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Synthesis and Properties of d(ATACGCGTAT) and Its Derivatives Containing One and Two 5-Methylcytosine Residues. Effect of the Methylation on Deoxyribonucleic Acid Conformation

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In order to elucidate the effects of cytosine methylation on deoxyribonucleic acid (DNA) conformation, three self-complementary deoxyribodecanucleotides, d(ATACGCGTAT), d(ATACGm^{5}CGTAT) and d(ATAm^{5}CGm^5 CGTAT), were synthesized by a modified phosphotriester method and their conformational properties were examined by ultraviolet (UV) and circular dichroism (CD) spectroscopy. The two modified decamers were completely resistant to restriction endonuclease *Mlu*I, whose recognition sequence is ACGCGT, while the unmodified decamer was completely cleaved by the enzyme. All the decamers were assumed to exist as a B-form duplex in 0.1 M NaCl. The UV-temperature profiles suggested that the modified decamer duplexes have slightly higher stability to thermal perturbation but their melting processes are significantly less cooperative, as compared to the case of the unmodified decamer. All three decamers showed similar CD spectra under various salt conditions, including 4 M NaCl and 6 M CsF. However, substantial differences in CD spectra were observed in 8 M $NaClO_4$ at −20 °C. Both modified decamers were assumed to exist as a Z-form duplex under these extreme conditions, and they showed similar UV- and CD-temperature profiles which were quite different from those of the unmodified decamer.

Keywords——DNA; oligonucleotide; 5-methylcytosine; UV; CD; restriction endonuclease; Z-DNA; conformation

5-Methylcytosine (m^{5}C) is a minor base in deoxyribonucleic acid (DNA) of both prokaryotes and eukaryotes. In prokaryotes, m^{5}C is known to be involved in restriction–modification systems. Most restriction endonucleases cannot cleave DNA at methylated sequences. In eukaryotes, m^{5}C is assumed to be involved in the regulation of gene expression and also in cell differentiation.[1,2] m^{5}C is the only modified base in eukaryotic DNA and occurs predominantly in the sequence CpG (average of 70% of all CpG sequence in animal DNA).[2] On the other hand, m^{5}C is known to promote B–Z transition of DNA containing alternating pyrimidine–purine sequences.[3]

In order to elucidate the effect of 5-methylation of cytosine residues on the conformation and function of NDA, we have synthesized three self-complementary deoxyribodecanucleotides, d(ATACGCGTAT), d(ATACGm^{5}CGTAT) and d(ATAm^{5}CGm^{5}CGTAT) that contain no, one and two m^{5}C residues in a decamer strand, respectively. These decamers have perfectly alternating purine–pyrimidine sequences and contain a CGCG core flanked by ATA and TAT segments. In this paper, we report the synthesis, enzymatic activity and optical properties of these decamers and discuss the effects of methylation.

Materials and Methods

General Procedures——Thin layer chromatography (TLC) was performed on a sheet of Silica gel $60F_{254}$

(Merck) with a mixture of $CHCl_3$–methanol. For reverse-phase TLC (RTLC), Silica gel $60F_{254}$ silanized (Merck) or HPTLC RP-$18F_{254S}$ (Merck) was used with a mixture of acetone–water. For column chromatography, silica gel (type 60, Merck), alkylated silica gel (C-18, Waters) and DEAE-cellulose (DE52, Whatman) were used. Analytical high-pressure liquid chromatography (HPLC) was performed on a column of alkylated silica gel (μ Bondapak C-18).

Ultraviolet (UV) spectra were recorded on a JASCO UVIDEC 610C spectrophotometer. UV-temperature profiles in 0.1 M NaCl were recorded on a Beckman DU-8B spectrophotometer with a Tm accessory. Circular dichroism (CD) spectra were measured on a JASCO J-500A spectrometer. The molar absorption coefficient, ε, and molar ellipticity, $[\theta]$, are presented in terms of per base residue.

Nuclease P1 was obtained from Yamasa Shoyu Co. (Choshi, Japan). Restriction endonuclease *Mlu*I was purchased from Takara Shuzo Co. (Kyoto, Japan). 5-Methyldeoxycytidine was synthesized according to the published procedure.[4]

d-bzm^{5}C——Benzoyl chloride (20 mmol) was added dropwise to a stirred suspension of d-m^{5}C–HCl (5 mmol) in pyridine (25 ml) at 0 °C, and the mixture was stirred at 0 °C for 10 min, then at room temperature for 3 h. Disappearance of the starting material was checked by TLC ($CHCl_3$–MeOH, 50 : 1). Aqueous pyridine (20%, 20 ml) was added to the reaction mixture cooled at 0 °C, and the whole was diluted to 70 ml with water and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 5% $NaHCO_3$ and water and evaporated to dryness. The residue was dissolved in pyridine (30 ml)–ethanol (20 ml). A mixture of 2 N NaOH (30 ml)–ethanol (10 ml) was added to the solution at 0 °C with stirring, and the reaction mixture was stirred at 0 °C for 7 min, then at room temperature for 8 min. The mixture was again cooled to 0 °C and added to a stirred suspension of pyridinium Dowex 50 resin (100 ml) in water. The whole mixture was poured into a column containing additional pyridinium Dowex 50 resin. The column was washed with 20% aqueous pyridine (bed volume × 3). The effluents were evaporated, and the residue was crystallized from 10% aqueous pyridine to give 3.64 mmol of d-bzm^{5}C (73%); TLC ($CHCl_3$–MeOH, 5 : 1) *Rf* 0.53; RTLC (60 F_{254} silanized, acetone–water, 6 : 4) *Rf* 0.63.

d-[(MeO)$_2$Tr]bzm^{5}C——d-bzm^{5}C (3.64 mmol) was dried by repeated evaporation with pyridine and dissolved in pyridine (5 ml) (sometimes the mixture remained partially as a suspension). Dimethoxytrityl chloride (4.6 mmol, 1.26 eq) was added. After evaporation of a small volume of the solvent, the reaction mixture was kept at room temperature for 2 h. Disappearance of the starting material was checked by TLC ($CHCl_3$–MeOH, 10 : 1). Aqueous pyridine (20%) was added at 0 °C, then the mixture was diluted to 40 ml with water and the whole was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with water and evaporated to dryness. The residue was dried by evaporation with toluene and chromatographed on a column (6.5 × 5.5 cm) of Silica gel 60. Elution was carried out stepwise with $CHCl_3$–MeOH mixtures (100 : 1, 75 : 1 and 50 : 1). The appropriate fractions were pooled and evaporated. d-(MeO)$_2$Tr bzm^{5}C was precipitated with *n*-pentane (200 ml) from its solution in $CHCl_3$ (10 ml). The yield was 2.58 mmol (71%); TLC ($CHCl_3$–MeOH, 10 : 1 *Rf* 0.42; RTLC ($60F_{254}$ silanized, acetone–water, 7 : 3) *Rf* 0.42.

d-[(MeO)$_2$Tr]bzm^{5}Cṗ (4m)[5]——*p*-Chlorophenyl phosphorodichloridate (5.24 mmol) was added dropwise to a suspension of triazole (11.6 mmol) in dioxane (12 ml)–triethylamine (1.6 ml, 11.5 mmol) at 0 °C with stirring, and the reaction mixture was stirred for 1.5 h. The precipitated triethylammonium hydrochloride was removed by filtration. The filtrate was added to a solution of d-[(MeO)$_2$Tr]bzm^{5}C (2.58 mmol) in pyridine (8 ml) which had been dried by evaporation with pyridine. The mixture was concentrated to *ca.* 8 ml under reduced pressure and kept at room temperature for 3 h. Disappearance of the nucleoside component was checked by TLC ($CHCl_3$–MeOH, 10 : 1) and RTLC (acetone water, 7 : 3). Aqueous pyridine (20%) was added at 0 °C, and the mixture was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 0.1 M triethylammonium bicarbonate ($Et_3NH_2CO_3$) and evaporated. The residue was dried by repeated evaporation with pyridine and used for the condensation reactions without further purification.

Synthesis of d-[(MeO)$_2$Tr]bzm^{5}CṗibGṗan (8m) by Condensation——A mixture of **4m** (2.58 mmol) and d-ibGṗan (**5**, 1.96 mmol) was dried by evaporation with pyridine. After addition of 1-mesitylenesulfonyl-4-nitroimidazole (7.74 mmol) and evaporation of a small volume of the solvent, the reaction mixture was kept at room temperature for 1 h. Disappearance of **5** was checked by TLC ($CHCl_3$–MeOH, 10 : 1) and RTLC (60 F_{254} silanized, acetone–water, 7 : 3). Aqueous pyridine (20%) was added to the mixture cooled at 0 °C, and the whole was diluted to 40 ml with water, then extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 0.1 M triethylammonium bicarbonate buffer and evaporated to dryness. The residue was evaporated with pyridine then with toluene, and chromatographed on a column (6.5 × 5.6 cm) of Silica gel 60 (100 g). Elution was carried out stepwise with $CHCl_3$–MeOH mixtures (100 : 1, 75 : 1, 60 : 1, 50 : 1, 40 : 1 and 20 : 1). The appropriate fractions were pooled and evaporated. Compound **8m** was precipitated with *n*-pentane (300 ml) from its solution in $CHCl_3$ (15 ml). The yield was 1.79 mmol (91%): TLC ($CHCl_3$–MeOH, 10 : 1) *Rf* 0.46; RTLC (60 F_{254} silanized, acetone–water, 7 : 3) *Rf* 0.14.

Synthesis of d-[(MeO)$_2$Tr]bzAṗTṗbzAṗbzm^{5}CṗibGṗ (14m) by Removal of the *p*-Anisido Group——d-[(MeO)$_2$Tr]bzAṗTṗbzAṗbzm^{5}CṗibGṗan (0.163 mmol), which had been dried over P_2O_5, was dissolved in a mixture of pyridine–acetic acid (5 : 4, 8.7 ml) and triethylamine (0.21 ml, 1.51 mmol). Isoamyl nitrite (0.87 ml, 6.54 mmol) was added, and the mixture was shaken at 25—30 °C for 8.5 h. A mixture of pyridine–0.2 M triethylammonium bicarbonate buffer (1 : 1, 13 ml) was added to the stirred reaction mixture at 0 °C, and the whole was stirred at room

temperature for 3 h then cooled to 0 °C. Enough 0.2 M triethylammonium bicarbonate was added to obtain a slightly turbid solution, and the mixture was washed with *n*-pentane–ether (1 : 1) and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 0.2 M triethylammonium bicarbonate buffer and evaporated. The residue was dried by repeated evaporation with pyridine and used for the condensation reaction: TLC ($CHCl_3$–MeOH, 10 : 1) *Rf* 0.0; RTLC (HPTLC RP-18, acetone–0.02 M triethylammonium acetate, 7.5 : 2.5) *Rf* 0.56.

Preparation of d-bzm^{5}CṗibGṗTṗbzAṗTOBz (15m) by Removal of the Dimethoxytrityl Group——Benzenesulfonic acid monohydrate in MeOH (16.5 g/500 ml, 9 ml) was added to a stirred solution of d-[$(MeO)_2$Tr]-bzm^{5}CṗibGṗTṗbzAṗTOBz (obtained by condensation of **12** (0.4 mmol) and **13** (0.3 mmol)) in $CHCl_3$ (19 ml) at 0 °C. The reaction mixture was stirred at room temperature for 1 h, then 5% $NaHCO_3$ (19 ml) was added to the cooled mixture at 0 °C with stirring, and the whole was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 5% $NaHCO_3$ then with water, dried by evaporation with pyridine, and chromatographed on a column of Silica gel 60. Compound **15m** was precipitated with *n*-pentane: TLC ($CHCl_3$–MeOH, 10 : 1) *Rf* 0.25, RTLC (HPTLC RP-18, acetone–0.02 M triethylammonium acetate, 7.5 : 2.5); *Rf* 0.38.

Preparation of d-[$(MeO)_2$Tr]bzAṗTṗbzAṗbzm^{5}CṗibGṗbzm^{5}CṗibGṗTṗbzAṗTOBz (16 mm)——A mixture of **14m** (0.163 mmol) and **15m** (0.117 mmol) was dried by repeated evaporation with pyridine and dissolved in pyridine (2.1 ml). After addition of 1-mesitylenesulfonyl-1,2,3,4-tetrazole (0.652 mmol, 4 eq to **14m**) and evaporation of a small volume of the solvent, the reaction mixture was shaken at 30 °C for 25 min then cooled to 0 °C. Aqueous pyridine (50%)–0.2 M triethylammonium bicarbonate (1 : 1) was added, and the whole was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 0.05 M triethylammonium bicarbonate buffer and evaporated. The residue was dried by evaporation with pyridine and chromatographed on a column (3 × 5.5 cm) of alkylated Silica gel C-18. Elution was carried out stepwise with acetone–1.5% aqueous pyridine mixtures (65 : 35, 72.5 : 27.5 and 75 : 25). The appropriate fractions were pooled and evaporated. The residue was dried by evaporation with pyridine and precipitated with *n*-pentane. The yield was 0.0977 mmol (83.5% from **15m**): TLC ($CHCl_3$–MeOH, 8 : 1) *Rf* 0.80; RTLC (HPLC RP-18, acetone–0.02 M triethylammonium acetate, 7.8 : 2.2) *Rf* 0.30.

d(ATAm^{5}CGm^{5}CGTAT) (17mm)——**16mm** (28.1 μmol) was dissolved in 1 M tetraethylguanidinium *syn*-pyridine-2-aldoximate in dioxane (13.5 ml), then water was added, and (13.5 ml) the mixture was shaken at 30 °C for 49 h. Conc. NH_4OH (36 ml) was added. The mixture was kept at 55 °C for 4.5 h and evaporated. The residue was evaporated with 20% aqueous pyridine, dissolved in 20% aqueous pyridine (40 ml) and cooled to 0 °C. Dowex 50 resin (pyridinium form, 50 ml) was added to the stirred solution, and the mixture was shaken at room temperature for 10 min then filtered. The filtrate was evaporated. A solution of the residue in dilute aqueous pyridine (100 ml) was washed with ethyl acetate and evaporated. The residue was evaporated with toluene and treated with 80% aqueous acetic acid (38 ml) at 30 °C for 25 min with shaking. The residue was evaporated with water. Dilute aqueous pyridine (50 ml) was added, the mixture was extracted with ethyl acetate, and the aqueous fraction was evaporated. After evaporation with water, the residue was dissolved in 7 M urea–20 mM Tris–HCl (pH 8.0) (45 ml) and applied to a column (2.5 × 61 cm) of DEAE-cellulose (chloride form) equilibrated with the same 7 M urea–buffer mixture. Elution was carried out with a linear gradient of NaCl (0.08—0.35 M, total 6 l) in 7 M urea–20 mM Tris–HCl (pH 8.0), and 1380 A_{260} units (16.8 μmol, 60%) of pure **17mm** were eluted at around 0.26—0.27 M NaCl. The appropriate fractions were pooled, diluted 5-fold with water and passed through a small column of DEAE-cellulose. The column was thoroughly washed with 50 mM triethylammonium bicarbonate, then eluted with 1 M triethylammonium bicarbonate. After being desalted by repeated evaporation with water, the decanucleotide was treated successively with columns of Dowex 50 (pyridinium form) (2 ml), Dowex 50 (Na form) (1.5 ml) and Chelex 100 (Na form) (1.5 ml) and lyophilized to give the Na salt of **17mm** which was suitable for nuclear magnetic resonance (NMR) measurement or for crystallization.

d(ATACGCGTAT) (17)——The fully protected decamer (**16**, 25.8 μmol) was successively treated with the oximate, conc. NH_4OH and 80% acetic acid as described above, and the free decamer was chromatographed on a column (2.5 × 70 cm) of DEAE-cellulose (chloride form) using the 7 M urea system. Elution was carried out as described above. Fractions containing the pure decamer (1020 A_{260} units) were worked up as described above. Fractions containing the decamer and some impurities, which were eluted before and after the pure fractions, were pooled and desalted with a small column of DEAE-cellulose. The decamer was purified by reverse-phase chromatography on a column (1.1 × 26 cm) of alkylated Silica gel C-18. Elution was carried out with a linear gradient of aqueous acetonitrile (10%—20%) in 0.1 M triethylammonium acetate (total 400 ml), and the appropriate fractions were pooled, evaporated and desalted with a small column of DEAE-cellulose. The total yield of **17** was 1700 A_{260} units (20.3 μmol, 79%). The decamer was converted to the Na salt as described above.

d(ATACGm^{5}CGTAT) (17m)——The fully protected decamer (**16m**, 9.43 μmol) was deprotected as described above. The free decamer was chromatographed on a column (1.6 × 50 cm) of DEAE-cellulose (chloride form) using the 7 M urea system. Elution was carried out with a linear gradient of NaCl (0.05—0.35 M, total 2 l) in the same urea–buffer solution as described above. Fractions containing the pure decamer (630 A_{260} units, 7.95 μmol, 84%) were worked up as described above.

Cleavage of d(ATACGCGTAT) with Restriction Endonuclease *Mlu*I——d(ATACGCGTAT) was labeled with a [^{32}P]phosphate group at the 5′-end using polynucleotide kinase and purified by gel filtration on Sephadex G-50. The decamer (0.5—2.0 μM) in 10 mM Tris–HCl (pH 7.5), 7 mM $MgCl_2$, 150 mM NaCl, 7 mM 2-mercaptoethanol was

annealed by heating to 60—70 °C followed by incubation at 30 °C for 1 h. Then the decamer was incubated with *Mlu*I (0.5 unit/μl) at 30 °C (total volume 20 μl). An aliquot (3 μl) was removed after a certain time, mixed with 25 mM sodium EDTA (pH 7.0, 4 μl), heated at 90 °C for 2 min and analyzed by homochromatography[6] with homomix III.[7] The extent of cleavage reaction was determined by measuring the radioactivities of the remaining decamer and d(pATA) eluted from the corresponding spots.

Results and Discussion

Synthesis of the Decanucleotides

The deoxyribodecanucleotides, d(ATACGCGTAT) (**17**), d(ATACGm^{5}CGTAT) (**17m**) and d(ATAm^{5}CGm^{5}CGTAT) (**17mm**), were synthesized by a modified phosphotriester method with a *p*-methoxyanilido group for temporary protection of the 3′-terminal phosphate groups.[8] The block condensation method was employed, as shown in Fig. 1. The intermediate oligonucleotide blocks and the fully protected decanucleotides were purified by chromatography on silica gel or alkylated silica gel columns. The yields of the condensation reactions are summarized in Table I. The fully protected decamers were deprotected by successive treatment with tetramethylguanidinium pyridine-2-carboxyaldoximate,[9] concd. NH_4OH and 80% aqueous acetic acid. The free decamers were purified by chromatography on a DEAE-cellulose column in the 7 M urea system. Each decamer thus obtained gave a single peak on analysis by HPLC on ion exchange resin and alkylated silica gel columns. The base sequence of the decamers was confirmed by mobility shift analysis[7] (see Fig. 2) after partial digestion with nuclease P1[10] and identification of the 5′-terminal nucleotide residues. The molar absorption coefficients and hypochromicities were determined by complete digestion experiments (Table II).

Cleavage of d(ATACGCGTAT) with Restriction Endonuclease *Mlu*I

Restriction endonuclease *Mlu*I, isolated from *Micrococcus luteus*, recognizes the -ACGCGT- sequence and cuts the phosphodiester bond between dA and dC residues.[11] In

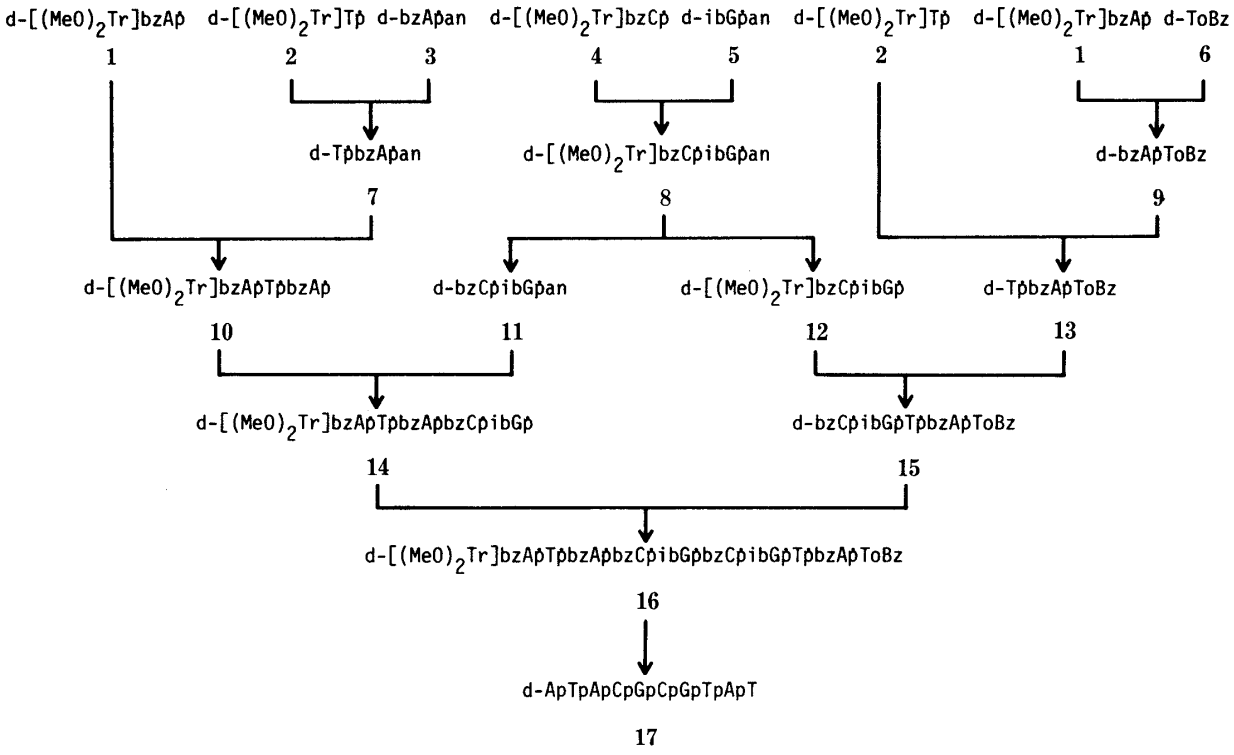

Fig. 1. Synthetic Scheme for d(ATACGCGTAT)

ṗ, *p*-chlorophenyl phosphate; ṗan, *p*-chlorophenyl phosphoro-*p*-anisidate.

TABLE I. Conditions and Yields of Condensation Reactions

3′-Phosphodiester component (mmol)		5′-Hydroxyl component (mmol)·		Product	Yield (mmol)	
2	5.50	**3**	4.41	d-[$(MeO)_2$Tr]TṗbzAṗan	3.94	89%
4	5.02	**5**	4.00	d-[$(MeO)_2$Tr]bzCṗibGṗan	3.68	92%
1	9.10	**6**	7.00	d-[$(MeO)_2$Tr]bzAṗToBz	5.34	76%
1	3.13	**7**	2.38	d-[$(MeO)_2$Tr]bzAṗTṗbzAṗan	1.71	72%
2	5.50	**9**	4.36	d-TṗbzAṗToBz **13**	3.33	76%
10	0.400	**11**	0.302	d-[$(MeO)_2$Tr]bzAṗTṗbzAṗbzCṗibGṗan	0.207	69%
12	0.400	**13**	0.205	d-bzCṗibGṗTṗbzAṗToBz **15**	0.121	59%
14	0.121	**15**	0.0645	**16**	0.0513	80%
4m	2.58	**5**	1.96	d-[$(MeO)_2$Tr]bzm^{5}CṗibGṗan	1.79	91%
12m	0.408	**13**	0.300	d-bzm^{5}CṗibGṗTṗbzAṗToBz **15m**	0.203	68%
14	0.0863	**15m**	0.0608	**16m**	0.0428	70%
10	0.250	**11m**	0.194	d-[$(MeO)_2$Tr]bzAṗTṗbzAṗbzm^{5}CṗibGṗan	0.163	84%
14m	0.163	**15m**	0.117	**16mm**	0.0977	84%

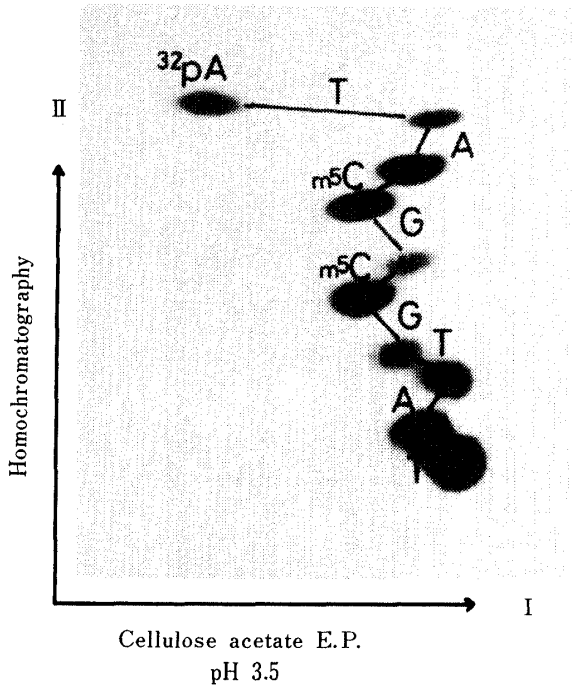

Fig. 2. Mobility Shift Analysis of d(ATAm^5-CGm^{5}CGTAT)

The 5′-labeled decamer was partially digested with nuclease P1. Homomix III was used in the second dimension.

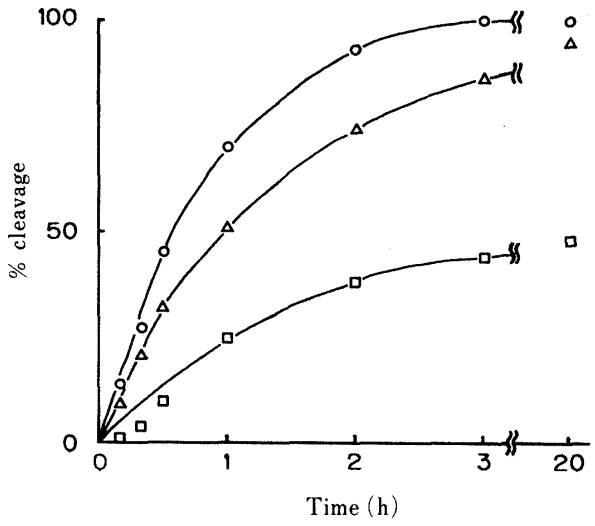

Fig. 3. Cleavage Reaction of d(ATACG-CGTAT) with Restriction Endonuclease *Mlu*I

The decamer (0.5 μM, ○; 1.0 μM, △; 2.0 μM, □) was incubated with *Mlu*I (0.5 unit/μl) at 30 °C. For details, see Materials and Methods.

order to determine the effect of a 5-methyl group on the dC residue on the nuclease activity, the decamers were treated with *Mlu*I at 26 and 30 °C. d(ATACGm^{5}CGTAT) and d(ATAm^{5}CGm^{5}CGTAT) were completely resistant to the nuclease for at least 20 h. The unmodified decamer was cleaved with *Mlu*I. The time courses of the cleavage at 30 °C with various substrate/enzyme ratios are shown in Fig. 3. These results suggest that the site of methylation by the methylase corresponding to *Mlu*I, which is unknown at present, may be the fourth cytosine residue of the *Mlu*I recognition sequence, ACGCGT. These results also suggest that d(ATACGCGTAT) forms a self-duplex even at 0.5×10^{-6} M strand concentration.

TABLE II. UV Absorption Properties of Decadeoxyribonucleotides[a)]

	λ_{max} (nm) (ε_{max})	λ_{min} (nm) (ε_{min})	Hypochromicity at 260 nm
d-ATACGCGTAT	258 (8400)	230 (3500)	25%
d-ATACGm^{5}CGTAT	258 (8000)	230 (3100)	28%
d-ATAm^{5}CGm^{5}CGTAT	258 (8200)	230 (3700)	24%

a) The ε per residue and hypochromocity were calculated from the results of digestion experiments with nuclease P1. The UV spectra were measured in 0.1 M NaCl, 10 mM sodium cacodylate buffer (pH 7.0) at 20 °C.

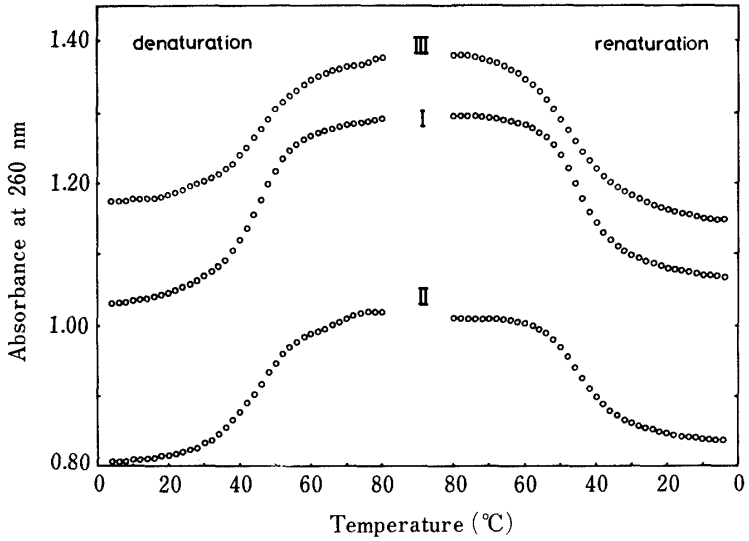

Fig. 4. UV-Temperature Profiles for d(ATACGCGTAT) (I), d(ATACGm^5-CGTAT) (II) and d(ATAm^{5}CGm^{5}CGTAT) (III) in 0.1 M NaCl, 10 mM Sodium Cacodylate Buffer (pH 7.0)

UV and CD Spectral Properties of the Decamers

All the decamers showed almost the same UV spectra (Table II). They also showed similar hypochromicity (24—28% at 260 nm). UV-temperature profiles for these decamers (1.1—1.4 × 10^{-5} M strand concentration) are shown in Fig. 4. All the decamers exhibited relatively sharp absorbance changes over a temperature range of about 20 °C. These results suggest that these decamers form a self-duplex at low temperature. The Tm's for d(ATACGCGTAT), d(ATACGm^{5}CGTAT) and d(ATAm^{5}CGm^{5}CGTAT) were found to be 45, 46 and 48 °C, respectively. The slope of the absorbance change around Tm for d(ATACGCGTAT) was significantly steeper than those for the m^{5}C-containing decamers. It seems that methylation of the cytosine residues stabilizes the duplex against thermal perturbation but reduces the cooperativity of the duplex melting process. These phenomena could be explained in terms of increased hydrophobic attractive interactions between the m^{5}C residues and neighboring bases. It is known that methylation of base residues in oligonucleotides stabilizes the stacking interactions.[12,13)]

CD spectra of d(ATAm^{5}CGm^{5}CGTAT) at 2 °C in various salt solutions are shown in Fig. 5. The other decamers showed similar CD spectra under corresponding conditions. The CD spectrum in 0.1 M NaCl shows two positive bands at 275 nm ($[\theta] = 1.05 \times 10^4$) and 222 nm ($[\theta] = 0.72 \times 10^4$) and a negative band at 250 nm ($[\theta] = -1.80 \times 10^4$). The positive band at

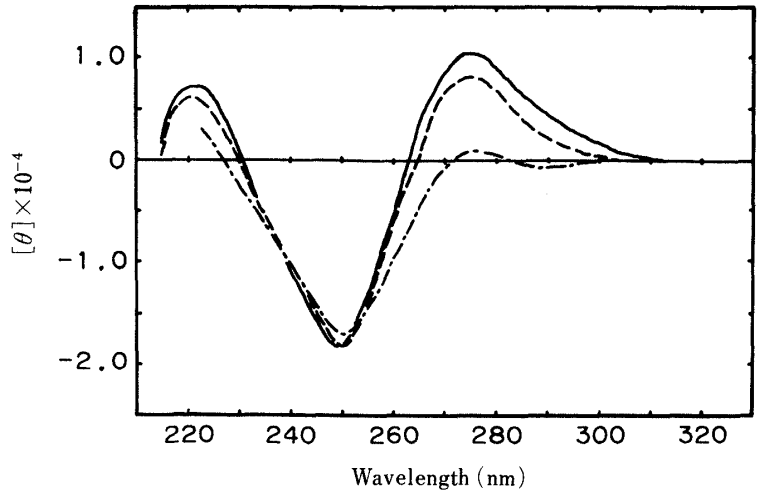

Fig. 5. CD Spectra of d(ATAm^{5}CGm^{5}CGTAT) in 0.1 M NaCl (——), 4.0 M NaCl (– – – –) and 6.0 M CsF (—·—) Containing 10 mM Sodium Cacodylate Buffer (pH 7.0) at 2 °C

around 220 nm may originate from the ATA and TAT sequences, since this band is not observed in d(CGCG)[14] but is observed in d(AT) and d(TA).[15] The CD spectrum in 4.0 M NaCl, where oligo(dC-dG) forms a Z-DNA duplex and shows a reversed CD pattern in the 240—310 nm region,[14] is very similar to that in 0.1 M NaCl, although the intensity of the positive band is significantly reduced. These results suggest that these decamers containing perfectly alternating purine–pyrimidine sequences form a B-form (or modified B-form) duplex even in 4 M NaCl and even with m^{5}C residues, which promote a B–Z transition.[14] CD spectra similar to that in 0.1 M NaCl were also observed in 0.1 M NaCl–1.0 M $MgCl_2$ and in 0.1 M NaCl–0.1 mM $Co(NH_3)_6Cl_3$. CD spectra similar to that in 4 M NaCl were also observed in 0.1 M NaCl–0.1 mM spermine and in 56% ethanol.

The positive band at around 275 nm is missing in the CD spectrum in 6.0 M CsF while the negative band at around 250 nm remains unchanged. In the case of d(ATACGCGTAT), a small negative band ($[\theta]) = -0.3 \times 10^4$) was observed at around 285 nm. The same phenomena were observed for poly(dG-dC) in 7.1 M CsCl.[16] Similar phenomena were also found for poly(dA-dT) in 4—6 M CsF.[17,18] In the latter case, a positive band at around 260 nm in a low salt solution changed into a large negative band in 5—6 M CsF. The decamers may undergo the same conformational change as the polymers in a high CsF solution.

The CD spectra of d(ATACGCGTAT) and d(ATAm^{5}CGm^{5}CGTAT) at various temperatures are shown in Fig. 6. d(ATACGm^{5}CGTAT) also showed similar CD spectral changes. Upon duplex melting, the largest change in intensity occurs in the negative band at around 250 nm. Only a small change is seen in the positive band at around 250 nm. Similar phenomena are observed for oligo(dC-dG) duplexes in 0.1 M NaCl.[13] When the spectra in 0.1 M NaCl and 4 M NaCl are compared, it is noted that major change in CD intensity of the positive band at around 275 nm occur in different parts of the 280 nm band (below 280 nm in 0.1 M NaCl and above 280 nm in 4 M NaCl).

All the decamers showed similar CD spectra under various conditions as mentioned above. In 8 M $NaClO_4$ at −20 °C, however, substantially different CD spectra were observed (Fig. 7). It should be noted that the spectra of the decamers containing m^{5}C residues are almost identical but are different from that of the unmodified decamer. UV and CD spectra of d(ATAm^{5}CGm^{5}CGTAT) at −20 and 20 °C are shown in Fig. 8. When the two CD spectra are compared, the spectrum at −20 °C shows changes in a negative direction over the 278—310 nm region and in a positive direction over the 220—278 nm region. The largest negative

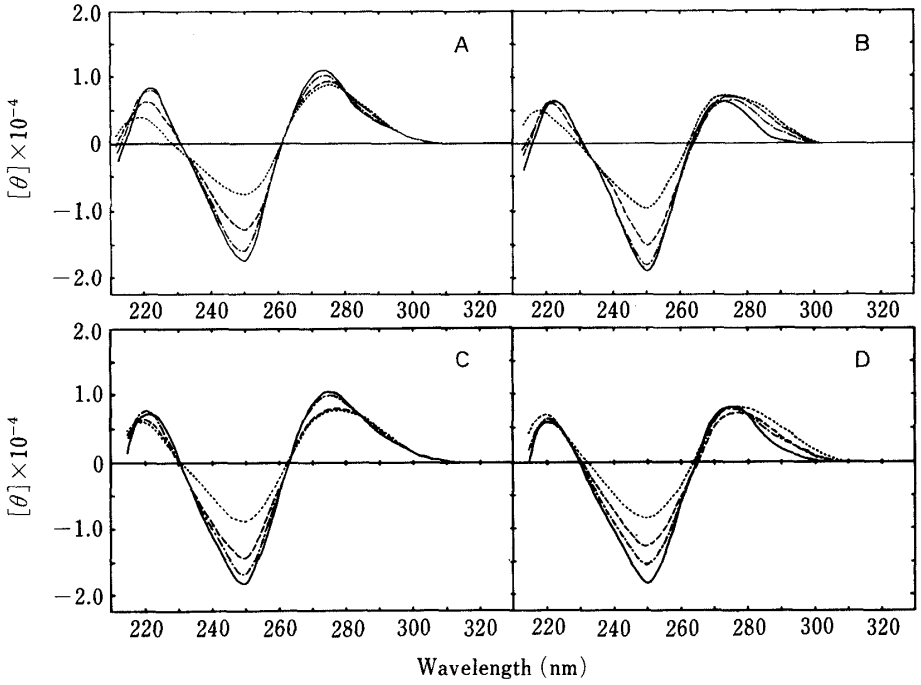

Fig. 6. CD Spectra of d(ATACGCGTAT) (A and B) and d(ATAm^{5}CGm^5-CGTAT) (C and D) in 0.1 M NaCl (A and C) and 4.0 M NaCl (B and D) at 0 °C (——), 20 °C (—·—), 40 °C (– – –) and 57 °C (– – – –)

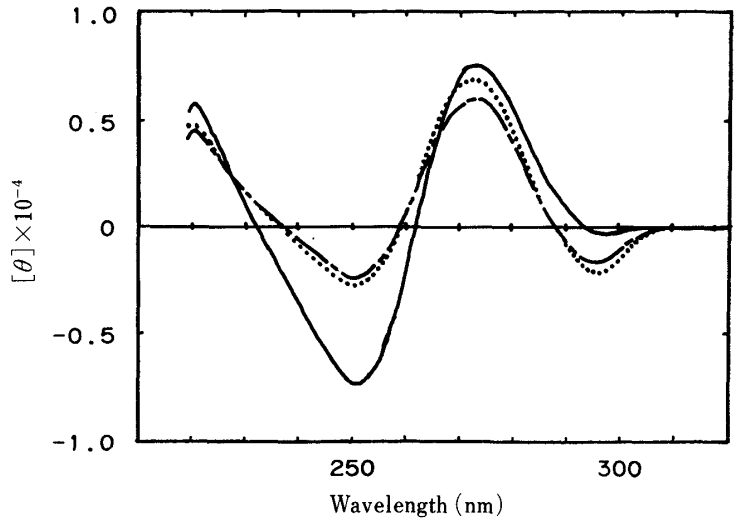

Fig. 7. CD Spectra of d(ATACGCGTAT) (——), d(ATACGm^{5}CGTAT) (—·—) and d(ATAm^{5}CGm^{5}CGTAT) (– – – –) at −20 °C in 8 M $NaClO_4$, 10 mM Sodium Cacodylate Buffer (pH 7.5)

The decamer concentration was 1.5 A_{260} units/ml.

change in the former region occurs at around 290—295 nm and the largest positive change in the latter region occurs at around 250 nm. Similar CD changes were observed in the duplex melting transition of a Z-form duplex of oligo(dC-dG)'s.[14] When the two UV spectra are compared, the spectrum at −20 °C shows hyperchromism at around 295 nm. It is known that poly(dG-dC) shows hyperchromism at around 290 nm upon B–Z transition.[16,19] These results strongly suggest that the decamers containing m^{5}C residues form a Z-DNA duplex in 8 M $NaClO_4$ and at −20 °C. It is known that even poly(rG-rC)[20] and oligo(rC-rG) (H. Urata, unpublished work) can form a Z-form duplex in 6 M $NaClO_4$. UV- and CD-temperature

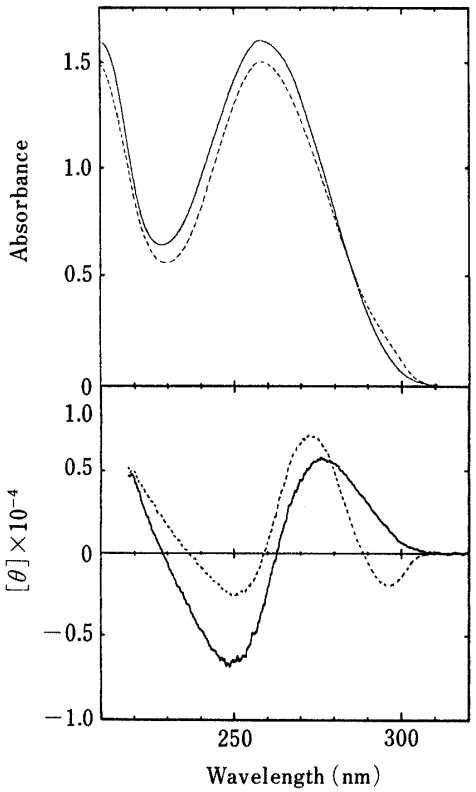

Fig. 8. UV and CD Spectra of d(ATAm^5-CGm^{5}CGTAT) in 8 M $NaClO_4$, 10 mM Sodium Cacodylate Buffer (pH 7.0) at 20 °C (——) and −20 °C (----)

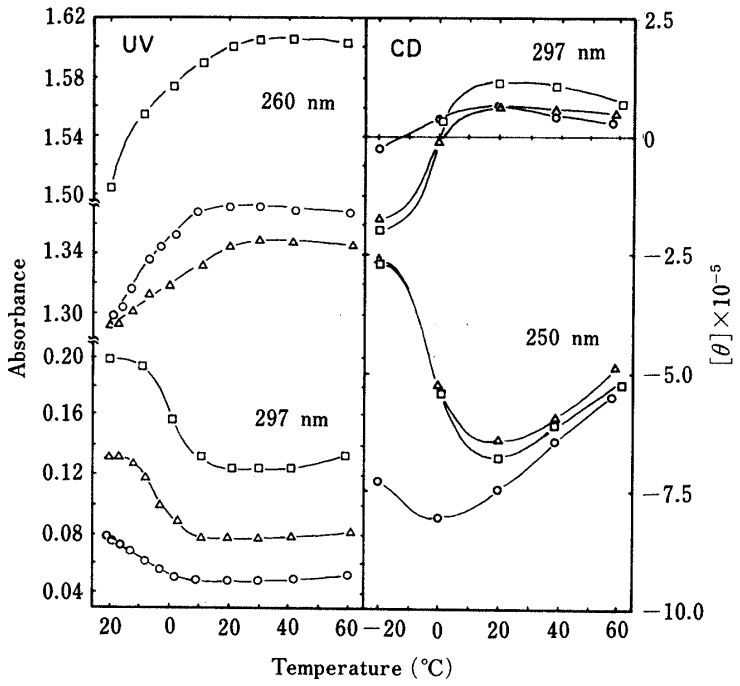

Fig. 9. UV- and CD-Temperature Profiles for d(ATACGCGTAT) (○), d(ATACGm^{5}CGTAT) (△) and d(ATAm^{5}CGm^{5}CGTAT) (□) in 8 M $NaClO_4$, 10 mM Sodium Cacodylate Buffer (pH 7.0)

profiles for the three decamers in 8 M $NaClO_4$ are shown in Fig. 9. It is readily seen that the profiles for the modified decamers are very similar to each other but are different from those for the unmodified decamer. The transition for the modified decamers occurs at around 0 °C, and may involve a double strand–single strand transition rather than a duplex–duplex transition, since no second transition is visible up to 60 °C and the magnitudes of the CD

bands at high temperature are too small for those of a duplex. It is assumed that B-DNA duplexes are destabilized by high salt concentration[14] and an alternative conformation (Z-form or other form), which can be stabilized by high salt, becomes predominant.

Conclusions

Three deoxyribodecanucleotides, d(ATACGCGTAT), d(ATACGm^{5}CGTAT) and d(ATAm^{5}CGm^{5}CGTAT), were synthesized and their enzymatic activity and optical properties were examined. These decamers have perfectly alternating purine–pyrimidine sequences and contain a CGCG core which is prone to form a Z-DNA duplex. The m^{5}C residues are known to promote B–Z transition[3] of C-containing duplexes.

From CD spectral studies, it is concluded that the three decamers have very similar conformations, especially in terms of base stacking geometry, under various salt conditions. Therefore it is rather surprising that even d(ATACGm^{5}CGTAT) is completely resistant to restriction endonuclease *Mlu*I while the unmodified decamer can be completely cleaved. The enzyme must recognize the extra methyl group itself for exclusion of the decamers as a substrate. The m^{5}C-containing decamers show significant differences from the unmodified decamer in the slope of UV-temperature profiles involving a duplex melting process. This phenomenon may be explained by differences in the dynamic nature of base stacking with respect to thermal perturbation, due to greater hydrophobic interactions induced by the extra methyl groups. It should be noted that one extra methyl group in a decamer strand is enough to cause this difference. The same is true for the behavior of the decamers in 8 M $NaClO_4$. The two modified decamers showed the same CD spectra at $-20\,^{\circ}C$ and gave similar UV- and CD-temperature profiles which are quite different from that of the unmodified decamer. The modified decamers are assumed to form a Z-DNA duplex under extreme conditions, in 8 M $NaClO_4$ and at $-20\,^{\circ}C$. The extra methyl groups may stabilize the Z-form structure as a result of the greater hydrophobic interactions. Quite recently it has been shown that deoxyoligonucleotides containing TA sequences can form a Z-DNA duplex in crystals.[21,22]

References and Notes

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