Synthesis and Properties of CpG Analogues Containing an 8-Bromoguanosine Residue. Evidence for Z-RNA Duplex Formation†

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ABSTRACT: Three dinucleoside monophosphates containing 8-bromoguanosine (br^8G), (2′–5′)C-br^8G, (3′–5′)C-br^8G, and dC-br^8G, were synthesized and characterized by UV absorption, CD, and ^1H NMR spectroscopy. The ^1H NMR data show that all the br^8G residues in these dimers take a syn glycosidic conformation. At low dimer strand concentration (5×10^{-5} M), the UV hypochromicity data suggest that the degree of base stacking decreases in the following order, (2′–5′)C-br^8G > C-G ≈ dC-br^8G > (3′–5′)C-br^8G. The CD data also suggest little stacking in (3′–5′)C-br^8G. At high dimer strand concentration (5×10^{-3} M), only (3′–5′)C-br^8G shows duplex formation in 0.1 M NaCl. The duplex is assumed to take a left-handed helical structure similar to that of Z-DNA. The T_m of this duplex is surprisingly high for a dimer (about 35 and 45 °C at 5×10^{-3} and 10^{-2} M dimer strand concentration, respectively). The above results and the similarity between the CD spectra of (3′–5′)C-br^8G and poly(G-C) suggest the possible existence of Z-form structure in ribooligo- and ribopolynucleotides with alternating purine–pyrimidine sequences.

Recently a left-handed double helical form of DNA has been found in crystals of the oligomers d(C-G-C-G-C-G) and d(C-G-C-G) (Wang et al., 1979, 1981; Drew et al., 1980; Crawford et al., 1980) and in fibers of polymers containing an alternating pyrimidine–purine base sequence (Arnott et al., 1980). In these duplexes, each deoxyguanosine residue takes a syn glycosidic conformation compared with an anti conformation in right-handed duplexes. It has been postulated that this unusual DNA structure, the so-called Z-form, might play some biological role in regulation of DNA functions. We have studied the solution conformations of d(C-G), d(C-G-C-G), and d(C-G-C-G-C-G) by UV, CD, and ^1H NMR[1] methods and reach the following conclusions (Uesugi et al., 1981). At relatively high oligomer concentration (above 3×10^{-4} M strand concentration) and high salt concentration (4 M NaCl), where the right-handed duplex structure is destabilized, the tetramer and hexamer can form a left-handed duplex. At low salt concentration (0.1 M NaCl), the tetramer and hexamer form a right-handed duplex, though crystals of the left-handed duplex, but not the right-handed duplex, can be obtained under similar conditions (Wang et al., 1979, 1981; Crawford et al., 1980). The dimer does not display any duplex formation under the conditions studied. From these results, it was assumed that some factor which destabilizes the B-form structure and/or flips the glycosidic conformation of the deoxyguanosine residues from the anti to syn form may be needed for an alternating C-G sequence of DNA to adopt the Z-form structure under physiological conditions.

We have synthesized some C-G analogues containing 8-bromoguanosine which has a tendency to take a syn conformation (Tavale & Sobell, 1970; Ikehara et al., 1972a,b) and examined the conformational properties of the dinucleoside monophosphates by UV, CD, and ^1H NMR spectroscopy. The results suggest that (3′–5′)C-br^8G can form a left-handed duplex.

Materials and Methods

General Procedures. Paper chromatography was performed by descending technique on Whatman No. 1 paper using the following solvent systems: solvent A, 2-propanol–concentrated NH_4OH–water (7:1:2); solvent B, ethanol–1 M ammonium acetate (pH 7) (7:3); solvent C, 1-butanol–acetic acid–water (5:2:3); solvent D, 1-propanol–concentrated NH_4OH–water (55:10:35). Paper electrophoresis was performed for 1 h with a voltage gradient of 35 V/cm on Toyo filter paper no. 51A using 0.05 M triethylammonium bicarbonate ($Et_3NH_2CO_3$) buffer (pH 7.5).

UV absorption spectra were recorded on a Hitachi 200-10 spectrophotometer. For temperature variation experiments, a Komatsu Solidate SPD-H-124 thermostated cell was used. The temperature within the cell was measured with a Shibaura MGB-III thermistor. CD spectra were recorded on a Jasco ORD/UV-5 spectropolarimeter equipped with a CD attachment. For temperature variation experiments, a thermojacketed cell and a Neslab RTE-8 circulating bath were used. The temperature of the cell was measured with the same thermistor. For measurement of high dimer concentration samples, cells of 0.1- and 0.05-mm light path length were used. The molar absorption coefficient, ϵ, and the molar ellipticity, $[\theta]$, are presented in terms of per base residue values. Phosphorus analysis was carried out by a combination of the methods of Baginski et al. (1967) for ashing and Chen et al. (1956) for color development.

Snake venom phosphodiesterase was purchased from Boehringer-Mannheim Co., and incubation was carried out in 0.1 M $Et_3NH_2CO_3$ (pH 7.5) at 37 °C for 3 h with the enzyme (25 μg/mL). Alkaline hydrolysis was performed in 0.3 N KOH at 37 °C for 18 h. In each digestion experiment, about 5 A_{max} units of the dimer was used. 8-Bromoguanosine (Long et al., 1968), its 5′-phosphate (Ikehara et al., 1969), and N^4-benzoyl-5′-*O*-(dimethoxytrityl)deoxycytidine (**5**) (Schaller et al., 1963) were synthesized according to published procedures.

N^4,5′-O-Diacetylcytidine 2′,3′-Phosphate (**1**). To a suspension of cytidine 2′(3′)-phosphate (free acid monohydrate, 1 mmol) in pyridine (15 mL) were added acetic anhydride (3 mL) and tri-*n*-butylamine (0.48 mL, 2 mmol), and the mixture

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[1] Abbreviations: br^8G, 8-bromoguanosine; UV, ultraviolet; CD, circular dichroism; NMR, nuclear magnetic resonance; Ac, acetyl; Bz, benzoyl; Ib, isobutyryl; $(MeO)_2Tr$, dimethoxytrityl; DMF, dimethylformamide; PC, paper chromatography; PEP, paper electrophoresis; T_m, melting temperature; other abbreviations principally follow the recommendation of the IUPAC–IUB Commission on Biological Nomenclature (1970, 1977).

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was heated at 85 °C for 2 h with stirring. After addition of triethylamine (0.07 mL, 0.5 mmol), the mixture was further heated at 80 °C for 1 h. Disappearance of the starting material was monitored by paper electrophoresis. Methanol (6 mL) was added, and the mixture was kept at room temperature for 4.5 h. The solvent was evaporated with dimethylformamide (DMF) (2 mL × 6) at 40–50 °C in vacuo. The product isolated by paper chromatography in solvent B showed $\lambda_{max}^{H_2O}$ 247, 297 nm; PC R_f(B) 0.71 (Cp 0.28, C 0.65); PEP R_m-(Cp-C) 0.53. The gum was finally dissolved in DMF (5 mL), and 4 mL of this solution was used in the following reaction.

(2′–5′)- and (3′–5′)C-br^{8}G (**3** *and* **4**). **1** in DMF (4 mL) was cooled in an ice bath, and triisopropylbenzenesulfonyl chloride (727 mg, 2.4 mmol) was added with stirring. After about 15 min at 0 °C, the solid had mostly dissolved. To this solution was added 8-bromoguanosine (579 mg, 1.6 mmol). The mixture was stirred at 0 °C for 3 h to give a clear solution and kept at 0 °C for a further 5 h. Triethylamine (2 mL)–water (5 mL) was added with cooling and the resulting solution kept at room temperature for 1.5 h and then at 0 °C for 3 h. The solution was diluted with water (40 mL) and washed with ether (25 mL × 3). The aqueous phase, to which 1-butanol (5 mL) was added to prevent foaming, was evaporated. The residue was treated with methanolic ammonia (70 mL) for 24 h at room temperature. The solvent was removed with an aspirator. To the residue, water (100 mL) and $CHCl_3$ (20 mL) were added and shaken. The aqueous layer was separated and washed with $CHCl_3$ (20 mL × 2). The combined organic fractions were back-extracted with water (15 mL). These combined aqueous fractions were evaporated to a small volume and neutralized with 2 N NH_4OH. Solid materials were removed by filtration. The filtrate was diluted with water to 400 mL and chromatographed on a column (2.6 × 20 cm) of DEAE-cellulose (DE23, HCO_3^- form). The column was washed with 0.005 M $Et_3NH_2CO_3$ (360 mL) and eluted with 0.005–0.10 M $Et_3NH_2CO_3$ (total 5 L). Fractions of 20 mL were collected at a flow rate of 200 mL/h. The second (tubes 81–100) and third (tubes 101–121) peaks (eluted at 0.024 and 0.034 M salt concentrations, respectively) contained dimers **3** and **4**. Pooled fractions from each peak were evaporated and $Et_3NH_2CO_3$ was removed by repeated evaporation with water.

3. The residue from the second peak was dissolved in water (50 mL) and chromatographed on a column (1.0 × 39.5 cm) of Dowex 1-X2 resin ($HCOO^-$ form). The column was washed with water and eluted with 0.03–0.18 M HCOOH (total 6 L) at a flow rate of 108 mL/h. **3** was eluted at around 0.056 M HCOOH concentration. The combined fractions were passed through a column of chromatography charcoal (5 mL). The column was washed thoroughly with water and eluted with ethanol–water–concentrated NH_4OH (50:48:2). The fraction containing **3** was evaporated and dissolved in water. The solution was applied to a small column (1.5 × 7 cm) of DEAE-cellulose (HCO_3^- form). After the column was washed with water, elution was carried out with 1 M $Et_3NH_2CO_3$. The fraction containing **3** was evaporated and desalted by repeated evaporation with water. **3** was isolated as a powder by lyophilization. The yield was 4323 A_{261} units (0.22 mmol, 27%). The chromatographic properties and UV spectral data are presented in Tables I and II. This compound was completely hydrolyzed with snake venom phosphodiesterase to give C and pbr^{8}G in a ratio of 1:1. It was also hydrolyzed with 0.3 N KOH but resistant to RNase A.

4. The residue from the third peak was dissolved in water (100 mL) and chromatographed on a column (1.0 × 55 cm) of Dowex 1-X2 ($HCOO^-$ form) resin. The column was washed with water and eluted with 0.02–0.12 M HCOOH (total 5 L) and 0.12–0.22 M HCOOH (total 2 L). **4** was eluted at around 0.12 M HCOOH concentration. The combined fractions containing **4** were passed through a charcoal column and then through a small DEAE-cellulose column as described above. Lyophilization gave **4** as a powder (2385 A_{265} units, 0.092 mmol, 12%). The chromatographic properties and UV spectral data are presented in Tables I and II. This compound was completely hydrolyzed with RNase A to give Cp and br^{8}G in a ratio of 1:1. It was also completely hydrolyzed with snake venom phosphodiesterase and with 0.3 N KOH.

Table I: Chromatographic Properties of the Dimers

compound	PC[a] R_f in solvent A	B	C	D	PEP[a] R_m-(dCp-dC)[b]
(2′–5′)C-br^{8}G	0.25	0.35	0.17	0.38	0.49
(3′–5′)C-br^{8}G	0.21	0.29	0.16	0.37	0.43
dC-br^{8}G	0.23	0.32	0.16	0.41	0.39
C-G	0.20	0.27	0.10	0.38	0.47
dC	0.65	0.72	0.42	0.71	0.00
dCp	0.18	0.26	0.20	0.40	1.00
A	0.60	0.58	0.42	0.67	−0.10
pA	0.16	0.18	0.20	0.36	0.89

[a] Compositions of the solvent and buffer systems are given under Materials and Methods. PC, paper chromatography; PEP, paper electrophoresis. [b] Relative mobility to dCp (1.0) and dC (0.0).

Table II: Ultraviolet Absorption Data of the Dimers

compound	λ_{max} (nm) [ϵ (P)][a] pH 2[c]	pH 7[d]	pH 12[e]	hypochromicity[b] (%)
(2′–5′)-C-br^{8}G	262.5 (20 400)	261 (19 700)	262.5 (18 800)	12
(3′–5′)-C-br^{8}G	273.5 (21 400)	265 (20 900)	266 (20 400)	4
dC-br^{8}G	273 (20 900)	263.5 (20 200)	269.5 (18 700)	7
C-G	275 (20 300)	253 (19 500)[f]	255.5 (18 600)	8

[a] Determined by phosphorus analysis and given in ϵ per phosphate residue. UV spectra were measured at room temperature (≈20 °C). [b] Calculated from hydrolysis experiment with snake venom phosphodiesterase, and the value at pH 7 is given. The absorbances at λ_{max}'s before and after hydrolysis are compared. [c] Measured in 0.01 N HCl–0.1 M NaCl. [d] Measured in 0.01 M sodium cacodylate–0.1 M NaCl. [e] Measured in 0.01 N NaOH–0.1 M NaCl. [f] Calculated from the data of Warshaw (1975).

N^2,2′-O,3′-O-Triisobutyl-8-bromoguanosine 5′-Phosphate (**6**). A suspension of pyridinium 8-bromoguanosine 5′-phosphate (1960 A_{260} units, 0.126 mmol) in pyridine (2 mL) was cooled in an ice bath, and isobutyryl chloride (0.2 mL, 1.9 mmol) was added with stirring. The mixture was stirred at room temperature for 2 h. Ice–water was added, and the mixture was extracted with $CHCl_3$ (10 mL × 5). The combined $CHCl_3$ fractions were washed with water (25 mL × 2) and evaporated. The residue was treated with 50% aqueous pyridine at room temperature overnight, and the solvent was evaporated. The residue was dried by coevaporation with pyridine and dissolved in a small volume of pyridine. **6** was precipitated with ether–hexane (3:1, 80 mL). The yield was 84 mg (0.108 mmol, 86%): $\lambda_{max}^{H^+}$ 261.5, 283 nm (sh); $\lambda_{max}^{H_2O}$ 264.5, 283 nm (sh); $\lambda_{max}^{OH^-}$ 270 nm; PC R_f(C) 0.78 (pbr^{8}G 0.02, A 0.33); PEP R_m(pA-A) 0.88.

dC-br^{8}G (**7**). A mixture of **6** (pyridinium salt, 0.108 mmol), N^4-benzoyl-5′-O-(dimethoxytrityl)deoxycytidine (60 mg, 0.095

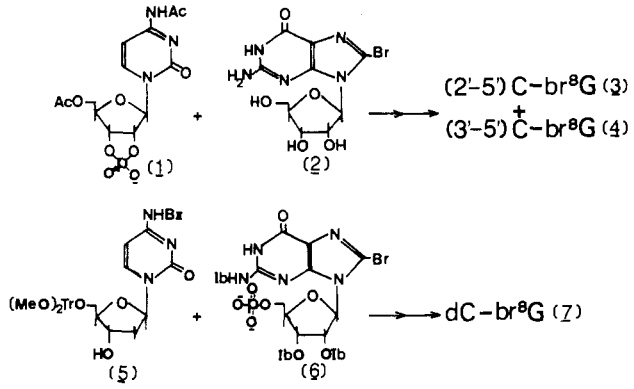

FIGURE 1: Synthetic scheme for the preparation of the dinucleoside monophosphates containing an 8-bromoguanosine residue.

mmol), and pyridinium Dowex 50 resin (122 mg) was dried by repeated evaporation with pyridine. The residue was finally dissolved in pyridine (2 mL) and dicyclohexylcarbodiimide (111 mg, 5 equiv) was added. The mixture was kept at 32 °C for 28 h; 50% aqueous pyridine (15 mL) was added, and the mixture was kept at room temperature overnight. The solid materials were filtered off, and the filtrate was evaporated. After evaporation with toluene, the residue was treated with 80% aqueous acetic acid (20 mL) at room temperature for 20 min, and the solvent was evaporated. After evaporation with water–1-butanol, the residue was treated with methanolic ammonia (15 mL) at room temperature for 20 h, and the solvent was evaporated. The residue was dissolved in 10% aqueous pyridine and washed with ether. The solvent was evaporated, and a solution of the residue in water was chromatographed on a column (1 × 32 cm) of DEAE-cellulose (HCO_3^- form). The column was washed with water and eluted with 0–0.15 M $Et_3NH_2CO_3$ (total 2.25 L) at a flow rate of 45 mL/h. **7** was eluted at around 0.04 M salt concentration. The combined fractions containing **7** were evaporated and desalted by repeated evaporation with water. The yield was 1295 $A_{263.5}$ units (0.056 mmol, 59%). The chromatographic properties and UV spectral data are presented in Tables I and II. This compound was completely hydrolyzed by snake venom phosphodiesterase to give dC and pbr^{8}G in a ratio of 1:1.

^1H NMR Measurements. The dimers were purified by paper electrophoresis at pH 7.5 on Whatman 3MM paper. Each dimer sample was passed through columns of Dowex 50 (Na^+ form) and Chelex-100 resins, successively, and lyophilized 3 times from D_2O solution. (2′–5′)- and (3′–5′)C-br^{8}G were measured in 0.01 M sodium phosphate buffer (pD 7.5)–0.1 M NaCl. Other dimers were measured without buffer and salt, but the pD was adjusted to 7.5. The concentrations of the dimers and component monomers were 100 A_{max} units/10.4 mL and 50 A_{max} units/0.4 mL, respectively. The pD of the monomer solution was adjusted to 5.5. ^{1}H NMR spectra were recorded with a Hitachi R-900 spectrometer (90 MHz) operating in the Fourier transform mode. The temperature was measured with a copper–constantan thermocouple. The ^{1}H chemical shifts were determined relative to internal 2-methyl-2-propanol, which had in turn been referenced to DSS [sodium 3-(trimethylsilyl)propane-1-sulfonate]. The HDO signal was suppressed with the 180°–τ–90° pulse sequence when necessary. The ^{1}H–^{1}H decoupling experiments were performed with a Bruker WM360 wb spectrometer (360 MHz) operating in the Fourier transform mode.

Results and Discussion

Preparation of the Dinucleoside Monophosphates. Synthetic schemes for (2′–5′)- and (3′–5′)C-br^{8}G (**3** and **4**) and dC-br^{8}G (**7**) are illustrated in Figure 1. **3** and **4** were synthesized by a modification of Michelson's method (Miura & Ueda, 1971; Miura et al., 1973). Thus N^4,5′-*O*-diacetylcytidine 2′,3′-phosphate (**1**) and unprotected 8-bromoguanosine (**2**) were treated with triisopropylbenzenesulfonyl chloride in DMF at 0 °C for 8 h. The reaction temperature must be around 0 °C to avoid O^2,2′-cyclization of the cytidine residue which gives rise to an arabinosylcytosine derivative (Miura et al., 1973). **3** and **4** were partially separated by chromatography on a DEAE-cellulose column. Each dimer was further purified by rechromatography on a Dowex 1 resin column which gave a better separation of **3** and **4**. Three different digestion experiments (alkali, RNase A, and snake venom phosphodiesterase) proved the structures of the dimers to be correct. Thus **3** was not hydrolyzed with RNase A but completely hydrolyzed with snake venom phosphodiesterase and with 0.3 N KOH. On the other hand, **4** was completely hydrolyzed by all the reagents examined. The sensitivity of both dimers to alkaline hydrolysis suggests that they are not contaminated with the arabinosylcytosine derivative. dC-br^{8}G was synthesized by condensation of **5** and **6** with dicyclohexylcarbodiimide in pyridine and isolated by chromatography on a DEAE-cellulose column. **7** was completely hydrolyzed with snake venom phosphodiesterase but was resistant to alkali and RNase A. The chromatographic properties of these dimers are presented in Table I.

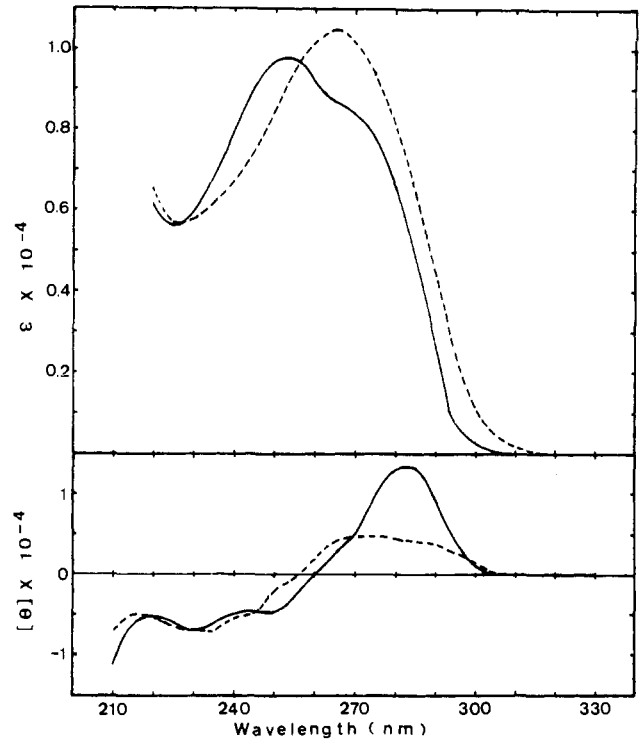

FIGURE 2: UV and CD spectra of C-G (—) and (3′–5′)C-br^{8}G (---) at 20 °C in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). The dimer strand concentration is 5 × 10^{-5} M.

Ultraviolet Absorption and Circular Dichroism Spectra at Low Dimer Concentration. UV data for the dimers (strand concentration, about 5 × 10^{-5} M) are shown in Table II. The data for C-G are also included for comparison. The UV spectra of C-G and (3′–5′)C-br^{8}G at pH 7 are shown in Figure 2. The λ_{max}'s of the dimers containing br^{8}G at pH 7 are at a longer wavelength than that of C-G by about 10 nm because of the long wavelength λ_{max} of br^{8}G (263 nm) with respect to G (252 nm). Similar differences in λ_{max}'s were also observed at pH 12. At pH 2, where the protonated cytosine residues make major contributions to absorption, (3′–5′)C-br^{8}G and

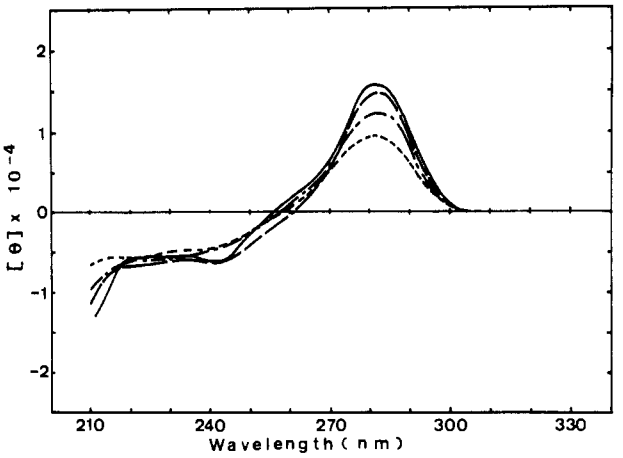

FIGURE 3: CD spectra of C-G (strand concentration 5 × 10^{-3} M) at 2 (—), 20 (– –), 40 (–·–), and 60 °C (– · –) in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0).

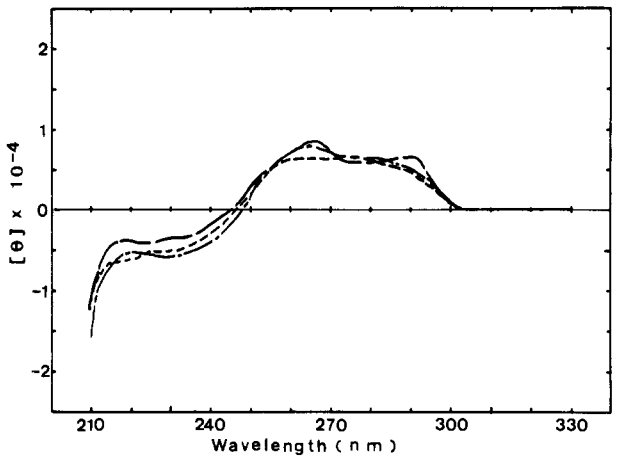

FIGURE 4: CD spectra of dC-br^{8}G (strand concentration 5 × 10^{-3} M) at 25 (– –), 40 (–·–) and 60 °C (– · –) in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0).

dC-br^{8}G show λ_{max}'s similar to that of C-G, but (2′–5′)C-br^{8}G exhibits a considerably different λ_{max}. The spectral differences between (3′–5′)- and (2′–5′)C-br^{8}G may be due to differences in the mode and stability of their base–base interactions. The hypochromicity of the dimers (Table II), which can be a measure of their base-stacking interactions, decreases in the following order, (2′–5′)C-br^{8}G > C-G ≈ dC-br^{8}G > (3′–5′)C-br^{8}G. This order should reflect the degree of base stacking in these dimers.

The CD spectra of C-G and (3′–5′)C-br^{8}G (strand concentration 5 × 10^{-5} M) at 20 °C and pH 7.0 are also shown in Figure 2. For the CD spectra of dC-br^{8}G and (2′–5′)C-br^{8}G, see Figures 3 and 4 which contain the spectra at high dimer concentration because the spectra at low and high dimer concentrations are very similar. C-G shows the largest band at around 283 nm. (2′–5′)C-br^{8}G shows a pair of positive and negative bands of medium magnitudes centered at around 280 nm. These CD spectral properties suggest that these dimers take a stacked conformation as deduced from the hypochromicity data (8% and 12%, respectively). However, (3′–5′)C-br^{8}G and dC-br^{8}G show only small CD bands, suggesting that their base-stacking interaction is very weak or that the stacking geometry is not favorable for induction of CD bands. The latter explanation may be valid for dC-br^{8}G because it has relatively large hypochromicity (7%).

In the case of natural dimers, larger UV hypochromicity and smaller CD bands are usually observed for the 2′–5′ isomer

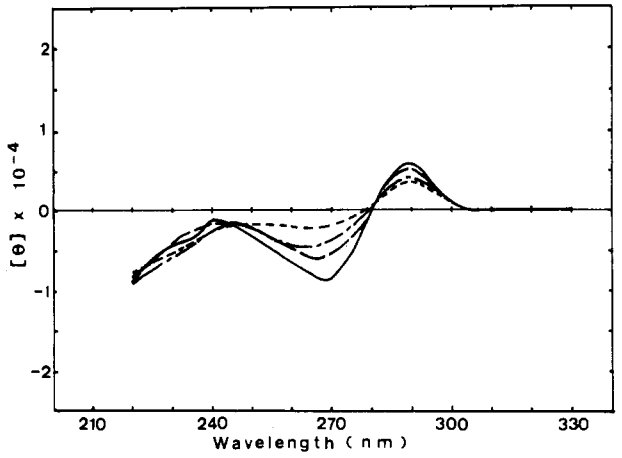

FIGURE 5: CD spectra of (2′–5′)C-br^{8}G (strand concentration 5 × 10^{-3} M) at 2 (—), 20 (– –), 40 (–·–), and 60 °C (– · –) in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0).

with respect to the 3′–5′ isomer when a pair of phosphodiester linkage isomers are compared (Kondo et al., 1970; Sussman et al., 1973). However, in the case of A-A analogues containing 8-substituted adenosine residues (br^{8}A and m^{8}A), larger hypochromicity and larger CD bands have been observed for the 2′–5′ isomers (Ikehara et al., 1972a,b, 1978). In this case, even A-A analogues with 2′–5′ phosphodiester linkages have rather weaker stacking interactions than A-A itself. Therefore the results herein suggesting more stable stacking in C-G and (2′–5′)C-br^{8}G than in (3′–5′)C-br^{8}G are understandable. These phenomena can be explained by steric repulsion between the syn base and the neighboring sugar phosphate residue as well as the greater distance between the two sugar residues in the 2′–5′ isomer.

CD Spectra at Higher Dimer Concentrations. It is known that base pairing involving oligonucleotides can be stabilized by increasing the oligomer concentration (Applequist & Damle, 1965). CD spectra were measured at 100-fold higher strand concentration (5 × 10^{-3} M) and at various temperatures (Figures 3–6) to detect duplex formation. C-G and (2′–5′)-C-br^{8}G show almost the same spectra as at the low dimer concentration at 20 °C and no sharp changes of the spectra over the temperature range 0–60 °C (Figures 3 and 5). The spectra of dC-br^{8}G are similar to those at the low dimer concentration and show essentially no change with temperature (25–60 °C; Figure 4).[2] No sign of duplex formation was observed for these dimers. However, (3′–5′)C-br^{8}G shows CD spectra entirely different in pattern and magnitude at relatively low temperature compared with that at the low dimer concentration (Figure 6A, 5 × 10^{-3} M; Figure 6B, 10 × 10^{-3} M). It also shows a sharp CD spectral transition upon temperature change, suggesting duplex formation at low temperature. The low temperature spectrum has two negative bands at around 300 and 225 nm and a positive band at around 270 nm. The spectral pattern in the long wavelength region (250–300 nm) is similar to that of d(C-G-C-G-C-G) in 4 M NaCl at low temperature, though the λ_{max} and λ_{min} are shifted to longer wavelength by about 5 nm and the magnitudes of the bands are several times larger in the case of (3′–5′)C-br^{8}G (Uesugi et al., 1981; Quadrifoglio et al., 1981). However, the spectral pattern in the short wavelength region (below 250 nm) is quite different from that of the high salt form of d(C-G-C-G-C-G) which has C-G and G-C base sequences. The high tempera-

[2] CD spectra at low temperature were not obtained because dC-br^{8}G forms precipitate from solution below 25 °C.

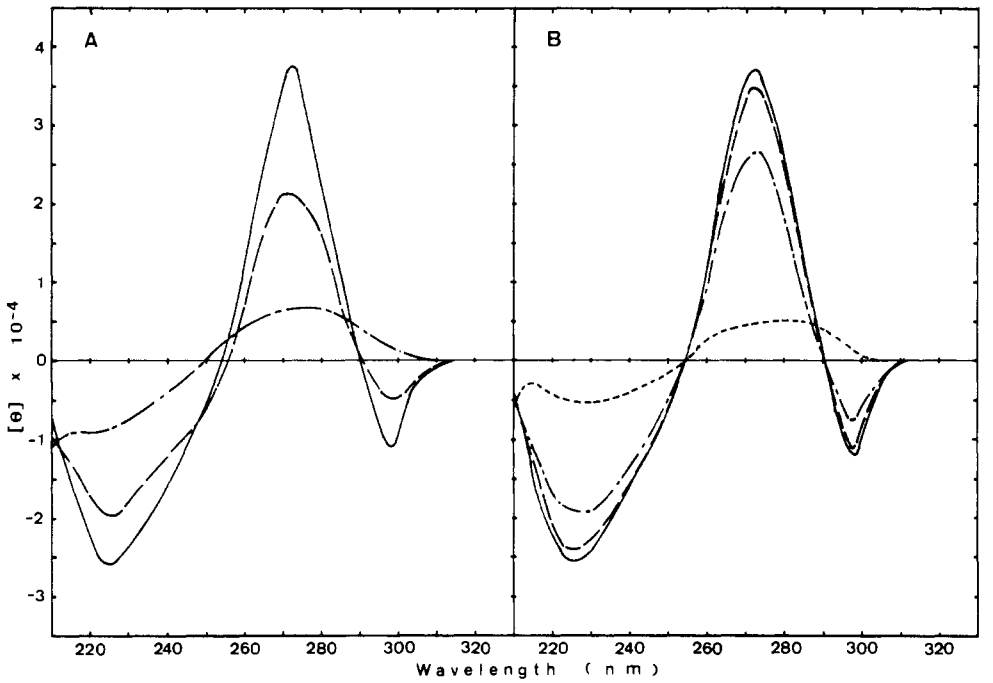

FIGURE 6: CD spectra of (3′–5′)C-br^{8}G at 2 (—), 20 (– –), 40 (–·–), and 60 °C (- - -) at a dimer strand concentration of 5×10^{-3} M (A) and 10×10^{-3} M (B) in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0).

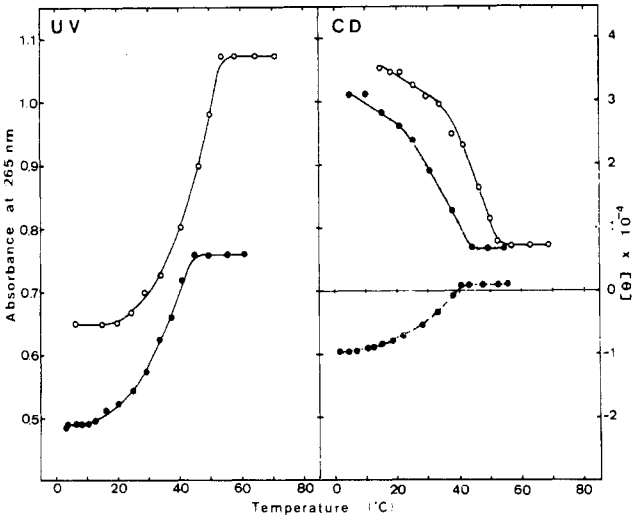

FIGURE 7: UV- and CD-temperature profiles for (3′–5′)C-br^{8}G at dimer strand concentrations of 5×10^{-3} M (●) and 10×10^{-3} M (○) in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). CD was monitored at 270 (—) and 300 nm (- - -).

ture spectrum of (3′–5′)C-br^{8}G (Figure 6A at 40 °C or Figure 6B at 60 °C) is almost the same as that at the low dimer concentration. These results suggest that (3′–5′)C-br^{8}G forms a duplex structure, presumably of a left-handed helical type, at high dimer concentration in 0.1 M NaCl.

UV- and CD-Temperature Profiles for (3′–5′)C-br^{8}G. The melting temperature (T_m) of the duplex of (3′–5′)C-br^{8}G was measured by UV and CD methods. The temperature profiles for two strand concentrations (5×10^{-3} M and 10×10^{-3} M) are shown in Figure 7. T_m's obtained by the UV method are 34.5 and 44.5 °C at 5×10^{-3} and 10×10^{-3} M strand concentrations, respectively. T_m's obtained by the CD method are 35 and 46.5 °C at the same concentrations (monitored at 270 nm). The $[\theta]_{300}$–temperature profile at 5×10^{-3} M dimer concentration gives a T_m of 33 °C which is close to that obtained at 270 nm (positive band). The T_m's obtained from both methods are almost identical. This result is in contrast

Table III: ^{1}H Chemical Shifts (ppm) and Coupling Constants (Hz) for the Dimers and Component Monomers[a]

compound		chemical shift[b] H(6) [H(8)]	H(5)	H(1′)	coupling constant $J_{1'2'}$
(2′–5′)C-br^{8}G	Cp-pbr^{8}G	7.37	5.57	5.98	4.1
				5.82	7.1
(3′–5′)C-br^{8}G	Cp-pbr^{8}G	7.67	5.97	5.82	4.8
				5.93	5.6
dC-br^{8}G	dCp-pbr^{8}G	7.58	5.94	6.07	8.4, 6.4
				5.91	5.2
C-G	Cp-pG	7.71	5.89	5.77	3.2
		8.00		5.88	4.9
Cp(2′)		7.77	6.05	5.99	4.8
Cp(3′)		7.86	6.07	5.92	4.0
dCp		7.89	6.09	6.27	7.0, 6.4
pbr^{8}G				5.96	6.3
pG		8.10		5.93	6.1

[a] Measured in D_2O at pD 7.5 [dimer, (12–13) × 10^{-3} M] or pD 5.5 [monomer, (9–14) × 10^{-3} M] and at 34 °C. The T_m of (3′–5′)C-br^{8}G is estimated to be about 50 °C. [b] Shifts are given relative to DSS.

to results for d(C-G-C-G) and d(C-G-C-G-C-G) in 4 M NaCl (Uesugi et al., 1981). In the case of oligo(dC-dG), the CD–temperature profile is quite different from the UV–temperature profile which is an ordinary sigmoidal curve. The $[\theta]$ of a long wavelength negative band increases linearly in a positive direction without a lower limit above 0 °C and reaches a plateau at around the T_m obtained by the UV method. The characteristic of the initial step of the CD–temperature profiles of oligo(dC-dG) is a decrease of the negative band magnitude without UV hyperchromism. A similar trend is noted in the low temperature region for (3′–5′)C-br^{8}G when UV- and CD–temperature profiles are compared (see the CD profiles for the positive band). These results suggest that the melting process in the (3′–5′)C-br^{8}G duplex is different from that of oligo(dC-dG) at least in the high temperature region (above T_m). The implications of this phenomenon are discussed below.

^{1}H NMR Spectra. ^{1}H NMR spectra were measured to obtain detailed information about the conformations of these

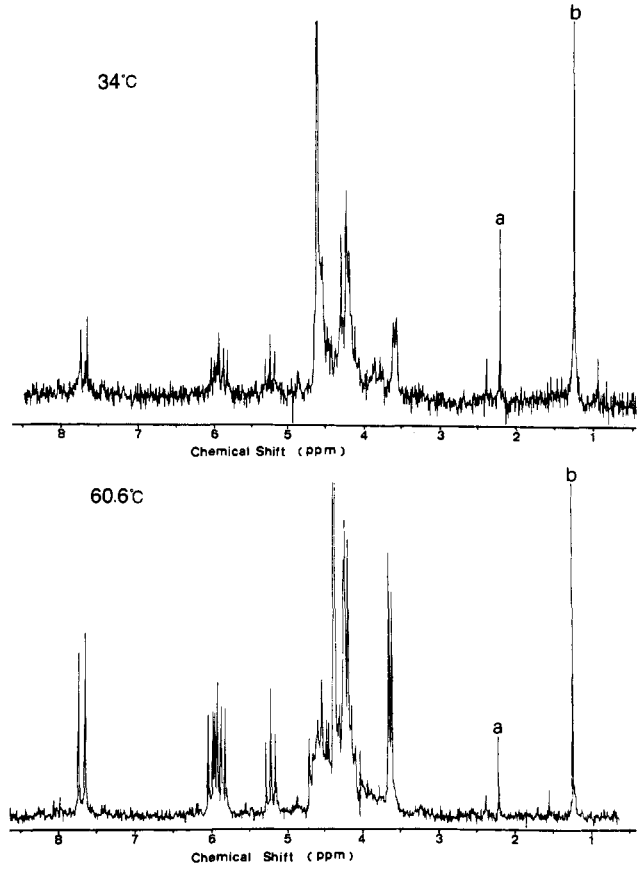

FIGURE 8: ^{1}H NMR spectra of (3′–5′)C-br^{8}G (strand concentration 12×10^{-3} M) at 34 and 61 °C in D_2O containing 0.1 M NaCl–0.01 M sodium phosphate (pD 7.5). (Signal a) Impurity from paper; (signal b) 2-methyl-2-propanol used as an internal reference.

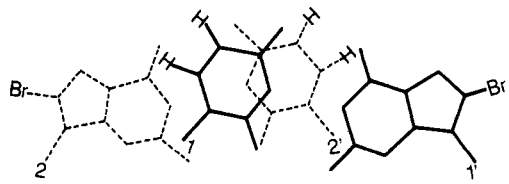

FIGURE 9: Base-stacking pattern of (3′–5′)C-br^{8}G duplex in the Z form. The geometry of the Z form is taken from Wang et al. (1979). Note that the cytosines of opposite strands overlap significantly.

dimers. The chemical shifts of the base and ribose 1′ protons and coupling constants between H(1′) and H(2′) ($J_{1'2'}$) are presented in Table III. The ^{1}H spectra of (3′–5′)C-br^{8}G are shown in Figure 8. The signals for C-G were assigned according to published results (Ezra et al., 1977). For dimers containing br^{8}G, the H(6) resonance of the Cp- or dCp- residue can be assigned easily because of its distinct chemical shift and coupling with H(5), and the H(5) resonance, in turn, can be assigned from its coupling constant with H(6). The H(2′) resonance of the -pbr^{8}G residue can also be easily assigned because of its distinctive chemical shift between the H(1′) and HDO regions (see Figure 9). The H(1′) resonance, in turn, can be assigned from its coupling constant with H(2′). These assignments were confirmed by homonuclear decoupling experiments. The remaining resonance in the region of H(1′) and H(5) was assigned to H(1′) of the Cp- or dCp- residue.

It is known that 8-substituted purine nucleoside derivatives show a large downfield shift of their H(2′) resonance with respect to the corresponding unsubstituted derivatives (Ikehara et al., 1972a,b; Sarma et al., 1974; Jordan & Niv, 1977). This phenomenon is considered to be associated with the syn conformation of these derivatives and caused by diamagnetic

Table IV: Dimerization Shifts (ppm)

compound		δ(monomer) − δ(dimer) H(6) [H(8)]	H(5)	H(1′)
(2′–5′)C-br^{8}G	Cp-	0.40	0.48	0.01
	-pbr^{8}G			0.14
(3′–5′)C-br^{8}G	Cp-	0.19	0.10	0.10
	-pbr^{8}G			0.03
dC-br^{8}G	dCp-	0.31	0.15	0.20
	-pbr^{8}G			0.05
C-G	Cp-	0.15	0.18	0.15
	-pG	0.10		0.05

anisotropy and polarizing effects of the N(3) atom which is close to H(2′) especially in 2′-endo puckering (Giessner-Prettre & Pullman, 1977). Thus H(2′) of pbr^{8}G (δ 5.27) shows a downfield shift of 0.54 ppm with respect to pG while H(1′) and H(3′) (δ 4.57) only show small downfield shifts (0.03 and 0.11 ppm, respectively). All the H(2′) resonances of the -pbr^{8}G residues in the dimers show approximately the same chemical shifts as that of pbr^{8}G [δ 5.31, 5.23, and 5.26 for (2′–5′)C-br^{8}G, (3′–5′)C-br^{8}G, and dC-br^{8}G, respectively]. These results suggest that all the -pbr^{8}G residues in the dimers take a syn conformation similar to that for pbr^{8}G and that their glycosidic torsion angles are similar.

Dimerization shifts, $\Delta\delta = \delta$(monomer) − δ(dimer), to exemplify base-stacking interactions in the dimers are calculated as shown in Table IV. An aromatic base residue can cause an upfield shift of nearby protons by ring-current and atomic diamagnetic anisotropy effects (Giessner-Prettre & Pullman, 1976), and therefore, Δδ can give a measure of stacking interaction. (2′–5′)C-br^{8}G shows large Δδ's on H(6) and H(5) of the Cp- residue [H(5) > H(6)], much smaller Δδ on H(1′) of the -pbr^{8}G residue, and negligible Δδ on H(1′) of the -Cp residue. It is known that 2′–5′ dimers generally show larger Δδ's than those for 3′–5′ dimers and that the Δδ of H(1′) of the -pN residue is larger than that for the Np- residue (Kondo et al., 1970; Ezra et al., 1976; Dhingra & Sarma, 1978). Dhingra & Sarma (1978) reported that (2′–5′)C-A exhibits large Δδ's for cytosine protons [0.65 ppm for H(5) and 0.39 ppm for H(6)]. Therefore, (2′–5′)C-br^{8}G may adopt a well-stacked conformation with a sugar phosphate backbone similar to that of natural 2′–5′ dimers but with a syn conformation in the -pbr^{8}G residue. The predominant backbone conformation of (2′–5′)C-br^{8}G may be that of a regular right-handed stack as proposed by Kondo et al. (1970).

dC-br^{8}G shows relatively large Δδ's, but the shielding pattern is different from that of (2′–5′)C-br^{8}G. Thus Δδ of H(6) is larger than that of H(5), and H(1′) of the dCp- residue shows a much larger Δδ than that of the -pbr^{8}G residue. The H(2′) protons of the dCp- residue show Δδ's of 0.41 and 0.08. This shielding pattern is very similar to that of d(C-G) (Cheng & Sarma, 1977). Moreover, the $J_{1'2'}$ of the dCp-residue is larger and $J_{1'2'}$ of the -pbr^{8}G residue is smaller with respect to those for the corresponding monomers. This means that the dCp- residue is more 2′-endo puckered and the -pbr^{8}G residue more 3′-endo puckered in the dimer. This trend is generally observed in deoxyribodinucleoside monophosphates with a pyrimidine–purine sequence (Cheng & Sarma, 1977). Therefore, dC-br^{8}G may adopt a sugar phosphate backbone structure very similar to those of natural deoxyribo dimers but with syn conformation in the -pbr^{8}G residue.

In the case of (3′–5′)C-br^{8}G, it should be noted that it forms a duplex at this dimer concentration (12 mM) and the T_m is estimated to be about 50 °C. We note that the ^{1}H spectra of this dimer at low temperature display relatively broad signals with small intensities, and the signals become sharper

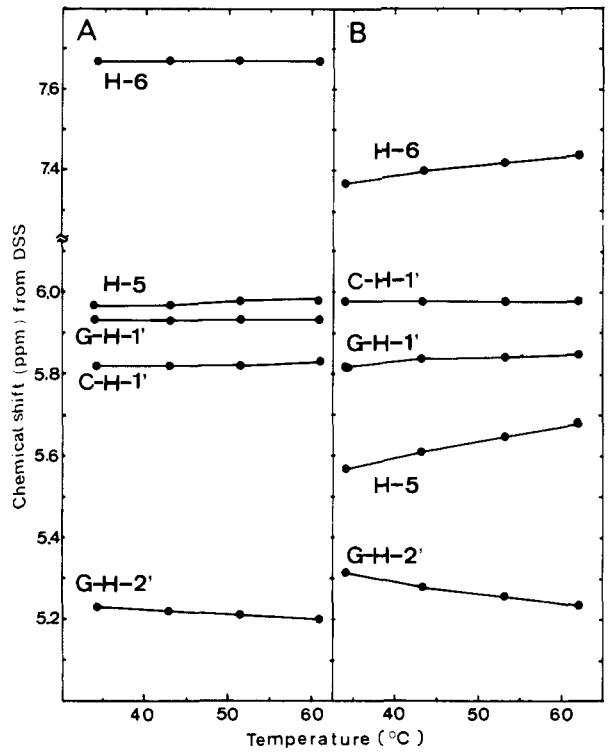

FIGURE 10: ^{1}H chemical shift–temperature profiles for (3′–5′)C-br^{8}G (A) and (2′–5′)C-br^{8}G (B). The dimer concentration and solvent are the same as described in Figure 8.

and larger upon increasing temperature (compare the spectra at 34 and 61 °C in Figure 8).[3] This phenomenon is consistent with duplex formation in (3′–5′)C-br^{8}G since broadening of NMR signals is generally observed for oligo- and polynucleotide duplexes. (3′–5′)C-br^8 shows $\Delta\delta$'s entirely different from those of the 2′–5′ isomer in both magnitude and pattern. H(6) of the Cp- residue shows a moderate $\Delta\delta$ (0.19 ppm), and H(5) shows an even smaller $\Delta\delta$ (0.10 ppm). The $\Delta\delta$ for H(1′) of the Cp- residue (0.10 ppm) is relatively small but still larger than that of the -pbr^{8}G residue (0.03 ppm). Moreover, the Cp- residue shows a larger $J_{1'2'}$ (increase in 2′-endo pucker) and the -pbr^{8}G residue shows a smaller $J_{1'2'}$ (increase in 3′-endo pucker) with respect to the corresponding monomers. These properties are consistent with the Z-form duplex structure (Wang et al., 1979) where there is no overlap between the cytosine and guanine bases, and H(6) and H(1′) of the Cp- residue are relatively closer to the pyrimidine part of the guanine base (see Figure 9) with respect to H(5) of the Cp- residue and H(1′) of the -pbr^{8}G residue, respectively.

^{1}H spectra of (2′–5′)- and (3′–5′)C-br^{8}G were measured at various temperatures (34–62 °C) to determine the effect of temperature on the chemical shift of each proton. The chemical shift–temperature profiles are shown in Figure 10. The result for (3′–5′)C-br^{8}G is surprising. All protons (Figure 10) show almost no chemical shift changes in this temperature range, whereas the duplex should have a T_m at around 50 °C. A temperature variation experiment was also performed for a sample at half this dimer concentration (6 mM; T_m is estimated to be 38 °C), but the same result was obtained (data not shown). This is in contrast to previous results for d(C-G-C-G) and d(C-G-C-G-C-G) in 4 M NaCl where all base protons show considerable downfield shifts upon duplex melting (Uesugi et al., 1981). In the case of oligo(dC-dG), the G-C as well as C-G sequence is involved, and syn to anti conversion of glycosidic conformation of the dG residues and subsequent transition of helix handedness from left to right are assumed to occur during duplex melting. The results for (3′–5′)C-br^{8}G suggest that the conformation of this dimer does not change greatly upon duplex melting, and therefore, the syn conformation of the br^{8}G residue and the sugar phosphate backbone conformation remain almost unchanged. The discrepancy between the CD–temperature profiles of oligo(dC-dG) and (3′–5′)C-br^{8}G above T_m, mentioned above, can be explained in this way. On the other hand, (2′–5′)C-br^{8}G shows considerable downfield shifts of H(6) and H(5) (0.07 and 0.11 ppm, respectively) and an upfield shift of H(2′) of the -pbr^{8}G residue (0.07 ppm) over the temperature range 34–62 °C. These types of gradual changes of the chemical shifts are generally observed for single-stranded oligonucleotides and caused by noncooperative destacking of the bases.

Conclusions

The results described here reveal that among three C-G analogues containing the br^{8}G residue, only (3′–5′)C-br^{8}G shows duplex formation at high dimer concentration (5×10^{-3} M) in 0.1 M NaCl. T_m's of the duplex are about 35 and 45 °C at dimer concentrations of 5×10^{-3} and 10×10^{-3} M. Although some dimers are known to form duplexes (Young & Krugh, 1975; Krugh et al., 1976; Dhingra et al., 1981), these T_m's of (3′–5′)C-br^{8}G are surprisingly high for the present dimer and salt concentrations. From the CD spectral pattern and syn conformation preference of the 8-bromoguanosine residue, this (3′–5′)C-br^{8}G duplex is assumed to have a left-handed backbone of the Z-form DNA type. The geometry of base stacking in the Z form for (3′–5′)C-br^{8}G (Figure 9) shows that the two cytosine residues of the opposite strands partially overlap, but there is no overlap between cytosine and guanine residues. The ^{1}H NMR data, that include the dimerization shifts, coupling constants, and chemical shift of H(2′) of the -pbr^{8}G residue, are consistent with the left-handed duplex structure. (3′–5′)C-br^{8}G shows little chemical shift changes upon duplex melting. This result confirms that there is no extensive base–base overlap in the duplex and the syn conformation of the -pbr^{8}G residue remains unchanged during melting. The latter presumption may explain the difference in CD–temperature profiles of oligo(dC-dG) and (3′–5′)C-br^{8}G in the high temperature region (above the T_m obtained by the UV method) as discussed in the previous section.

If (3′–5′)C-br^{8}G can form a Z-form duplex, then why cannot the other two dimers? In the case of (2′–5′)C-br^{8}G, the phosphodiester linkage is not favorable for a left-handed base arrangement of the Z-form type (see Figure 9), because the O(2′) of the Cp- residue is more distant from the O(5′) of the -pbr^{8}G residue than the O(3′). In the case of dC-br^{8}G, which does not show duplex formation at a dimer concentration of 10×10^{-3} M and above 25 °C, a possible explanation can be obtained from the difference in the conformations of dC-br^{8}G and (3′–5′)C-br^{8}G in the single-stranded state. The UV hypochromicity and ^{1}H NMR dimerization shift data suggest that dC-br^{8}G takes a much more stacked conformation than (3′–5′)C-br^{8}G (note that the former may take B-form type stacking which allows maximum overlap between the two bases, whereas the latter cannot because of the presence of the 2′-OH group). If the conformation of the monomeric dC-br^{8}G is well stabilized by stacking, there is no driving force to form the Z-form duplex where only the cytosine residues partially overlap. In contrast to this, (3′–5′)C-br^{8}G has little base–base overlap in the monomeric state, and when it forms the Z-form duplex, its conformation can be stabilized by cy-

[3] We also noted that the NMR sample of (3′–5′)C-br^{8}G was very viscous even at 34 °C.

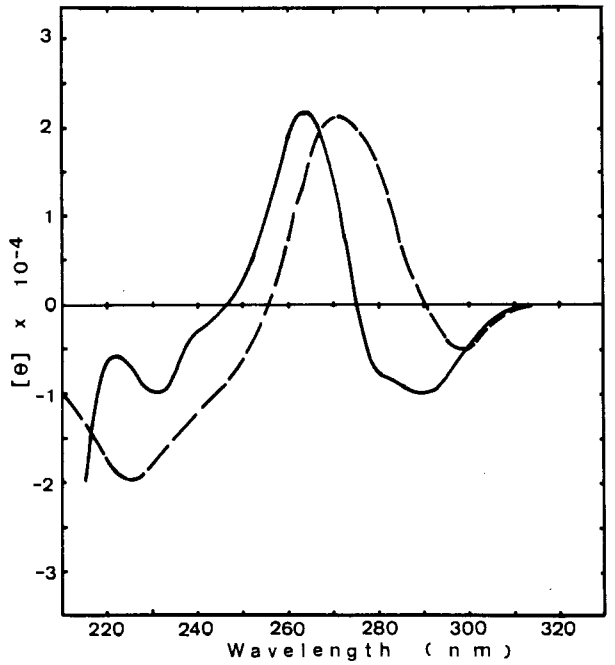

FIGURE 11: Comparison of the CD spectra of (3′–5′)C-br^{8}G (5×10^{-3} M strand concentration, 20 °C) and poly(G-C) (10^{-3} M Na^+, 20 °C). The latter spectrum was taken from Gray et al. (1972).

tosine–cytosine stacking and hydrogen bonding. A similar phenomenon has been observed in sequence isomers of self-complementary dinucleoside monophosphates containing 8,2′-*S*-cycloadenosine (A^S) and 6,2′-*O*-cyclouridine (U^O) residues (Dhingra et al., 1981). At low dimer concentration, A^S-U^O does not take a stacked conformation while U^O-A^S does. At high dimer concentration, A^S-U^O forms a left-handed duplex with interstrand adenine–adenine stacking but U^O-A^S does not.

Our present results suggest that not only DNA but also RNA can form Z-form duplexes. Examination of r(C-G-C-G) in the Z form with CPK molecular models suggests that a Z-RNA duplex is quite possible and the 2′-OH causes no steric hindrance. The 2′-OH groups are always outside the space between adjacent sugar residues. In this context, we note that the poly(G-C) duplex in low salt solution is reported to have a CD spectral pattern very similar to that of the (3′–5′)C-br^{8}G duplex not only in the long wavelength region but also in the short wavelength region (Gray et al., 1972). The two spectra are compared in Figure 11.

After preparation of this paper, we noted two recent papers which deal with closely related subjects. Gray et al. (1981) report that many synthetic, double-stranded polyribonucleotides containing regular purine–pyrimidine sequences show a negative CD band in the 290–300-nm region. At present it is not certain whether the similarity between the CD spectra of (3′–5′)C-br^{8}G and poly(G-C) duplexes is coincidental or not. Lafer et al. (1981) report that the brominated poly(dG-dC) duplex takes a Z-form structure even at low salt concentration. This result, apparently, seems to be inconsistent with our result on dC-br^{8}G. However, such direct comparison may not be adequate because the brominated poly(dG-dC) contains brominated dC as well as brominated dG residues and polymerization of dC-dG units may cooperatively stabilize its Z-form structure.

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Protein Binding Sites in Nucleation Complexes of Alfalfa Mosaic Virus RNA 4†

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ABSTRACT: The subgenomic coat protein messenger RNA 4 of alfalfa mosaic virus forms complexes with one and three coat protein dimers, which are designated complexes I and III, respectively. These complexes were separated, subjected to digestion with ribonuclease T_1, and filtered onto Millipore filters. Phenol extracts of the filters contained specific fragments of RNA 4, which were sequenced after electrophoretic separation on nondenaturing and denaturing polyacrylamide gels. Complex I yielded only a 68-nucleotide fragment including the 3′ terminus [fragment 814–881 according to the numbering of Brederode, F. Th., Koper-Zwarthoff, E. C., & Bol, J. F. (1980) *Nucleic Acids Res. 8*, 2213–2223]. Complex III yielded in addition to the former fragment also other, mostly extracistronic, fragments from the 3′-terminal region, as well as fragments from an intracistronic region, comprising positions 425–474, in the middle of RNA 4. The 3′-terminal region was subdivided by small gaps into three coat protein binding sites: 799–881, 759–787, and 667–753, designated sites 1, 2, and 3, respectively, and possibly representing the sites occupied by the three coat protein dimers. A similarity may exist between the secondary structure of sites 1 and 3, which both may have three hairpins, two of which flanked at their 3′ side by an AUGC sequence. Furthermore, a complementarity was noted between the loop of a large hairpin which can be drawn in the intracistronic site and the upper part of one of the three hairpins in the 3′-terminal site 1. These binding features have been combined in a model structure for the complex of RNA 4 with three coat protein dimers.

Recently evidence has been obtained that the genome of alfalfa mosaic virus becomes infectious only after each of the three genome parts (viz., the RNA species 1, 2, and 3) has bound a few coat protein molecules (Smit et al., 1981). Binding studies with specific fragments of RNA 4 (the subgenomic coat protein messenger RNA of alfalfa mosaic virus, which is homologous with the 3′ half of the smallest genome part, RNA 3) have strongly suggested that high-affinity binding sites for the coat protein are located close to the 3′ terminus of the molecule (Houwing & Jaspars, 1978; Stoker et al., 1980). In a preceding study we have isolated complexes of RNA 4 with one and three coat protein dimers and designated them complex I and complex III, respectively (Houwing & Jaspars, 1980). It was assumed that the coat protein in these complexes was bound specifically to the high-affinity sites of RNA 4, since in the complex forming reaction the coat protein was donated by virions. In this report we describe the sites on RNA 4 which are protected in complexes I and III against degradation by ribonuclease T_1. It appeared that these sites are not exclusively located in the 3′-terminal region of the molecule.

Materials and Methods

RNA 4, coat protein, and RNA 4/coat protein complexes were prepared as described previously (Houwing & Jaspars, 1980, and references therein). Immediately before ribonuclease digestion, complex I was found by analytical gel electrophoresis to contain about 30% free RNA 4; complex III contained about 35% free RNA 4 and lower complexes. The quantities of complexes are expressed in micrograms of their RNA contents.

Other Materials. Ribonucleases T_1 and U_2 were from Sankyo (via Calbiochem). T4 polynucleotide kinase was obtained from Boehringer Mannheim. [γ-^{32}P]ATP (3000 Ci/mmol) was from The Radiochemical Centre, Amersham, England. Dextran T 500 was obtained from Pharmacia, and 24-mm HAMK filters were from the Millipore Corp. Ultrapure urea was purchased from Schwarz/Mann and 2 times crystallized p.A. acrylamide from Serva (Heidelberg). All other chemicals were reagent grade. The concentration of glycerol refers to glycerol as supplied, with no correction for water contamination (about 13%).

Ribonuclease Digestion. RNA and ribonucleoprotein complexes I and III were digested at a concentration of 5–15

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