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Polynucleotides. LXIII.[1] Solution Conformation of Oligodeoxyribonucleotides containing an Alternating dC-dG Sequence which can form a Left-handed Double Helix

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To elucidate the conformational properties and their chain length dependency in solution of oligodeoxyribonucleotides containing an alternating C–G sequence, which have been shown to take a left-handed duplex structure in crystals, d(C–G), d(C–G–C–G) and d(C–G–C–G–C–G) were synthesized by a modified triester method and characterized by ultraviolet (UV) circular dichroism (CD) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. The effects of salt concentration (0.1 M and 4 M NaCl), oligomer strand concentration (10^{-5}—10^{-2} M) and temperature (0°—90°C) on the oligomer conformation were investigated. The dimer does not form a duplex under any conditions studied. The tetramer does form a right-handed duplex in 0.1 M NaCl even at 3×10^{-5} M strand concentration. In 4 M NaCl, however, it does not form any duplex at the same strand concentration, and left-handed duplex formation is observed at 10-fold higher concentration. The hexamer forms a duplex in either salt concentration even at 2×10^{-5} M strand concentration. The melting temperatures of these duplexes were measured by UV and CD methods. The CD-temperature profile in 4 M NaCl, which is not sigmoidal when monitored at the λ_{min} of the characteristic negative band, suggests that melting of the high salt duplex is a rather complex process that involves more than two species. The results of ^{1}H-NMR-temperature studies support left-handed duplex formation in a high salt solution.

Keywords——oligodeoxyribonucleotide; alternating CG sequence; left-handed double helix; melting temperature; solution conformation; CD; UV; ^{1}H-NMR

Recently a left-handed double helical form of deoxyribonucleic acid (DNA) has been found in crystals of oligomers, d(C–G–C–G–C–G)[2,3] and d(C–G–C–G)[4] and in fibers of polymers containing an alternating pyrimidine-purine base sequence.[5] This form, so-called Z-form, is entirely different from the right-handed double helical B-form or A-form which is commonly observed for DNA in fibers and in solution. It is assumed that this structure might play some biological role in the modification of DNA functional state. On the other hand, we have been working on oligonucleotides containing cyclonucleosides with a high *anti* glycosidic torsion angle.[6–8] These oligomers have a tendency to take a left-handed, stacked conformation forming a left-handed helix. A pair of complementary oligomers[9] and a self-complemetary dimer of the cyclonucleotides[10] were shown to form a left-handed double helix. Therefore, we are interested in the newly found left-handed double helical structure of Z-DNA. In this case, the deoxyguanosine residue takes a *syn* conformation around the glycosidic bond and the deoxycytidine residue takes an *anti* conformation. In this paper, we report the synthesis and characterization of a series of oligo(dG-dC) by ultraviolet (UV), circular dichroism (CD) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy to elucidate the conformational properties in solution. By a combination of these techniques, different features of the conformation and dynamic behavior of the oligomers can be seen.

Results

Synthesis of the Oligomers

The oligodeoxyribonucleotides, d(C–G), d(C–G–C–G) and d(C–G–C–G–C–G), were synthesized by a modified phosphotriester method.[11] The 3′-terminal phosphate group

was protected with *o*-chlorophenyl and *p*-methoxyanilido groups. The latter group can be easily removed by treatment with isoamyl nitrite in acetic acid–pyridine mixture without damage to the 5′-*O*-monomethoxytrityl group. The internal phosphodiester group was protected with an *o*-chlorophenyl group. The amino and hydroxyl group were protected with benzoyl (NH_2 of dC and 3′-OH of dG), isobutyryl (NH_2 of dG) and monomethoxytrityl (5′-OH) groups. The synthetic scheme for the hexamer is shown in Fig. 1. The condensation reactions were carried out with mesitylenesulfonyl 4-nitroimidazolide[12] in pyridine. The fully protected product was isolated by chromatography on a silica gel column. The deblocked oligomers were isolated by chromatography on a DEAE-cellulose column. In the case of the hexamer, elution was carried out in the presence of 7 M urea. For physical and enzymatic studies, the oligomers were further purified by paper electrophoresis on Whatman 3MM paper. The oligomers thus obtained were characterized by paper chromatography and paper electrophoresis as shown in Table I. These oligomers were completely hydrolyzed with nuclease P1[13] to give corresponding monomers in correct ratios.

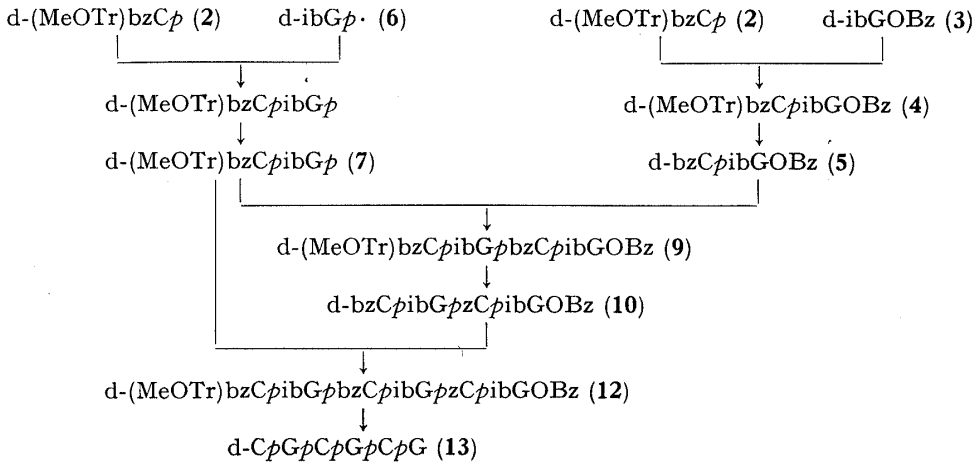

Fig. 1. Synthetic Scheme for Preparation of d(C–G–C–G–C–G)

TABLE I. Chromatographic Properties of the Oligomers[a]

Compound	Paper chromatography *Rf* in solvent			Paper electrophoresis R_m (dC*p*-dC)[b]
	A	B	C	
d(C–G)	0.17	0.47	0.46	0.49
d(C–G–C–G)	0.02	0.16	0.27	0.86
d(C–G–C–G–C–G)	0.00	0.06	0.17	1.01
dC	0.52	0.76	0.64	0.00
dC*p*	0.25	0.42	0.41	1.00

a) The compositions of the solvent and buffer (pH 7.5) systems are given in "Experimental."
b) Relative mobility with respect to dC*p* (1.0) and dC(0.0).

Ultraviolet Absorption Spectra

UV spectral data of the oligomers at three pH's are presented in Table II. Each oligomer shows spectral changes at acidic and alkaline pH's. At pH 2, large increase and decrease of ε are observed at around 280 nm and 240 nm, respectively. At pH 12, a shift of the λ_{max} (around 250 nm) to longer wavelength (around 265 nm) is observed, These characteristic changes suggest that the oligomer contains both cytosine and guanine residues. The ε data in Table II also show that the differences between the dimer and the higher oligomers are much larger than that between the tetramer and hexamer. A similar phenomenon can be clearly seen in

Table II. UV Absorption Data for the Oligomers[a)]

Compound	λ (nm) (ε) pH 2[b)]		pH 7[c)]		pH 12[d)]	
d(C–G)	275.5	(10100)	251	(9700)	265.5	(9100)
	261(sh.)	(9100)	266(sh.)	(8400)		
d(C–G–C–G)	276	(8800)	252	(8400)	265.5	(8300)
	262(sh.)	(8000)	268(sh.)	(7200)		
d(C–G–C–G–C–G)	275.5	(8700)	254	(8200)	265	(8100)
	262(sh.)	(8000)				

a) ε was calculated from the result of a nuclease P1 digestion experiment and is given as per base residue value. UV spectra of the oligomers (*ca.* 0.8 A_{max} unit/ml) were taken at room temperature (≈20°C).
b) 0.01 N HCl–0.1 M NaCl.
c) 0.01M sodium cacodylate buffer (pH 7.0)–0.1 M NaCl.
d) 0.01 N NaOH–0.1 M NaCl.

the difference spectra (with respect to the monomer mixture spectrum)shown in Fig. 2B. The original UV spectra of the hexamer at different salt concentrations are also included in Fig. 2A. The difference spectra of the tetramer and hexamer in 0.1 M NaCl have two peaks at around 240 nm and 275 nm, while the dimer shows only one peak at around 275 nm. This difference can be attributed to a lack of G–C spequence or a lack of duplex formation in the dimer. The latter problem will be discussed later. The difference spectrum of the hexamer in 4 M NaCl is significantly different both in pattern and magnitude from that in 0.1 M NaCl. It is known that at 4 M NaCl concentration the transition from a low salt form to a high salt form is complete for poly (dG–dC)[14] and oligo (dG–dC) with chain length longer than 16.[15]

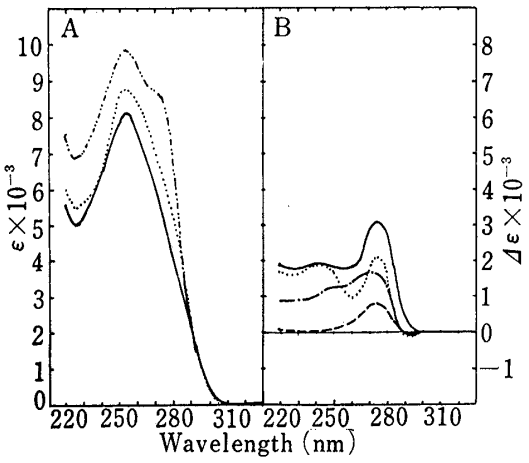

Fig. 2. UV Spectra of the Hexamer (A) and UV Difference Spectra for the Oligomers(B)

——,the hexamer in 0.1 M NaCl; ----, the hexamer in 4 M NaCl; -··-, after enzymic digestion of the hexamer; —·—, the tetramer in 0.1 M NaCl; – – –, the dimer in 0.1M NaCl at pH 7.0.

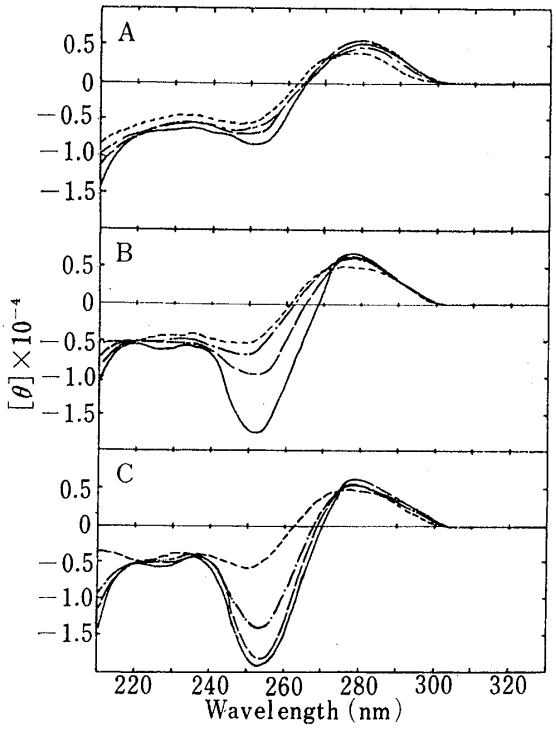

Fig. 3. CD Spectra of the Dimer (A), Tetramer (B) and Hexamer (C)

——,7°C; – –, 25°C; - · -, 45°C; ----, 65°C in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). Oligomer concentration: 1 A_{max} unit/ml.

Circular Dichroism Spectra

CD spectra of the oligomers (1 A_{max} unit/ml) at various temperatures in 0.1 M NaCl and 4 M NaCl are shown in Figs. 3 and 4, respectively. d(C–G) shows relatively small CD bands, a positive band at around 280 nm and a negative band at around 250 nm, and only a small change upon temperature change (7°C to 65°C). The spectrum at 25°C is the same as the published spectrum of d(C–G) measured under similar conditions.[16] The decrease of the CD magnitude with increasing temperature may be due to destacking of the bases, as is usually observed in single-stranded oligomers. In contrast to the dimer, d(C–G–C–G) and d(C–G–C–G–C–G) show marked changes of CD spectra with temperature in 0.1 M NaCl. At low temperature, the magnitude of the negative band at around 250 nm increases greatly. The spectra of the tetramer and hexamer at 65°C are nearly identical to that of the dimer at the same temperature. The absolute $[\theta]$ value does not increase linearly with decreasing temperature. The process seems to involve a cooperative transition and, therefore, to be associated with duplex formation of the self-complementary oligomer. It should be noted that the spectra of the tetramer and hexamer at 7°C are very similar to that of poly (dG–dC) in a low salt solution, where poly (dG–dC) is assumed to take a double-stranded B-form structure.[14]

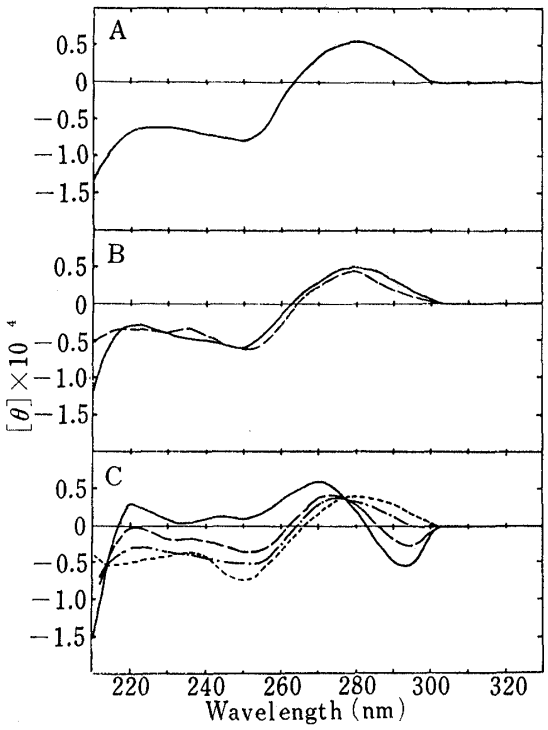

Fig. 4. CD Spectra of the Dimer (A), Tetramer (B) and Hexamer (C)

——, 7°C; — —, 25°C; - · -, 45°C; ——, 65°C in 4 M NaCl–0.01 M sodium cacodylate (pH 7.0). Oligomer concentration: 1 A_{max} unit/ml.

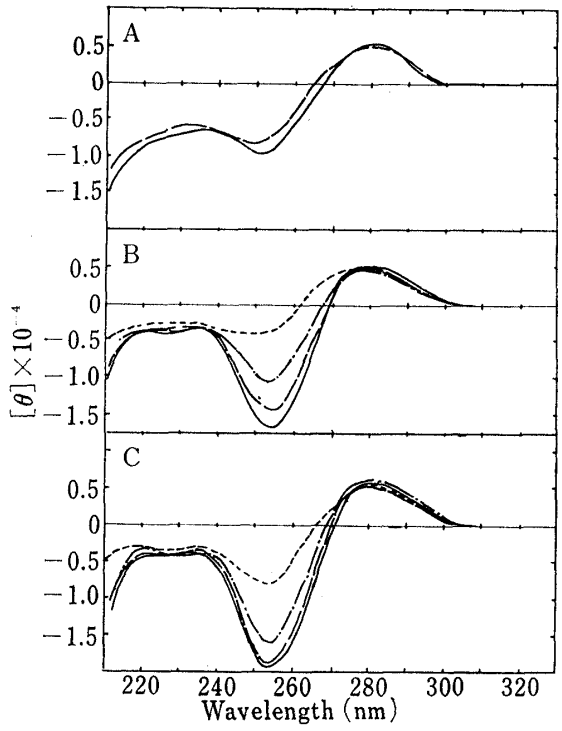

Fig. 5. CD Spectra of the Dimer (A), Tetramer (B) and Hexamer (C)

——, 7°C; — —, 25°C; - · -, 45°C; ——, 65°C in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). Oligomer concentration: 100 A_{max} units/ml.

In 4 M NaCl, not only the dimer but also the tetramer show the same spectra as that of the dimer in 0.1 M NaCl, the spectrum of a single-stranded form. Therefore, B–DNA duplex of the tetramer must be destabilized by a high concentration of the salt. On the other hand, the hexamer shows a CD spectrum entirely different from that in 0.1 M NaCl at 7°C. It shows a negative band at around 290 nm and a positive band at around 270 nm. The spectral pattern is apparently reversed and is very similar to that of the high salt form of poly(dG–dC).[14]

The $[\theta]$ value of the negative band shifts to a positive direction with increasing temperature. The spectrum at 65°C is nearly identical to those of the dimer and tetramer. From these results for low oligomer concentration (about $1.5—5\times10^{-5}$ M strand concentration), it is concluded that the tetramer and hexamer in 0.1 M NaCl take a right-handed duplex structure which is destabilized in high salt solution, and in 4 M NaCl only the hexamer takes a left-handed duplex structure.

To determine the effect of oligomer concentration, CD spectra of 100-fold concentrated solutions of the oligomers (100 A_{max} units/ml) were also measured and are shown in Figs. 5 and 6. In the case of 0.1 M solution, d (C–G) still does not show any sign of duplex formation, while the tetramer and hexamer show the characteristic spectra of the duplex. In the case of 4 M NaCl solution, not only the hexamer but also the tetramer show CD spectra characteristic of a left-handed duplex at low temperature. The dimer again shows no sign of duplex formation.

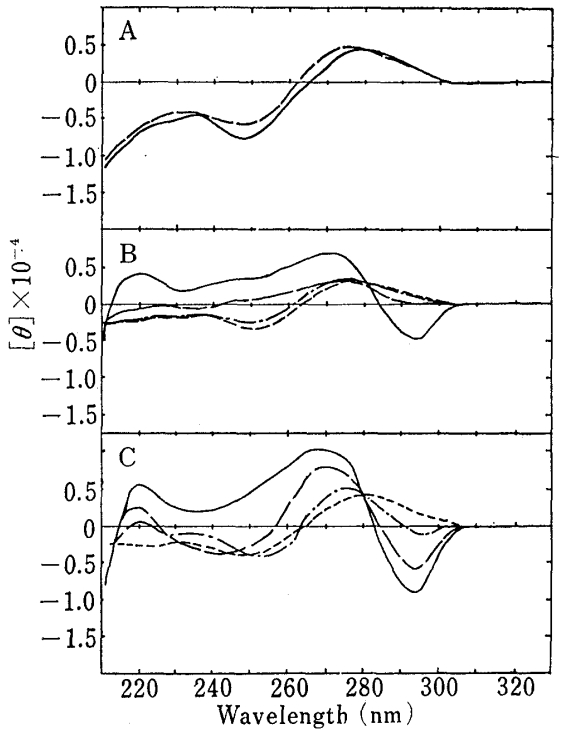

Fig. 6. CD Spectra of the Dimer(A), Tetramer (B) and Hexamer (C)

——, 7°C; — —, 25°C; - · -, 45°C; ----, 65°C in 4 M NaCl -0.01 M sodium cacodylate (pH 7.0). Oligomer concentration: 100 A_{max} units/ml.

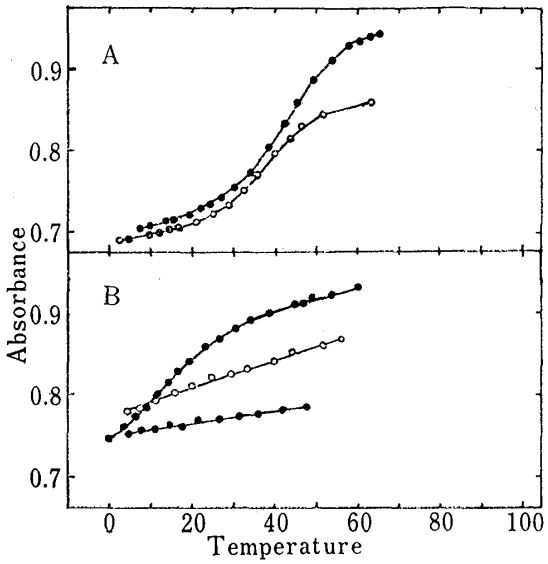

Fig. 7. UV-Temperature Profiles for the Hexamer (A), Tetramer (B, upper two curves) and Dimer (B, the bottom curve)

●, 0.1 M NaCl; ○, 4 M NaCl at pH 7.0. Oligomer concentration: 1 A_{max} unit/ml.

UV- and CD-Temperature Profiles

To confirm the duplex formation and examine the effect of oligomer concentration, melting temperature (T_m) was measured at three concentrations of the oligomers (about 1, 10 and 100 A_{max} units/ml) by UV and CD methods. The results are shown in Figs. 7—9. In the case of the lowest concentration (2×10^{-5} M strand concentration for the hexamer), the hexamer gives clear T_m's (41.5°C in 0.1 M NaCl and 36°C in 4 M NaCl) when monitored with UV absorption. The tetramer (3×10^{-5} M) shows a UV-temperature profile suggesting a cooperative transition at around 12°C in 0.1 M NaCl. As expected from the earlier results, the dimer (5×10^{-5} M) in 0.1 M NaCl and the tetramer in 4 M NaCl show no sign of cooperative melting. In the case of the medium concentration (2×10^{-4} M for the hexamer), the hexamer

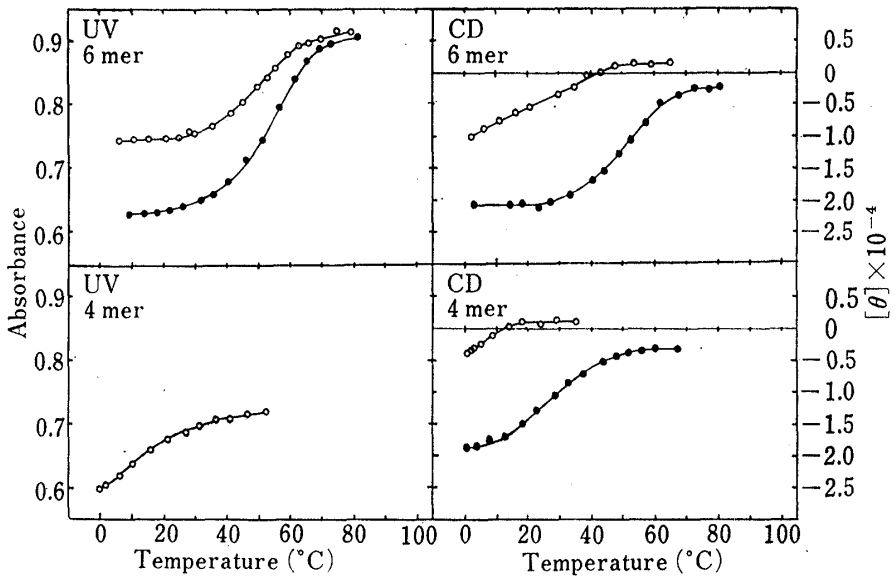

Fig. 8. UV- and CD-Temperature Profiles for the Hexamer and Tetramer

●, 0.1 M NaCl; ○, 4 M NaCl at pH 7.0. Oligomer concentration: 10 A_{max} units/ml.

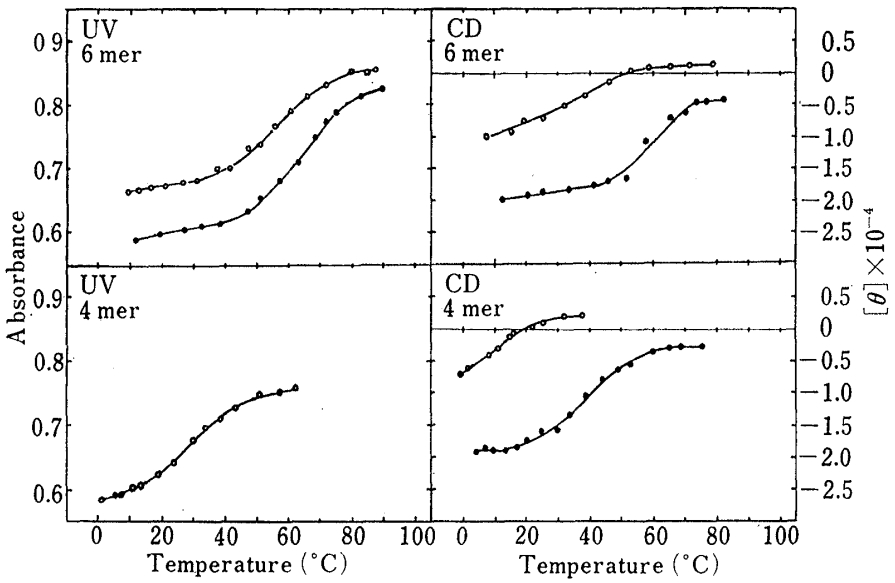

Fig. 9. UV- and CD-Temperature Profiles for the Hexamer and Tetramer

●, 0.1 M NaCl; ○, 4 M NaCl at pH 7.0. Oligomer concentration: 100 A_{max} units/ml.

shows T_m's (52.5°C in 0.1 M NaCl and 46.5°C in 4 M NaCl) when measured by the UV method. When monitored by the CD method, the hexamer shows a sigmoidal curve and gives almost the same T_m (51.5°C) in 0.1 M NaCl as that obtained by the UV method. However, the CD-temperature profile monitored at 294 nm in 4 M NaCl is not a sigmoidal curve. The $[\theta]_{294}$ goes up linearly with increasing temperature over the range of 0°C to 50°C and reaches a plateau at around 50°C, where melting of the duplex is only half-completed as judged from the UV-temperature profile. The same phenomenon is also observed in the case of the hexamer at the highest concentration (2×10^{-3} M) in 4 M NaCl (Fig. 9), and the inflection point coincides with the T_m obtained by the UV method. In contrast to this, in the case of the hexamer in 0.1 M NaCl, the temperature profiles of UV and CD are again both sigmoidal and the T_m's obtained from both methods (61.5°C and 60.5°C, respectively) are nearly identical. When

the UV- and CD-temperature profiles of the hexamer are compared, it should be noted that in the low temperature region, where the UV absorption decrease reaches a plateau, the $[\theta]_{294}$ is still going down linearly. These results may imply that the melting and structural organization of the oligomer duplex in 4 M NaCl involve a complex process which is reflected in the CD spectra but not in the UV spectra. A similar phenomenon can also be seen in the case of the tetramer in 4 M NaCl (Figs. 8 and 9). The T_m's of the tetramer in 0.1 M NaCl are 26.5°C and 38°C for strand concentrations of 3×10^{-4} and 3×10^{-3} M, respectively. The T_m of the tetramer in 4 M NaCl is 31°C for the highest concentration and is below 10°C for the medium concentration when monitored by the UV method. It should be noted generally that the T_m in 4 M NaCl is lower than that in 0.1 M NaCl at the same oligomer concentration.

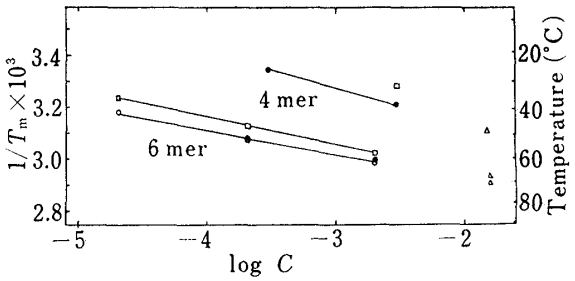

Fig. 10. Plot of the Reciprocal Melting Temperature (K) against Logarithim of the Oligomer Strand Concentration (M)

□, in 4 M NaCl from UV; ○, in 0.1 M NaCl from UV; ●, in 0.1 M NaCl from CD; △, the estimated T_m's at the oligomer strand concentration (15×10^{-3} M).

To determine the oligomer concentration effect on T_m, $1/T_m$(K) was plotted against the logarithm of the strand concentration (Fig. 10). A linear correlation between them is observed, as expected for a double-strand–single-strand equilibrium involving oligonucleotides.[17]

^{1}H-NMR Spectra

^{1}H-NMR spectra of d(C–G–C–G) (15×10^{-3} M) in 0.1 M NaCl and 4 M NaCl are shown in Fig. 11. In 0.1 M NaCl, T_m is estimated to be 49°C from Fig. 10. At 34°C, which is well below

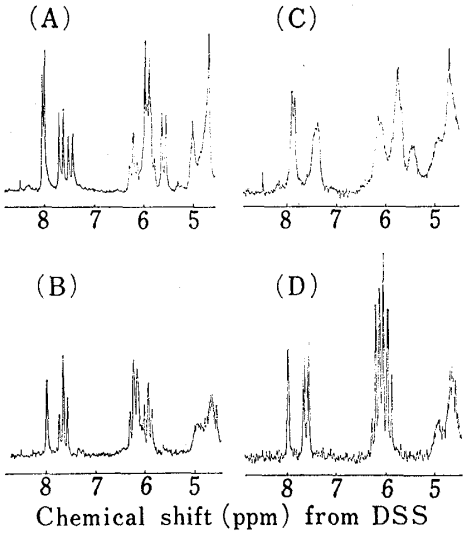

Fig. 11. ^{1}H-NMR Spectra of the Tetramer (15×10^{-3} M Strand Concentration) in 0.1 M NaCl (A, at 34°C; B, at 75°C) and 4 M NaCl (C, at 34°C; D, at 75°C) at pD 7.5

Recorded in the Fourier transform mode under conditions of HOD suppression.

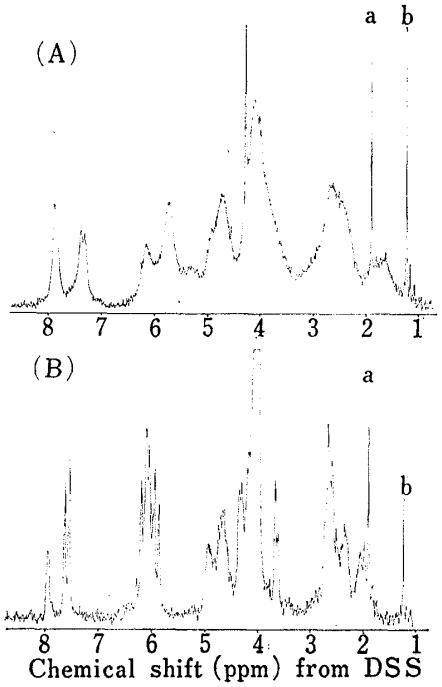

Fig. 12. ^{1}H-NMR Spectra of the Hexamer (15×10^{-3} M Strand Concentration) in 4 M NaCl at 49°C (A) and 88°C (B) at pD 7.5

Recorded in the Fourier transform mode under conditions of HOD suppression. Signal a, impurity from paper; signal b, *tert*-butanol used as an internal reference.

the T_m, the tetramer still shows relatively sharp signals. In 4 M NaCl, the T_m is assumed to be higher than 42°C because the T_m difference at 3×10^{-3} M strand concentration is 7°C. However the spectrum at 34°C is already very broad. With increasing temperature, the signals become sharper and at 75°C, after complete melting, nicely resolved signals can be observed as shown in Fig. 11. In the case of the hexamer at the same strand concentration, a similar phenomenon is observed and the spectrum in 4 M NaCl at 50°C (17°C below the estimated T_m) is as broad as that of the tetramer at 34°C (Fig. 12). Taking into account the chemical shift and coupling constant data of the component monomers and the oligomers with well-resolved spectra, proton signals of base residues and deoxyribose-1′ can be assigned and their temperature dependent chemical shift changes can be traced. In the case of the hexamer, the proton signals at low temperature can be monitored only as a group. Nevertheless, the temperature depedence of the chemical shifts of the tetramer and hexamer are shown in Figs. 13 and 14. The results for 0.1 M NaCl solutions are similar to those obtained by Patel under similar conditions.[18] The H-8 signals of guanine residues of d(C–G–C–G) in 0.1 M NaCl show only a small dependence on temperature. In the case of the H-6 and H-5 signals of the cytosine residues, one signal has nearly constant chemical shift but the others show a great downfield shift upon melting. In the case of the H-1′, one signal remains unchanged but others show large downfield shifts upon melting. In contrast to these results, both H-8 signals of the tetramer in 4 M NaCl show significant downfield shifts upon melting. Both H-6 signals show larger downfield shifts and, again, both H-5 signals show marked downfield shifts upon melting. These differences should be due to the difference in the duplex structures. Similar differences in melting behavior are also observed for the hexamer. The T_m's obtained from the curves for H-5 of the cytosine residues (about 69°C in 0.1 M NaCl and 67°C in 4 M NaCl) are about the same as those estimated from the concentration dependence experiment (71°C and 67°C, respectively). In 4 M NaCl, it was also noted that an H-5′ signal (presumably H-5′ of the terminal dC residue) and some H-2′ signals (presumably H-2′ of the dC residues) show relatively large downfield shifts upon melting (see Fig. 12). Thus, the H-5′ and H-2′ signals shift from 3.3 ppm and 1.7 ppm at 34°C to 3.6 ppm and 2.0 ppm at 75°C, respectively, in the case of the tetramer. The H-5′ and H-2′ signals of the hexamer shift from 3.0 ppm and 1.6 ppm at 34°C to 3.7 ppm and 2.0 ppm at 88°C, respectively.

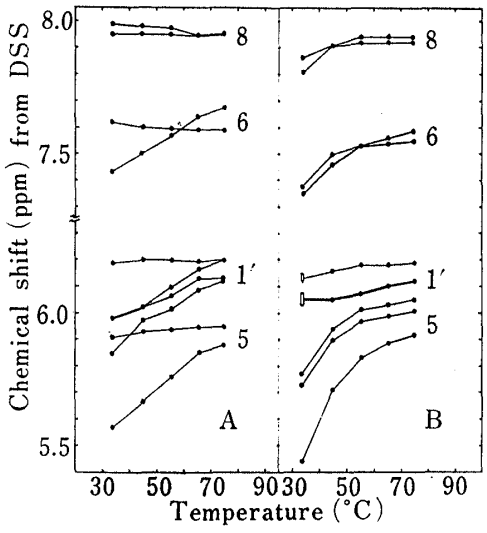

Fig. 13. Chemical Shift-Temperature Profiles for the Tetramer in 0.1 M NaCl (A) and 4 M NaCl (B) at pD 7.5

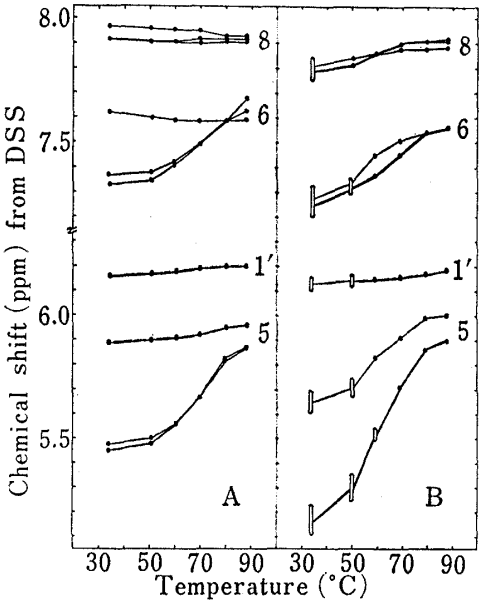

Fig. 14. Chemical Shift-Temperature Profiles for the Hexamer in 0.1 M NaCl (A) and 4 M NaCl (B) at pD 7.5

Discussion

The present studies described above reveal that d(C–G) does not form any kind of duplex under the conditions examined (5×10^{-5}—5×10^{-3} M strand concentration, 0.1 M and 4 M NaCl, above 0°C). d(C–G–C–G) forms a duplex which is assumed to be a B-form even at 3×10^{-5} M strand concentration in 0.1 M NaCl. In 4 M NaCl, the tetramer does not form a duplex at the same strand concentration but forms a different type of duplex, which is assumed to be a Z-form, at 3×10^{-4} M strand concentration. These results imply that the right-handed duplex is destabilized by a very high concentration of salt. On the other hand, d(C–G–C–G–C–G) forms stable duplexes in 0.1 M NaCl (T_m=41.5°C) as well as in 4M NaCl (T_m=36°C) at 2×10^{-5} M strand concentration. In 4M NaCl at three strand concentrations, the hexamer shows T_m's about 5°C lower than those in 0.1 M NaCl. The tetramer also shows lower T_m (by 7°C) in 4 M NaCl at 3×10^{-3} M strand concentration. These results suggest that the high salt duplex is not a particularly stable one and may only be an alternative when the low salt duplex structure is greatly destabilized.

Melting temperature measurement in 4 M NaCl reveals an apparent discrepancy between the results obtained by the UV and CD methods. The [θ]-temperature profile was monitored at 294 nm, where the characteristic negative band is observed. It does not show a sigmoidal curve but a linear change in a positive direction with increasing temperature, and levels off at around the T_m obtained by the UV method. This phenomenon can be divided into three steps. They are a positive increase of the [θ] without UV hyperchromism, a positive increase of the [θ] with UV hyperchromism and no apparent change of the [θ] with continuous UV hyperchromism. The presence of the first step can be seen more clearly in the [θ] change in the 250—260 nm region (compare the spectra at 7°C and 25°C of the hexamer in Figs. 4 and 6). Relatively sharp UV hyperchromism upon heating of a nucleic acid solution is thought to reflect a destacking of the bases due to a cooperative dissociation of a base-paired complex. Therefore, the first step should involve some intra-duplex conformational change. Crystallographic studies of oligomers and polymer containing repeating dC–dG sequences show that they can take a variety of conformations within a left-handed double helix. Those are designated by the authors as Z-form,[3a] Z′-form,[4a] Z_{II}-form[3b] and S-form.[5,19] DNA, in general, seems to be conformationally flexible, mainly because of the flexibility in sugar puckering and glycosidic conformation.[19,20] The second and third steps must involve a dissociation of a duplex or duplexes. However the dissociation process is not a simple one but may involve a rather complex mixture of species . In the simplest case, it may involve a left-handed duplex,a left-handed single helix and a right-handed single helix. It could even contain a right-handed duplex. If the left-handed single helix with dG residues in a *syn* form is relatively unstable and is converted rapidly to a right-handed single helix with dG residues in an *anti* form, cancellation of CD bands of the left-handed duplex will occur in the melting process and result in an apparent leveling off of the positive [θ] increase at 294 nm before complete melting. Interconversion between a right-handed duplex and a left-handed helix has been reported in the case of poly(dG–dC) in very low salt solution (0.2 mM NaCl) at around the melting temperature of the right-handed duplex.[21]

^{1}H-NMR study reveals that the conformations in the high salt and low salt duplexes are quite different. The difference is apparent in the broadness of the signal linewidth and the pattern of temperature-dependent chemical shift change of the signals. For the low salt form, one the H-6 or H-5 signals of the cytosine residues remains almost unchanged and the H-8 of the guanine residues shows no downfield shift upon heating. For the high salt form, all of the base protons of the cytosine residues show considerable downfield shifts and H-8 signals of the guanine residues show significant downfield shifts. These phenomena can be explained in terms of a B–DNA model for the low salt form and a Z-form for the high salt form. The base stacking patterns for d(C–G–C–G) in the Z-form and B-form

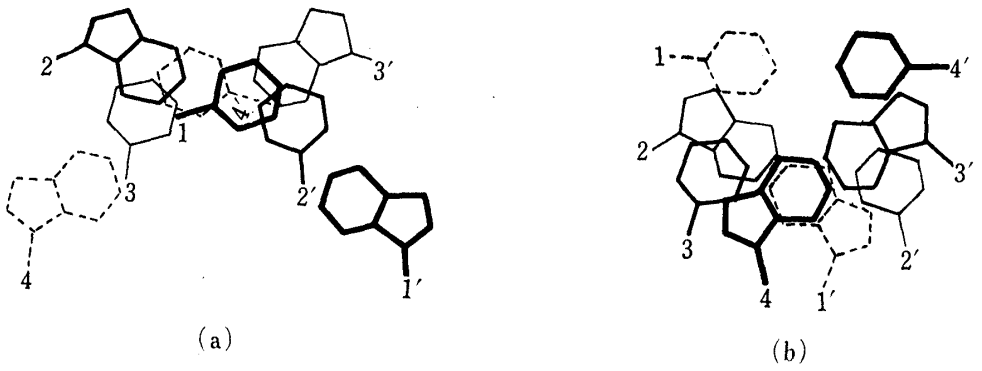

Fig. 15. The Base Stacking Patterns of d(C–G–C–G) for Z-form (a) and B-form (b) Duplexes

The geometries of the both forms are taken from ref. 3*a*). The bases are numbered from the 5′-end as 1—4 for one strand and as 4′—1′ for the other strand, and, therefore, the bases n and n′ make a base-pair.

are illustrated in Fig. 15. The geometries of the base pairs in the two forms are taken from the paper of Wang *et al.*[3a] It can be readily seen from Fig. 15b that the 5′-terminal cytosine residues (designated as 1 and 4′) are not stacked on any other bases and, therefore, their H-5 and H-6 may not be shielded by the ring-current effect. On the other hand, the internal cytosine residues (designated as 3 and 2′) are stacked on the nearest-neighbor guanine bases (bases 2 and 3′) and therefore, their base protons may be shielded. A similar conclusion has been obtained by calculation of the ring-current upfield shift contribution[18] using the contour maps of Giessner–Prettre and Pullman.[22] In the case of Z-form structure (Fig. 15a), the 5′-terminal cytosine residues (bases 1 and 4′) are stacked with the nearest-neighbor cytosine residues (bases 2′ and 3, respectively) and the next-neighbor guanine residues (bases 3′ and 2, respectively) of the opposite strand. The internal cytosine residues (bases 3 and 2′) are stacked with the nearest-neighbor guanine residues (bases 2 and 3′, respectively) of the same strand and next-neighbor cytosine residues (bases 4′ and 1, respectively) of the opposite strand. Therefore both terminal and internal cytosine protons may receive a considerable shielding. It should be noted that the guanine residues are arranged in the outer part of the helix in the Z-form structure and the H-8 protons are expected to receive no shielding. The experimentally observed upfield shifts of the guanine H-8 signals upon duplex formation may be associated with the change of the glycosidic conformation from *anti* to *syn*. It is known that H-8 of purine 5′-nucleotides in the *anti* conformation is deshielded by an anisotropic effect of the 5′-phosphate group.[23] Theoretical calculation of the chemical shift dependence on glycosidic conformation in 5′-nucleotides supports this trend.[24] Conformational change from *anti* to *syn* conformation, where this deshielding effect is absent, will bring about an apparent upfield shift of H-8.

When we consider the present results on the stability of the left-handed duplex, it is surprising that crystals of d(C–G–C–G–C–G) and even d(C–G–C–G) in Z-form structure have been obtained from a solution of low salt concentration (15 mM $MgCl_2$, 30 mM sodium cacodylate).[3,4b] There is no doubt that the major species in such a low salt solution is the right-handed duplex, though crystals of that form are not obtained. Therefore the Z-form duplex must be much more crystallizable than the B-form duplex, presumably because of the more stable packing of the molecule in the crystal. Some kind of factor which destabilizes the B-form structure and/or flips the glycosidic conformation of the guanine residues from *anti* to *syn* form may be needed for an alternating CG sequence in DNA to take a Z-form structure under physiological conditions. In fact, we have obtained evidence which suggests that even a dimer can form a Z-form duplex when a *syn* conformation of the guanine residue is

stabilized by the introduction of a bromo group at C-8.[25] This results will be published elsewhere.

Experimental

Materials and General Procedures——Paper chromatography was performed by a descending technique on Whatman No. 1 paper using the following solvent systems: solvent A, *n*-butanol–acetic acid–water (5: 2: 3); solvent B, ethanol–1 M ammonium acetate (pH 7) (7: 3); solvent C, *n*-propanol–conc. 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 buffer (pH 7.5) or 0.05 M ammonium acetate buffer (pH 3.7). Silica gel column chromatography was carried out with Merck silica gel 60H or silica gel 60H silanized. Thin-layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ using $CHCl_3$–methanol mixture or silica gel $RP\text{-}18F_{254}$ pre-coated sheets using acetone–water mixture. Nuclease P1[13] was obtained from Yamasa Shoyu Co. and incubation was carried out in 0.05 M ammonium acetate (pH 5) at 37°C for 2 h with the enzyme (5 μg/ml).

Ultraviolet absorption (UV) spectra were recorded on a Hitachi 124 spectrophotometer. For temperature variation experiments, a Komatsu Solidate SPD-H-124 thermostated cell was used. The temperature within the cell was measured by a Shibaura MGB-III thermister. Circular dichroism (CD) spectra were recorded on a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. For temperature variation experiments, a thermo-jacketed cell and a Neslab RTE-8 circulating bath were used. The temperature of the cell was measured by the same thermister. For measurement of high concentration samples, cells of 1, 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.

N-Benzoyl-5′-O-monomethoxytrityldeoxycytidine (d-(MeOTr)bzC, **1**)[26] and N-isobutyryl-3′-O-benzoyldeoxyguanosine (d-ibGOBz, **3**) were synthesized according to published procedures.[26,27] N-Isobutyryl-3′-O-(*o*-chlorophenyl)(*p*-methoxyanilido)phospho-deoxyguanosine (d-ibG*p*·, **6**) was synthesized by a modification of the procedure of Ohtsuka *et al.*[11]

d-bzC*p*ibGOBz (**5**)——*o*-Chlorophenylphosphorodichloridate[28] (9.32 g, 38 mmol), 1,2,4-triazole (5.28 g, 76.5 mmol), triethylamine (10.75 ml) and dioxane (100 ml) were mixed under cooling in an ice-bath and the mixture was stirred at room temperature for 1 h. The solid materials (triethylammonium hydrochloride) were filtered off and washed with a small volume of dioxane. The filtrate and washing were combined and d-(MeOTr)bzC (**1**, 7.22 g, 11.96 mmol) in pyridine (50 ml) was added. The mixture was kept at room temperature for 2 h and 50% aqueous pyridine was added. d-(MeOTr)bzCp (**2**) was extracted with $CHCl_3$ (150 ml) and washed with 0.1 M triethylammonium bicarbonate ($Et_3NH_2CO_3$, 8 ml × 5) and water (100 ml × 2). The solvent was evaporated off. The residue was evaporated with pyridine (three times) and dissolved in pyridine (30 ml). A 10 ml portion of this solution was used in the following step. **2** (3.99 mmol) in pyridine (10 ml), d-ibGOBz (**3**, 1.24 g, 2.8 mmol), pyridine (25 ml) and mesitylenesulfonyl-4-nitroimidazolide[12] (MSNI, 1.77 g, 6.0 mmol) were mixed and kept at 30°C for 15 h. The volume of the reaction mixture was reduced to about one-third and further MSNI (1.77 g) was added. The mixture was kept at 30°C for 18 h. The reaction was checked for disappearance of the nucleotide component at the origin by TLC on a silica gel plate using $CHCl_3$–ethanol (10: 1). 50% aqueous pyridine was added. The product was extracted with $CHCl_3$ (100 ml) and washed with 0.1 M $Et_3NH_2CO_3$ (200 ml × 2) and water (200 ml × 3). The solvent was evaporated off and the residue was evaporated with pyridine and toluene. The resulting gum was dissolved in $CHCl_3$; this solution was applied to a column of silica gel H (40 g) and eluted stepwise with $CHCl_3$–95% ethanol mixtures (98: 2—96: 4, about 300 ml). The flow rate was increased by suction with an aspirator. The fractions containing the dimer were combined and concentrated. The residue was evaporated with pyridine and toluene. d-(MeOTr)bzC*p*ibGOBz (**4**) thus obtained was treated with 2% benzenesulfonic acid in $CHCl_3$–methanol (7: 3, 400 ml) at 0°C for 40 min. The mixture was neutralized with saturated $NaHCO_3$ and washed with the same solutions (200 ml × 2) and water (200 ml × 3). The organic fraction was concentrated and the residue was evaporated with pyridine and toluene. The gum was dissolved in $CHCl_3$ and chromatographed on a column of silica gel H (40 g). Elution was carried out in the same manner as described above. **5** was isolated by precipitation from hexane. The yield was 1.11 g (1.18 mmol, 42% from **3**).

d-(MeOTr)bzC*p*ibG*p* (**7**)——A solution of **2** (7.97 mmol) in pyridine (20 ml) prepared as above, pyridine (50 ml), d-ibG*p*· (**6**, 3.51 g, 5.55 mmol) and MSNI (3.55 g, 12 mmol) were mixed and kept at 30°C for 19 h. The volume of the reaction mixture was reduced to the one-third by evaporation and further MSNI (3.56 g) was added. The reaction mixture was kept at 30°C for 15 h. The reaction was checked by TLC as described earlier. 50% aqueous pyridine was added and the product was extracted with $CHCl_3$ (150 ml). The organic layer was washed with 0.1 M $Et_3NH_2CO_3$ (100 ml × 4) and water (100 ml × 3). The solvent was evaporated off and the residue was evaporated with pyridine and toluene. The gum was dissolved in $CHCl_3$ and chromatographed on a column of silica gel H (40 g). Elution was carried out as described earlier. The crude product was further purified by re-chromatography on the same column. d-(MeOTr)bzC*p*ibG*p*· thus obtained was treated with isoamyl nitrite (24 ml) in pyridine–acetic acid (5: 4, 120 ml) at room temperature for

4 h with stirring. 10% aqueous pyridine was added and the mixture was washed with ether–hexane (1: 1). The product was extracted with $CHCl_3$ (100 ml and 50 ml) and washed with 0.2 M $Et_3NH_2CO_3$ (100 ml × 5) and water (100 ml × 3). The organic fraction was concentrated and the residue was evaporated with pyridine. TLC on a silanized silica gel plate using acetone–water (7: 3) showed the presence of some by-product, presumably the 5′-sulfonate compound. The gum was dissolved in ethyl acetate (60 ml) and washed with 50% aqueous pyridine. The aqueous layer was back-extracted with ethyl acetate–ether (3: 1 and 1: 1). The fractions containing the product were combined and concentrated. The resulting gum was dissolved in acetone–water (6: 4) and chromatographed on a column (3 × 30 cm) of silanized silica gel. Elution was carried out with acetone–water (6: 4). The fractions containing the desired product was combined and concentrated. The residue was evaporated with pyridine and toluene and dissolved in $CHCl_3$. **7** was isolated by precipitation from ether–hexane (1: 1, 200 ml). The yield was 2.11 g (1.50 mmol, 27% from **6**).

d(C-G) (8)——**4** (360 mg, 0.3 mmol) was treated with concentrated NH_4OH (20 ml) in pyridine (10 ml) at 60°C for 5 h. Ammonia was largely removed by bubbling nitrogen gas through the solution. After addition of a small volume of *n*-butanol, the solvent was evaporated off. The residue was treated with 80% aqueous acetic acid (50 ml) at room temperature for 2 h and the solvent was evaporated off. The residue was dissolved in 20% aqueous pyridine (60 ml) and washed with ether (50 ml). After evaporation of the solvent, the residue was dissolved in water and chromatographed on a column (2.3 × 40.7 cm) of diethylaminoethyl (DEAE)-cellulose (DE-23, bicarbonate form). The column was washed with water (1 l) and eluted with 0—0.2 M $Et_3NH_2CO_3$ (total 4 l) at a flow rate of 85 ml/h. **8** was eluted at around 0.056 M salt concentration. The yield was 4020 A_{250} units (0.21 mmol, 70%). The chromatographic properties, UV data and CD spectra are presented in Tables I and II and Figs. 2—4. This compound was completely hydrolyzed with nuclease P1 to give dC and d-pG in a 1.0: 1.0 ratio.

d-bzC*p*ibG*p*bzC*p*ibGOBz (10)——A solution of **7** (1.53 g, 1.09 mmol) and **5** (947 mg, 1.0 mmol) in pyridine (10 ml) was treated with MSNI (484 mg, 1.64 mmol) at 30°C for 13.5 h. After appropriate work-up as described for the preparation of **5**, d-(MeTr)bzC*p*ibG*p*bzC*p*ibGOBz (**9**) was isolated by chromatography on a column of silica gel H (10 g). **9** was treated with 2% benzenesulfonic acid in $CHCl_3$–methanol (7: 3, 317 ml) at 0°C for 1.5 h. After appropriate work-up and chromatography on a column of silica gel H (30 g) as described for the preparation of **5**, **10** was isolated by precipitation from ether–hexane (1: 3, 200 ml). The yield was 1.56 g (0.80 mmol, 80% from **5**).

d(C-G-C-G) (11)——**9** (0.5 mmol) prepared as above and isolated by precipitation from ether–hexane was treated with concd. NH_4OH and 80% aqueous acetic acid successively as described for the preparation of **8**. The tetramer was isolated by chromatography on a column (2.3 × 41 cm) of DEAE-cellulose (DE-23, bicarbonate form). The column was washed with water (1 l) and eluted with 0.1—0.3 M $Et_3NH_2CO_3$ (total 4 l) at a flow rate of 85 ml/h. **11** was eluted at around 0.26 M salt concentration. The yield was 9070 A_{250} units (0.27 mmol, 54%). The chromatographic properties, UV spectral data and CD spectra are presented in Tables I and II and Figs. 2—4. This compound was completely hydrolyzed with nuclease P1 to give dC, d-pC and d-pG in a ratio of 1.0: 1.1: 2.0 as examined by paper electrophoresis at pH 3.7.

d-(MeTr)bzC*p*ibG*p*bzC*p*ibG*p*bzC*p*ibGOBz (12)——A solution of **7** (491 mg, 0.35 mmol) and **10** (590 mg, 0.30 mmol) in pyridine (3.5 ml) was treated with MSNI (155 mg, 0.52 mmol) at 30°C for 17.5 h. After appropriate work-up as described for the preparation of **5**, **12** was isolated by chromatography on a column of silica gel H (20 g). Precipitation from ether–hexane (3: 1, 100 ml) gave 759 mg of **12** (0.23 mmol, 78%).

d(C-G-C-G-C-G) (13)——**12** (393 mg, 0.21 mmol) was treated in the same manner as described for the preparation of **5**. The final residue was dissolved in 0.02 M Tris-HCl (pH 8.0)–7 M urea (100 ml) and chromatographed on a column (2.3 × 36 cm) of DEAE-cellulose (DE-23, chloride form). The column was washed with the same buffer and eluted with 0.15—0.25 M NaCl in 0.02 M Tris–HCl (pH 8.0)–7 M urea (total 3 l) at a flow rate of 75 ml/h. **13** was eluted at around 0.21 M NaCl concentration. The yield was 3230 A_{250} units (0.06 mmol, 55%). The chromatographic properties, UV spectra, UV spectral data and CD spectra are presented in Tables I and II and Figs. 1—5. This compound was completely hydrolyzed with nuclease P1 to give dC, d-pC and d-pG in a ratio of 1.0: 1.9: 2.9 as examined by paper electrophoresis at pH 3.7.

^{1}H-NMR Measurement——The tetramer and hexamer were purified by paper electrophoresis at pH 7.5 on Whatman 3MM paper. Each oligomer sample was passed through columns of Dowex 50 (Na^+ form) and Chelex 100 resins, successively, and lyophilized three times from D_2O solution. To the residue, D_2O solutions of sodium phosphate buffer (pD 7.5) and NaCl were added to make 0.01 M sodium phosphate–0.1 M NaCl or 0.01 M sodium phosphate–4 M NaCl solution containing the oligomer (15×10^{-3} M) (0.4 ml). ^{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 Cu-Constantan thermocouple. The ^{1}H chemical shifts were determined relative to internal *tert*-butanol, which had in turn been referenced to DSS (sodium 1-trimethylsilyl-propyl-3-sulfonate). The HDO signal was suppressed with the 180°–τ–90° pulse sequence.

References and Notes

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2) Abbreviations used: d-N*p*N, a deoxyribooligonucleotide *o*-chlorophenyl phosphotriester; d-N*p*·, a deoxyribonucleoside with a 3′-*O*-(*o*-chlorophenyl)(*p*-methoxyanilido)phospho group; MeOTr, 5′-*O*-monomethoxytrityl; bz, N-benzoyl; ib, N-isobutyryl; OBz, 3′-*O*-benzoyl; MSNI, mesitylenesulfonyl 4-nitro-imidazolide. Other abbreviations principally follow recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, **9**, 4022 (1970), *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2222 (1977)].
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