incremental Analysis[a] of CACUG in D_2O[b] at 70 °C

proton	CA	CAC	CACU	CACUG
C(1) H-6	7.660	7.679	7.688	7.672
A(2) H-8	8.377	8.369	8.356	8.352
A(2) H-2	8.260	8.222	8.199	8.183
C(3) H-6		7.741	7.711	7.710
U(4) H-6			7.770	7.710
G(5) H-8				7.978

[a] Chemical shifts are in parts per million relative to DSS, using *tert*-butyl alcohol-*d* as an internal reference, and accurate to ±0.005 ppm. [b] pD 7.0. Concentrations: CA, 16 mM; CAC, 23 mM; CACU, 11 mM; CACUG, 5.3 mM.

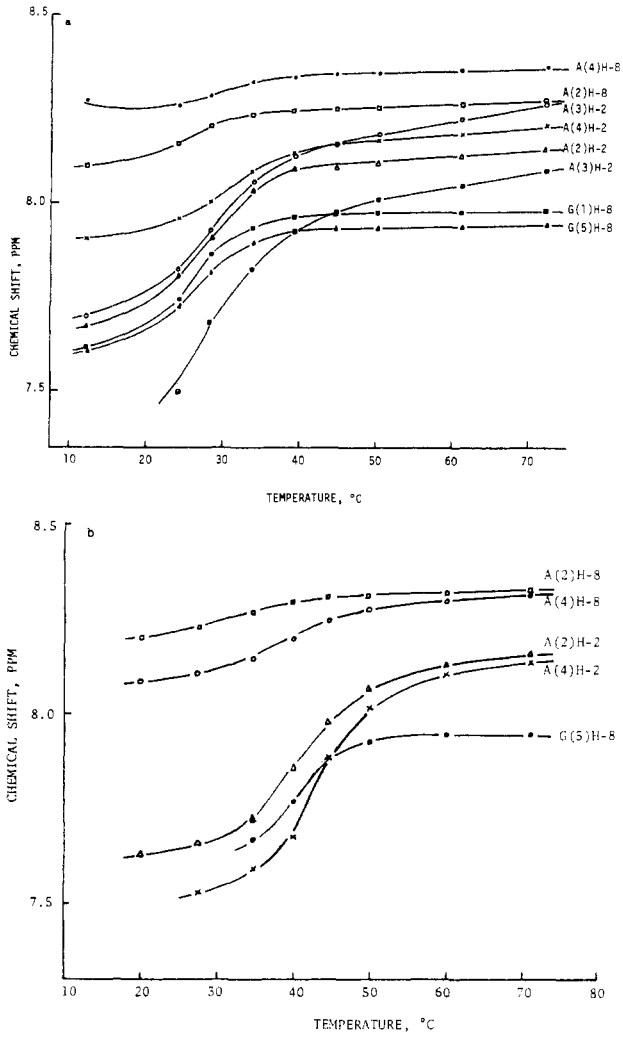


FIGURE 4: Chemical shift vs. temperature plots for the aromatic base protons of (a) the duplex CAAUG:CAUUG (11 mM) and (b) CAUAUG self-complementary duplex (9.9 mM).

When the spectrum of the mixed complementary sequences was recorded at 70 °C, the purine base protons displayed chemical shifts which were essentially identical with those in the single-strand spectra at the same temperature. However, the pyrimidine H-6 signals could not be assigned directly because of the overlap of these resonances. This problem was solved by the technique of spectral subtraction and is illustrated in Figure 5 by the sequence CAAUG. Computer subtraction of the CAUUG 70 °C spectrum from that of the mixture CAAUG:CAUUG produced the difference spectrum of CAAUG from which its chemical shifts could be determined by comparison to the original 70 °C spectrum of CAAUG. Spectral subtraction of mixtures of complementary oligoribonucleotides is limited to only the high-temperature spectra since

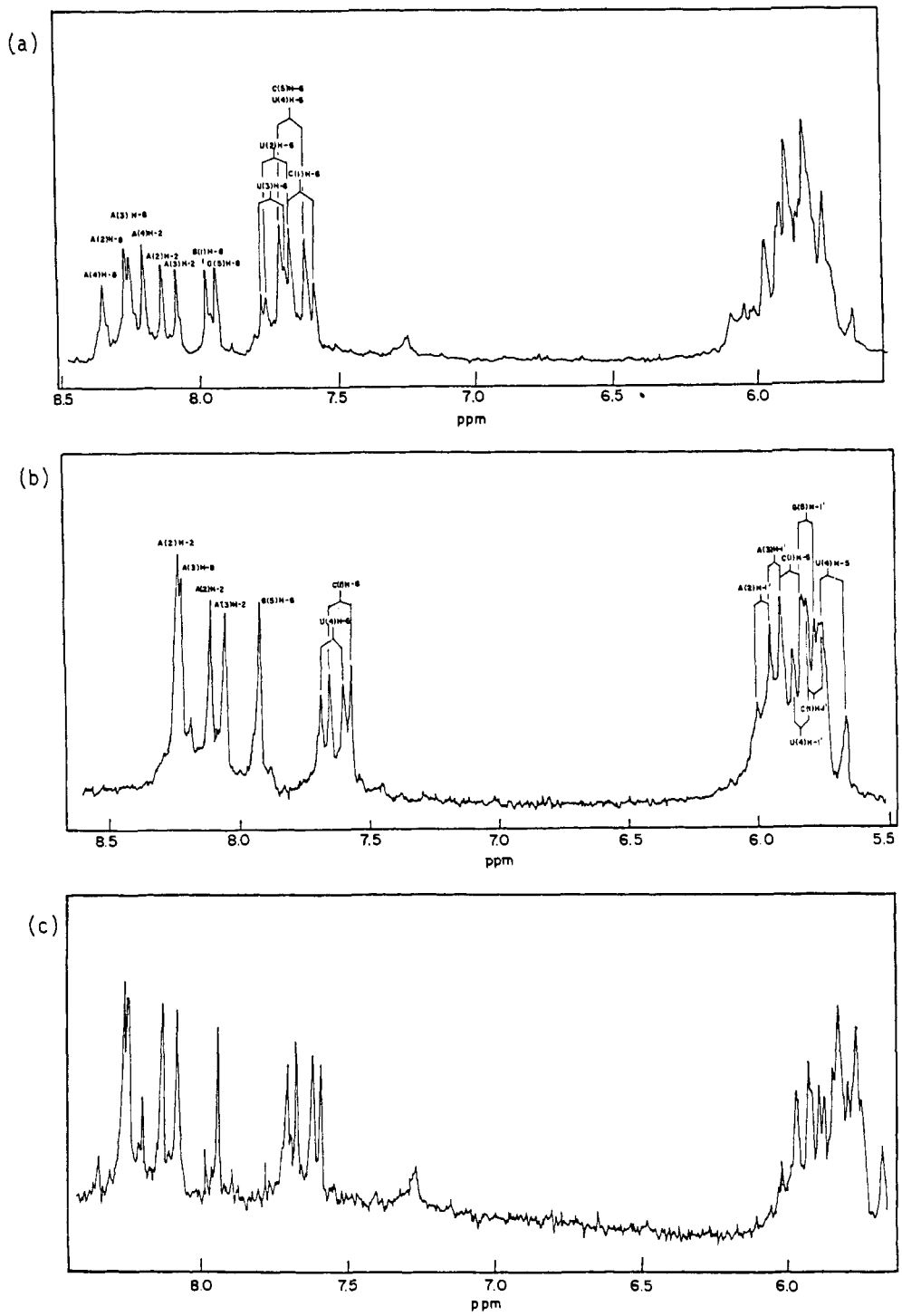


FIGURE 5: Spectral subtraction of CAUUG 70 °C spectrum from the CAAUG:CAUUG 70 °C spectrum (a) yields the spectrum shown in (c). Comparison of (c) with the 70 °C spectrum of CAAUG (b) allowed complete assignment of the aromatic base proton resonances in the 70 °C spectrum of the duplex set (a).

both the intrastrand base stacking and interstrand base pairing are at a minimum under these conditions.

The self-complementary hexaribonucleotide CAUAUG was also studied since it represents an extension of the CAUG duplex with the incorporation of two additional A·U base pairs. The chemical shift vs. temperature plots for the purine aromatic resonances are shown in Figure 4b. The average T_m, as determined from the sigmoidal lines, is 41.5 ± 1 °C, representing a 17 °C increase over the T_m of CAUG at about the same concentration (Table V). Evidence of fraying of the terminal G·C base pairs is not observed, as was the case for CAUG (Romaniuk et al., 1978b).

The effect of an additional internal G·C base pair on the duplex stability of CAUG was studied by using the complementary set CAGUG:CACUG. The results of this experiment was shown in Figure 6. The average T_m of the duplex was 38.4 ± 0.6 °C, representing a large increase over the T_m of CAUG (Table V).

Formation of a G·U Base Pair within a Watson–Crick Double Helix. By mixing the two single strands CAGUG and CAUUG together, we were able to observe, using ^{1}H NMR spectroscopy, the formation of a RNA double helix containing an internal G·U base pair surrounded by regular Watson–Crick A·U and G·C base pairs. The chemical shift vs. tem-

Table V: Melting Temperatures and Concentrations of the Based-Paired Duplexes

duplex	T_m (°C)	concn (M)
CAUG GUAC	24.0 ± 1.0	9.2×10^{-3}
CAGUG GUUAC	23.4 ± 2.0	1.8×10^{-2}
CAAUG GUUAC	28.5 ± 2.1	1.1×10^{-2}
CAGUG GUCAC	38.4 ± 0.6	3.2×10^{-3}
CAUAUG GUAUAC	41.5 ± 1.1	9.9×10^{-3}

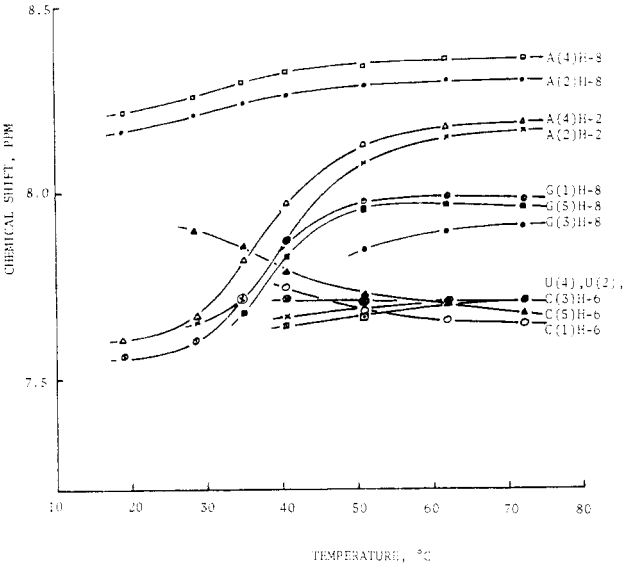


FIGURE 6: Chemical shift vs. temperature plots for the aromatic base protons of the duplex CAGUG:CACUG (3.2 mM).

perature plots are shown in Figure 7. The average T_m of the duplex formed is 23.4 ± 2.0 °C and represents neither an increase nor a decrease in stability with respect to CAUG, the reference duplex (Table V). In fact, the T_m values for the aromatic resonances of the A and G bases involved in Watson–Crick base pairs (Figure 7) are in very good agreement with those for the same base pairs in CAUG (Romaniuk et al., 1978b). By using NMR techniques, it was possible to follow the G(3) H-8 separately, and the T_m of the guanine of the G·U base pair is 20 °C, which is significantly lower than the T_m values of the other base pairs in this duplex. It appears that the G·U base pair is a region of local instability within the duplex.

Discussion

One of the interests arising from the sequencing of natural RNA molecules has been the determination of possible secondary structures for these molecules (Fiers et al., 1975, 1976; Gross et al., 1978; Sitz et al., 1978). The secondary structure of an RNA could play an important role in the initiation of translation (Fiers et al., 1975, 1976), the interaction of proteins with RNA (Krol et al., 1978), and RNA–RNA interactions (Sitz et al., 1978) and be related to a pathogenic function (Gross et al., 1978). In the case of tRNAs, tertiary structure is also important to functionality.

The ability to predict and verify secondary structures for naturally occurring RNAs other than tRNAs is not well developed at present. Two main problems are the larger size of most RNAs of interest (viz. tRNA) and their diversity of functions. Present methods for determining the secondary structure of RNAs include clever digestion techniques (Fiers et al., 1975, 1976), maximizing base pairing of the primary structure (Gross et al., 1978), and employing rules derived from model studies (Tinoco et al., 1971, 1973; Studnicka et al., 1978). It is apparent that further information about the factors which affect the stability of RNA secondary structure is required in order to improve these predictive methods (Tinoco et al., 1973).

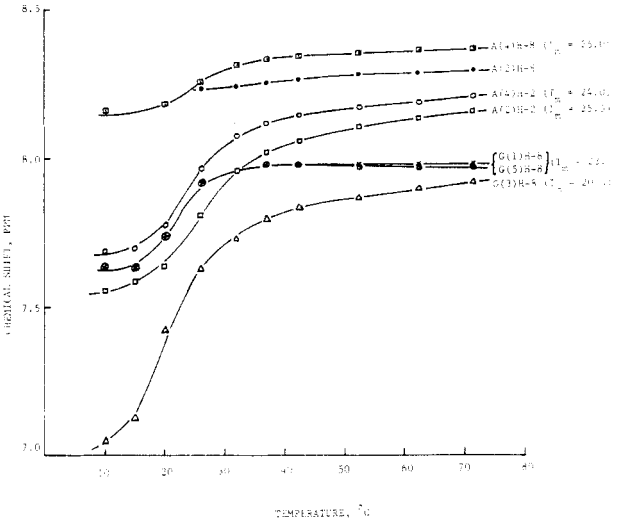


FIGURE 7: Chemical shift vs. temperature plots for the aromatic base protons of the duplex CAGUG:CAUUG (18 mM).

One approach to this problem has been the investigation of model duplex systems by NMR spectroscopy. Both deoxyribonucleotide (Cross & Crothers, 1971; Crothers et al., 1973; Patel, 1976, 1977, 1979; Early et al., 1978) and ribonucleotide sequences (Arter et al., 1974; Borer et al., 1975; Kan et al., 1975; Hughes et al., 1978; Romaniuk et al., 1978a,b) have been studied. In sufficiently simple sequences, each nucleotide is identifiable by several resonances, and, by the nature of an NMR experiment, the helix to coil transition of each base pair in the duplex can be monitored.

The helix to coil transition of the hexaribonucleotide A_2GCU_2 was studied by both the exchangeable and nonexchangeable proton resonances (Borer et al., 1975; Kan et al., 1975). Complete assignment of the nonexchangeable base and ribose anomeric proton resonances was realized through the incremental analysis technique (Borer et al., 1975). The T_m of the duplex, measured by the NMR experiment, was comparable to a value extrapolated from optical data. This result established the validity of T_m measurements determined from NMR data.

We have begun a series of experiments which probe various aspects of RNA secondary structure using an assortment of short oligoribonucleotides (Hughes et al., 1978; Romaniuk et al., 1978a,b). We have reported previously on the stabilizing effect of a dangling base on a neighboring duplex (Romaniuk et al., 1978a). By using ^1H NMR spectroscopy, we were able to demonstrate that a dangling base can increase the stability of a duplex which does not fray at its terminus, and it does so by a base-stacking interaction (Romaniuk et al., 1978a). This could serve as a model for the effect large loops have on adjacent duplex regions, a possibility currently under investigation.

In this communication we report on studies designed to examine systematically, using a model system, the effect very

small loops have on short neighboring duplex regions and the relative stability of a G·U base pair within a RNA double helix containing regular A·U and G·C base pairs. The elegance of this study lies in the fact that a maximum number of experiments were generated from a relatively small amount of synthetic material. This allowed for the conductance of seven studies on diverse aspects of RNA secondary structure using four pentaribonucleotides. This was possible because of the chemical synthesis methods described here, in combination with NMR techniques.

Each single pentaribonucleotide allowed for the study of duplex formation of a CAUG-type duplex (Romaniuk et al., 1978b) containing a nonbonding base opposition in the center of the duplex. Studies on loops of this type employed longer A_nU_n-type duplexes and optical techniques, which could give little conformational information (Uhlenbeck et al., 1971). It is now generally accepted that even a U-U nonbonding opposition will take an extrahelical conformation, allowing for stacking of duplex regions, and for most loops studied there is evidence of an extrahelical conformation (Lomant & Fresco, 1975). NMR evidence of looping out is a downfield shift with decreasing temperature as compared to the upfield shifts usually observed under these conditions (Shum, 1977). In Figures 2 and 3, the chemical shift vs. temperature plots for the aromatic base protons are not the sharp sigmoidal shapes associated with duplex formation, although the upfield shifts are somewhat larger than those usually associated with single-stranded base stacking (Romaniuk et al., 1978b). Even more important, however, is the observation that the plot for the aromatic proton of the middle base of each sequence shows an *upfield* trend with decreasing temperature. We interpret these results to mean that the base which would be involved in a nonbonding base opposition is *not* taking an extrahelical conformation. Under the conditions where CAUG has a T_m of 24 ± 1 °C (Romaniuk et al., 1978b), these pentanucleotides do not form a stable duplex with a small loop but prefer to maintain a single-stranded stacked conformation. This result is particularly surprising in the case of CAUUG since looping out of the U(3) would only break a U-U stacking interaction, and a A-U stacking interaction would be maintained in the duplex conformation (Lomant & Fresco, 1975). Perhaps, in naturally occurring RNA molecules, short duplex regions separating very small loops (one to two bases) may open up under suitable conditions to give larger loops which are stabilized by increased base-stacking interactions.

The effect of adding additional internal A·U or G·C base pairs was studied by using the following duplexes: CAAUG:CAUUG, CAUAUG (self-complementary), and CUG. Average T_m values were determined from the sigmoidal chemical shift vs. temperature curves of the aromatic base protons, and the results are summarized in Table V. In each case, the duplex T_m is increased relative to that of CAUG, a result which is not surprising. It is interesting to note that the increase in T_m for CAAUG:CAUUG is comparable to the T_m for CAUGU (Romaniuk et al., 1978a): a dangling U adjacent to the CAUG duplex stabilizes the duplex as much as an additional internal A·U base pair. These duplexes act as a second set of references in our model system and allow for further comparisons to be made between various features of secondary structure.

Figure 7 shows the chemical shifts vs. temperature plots for the CAGUG:CAUUG duplex set. The sharp sigmoidal nature of the curves is indicative of duplex formation and a T_m can be determined. It is important to remember that each single strand of this duplex has been studied in two different ways. As a single-strand study, neither strand showed evidence of formation of a self-complementary duplex containing a loop but rather maintained a stacked structure. Each strand has demonstrated its ability to form a Watson–Crick double helix with the appropriate complementary strand. On the basis of this evidence, it is apparent that the set CAGUG:CAUUG has formed a double helix which contains a G·U base pair. The stability of this base pair is measurable from the sigmoidal curve for the G(3) H-8 (Figure 7). This is the first study of the formation and relative stability of a G·U base pair within a double helix containing regular Watson–Crick A·U and G·C base pairs.

The exchangeable ring N–H hydrogen-bonded proton resonances were studied in an attempt to determine the hydrogen-bonding scheme of the G·U base pair. At temperatures of 0–5 °C, the resonance line widths were extremely broad, and individual resonances could not be identified. Previous NMR studies of exchangeable proton resonances have indicated that these resonances can be observed as sharp singlets only at temperatures of 40–50 °C *lower* than the T_m of the duplex studied (Arter et al., 1974; Kan et al., 1975; Rordorf et al., 1976; Patel, 1976, 1977, 1979). Apparently, the N–H protons of the CAGUG:CAUUG duplex are experiencing considerable exchange with the solvent at low temperatures.

The orientation of the bases in a wobble G·U base pair differs from the normal Watson–Crick base-pairing configuration (Mizuno & Sundaralingam, 1978). The shielding effect exerted by U(3) on the H-2 resonance of its 5′ nearest neighbor, adenosine, was compared for the CAAUG:CAUUG duplex and the CAGUG:CAUUG duplex. This A H-2 resonance is shielded –0.25 ppm in the former duplex and –0.45 ppm in the latter duplex. Little change was found for the shielding pattern exerted by the G(3) residue. The increased shielding of –0.20 ppm of the nearest-neighbor A H-2 resonance by U(3) in the CAGUG:CAUUG duplex must result from an altered base overlap when a G·U base pair is formed. This perturbation is consistent with the formation of a wobble G·U base pair but not with the formation of a tautomeric G·U base pair.

The results from the duplex studies reported here are summarized in Table V. Each single strand in the CAGUG:CAUUG duplex has the same stacking interactions as in the corresponding CAGUG:CACUG and CAAUG:CAUUG duplexes. The G·U pair most likely being formed is the wobble pair with two hydrogen bonds (Rordorf et al., 1976; Early et al., 1978). A comparison of the duplexes in Table V shows that the G·U pair is less stable than the A·U pair, and there is no increase of stability throughout the duplex, as there is for the CAAUG:CAUUG and CAGUG:CACUG duplexes when compared to CAUG. In fact, the T_m values for the A·U and G·C base pairs of the CAGUG:CAUUG duplex are identical with those found for CAUG (Romaniuk et al., 1978b), and the G·U base pair is significantly less stable (T_m = 20.0 °C) than the rest of the duplex (T_m = 24 ± 1 °C).

Several mechanisms could account for this decreased stability. The hydrogen bonding in a wobble G·U base pair requires a shift in the glycosyl torsion angles from the angles usually associated with Watson–Crick base pairing (Mizuno & Sundaralingam, 1978). This displacement of the bases can be easily accommodated with little perturbation of the backbone conformation (Mizuno & Sundaralingam, 1978). However, although stacking interactions are only slightly changed, this change might lead to an overall decrease in stability throughout the duplex. An alternate explanation arises from the observation that a G·U pair in the wobble position of a codon–anticodon interaction is as stable as an A·U pair in the

same position (Uhlenbeck et al., 1970). It is possible that the G·U pair cannot adopt its preferred conformation within a duplex and is therefore a region of local instability, perhaps affecting the nucleation and closing of the entire duplex. It is entirely plausible that both situations exist in solution and contribute to the observed effect.

Acknowledgments

The authors thank Dr. Mark Henrichs of Eastman Kodak for some low-temperature 270-MHz spectra.

References

Arter, D. B., Walker, G. C., Uhlenbeck, O. C., & Schmidt, P. G. (1974) *Biochem. Biophys. Res. Commun. 61*, 1089.

Borer, P. N., Kan, L. S., & Ts'o, P. O. P. (1975) *Biochemistry 14*, 4847.

Crick, F. H. C. (1966) *J. Mol. Biol. 19*, 548.

Cross, A. D., & Crothers, D. M. (1971) *Biochemistry 10*, 4015.

Crothers, D. M., Hilbers, C. W., & Schulman, R. G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2899.

Early, T. A., Olmsted, J., III, Kearns, D. R., & Lezius, A. G. (1978) *Nucleic Acids Res. 5*, 1955.

England, T. E., & Neilson, T. (1976) *Can. J. Chem. 54*, 1714.

Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Merregaert, J., Min Jou, W., Raeymaekers, A., Volckaert, G., Ysebaert, M., Van de Kerckhove, J., Nolf, F., & Van Montagu, M. (1975) *Nature (London) 256*, 273.

Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Tserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Volckaert, G., & Ysebaert, M. (1976) *Nature (London) 260*, 500.

Ganoza, M. C., Fraser, A. R., & Neilson, T. (1978) *Biochemistry 17*, 2769.

Giessner-Prettre, C., Pullman, B., Borer, P. N., Kan, L.-S., & Ts'o, P. O. P. (1976) *Biopolymers 15*, 2277.

Gray, D. M., & Ratliff, R. L. (1977) *Biopolymers 16*, 1331.

Gray, D. M., Tinoco, I., Jr., & Chamberlin, M. J. (1972) *Biopolymers 11*, 1235.

Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H., & Saenger, H. L. (1978) *Nature (London) 273*, 203.

Hughes, D. W., Bell, R. A., England, T. E., & Neilson, T. (1978) *Can. J. Chem. 56*, 2243.

IUPAC–IUB Commission (1970) *Biochemistry 9*, 4022.

Kan, L. S., Borer, P. N., & Ts'o, P. O. P. (1975) *Biochemistry 14*, 4864.

Kearns, D. R. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 18*, 91.

Krol, A., Machatt, M. A., Branlant, C., & Ebel, J. P. (1978) *Nucleic Acids Res. 5*, 4933.

Krugh, T. R., & Young, M. A. (1975) *Biochem. Biophys. Res. Commun. 62*, 1025.

Lezius, A. G., & Domin, E. (1973) *Nature (London), New Biol. 244*, 169.

Lomant, A. J., & Fresco, J. R. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 185.

Mizuno, H., & Sundaralingam, M. (1978) *Nucleic Acids Res. 5*, 4451.

Neilson, T., & Werstiuk, E. S. (1974) *J. Am. Chem. Soc. 96*, 2295.

Patel, D. J. (1976) *Biopolymers 15*, 533.

Patel, D. J. (1977) *Biopolymers 16*, 1635.

Patel, D. J. (1979) *Biopolymers 18*, 553.

Rich, A. (1977) *Acc. Chem. Res. 10*, 388.

Robillard, G. T., Hilbers, C. W., Reid, B. R., Gangloff, J., Dirheimer, G., & Schulman, R. G. (1976) *Biochemistry 15*, 1883.

Romaniuk, P. J., Hughes, D. W., Gregoire, R. J., Neilson, T., & Bell, R. A. (1978a) *J. Am. Chem. Soc. 100*, 3971.

Romaniuk, P. J., Neilson, T., Hughes, D. W., & Bell, R. A. (1978b) *Can. J. Chem. 56*, 2249.

Rordorf, B. F., Kearns, D. R., Hawkins, E., & Chang, S. H. (1976) *Biopolymers 15*, 325.

Shum, B. W.-K. (1977) Ph.D. Thesis, Yale University, New Haven, CT.

Sitz, T. O., Kuo, S.-C., & Nazar, R. N. (1978) *Biochemistry 17*, 5811.

Studnicka, G. M., Rahn, G. M., Cummings, T. W., & Salser, W. A. (1978) *Nucleic Acids Res. 5*, 3365.

Tinoco, I., Jr., Uhlenbeck, O. C., & Levine, M. (1971) *Nature (London) 230*, 362.

Tinoco, I., Jr., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., & Gralla, J. (1973) *Nature (London), New Biol. 246*, 40.

Uhlenbeck, O. C., Baller, J., & Doty, P. (1970) *Nature (London) 225*, 509.

Uhlenbeck, O. C., Martin, J. H., & Doty, P. (1971) *J. Mol. Biol. 57*, 217.

Werstiuk, E. S., & Neilson, T. (1976) *Can. J. Chem. 54*, 2689.