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Effects of 3′ Dangling End Stacking on the Stability of GGCC and CCGG Double Helices†

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ABSTRACT: Thermodynamic properties for helix formation of two core molecules, GGCC and CCGG, and pentanucleotides containing either core plus a 3′ dangling nucleotide were measured spectrophotometrically. In 1 M Na^+, the order of stability is GGCC ≈ GGCCp < pGGCC < GGCCC ≈ GGCCCp < GGCCUp < GGCCAp ≈ GGCCGp ≈ GGCCϵAp and CCGG < pCCGG < CCGGCp < CCGGUp < CCGGAp ≈ CCGGGp. In 0.01 M Na^+, the order of stability for the GGCC family does not change except GGCC is more stable than the tetramers with a terminal phosphate. Thermodynamic parameters obtained by using a two-state model demonstrate the stabilizing effect of a 3′ dangling end is enthalpic. The results indicate stacking is an important contributor to nucleic acid stability. Sedimentation equilibrium experiments at 3 °C on GGCCGp in 1 M Na^+ and GGCCAp in 0.01 M Na^+ indicate no aggregation of pentanucleotide helices at strand concentrations as high as 2 mM.

Base stacking is thought to play an important role in the stabilization of nucleic acid helices (Cantor & Schimmel, 1980; Bloomfield et al., 1974; Turner et al., 1981). Stacking in single-strand helices has been extensively studied (Felsenfeld & Miles, 1967; Adler et al., 1967; Brahms et al., 1967a,b; Stannard & Felsenfeld, 1975; Breslauer & Sturtevant, 1977; Filimonov & Privalov, 1978; Suurkuusk et al., 1977; Freier et al., 1981; Dewey & Turner, 1979, 1980; Pörschke, 1973, 1976, 1978). Stacking in double-strand helices can be modeled by addition of a terminal unpaired nucleotide (dangling end) to an oligonucleotide double helix. Addition of a dangling end to an RNA helix stabilizes the helix (Martin et al., 1971; Romaniuk et al., 1978; Neilson et al., 1980; Alkema et al., 1981a,b; Petersheim & Turner, 1983a). In this paper, we report the changes in thermodynamic properties of helix formation associated with attaching various 3′ dangling ends to two core helices, GGCC and CCGG.[1] The results provide insight into the forces stabilizing double helices and should help improve predictions of RNA secondary structure from sequence (Borer et al., 1974).

Materials and Methods

Oligonucleotide Synthesis. GGCC and GGCCC were synthesized from GG (Sigma) by using primer-dependent polynucleotide phosphorylase kindly provided by David Koh. The conditions were similar to those described by Petersheim & Turner (1983a), but no nuclease was added to the reaction mixture. Incubation at 37 °C for 2 days gave the highest yield of tetramer.

CCGG and GGCC were obtained from Collaborative Research. The chromatographic properties and melting curves of the commercial GGCC were identical with those of GGCC

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[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; TEAB, triethylammonium bicarbonate; Tris, tris(hydroxymethyl)-aminomethane; ϵA, 1,N^6-ethenoadenosine. For oligonucleotides, internal phosphates are not denoted; GGC is GpGpC. If a molecule contains a terminal phosphate, it is explicitly indicated.

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synthesized from GG. The identity and purity of the commercial CCGG were confirmed previously (Petersheim & Turner, 1983a,b).

pGGCC and pCCGG were synthesized from the respective tetranucleoside triphosphates with T4 polynucleotide kinase (P-L Biochemicals). The reaction contained 0.2 M Tris-HCl, pH 9.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5 mM ATP, 0.7–1.0 mM tetramer, 50 μg/mL BSA, and 10 units/mL polynucleotide kinase. Reactions were complete after 1 h at 37 °C.

The pentanucleotides GGCCXp and CCGGYp (X = A, ϵA, C, G, or U; Y = C or G) were synthesized from GGCC or CCGG with T4 RNA ligase (P-L Biochemicals) (Uhlenbeck & Cameron, 1977; England & Uhlenbeck, 1978). The reaction conditions were 0.2 M Tris-HCl, pH 8.5, 20 mM $MgCl_2$, 7.5 mM ATP, 5 mM DTT, 4 mM nucleoside 5′,3′-bisphosphate, 0.7–1.0 mM tetranucleoside triphosphate, 25 μg/mL BSA, and 25 units/mL T4 RNA ligase. Reaction was complete after 5 h at 37 °C.

GGC was prepared by using phosphotriester synthesis (England & Neilson, 1976; Werstiuk & Neilson, 1976; Alkema et al., 1981a). Identity was confirmed by NMR spectroscopy, and purity was confirmed by HPLC. GGCCp was synthesized from GGC under conditions described above for pentamer synthesis. Enzyme activity was 10 units/mL, and reaction was complete after 1 h.

All reactions were monitored by HPLC. The yields of the kinase and ligase reactions were nearly 100%.

Purification of Oligonucleotides. Reaction products were separated on DEAE-Sephadex in 0.01 M Tris-HCl, pH 8.2, and 7 M urea by using a gradient from 0.05 to 0.5 M NaCl. Product peaks were diluted fourfold and desalted by application to a DEAE-Sephadex column, washing with 0.5 L of 0.1 M TEAB, and elution with 1.5 M TEAB. The TEAB was removed by rotary evaporation. Addition of methanol facilitated complete removal of the TEAB. Purities of all oligomers were confirmed by HPLC. The identities of GGCC and GGCCAp were confirmed by NMR spectroscopy at 70 °C.

Oligonucleotide Solutions. Oligonucleotide concentrations were measured by absorbance and are strand concentrations. Extinction coefficients at 280 nm, 90 °C, were calculated from published dimer and monomer extinction coefficients (Richards, 1975) by using the nearest-neighbor approximation (Borer, 1975). In units of 10^4 M^{-1} cm^{-1}, they are the following: GGCC, 2.35; GGCCAp, 2.51; GGCCCp, 2.86; GGCCϵAp, 2.52; GGCCGp, 2.91; GGCCUp, 2.64; pCCGG, 2.44; CCGGCp, 2.78; CCGGGp, 3.17. Extinction coefficients at 90 °C were assumed to be independent of salt concentration and unaffected by addition of a terminal phosphate. Most melting curves were measured in 0.01 M sodium cacodylate and 0.001 M EDTA, pH 7, either with or without 1 M NaCl. Molar activity coefficients were calculated from molal activity coefficients (Robinson & Stokes, 1959) and solution densities (Picker et al., 1971).

Sedimentation Equilibrium. Sedimentation equilibrium measurements were performed with a Beckman Model E analytical ultracentrifuge with schlieren optics by using an aluminum An-D rotor and a 12-mm double sector cell equipped with quartz windows and an aluminum filled Epon centerpiece. Each run was allowed to equilibrate 48 h.

The schlieren position, Z, is proportional to dc/dr where c is the concentration and r the distance from the axis of rotation. The apparent Z-average molecular weight, M_Z, was obtained from the slope of a plot of Z/r vs. $\int_{r_i}^{r} dr' Z$

$$\text{slope} = \frac{d}{dc}\left(\frac{1}{r}\frac{dc}{dr}\right) = M_Z\frac{\omega^2(1-\bar{v}\rho)}{RT} \quad (1)$$

or a plot of $\log (Z/r)$ vs. r^2

$$\text{slope} = \frac{d}{dr^2}\left[\log\left(\frac{1}{r}\frac{dc}{dr}\right)\right] = \frac{M_Z\omega^2(1-\bar{v}\rho)}{4.606RT} \quad (2)$$

where r_i is the position of the inner meniscus, ω is the rotor speed, $\bar{v}$ is the partial specific volume of the oligomer, ρ is the solution density, R is the gas constant, and T is the temperature (Chervenka, 1969).

The densities of the buffers were measured by comparison of the weight of a volume of buffer to that of an equal volume of water.

Melting Curves. Absorbance vs. temperature curves were measured at 280 nm in a Gilford 250 spectrometer equipped with a Gilford 2527 thermoprogrammer. Each melting curve consisted of at least 350 absorbance vs. temperature points, and at least 12 melting curves were measured for each oligomer.

Thermodynamic Parameters. Thermodynamic parameters were obtained from absorbance vs. temperature curves as described previously (Petersheim & Turner, 1983a; Freier et al., 1983). "Temperature-independent" values are the average of two methods: (1) averaging the enthalpies and entropies obtained from fitting melting curves to a two-state model with linear sloping base lines (Petersheim & Turner, 1983a; Freier et al., 1983) and (2) plots of the concentration dependence of the melting temperature (Borer et al., 1974):

$$T_m^{-1} = \frac{2.3R}{\Delta H^\circ}\log (c_T) + \frac{\Delta S^\circ}{\Delta H^\circ} \quad (3)$$

where c_T is the total strand concentration and T_m is the temperature at which half the strands are in the double-helical state. The T_m's were derived from the best-fit thermodynamic parameters for each curve.

Temperature-dependent thermodynamic parameters were obtained from plots of ΔH° vs. T_m and ΔS° vs. $\ln (T_m)$ where ΔH°, ΔS°, and T_m are the values obtained from fitting each melting curve to a two-state model with linear sloping base lines.

Results

Temperature-Independent Thermodynamic Parameters. Plots of T_m^{-1} vs. $\log c_T$ are reported in Figures 1 and 2. As described under Materials and Methods, temperature-independent enthalpies, ΔH°, and entropies, ΔS°, were obtained from these plots and from fits of the melting curves to the two-state model with sloping base lines.

In 1 M NaCl, both the concentration dependence of T_m and the averages from the fits yielded thermodynamic properties that differed by less than 6%. Therefore, the average results of these two methods are listed in Table I. Duplicate sets of measurements yielded parameters that differed by 3% so we estimate the error in ΔH° and ΔS° to be about ±5%. The large correlation of ΔH° and ΔS° makes the error limits on ΔG° near the T_m roughly 1–2%.

In 0.01 M Na^+, plots of T_m^{-1} vs. $\log c_T$ yielded enthalpies and entropies of helix formation that were up to 30% less negative than those obtained from fits. Therefore, Table II lists thermodynamic properties of helix formation in 0.01 M Na^+ determined by each method as well as the average of the two methods. The disagreement between the two methods may mean the helix to coil equilibrium is less two state in low salt than in high salt. In general, salt effects on short duplexes are not well understood. For example, with

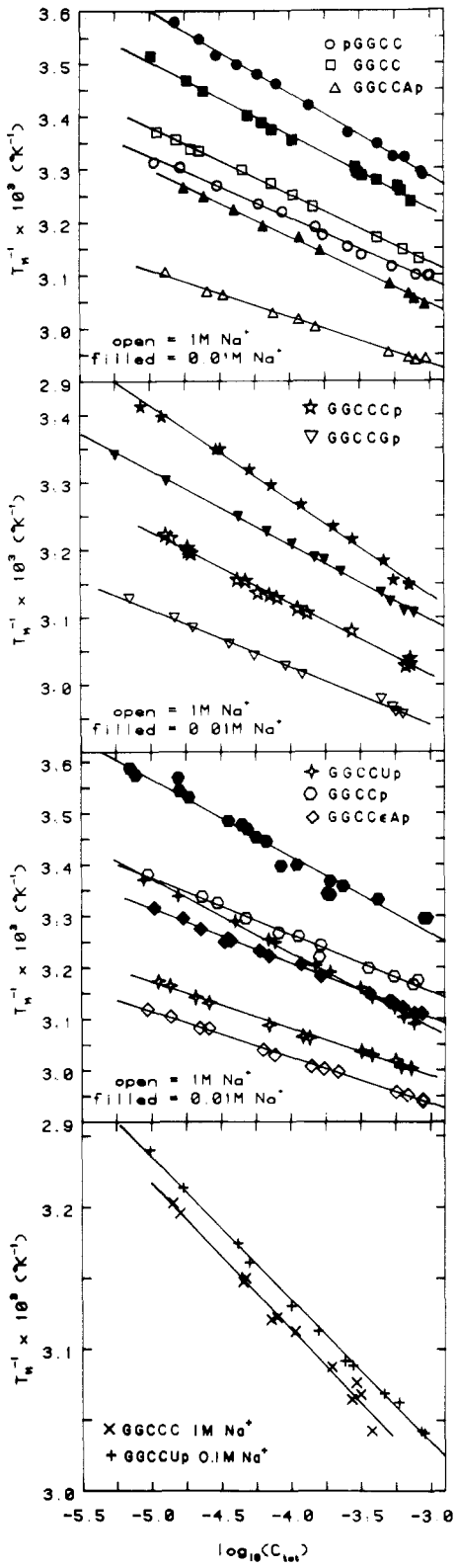

FIGURE 1: Reciprocal melting temperature vs. log (concentration) for GGCC and related oligoribonucleotides. The solid symbols are low salt (0.01 M sodium cacodylate and 0.001 M EDTA, pH 7); the open symbols are high salt (1 M NaCl, 0.01 M sodium cacodylate, and 0.001 M EDTA). (○) pGGCC; (□) GGCC; (△) GGCCAp; (stars) GGCCCp; (▽) GGCCGp; (clovers) GGCCUp; (hexagons) GGCCp; (◇) GGCCεAp; (×) GGCCC in high salt buffer; (+) GGCCUp in 0.1 M NaCl, 0.01 M sodium cacodylate, and 0.001 M EDTA, pH 7.

dCGCGAATTCGCG, calorimetric data indicate the helix-to-coil transition is more two state in 0.01 M Na^+ than in 0.1 M Na^+, whereas optical data suggest the opposite (Patel et al., 1982a; Marky et al., 1983).

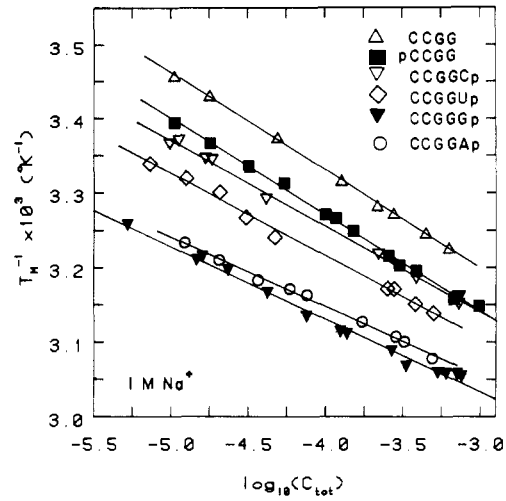

FIGURE 2: Reciprocal melting temperature vs. log (concentration) for CCGG and related oligoribonucleotides in 1 M NaCl, 0.01 M sodium cacodylate, and 0.001 M EDTA, pH 7. (△) CCGG; (■) pCCGG; (▽) CCGGCp; (◇) CCGGUp; (▼) CCGGGp; (○) CCGGAp. The data for CCGG, CCGGUp, and CCGGAp are from Petersheim & Turner (1983a).

Table I: Temperature-Independent Thermodynamic Parameters[a] for Helix Formation in 1 M NaCl, 0.01 M Sodium Cacodylate, and 0.001 M EDTA, pH 7

oligonucleotide	$-\Delta H^\circ$ (kcal/mol)[b]	$-\Delta S^\circ$ (eu)[b]	T_m at 1×10^{-4} M (°C)
GGCC	36.0	98	35.0
pGGCC	39.4	108	38.8
GGCCp	39.0	109	33.5
GGCCC	43.2	116	48.1
GGCCCp	41.7	112	47.5
GGCCUp	48.3	130	51.5
GGCCGp	52.3	140	57.5
GGCCAp	51.5	137	58.0
GGCCεAp	50.5	134	57.5
CCGG[c]	34.5	96	27.4
pCCGG	36.4	101	32.9
CCGGCp	40.3	113	34.4
CCGGUp[c]	42.1	117	38.0
CCGGGp	48.3	133	46.5
CCGGAp[c]	48.1	133	44.7

[a] Obtained from absorbance vs. temperature profiles as described under Materials and Methods. [b] Although the errors in ΔH° and ΔS° are ±5%, additional significant figures are given to allow accurate calculation of ΔG° vs. temperature. [c] From Petersheim & Turner (1983a).

Although the enthalpy and entropy changes on helix formation at 0.01 M Na^+ depend on the method of analysis, the free energies and T_m's are much less sensitive to method. The T_m's in Table II can, therefore, be compared to those in Table I. All the oligomers are less stable at 0.01 M Na^+ than 1 M Na^+.

For all the molecules studied, addition of a 3′ dangling end stabilizes the double helix. The T_m's at 10^{-4} M are 7–24 °C higher than for the tetramer cores. The order of increasing stability for both CCGG and GGCC cores is C < U < G, A. It is surprising that a dangling cytidine stabilizes the helix less than a dangling uridine. Poly(C) stacks into a single-stranded helix with a T_m of about 50 °C (Adler et al., 1967; Brahms et al., 1967a; Pörschke, 1976; Freier et al., 1981), whereas poly(U) is a random coil above 15 °C (Young & Kallenbach, 1978; Neumann & Tran-Dinh, 1982). This and studies on stacking of nucleotides (Solie & Schellman, 1968) and di-

Table II: Temperature-Independent Thermodynamic Parameters[a] for Helix Formation in 0.01 M Sodium Cacodylate and 0.001 M EDTA, pH 7

	average from fits[b]			$1/T_m$ vs. log (concentration)[b]			average of both methods		
oligonucleotide	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m[c] (°C)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m[c] (°C)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m[c] (°C)
GGCC	33.5	94	24.7	32.7	92	24.2	33.1	93	24.5
pGGCC	30.3	86	18.2	29.0	81	17.4	29.7	84	17.8
GGCCp	32.8	93	17.0	30.4	86	15.8	31.6	89	16.4
GGCCCp	40.7	115	32.8	32.5	88	32.4	36.6	101	32.6
GGCCUp	45.0	127	37.4	31.7	84	36.8	38.3	105	37.2
GGCCGp	46.3	130	39.1	41.2	114	38.7	43.8	122	38.9
GGCCAp	50.1	140	43.0	36.1	96	42.1	43.1	118	42.6
GGCCεAp	47.3	133	38.9	43.0	120	38.6	45.1	126	38.8

[a] Although errors in $\Delta H°$ and $\Delta S°$ are ±5%, additional significant figures are given to allow accurate calculation of $\Delta G°$ vs. temperature. For oligomers with T_m less than 25 °C (at 10^{-4} M) error limits on $\Delta H°$ and $\Delta S°$ are ±10%. [b] The methods used to obtain $\Delta H°$ and $\Delta S°$ are described under Materials and Methods and by Petersheim & Turner (1983a). [c] Calculated for 1×10^{-4} M strands.

nucleoside phosphates (Brahms et al., 1967b) have led to the conclusion that C stacks more than U (Bloomfield et al., 1974). Apparently, this generalization is not valid for 3′ dangling ends on GGCC and CCGG. Broido & Kearns (1982) suggest single-stranded poly(C) forms a left-handed helix. If single-stranded cytosines prefer an unusual geometry, the double helix may prevent a 3′-dangling C from assuming this geometry, thus preventing helix stabilization.

Temperature-Dependent Thermodynamic Parameters. Although the two-state model fits the high-salt data well, sloping base lines above and below the transition region indicate temperature-dependent equilibria other than the helix to coil transition. Single-strand unstacking is almost certainly involved and is expected to give a temperature-dependent final state resulting in a temperature-dependent $\Delta H°$. When the enthalpy and entropy changes obtained from fits are plotted vs. T_m or ln (T_m), respectively, an increase in $\Delta H°$ or $\Delta S°$ with T_m is observed. The slopes of these plots yield the heat capacity difference, $\Delta C_p°$, between products and reactants:

$$\Delta H°(T_m) = \Delta H°(T_0) + \Delta C_p°(T_m - T_0) \quad (4)$$

$$\Delta S°(T_m) = \Delta S°(T_0) + \Delta C_p° \ln (T_m/T_0) \quad (5)$$

Slopes from eq 4 and 5 agree within a few percent, and the heat capacity changes are listed in Table III. The plots of eq 4 and 5 have significant scatter. This is particularly true for oligomers with low T_m's where the slopes of the lower base lines are not well determined leading to uncertainty in the fitted parameters. This results in a precision of only ±30% for the slopes in Table III.

The heat capacity changes measured calorimetrically for the double helix transition of poly(A)·poly(U) range from −57 to −136 cal K^{-1} (mol of base pairs)$^{-1}$ (Filimonov & Privalov, 1978; Rawitscher et al., 1963; Suurkuusk et al., 1977). The heat capacities reported in Table III are of the same order. However, the $\Delta C_p°$ for GGCC is significantly larger than for the other molecules. The value was reproducible within 10%, and neither pGGCC or GGCCp exhibits as large a $\Delta C_p°$. One possible rationale is that GGCC aggregates at 1 M Na^+, and the terminal phosphates prevent this aggregation.

For comparison purposes, the heat capacities listed in Table III can be used to extrapolate the thermodynamic properties of all the oligomers to a common temperature. This must be done with caution since the temperature-dependent enthalpy and entropy changes are most precise near the T_m of each oligonucleotide. In Tables IV and V, the extrapolated thermodynamic parameters in 0.01 and 1 M Na^+ are listed at 25 and 50 °C, respectively, which requires the least extrapolation, and at 37 °C, a more physiologically relevant temperature.

Table III: Heat Capacity Changes[a] upon Helix Formation

oligonucleotide	$-\Delta C_p°$ (1 M Na^+)[b]	$-\Delta C_p°$ (0.01 M Na^+)[c]
GGCC	700	330
pGGCC	260	330
GGCCp	400	550
GGCCC	290	
GGCCCp	360	160
GGCCUp	210	100
GGCCGp	380	280
GGCCAp	260	190
GGCCεAp	90	340
CCGG[d]	380	
pCCGG	400	
CCGGCp	300	
CCGGUp[d]	360	
CCGGGp	200	
CCGGAp[d]	260	

[a] Average $\Delta C_p°$ obtained from eq 4 and 5. Estimated errors are ±30%. $-\Delta C_p°$ units: cal (mol of duplex)$^{-1}$ K^{-1}. [b] 1 M NaCl, 0.01 M sodium cacodylate, and 0.001 M EDTA, pH 7. [c] 0.01 M sodium cacodylate and 0.001 M EDTA, pH 7. [d] From Petersheim & Turner (1983a).

Ionic Strength Dependence of Helix Stability. The ionic strength dependence of the thermodynamic parameters of GGCCUp helix formation is listed in Table VI. Since the melting temperatures depend on ionic strength, it is probably best to compare the data at a common temperature. By use of the temperature-dependent values at 37 °C listed in Table VI, $\Delta H°$ is independent of [Na^+], and the salt dependence of T_m is an entropic effect. Comparison of the temperature-dependent enthalpies at 0.01 and 1 M Na^+ (Tables IV and V) results in the same conclusion for the other pentamers in the GGCC family. The independence of $\Delta H°$ on ionic strength has been theoretically predicted and experimentally observed (Manning, 1978; Record et al., 1978; Breslauer et al., 1975).

The uptake of counterions upon helix formation can be obtained from a plot of $\Delta G°$ vs. ln [Na^+] (Record et al., 1981):

$$\frac{d\Delta G°}{d \ln [Na^+]} = 2RT(\psi_c - \psi_h) \quad (6)$$

where $\Delta G°$ is the free energy (per helix) of helix formation and ψ_c and ψ_h are the number of counterions thermodynamically bound (per strand) to the coil and helix, respectively. The theory used to derive eq 6 is most appropriate for 10^{-4} M $\leq$ [Na^+] $\leq$ 0.1 M (Manning, 1978; Record et al., 1978). However, the plot of $\Delta G°$ (37 °C) vs. ln [Na^+] for GGCCUp is nearly linear from 0.01 to 1 M Na^+ (Figure 3). This suggests the thermodynamic data at 0.01 and 1 M Na^+ can be used to obtain a reasonable estimate of $\psi_c - \psi_h$ for each

Table IV: Temperature-Dependent Thermodynamic Properties[a] of Helix Formation in 1 M NaCl, 0.01 M Sodium Cacodylate, and 0.001 M EDTA, pH 7

oligonucleotide	$-\Delta H^\circ$ (50 °C) (kcal/mol)	$-\Delta S^\circ$ (50 °C) (eu)	$-\Delta G^\circ$ (50 °C) (kcal/mol)	$-\Delta H^\circ$ (37 °C) (kcal/mol)	$-\Delta S^\circ$ (37 °C) (eu)	$-\Delta G^\circ$ (37 °C) (kcal/mol)
GGCC	47.8	136	3.9	38.6	107	5.4
pGGCC	42.3	117	4.5	38.9	106	5.9
GGCCp	42.8	122	3.5	37.7	105	5.2
GGCCC	42.5	114	5.6	38.8	102	7.2
GGCCCp	41.8	112	5.6	37.2	97	7.0
GGCCUp	46.3	124	6.2	43.6	115	7.8
GGCCGp	48.3	128	7.1	43.5	112	8.8
GGCCAp	48.9	129	7.1	45.5	118	8.8
GGCCϵAp	49.4	131	7.1	48.3	127	8.8
CCGG[b]	43.4	125	3.1	38.3	109	4.5
pCCGG	44.1	125	3.8	38.8	108	5.2
CCGGCp	45.4	129	3.8	41.5	117	5.3
CCGGUp[b]	47.8	135	4.3	43.1	120	5.8
CCGGGp	50.1	139	5.4	47.5	131	7.1
CCGGAp[b]	48.8	135	5.1	45.4	124	6.8

[a] Obtained from plots of ΔH° vs. T_m and ΔS° vs. $\ln(T_m)$. ΔH° and ΔS° are obtained by fitting each melting curve to a two-state model with sloping base lines. See Petersheim & Turner (1983a) for details. [b] From Petersheim & Turner (1983a).

Table V: Temperature-Dependent Thermodynamic Parameters for Helix Formation in 0.01 M Sodium Cacodylate and 0.001 M EDTA, pH 7

oligonucleotide	$-\Delta H^\circ$ (25 °C) (kcal/mol)	$-\Delta S^\circ$ (25 °C) (eu)	$-\Delta G^\circ$ (25 °C) (kcal/mol)	$-\Delta H^\circ$ (37 °C) (kcal/mol)	$-\Delta S^\circ$ (37 °C) (eu)	$-\Delta G^\circ$ (37 °C) (kcal/mol)
GGCC	33.2	93	5.4	37.1	106	4.2
pGGCC	32.2	92	4.7	36.1	105	3.5
GGCCp	36.6	106	5.0	43.3	127	3.7
GGCCCp	39.2	111	6.2	41.4	117	5.2
GGCCUp	43.2	123	6.6	44.8	126	5.7
GGCCGp	42.1	117	7.2	45.5	128	5.9
GGCCAp	45.9	130	7.3	48.5	136	6.3
GGCCϵAp	42.4	118	7.3	46.5	131	5.9

Table VI: Salt Dependence of the Thermodynamic Properties of Helix Formation for GGCCUp

	temperature independent[a]			temperature dependent[a]			
$[Na^+]$[b] (M)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (cu)	T_m (°C) (1×10^{-4} M)	$-\Delta H^\circ$ (37 °C) (kcal/mol)	$-\Delta S^\circ$ (37 °C) (eu)	$-\Delta G^\circ$ (37 °C) (kcal/mol)	$-\Delta C_p^\circ$ (cal deg^{-1} mol^{-1})
0.01	38.3	105	37.2	44.8	126	5.7	100
0.1	46.2	126	46.0	44.8	122	6.9	180
1	48.3	130	51.5	43.6	115	7.8	210

[a] See text for explanation of methods used to determine thermodynamic properties. [b] All solutions contained 0.01 M sodium cacodylate and 0.001 M EDTA, pH 7.

oligomer. The slight curvature in Figure 3 suggests the calculated ($\psi_c - \psi_h$) may be 20% lower than the value apropriate at $[Na^+] \leq 0.1$ M. Equation 6 and the data in Tables IV and V were used to calculate $\Delta\psi$. For the oligomers in Table V, $\Delta\psi$ was calculated to be between 0.06 and 0.11 ion released per phosphate. These values are less than the 0.16–0.17 ion per phosphate observed for poly(A)·poly(U) denaturation (Record et al., 1978; Record & Lohman, 1978). For DNA, $\Delta\psi$ decreases with increasing fraction of GC pairs (Frank-Kamenetskii, 1971; Blake & Haydock, 1979). If this is also true for RNA, the value of $\Delta\psi$ observed for these GC-rich oligomers would be expected to be less than the value for poly(A)·poly(U). In addition, Record & Lohman (1978) predict $\Delta\psi$ should decrease with chain length. This could also explain the relatively small number of ions released per phosphate upon denaturation of these very small helices.

Concentration Dependence of Molecular Weight. Nelson et al. (1981) suggest that aggregation of helices may affect thermodynamic measurements and that a concentration-dependent hypochromicity may indicate aggregation. The melting curves measured in this study do exhibit a concentration-dependent hypochromicity. Therefore, sedimentation

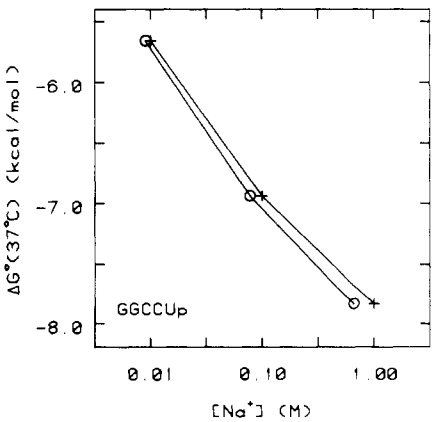

FIGURE 3: Free energy of helix formation vs. ln $[Na^+]$ for GGCCUp in 0.01 M sodium cacodylate and 0.001 M EDTA. (+) No activity corrections; (○) the Na^+ concentrations were adjusted for activity.

experiments were conducted to check for aggregation.

A plot of Z/r vs. $\int_{r_1}^{r} dr'/Z$ for GGCCGp in 1 M NaCl, 0.01 M sodium cacodylate, and 0.001 M EDTA [see supplementary material (see paragraph at end of paper regarding supple-

Table VII: Excess Stabilization Added by a 3′ Dangling End in 1 M NaCl

3′-terminal base	$-\Delta\Delta G^\circ$ (37 °C)[a] (kcal/mol)		$\Delta\Delta G^\circ$ (25 °C) (dangling end)[a]/$\Delta\Delta G^\circ$ (25 °C) (base pair)[b]	
	CCGG	GGCCp	CCGG	GGCCp
Cp	0.4	0.9	0.11	0.20
Up	0.6	1.3	0.35 (0.33)[c]	0.68
Gp	1.3	1.8	0.31	0.68
Ap	1.1	1.9	0.64	0.97
ϵAp		1.8		

[a] $\Delta\Delta G^\circ$ is half the difference between the observed ΔG° for the pentamer and that for the tetramer core. The data in Table I were used to calculate $\Delta\Delta G^\circ$. [b] From the parameters of Borer et al. (1974). [c] The value in parentheses was experimentally measured by Petersheim & Turner (1983a).

mentary material)] is linear, indicating apparent molecular weight is independent of concentration between 5×10^{-5} and 2×10^{-3} M oligonucleotide. Assuming $\bar{v}$ is 0.563 mL/g, the value for DNA in 1 M NaCl (Cohen & Eisenberg, 1968) and by use of 1.05 g/mL for ρ, the plot yields $M_Z = 4.1 \times 10^3$ g/mol. This is in reasonable agreement with the calculated molecular weight of 3.5×10^3 for a GGCCGp helix with one bound Na^+ per phosphate. Similar results were obtained from a plot of log (Z/r) vs. r^2. Sedimentation equilibrium experiments on GGCCAp at 0.01 M Na^+ yielded a concentration-independent molecular weight of 2.4×10^3 between 2×10^{-4} and 3.7×10^{-3} M. The discrepancies between measured and calculated molecular weights may be due to inappropriate choices of $\bar{v}$ or to different extents of solvation and ion binding for the two helices. There is no evidence in either case of extensive aggregation such as that reported for $CA_7G \cdot CU_7G$ where the average molecular weight at 10^{-3} M strands is 8 times the helix molecular weight (Nelson et al., 1981).

Discussion

Dangling ends greatly increase the stability of RNA duplexes (Martin et al., 1971; Romaniuk et al., 1978; Neilson et al., 1980; Alkema et al., 1981a,b; Petersheim & Turner, 1983a). As shown in Table I, addition of a 3′-dangling end to GGCC or CCGG increases the T_m by 7–24 °C for a 10^{-4} M solution. The free energy increment for stacking a single 3′ base on a CCGG or GGCC helix is half the difference between the free energies of helix formation for a pentamer and the corresponding tetramer core. These $\Delta\Delta G^\circ$'s are listed in Table VII. For both CCGG and GGCC, the stabilization is sequence dependent with the following order of increasing stability: C < U < G, A. A given 3′ dangling end, X, is more stabilizing when added to GGCC rather than CCGG.

Comparison of 3′ Dangling Ends to Terminal Base Pairs. The thermodynamic properties of 3′ dangling ends provide insight into the forces determining RNA structure. Presumably, the large stabilization from dangling ends does not involve hydrogen bonding and thus provides evidence for the importance of stacking interactions. It is interesting to compare the stabilization caused by stacking of a 3′ dangling end to that caused by the corresponding full base pair. Neglecting the effect of 3′-terminal phosphates at 1 M NaCl, the free energy increment from adding terminal base pairs to GGCCp or CCGG can be estimated from the nearest-neighbor free energies of Borer et al. (1974). Ratios of measured stabilizing free energies of 3′ dangling ends to predicted free energies of the corresponding full base pairs are listed in Table VII. The fractional stabilization of the 3′ dangling end depends on sequence, but certain generalizations can be made. A 3′-dangling G or U contributes 30–70% as much free energy as the corresponding full base pair, a 3′-dangling C contributes only 10–20%, and a 3′-dangling A contributes 65–95%. Any stacking free energy from the 5′-terminal base will increase the stacking contribution to the stability of the base pair. It should also be noted that our definition of stacking free energy underestimates pure stacking by including the unfavorable entropy change associated with restriction of backbone bonds (Dewey & Turner, 1979). Thus, stacking must provide a major source of base pair stability in RNA.

Origins of Stacking. The above results also provide insight into the origins of stacking interactions. In this regard, two observations are noteworthy. First, the interactions are sequence dependent. For example, a 3′ Up stabilizes GGCC and CCGG by 1.2 and 0.6 kcal/mol, respectively, at 37 °C. In all cases measured, a 3′ dangling end, X, is more stabilizing in the sequence CX than GX. Second, the stabilization is associated with a more favorable ΔH° and less favorable ΔS° of helix formation (see Tables I–V). The only exceptions are the temperature-dependent thermodynamic parameters for GGCCC and GGCCCp. These exceptions may be due to experimental uncertainty, to the increased number of base pairing possibilities available for this sequence, or to unusual properties for repeating C's suggested by the work of Broido & Kearns (1982).

The above observations are consistent with base–base interactions being the source of stacking free energy. Calculations of such interactions predict a favorable ΔH° for stacking and a sequence dependence (Pullman & Pullman, 1969; 1968; Ornstein et al., 1978; Kollman et al., 1981). Presumably, the stabilization of double helices by 3′ dangling bases results from interactions of the 3′ dangling base with the 5′-terminal base of the opposite strand. In geometries proposed for RNA helices (Arnott, 1972; Cruz et al., 1982; Seeman et al., 1976; Rosenberg et al., 1976), there is more overlap between these two bases when the 5′-terminal base is a guanine rather than a cytosine. This may be the reason for the enhanced 3′ stabilization in CX relative to GX sequences. Of course, detailed calculations are required to confirm this speculation.

Another possible source of stacking free energy is base–solvent interactions, either as hydrophobic (Kauzmann, 1959; Tanford, 1973) or solvophobic bonding (Sinanoglu & Abdulnur, 1964, 1965; Sinanoglu, 1968, 1980, 1982). The ΔG° due to both of these effects is expected to depend on the change in solvent exposed surface area on stacking (Janin & Chothia, 1976; Chothia, 1974; Alden & Kim, 1979; Sinanoglu, 1968, 1980, 1982). NMR results on CCGGUp and CCGGAp indicate 3′ dangling ends are fully stacked in the double helix and are consistent with the 3′ dangling end continuing the RNA geometry (Petersheim & Turner, 1983b,c). For proposed RNA helix geometries (Arnott, 1972; Cruz et al., 1982; Seeman et al., 1976; Rosenberg et al., 1976), the exposed surface area of a 3′ dangling end is independent of sequence. Thus, hydrophobic or solvophobic bonding can account for the observed CX vs. GX sequence dependence only if the exposed surface areas of the 3′ bases in the single strands are sequence dependent. The fractions of these 3′ bases unstacked in the single strands at 37 °C can be estimated from the fraction of unstacking in dinucleoside monophosphates at 37 °C (Davis & Tinoco, 1968): CC (0.80), GC (0.81); CU (0.81), GU (0.82); CA (0.85), GA (0.73). Thus, most of the bases in the single strands are fully exposed to solvent at 37 °C. Therefore, it appears base–solvent interactions cannot account for the sequence dependence of 3′ dangling end stability and are

unlikely to be the dominant driving force for stacking. Alden & Kim (1979) reached a similar conclusion based on the sequence dependence of the stability of short RNA duplexes (Borer et al., 1974).

The $\Delta H°$ and $\Delta S°$ measured for 3′ dangling end stacking provide additional evidence that hydrophobic bonding is not the dominant driving force. Hydrophobic bonding is traditionally associated with an unfavorable $\Delta H°$ and favorable $\Delta S°$ of complex formation (Kauzmann, 1959; Tanford, 1973). For example, for the dimerization of benzene in water, $\Delta H°$ = 4 kcal/mol and $\Delta S°$ = 20 cal/(K·mol) (Tucker et al., 1981). The results in Tables I–V indicate 3′ dangling end stabilization of double helices is associated with a less favorable $\Delta S°$ and a more favorable $\Delta H°$ of helix formation. Thus, it appears dangling end stabilization is not primarily due to hydrophobic bonding.

Charge Effects. Electrostatic effects on nucleic acid stability are generally well understood (Manning, 1978; Record et al., 1978). Nevertheless, there are two surprising results in this study. First, addition of a 5′-phosphate to either GGCC or CCGG increases the T_m by 4–6°C at 1 M NaCl, in spite of the increased charge repulsion. Addition of a 3′-phosphate to GGCC, GGCCC, or CCGG lowers the T_m by 1.5, 0.6, and 4 °C, respectively [see Table I and Petersheim & Turner (1983a)]. In 0.01 M NaCl, the low T_m's of pGGCC and GGCCp are difficult to measure. However, they are both roughly 16 °C which is clearly less than the 25 °C T_m of GGCC (see Table II). Thus, only the effect of a 5′-terminal phosphate at 1 M NaCl is anomalous. The reason for the observed stabilization is unknown. However, it may be related to the observations that 5′ dangling ends stabilize less than 3′ dangling ends and also alter the temperature dependence of sugar conformations in the core helix (Alkema et al., 1981a,b; Petersheim & Turner, 1983a,c).

A second surprising result with possible electrostatic origins is that the thermodynamic data at 0.01 M NaCl are dependent on method of analysis whereas those at 1 M NaCl are not. In particular, $\Delta H°$ and $\Delta S°$ obtained from fitting curves are more negative than those from plots of eq 3 (see Table II). Discrepancies of this kind have been observed previously and attributed to non-two-state behavior (Freier et al., 1983; D. Hickey and D. H. Turner, unpublished experiments). In those previous cases, the fitted $\Delta H°$ was less negative than the values from eq 3. In addition, enthalpies obtained from transition widths or slopes at T_m are often less favorable than those from log c_T plots or differential scanning calorimetry (Nelson et al., 1981; Breslauer et al., 1975; Patel et al., 1982a,b; Martin et al., 1971). Again, this observation has been attributed to a non-two-state transition. In Table II, transition widths (fitted $\Delta H°$) are sharper than predicted from log c_T plots. Marky et al. (1983) have seen a similar effect for d-(CGCGAATTCGCG) at 0.01 M Na^+ where hairpin loops can form. Thus, this trend probably also indicates non-two-state behavior. The results suggest these molecules have some conformational diversity that is salt dependent.

Temperature Dependence of $\Delta H°$ and $\Delta S°$. In 1 M NaCl, the thermodynamic parameters obtained from plots of $1/T_m$ vs. log c_T agree with the average of those obtained from fits. This suggests the two-state model is appropriate. However, $\Delta H°$ and $\Delta S°$ values obtained from fitting melting curves are temperature dependent, suggesting the two-state model is an approximation. This raises the question of whether the two-state model is reasonable.

Use of eq 3 assumes $\Delta H°$ and $\Delta S°$ are independent of temperature over the range of T_m studied, about 20 °C. The two-state model used for the fits assumes $\Delta H°$ and $\Delta S°$ do not change over the range of temperatures where there are significant populations of helix and coil. Typically, this range is 35 °C for 5–95% of the reaction. If ΔC_p is 300 cal/(K·mol·duplex), $\Delta H°$ and $\Delta S°$ vary by ±5 kcal/(mol·duplex) and ±15 cal/(K·mol·duplex), respectively, over a 35 °C range. These variations are roughly ±10% of the total $\Delta H°$ and $\Delta S°$. Since fits of melting curves are excellent, this temperature dependence is apparently small enough that the two-state approximation is reasonable.

While the two-state model with constant $\Delta H°$ appears reasonable for analyzing individual melting curves, the dependence of $\Delta H°$ and $\Delta S°$ on T_m suggests the heat capacity change on helix dissociation is not zero. The average $\Delta C_p°$ from Table III is about −70 cal/(K·mol·base pair). This is in reasonable agreement with calorimetrically measured values for poly(A)·poly(U) and DNA which range from −57 to −136 cal/(K·mol·base pair) (Shiao & Sturtevant, 1973; Suurkuusk et al., 1977; Filimonov & Privalov, 1978; Rawitscher et al., 1963). This provides further support that the approximations inherent in the analysis are reasonable.

Improvement of Parameters for Prediction of RNA Secondary Structure. Although dangling ends greatly increase the stability of RNA helices, this stabilization is currently ignored by methods for predicting secondary structure from sequence (Tinoco et al., 1971, 1973; Pipas & McMahon, 1975; Salser, 1977; Zuker & Stiegler, 1981; Nussinov & Tinoco, 1981; Nussinov et al., 1982; Auron et al., 1982). The thermodynamic parameters reported in Table VII provide single measurements of the eight nearest-neighbor parameters necessary for including the free energy contribution of 3′ dangling ends adjacent to GC base pairs.

Algorithms for prediction of RNA structure generally assume that only nearest-neighbor interactions are important. Our results on the GGCC and CCGG series are consistent with this assumption. However, the melting temperatures of 5 mM GCA, GCC, and GCU determined by NMR are 33, 20, and <10 °C, respectively (Alkema et al., 1981b; T. Neilson, unpublished observations). The nearest-neighbor increments from Table VII predict the opposite order of stability for GCC and GCU, indicating additional factors may affect stability. Thus, further experiments on additional sequences are necessary to determine the general applicability of nearest-neighbor free energy increments.

Most RNA duplexes in vivo terminate with base mismatches. If the nearest-neighbor approximation is valid and each mismatch is independent, then a total of 48 parameters will be necessary to include them in RNA structure prediction. However, it is possible the stabilization of each base in a mismatch is roughly independent of the opposing base. If this is true, then complete inclusion of mismatches will require additional measurement of 8 parameters for 3′ stacking on AU base pairs and 16 parameters for 5′ stacking [one of which has been measured by Petersheim & Turner (1983a)].

The above results provide thermodynamic parameters for the stabilization of double helices by 3′ dangling ends adjacent to GC base pairs. The parameters suggest that the additional stabilization is due to base–base interactions and that these provide a major contribution to base pair stability. The results also indicate the effects of dangling ends, salt concentration, and temperature on the thermodynamic properties should be considered when predictions of RNA structure from sequence are made.

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Supplementary Material Available

Plot of $(1/r)\,dc/dr$ vs. concentration for GGCCGp in high salt buffer (1 page). Ordering information is given on any current masthead page.

Registry No. GGCC, 56399-78-1; pGGCC, 87640-15-1; GGCCp, 87640-16-2; GGCCC, 87640-17-3; GGCCCp, 87640-18-4; GGCCUp, 87640-19-5; GGCCGp, 87640-20-8; GGCCAp, 87640-21-9; GGCCϵAp, 87655-18-3; pCCGG, 76873-96-6; CCGGCp, 87640-22-0; CCGGGp, 87640-23-1.

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Purification of Avian Vitellogenin III: Comparison with Vitellogenins I and II†

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ABSTRACT: Vitellogenin is an egg yolk precursor protein synthesized by livers of oviparous vertebrates in response to estrogenic stimulation. Previous studies have shown that chicken vitellogenin consists of two major species that differ in amino acid composition, peptide maps, and immunological properties. A third vitellogenin (VTG III) has now been isolated and characterized. VTG III differs from VTG I and VTG II in 11 amino acids. VTG III is also a phosphoprotein but contains only 44 mol of P in comparison to 116 mol of P for VTG I or VTG II. Partial proteolysis mapping shows major differences among VTG I, VTG II, and VTG III. Immunoblot analysis shows no reactivity between anti-VTG I and either VTG II or VTG III, no reactivity between anti-VTG II and either VTG I or VTG III, and no reactivity between anti-VTG III and either VTG I or VTG II. Radioimmunoassay also shows no significant reactivity between anti-VTG III and either VTG I or VTG II. We conclude that VTG III is a distinct vitellogenin most likely encoded by a third vitellogenin gene. Immunological analysis of pulse-labeled hepatocytes shows a newly synthesized intracellular form of VTG III, pVTG III. As is the case with the precursors to VTG I and VTG II, pVTG III has a greater electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels than the respective fully phosphorylated plasma vitellogenin. Dephosphorylation of plasma VTG III increases its mobility to an apparent molecular weight of 180 000, which corresponds to the mobility of hepatocyte pVTG III. Thus, each vitellogenin has an immunologically distinct nonphosphorylated hepatocyte precursor.

Vitellogenin is an egg yolk precursor protein synthesized by livers of oviparous vertebrates in response to estrogenic stimulation (Bergink et al., 1974). After secretion from the liver and uptake into the developing oocyte, vitellogenin is cleaved into a family of lipovitellin polypeptides and at least two heavily phosphorylated phosvitins. These proteins function as transport mechanisms for the movement of lipid, phosphorus, and metals to the yolk and serve directly as nutrient sources in embryonic development. In addition to its important developmental role, vitellogenin has received considerable attention as a model for steroid-regulated gene expression (Bergink et al., 1974; Ryffel,

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