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Polynucleotides. LIX.[1] Synthesis and Properties of a Deoxyribohexanucleotide corresponding to the *Eco*RI Recognition Sequence

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A deoxyribohexanucleotide corresponding to a restriction endonuclease (*Eco* RI) recognition sequence, d-G-A-A-T-T-C, was synthesized by stepwise condensation of mononucleotides using dicyclohexylcarbodiimide (DCC) or 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) as the condensing reagent. Protected oligonucleotides were separated by ion-exchange chromatography or gel filtration and the deblocked hexanucleotide was purified by DEAE-cellulose column chromatography in the presence of 7 M urea. The product was characterized by enzymatic hydrolysis and its temperature–absorption profile was measured at high ionic strength.

Keywords——d-G-A-A-T-T-C; melting temperature; ion-exchange chromatography; homochromatography; restriction sequence; partial venom diesterase digestion

Restriction endonucleases, which are considered to be baceterial protection systems against foreign DNA, have been investigated extensively.[3] These enzymes are very useful tools for the fragmentation of DNA at specified sequences. Most restriction enzymes recognize sequences in double-stranded DNA and create cohesive ends. The minimum recognition site of *E. coli* restriction and modification enzyme (*Eco*RI) is a hexanucleotide characterized by 2-fold symmetry (Chart 1). Cleavage by the enzyme generates the 3′-hydroxyl of 2′-deoxyguanosine and the 5′-phosphate of 2′-deoxyadenosine.[4] Modification is a consequence of methylation at the N^6 position of adenine residues adjacent to the axis of symmetry, preventing cleavage by the endonuclease. Polydeoxynucleotides containing some restriction enzyme recognition sequences have been synthesized and joined with DNA ligase to DNA for insertion into or combination with DNA fragments.[5] It is important to investigate the thermal stability of self-complementary duplexes corresponding to restriction enzyme recognition sites in connection with joining them at the base-paired end (blunt end ligation) using T4 DNA ligase.[6a] In this paper we describe the synthesis and properties of a self-complementary hexanucleotide d(G–A–A–T–T–C)[6b] corresponding to the recognition site for *Eco*RI.

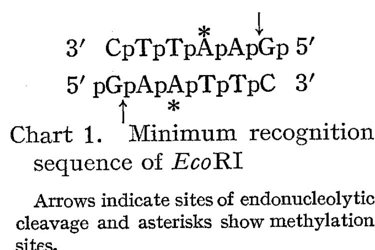

Chart 1. Minimum recognition sequence of *Eco*RI

Arrows indicate sites of endonucleolytic cleavage and asterisks show methylation sites.

1) Part LVIII of this series: E. Ohtsuka S. Tanaka, M. Hayashi, and M. Ikehara, *Biochim. Biophys. Acta* **565**, 192 (1979).
2) Location: *133-1 Yamadakami, Suita, Osaka, 565 Japan.*
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6*b*) Abbreviation are as recommended by the IUPAC-IUB commission, *J. Biol. Chem.*, **245**, 5171 (1970); *Proc. Nat. Acad. Sci. U.S.A.*, **74**, 2222 (1977); dicyclohexylcarbodiimide, TPS, 2,4,6-triisopropylbenzenesulfonyl chloride.

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Table I. Reaction Conditions and Yields

3′-OH component	mmol	Mononucleotide	mmol	Condensing reagent	mmol	Reaction time hr	Yield %
d(MeOTr)bzG	1.86	dpbzA(Ac)	2.16	DCC	14.5	120	62
d(MeOTr)bzG–bzA	0.441	dpbzA(Ac)	1.80	TPS	2.52	6	47
d(MeOTr)bzG–bzA–bzA	0.208	pT(Ac)	1.05	TPS	1.49	6	13
d(MeOTr)bzG–bzA–bzA–T	0.026	pT(Ac)	0.150	TPS	0.308	6	44
d(MeOTr)bzG–bzA–bzA–T–T	0.011	dpanC(Ac)	0.22	TPS	0.29	6	67

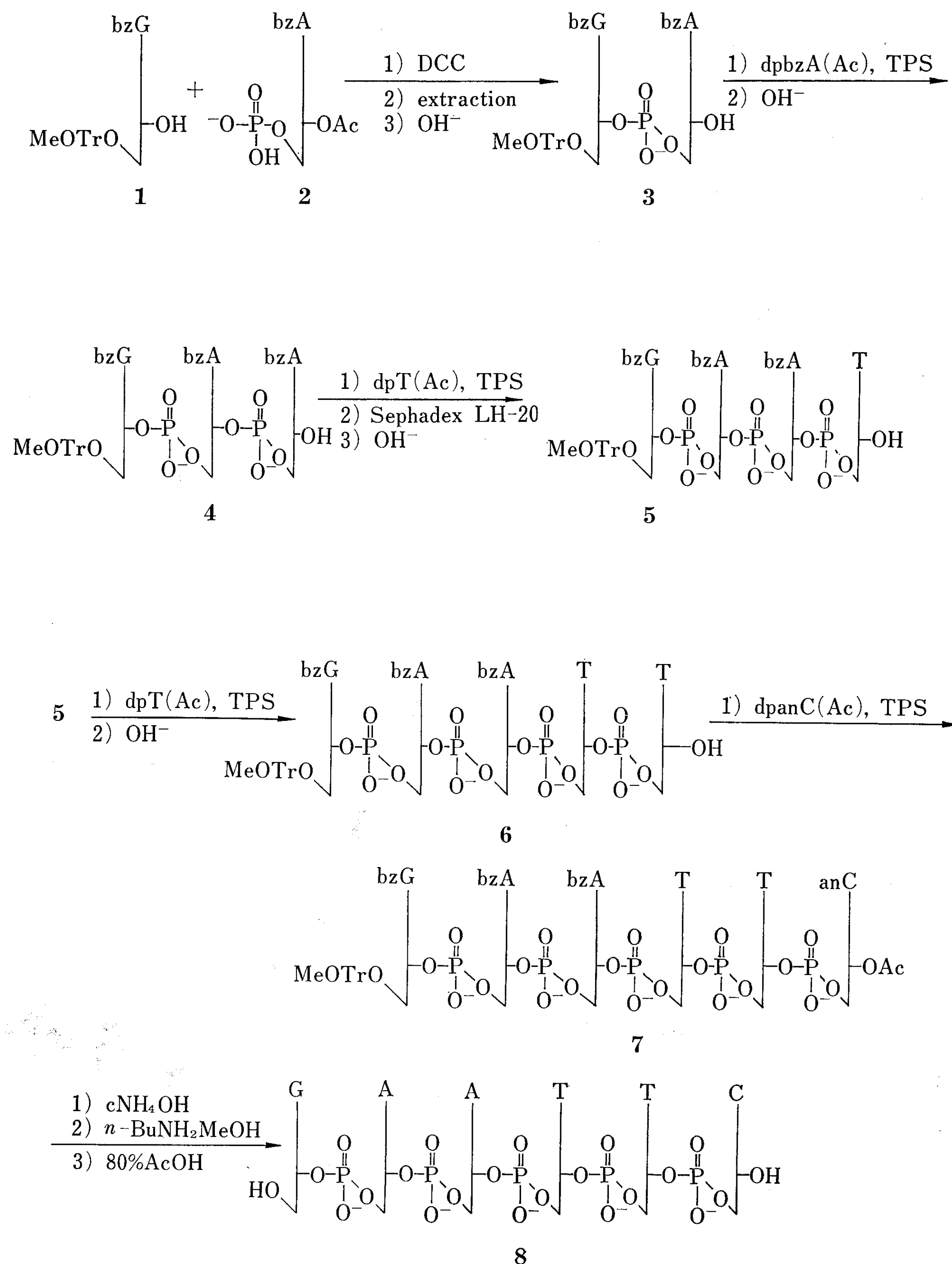

Chart 2

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Synthesis of d(G–A–A–T–T–C)

The hexanucleotide was synthesized by stepwise addition of protected mononucleotides to N,5′-O-protected 2′-deoxyguanosine (**1**), as shown in Chart 2. The reaction conditions and yields are summarized in Table I. The dimer (**3**) was obtained by activation of dpbzA (Ac) (**2**) with DCC and isolated by extraction with chloroform.[7] The 3′-acetyl moiety was removed by strong alkali treatment and **3** was precipitated with ether in a yield of 62%. TPS was used in the remaining steps. The trimer (**4**), pentamer (**6**) and hexamer (**7**) were isolated by ion-exchange chromatography on DEAE-cellulose. The isolation yields of **4**, **6** and **7** were 47%, 44% and 67%. Gel filtration on Sephadex LH-20 using 0.1 M triethylammonium bicarbonate in 30% ethanol was used for the separation of the tetramer **5**. An anomalous retardation and poor recovery of the tetramer were found, probably due to the low concentration of ethanol. However resolution of the oligonucleotides and mononucleotides was satisfactory. The deblocked product (**8**) was purified by ion-exchange chromatography on DEAE-cellulose in 7 M urea (Fig. 1) in a yield of 47%. Compound **8** was characterized by mobility shift analysis using cellulose-acetate electrophoresis with homochromatography[8] in the 2nd dimension after phosphorylation of the 5′-end with [γ-^{32}P]ATP and polynucleotide kinase and subsequent partial digestion with venom phosphodiesterase (Fig. 2).

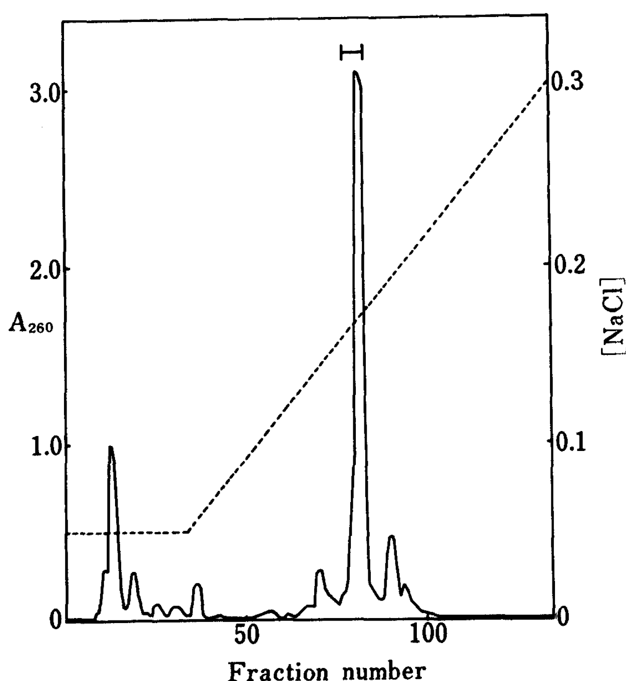

Fig. 1. Chromatography of d(G–A–A–T–T–C) on a Column (1.2×60 cm) of DEAE-cellulose (chloride form) equilibrated with 7 M Urea containing 0.05 M Sodium Chloride and 0.02 M Tris–HCl, pH 7.5

Elution was performed with a linear gradient of sodium chloride (0.05 M—0.3 M, total 1 l). Fractions of 9.5 ml were collected every 40 min. The main peak contained the product. The indicated fractions were combined.

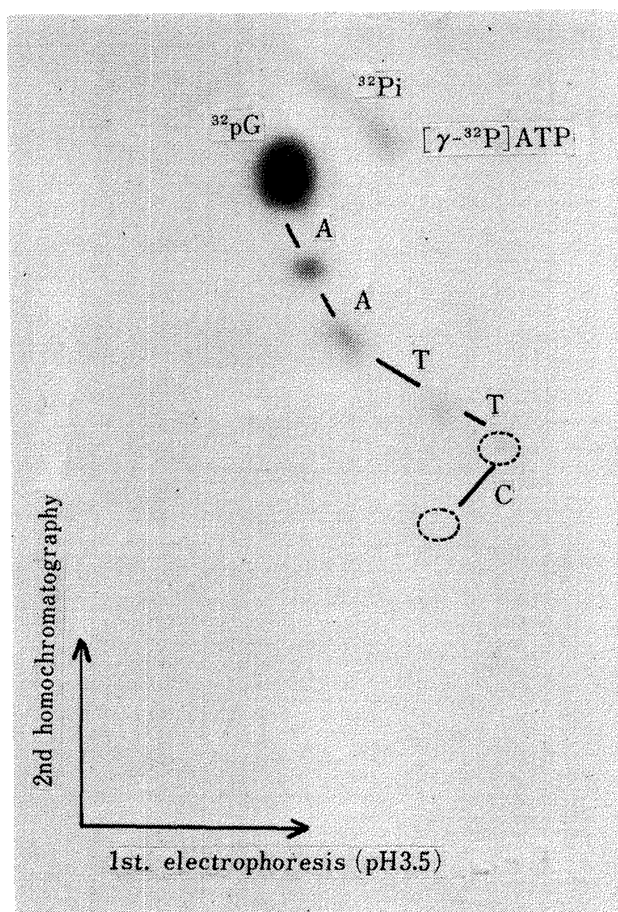

Fig. 2. Two-dimensional Homochromatography of Partially Digested d(G–A–A–T–T–C)

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Temperature-absorption profile of d(G–A–A–T–T–C)

The self-complementary hexamer d(G–A–A–T–T–C) should form double helices under certain conditions. It is necessary to obtain perfectly base-paired double helices for "blunt end ligation" with T4 DNA ligase.[6a] The temperature-absorption profile of the hexamer was measured in incubation media suitable for DNA ligase (Fig. 3a) The melting temperature (Tm) appeared to be below 5°. A shallower increase of absorption was observed above 12°. The hyperchromicity may be due to destacking of the bases of the single-stranded hexamer. In order to obtain a more stable complex, Tm was measured at higher ionic strength (0.3 M NaCl) (Fig. 3b). The profile showed a reproducible shoulder at 15° and Tm was apparently higher than that in Fig. 3a. Such stability of the double helices was anticipated on the basis of previous Tm measurements of oligodeoxynucleotides[9a] and oligoribonucleotides.[9b] It may be concluded that elongation beyond the hexamer is preferable for blunt end ligation, though DNA ligase is slightly active at 0°, well below the Tm of the hexamer.

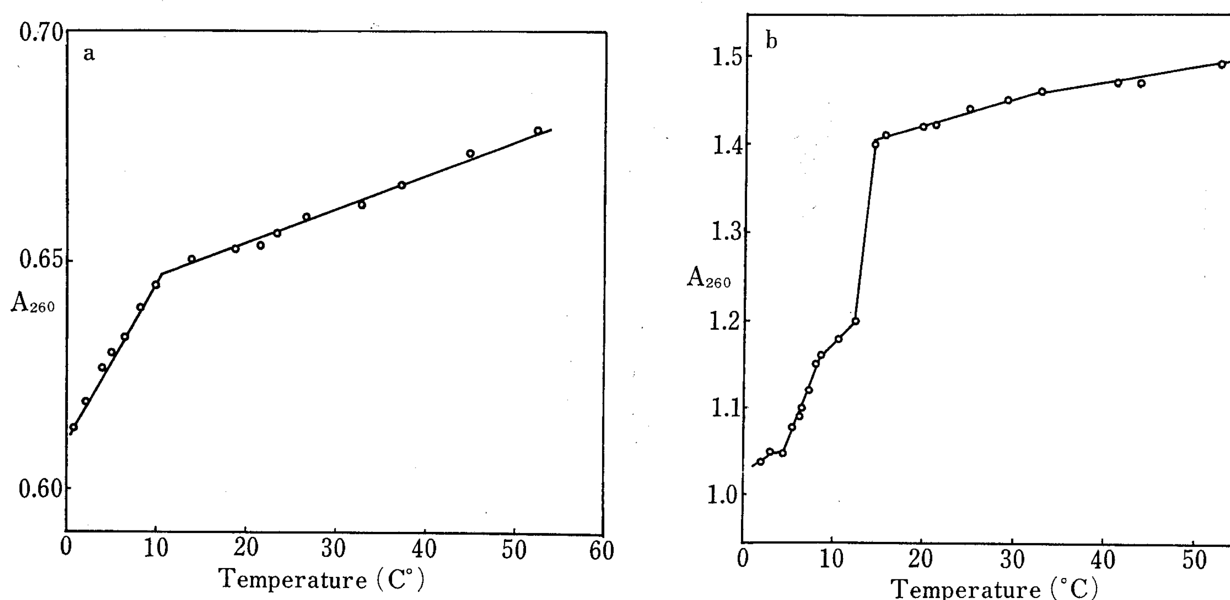

Fig. 3. Temperature-Absorption Profile of d(G–A–A–T–T–C) in (a) 50 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$; (b) 10 mM Tris-HCl (pH 7.5), 0.3 M NaCl and 0.1 mM EDTA

Experimental

General Methods——Paper chromatography was performed using the following solvent systems: A, propan-1-ol-c.ammonia-water (60: 10: 30 v/v); B, ethanol-1M ammonium acetate, pH 7.5 (7: 3, v/v); C, propan-1-ol–conc.ammonia–water (55: 15: 35, v/v). Paper electrophoresis was performed at 900V/40 cm in 0.05 M triethylammonium bicarbonate (pH 7.5) (Table II). Partial digestion with venom phosphodiesterase and 5′-phosphorylation using [γ-^{32}P]ATP were carried out as described previously.[10]

Tm was measured with a Hitachi 124 spectrophotometer equipped with a thermostated cell holder. The temperature inside the cell was measured with a thermocouple. d(MeOTr)bzG,[7] dpzA(Ac),[11] dpT(Ac)[12] and dpanC(Ac)[13] were prepared according to the literature. General methods for condensations and ion-exchange chromatography on DEAE-cellulose were as described previously.[7,14]

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Table II. Paper Chromatography and Electrophoresis Results

	Rf			R_m (dA–dpA) pH 7.5
	A	B	C	
d–G–A–A–T	0.10			0.88
d–G–A–A–T–T	0.09	0.02	0.44	0.90
d–G–A–A–T–T–C	0.03		0.35	1.00
d(MeOTr)G–A–A–T	0.70	0.38		0.76
d(MeOTr)G–A–A–T–T	0.57	0.23		0.85
d(MeOTr)G–A–A–T–T–C			0.78	0.96

d-(MeOTr)bzG-bzA-bzA-T(5)——d-(MeOtr)bzG-bzA-bzA[7] (4) and pT(Ac) were condensed as summarized in Table I. The reaction was stopped by adding 2 M diisopropylethylamine in pyridine (1.5 ml) and water (20 ml) to the solution in pyridine (6 ml) with cooling. The mixture was kept at room temperature overnight and TPS was removed by extraction with ethyl acetate (50 ml, two portions). The aqueous layer and washings were concentrated by coevaporation with added pyridine to 6 ml, and then applied to a column (2.4×79 cm) of Sephadex LH-20 equilibrated with 0.1 M triethylammonium bicarbonate in 30% ethanol. Elution was performed using the same buffer, and fractions of 4.5 ml were collected every 30 min. The tetramer was eluted in fractions 51—53 (identified by paper chromatography in solvent B and paper electrophoresis). The product was treated with 2 N sodium hydroxide (10 ml) in 50% aqueous pyridine (3 ml)—ethanol (4 ml) at 0° and neutralized with Dowex 50 (pyridinium form. 100 ml). The filtrate and washings of the resin were concentrated, and the product (1460 A_{280}) was precipitated with ether from its solution in pyridine.

d-(MeOTr)bzG-bzA-bzA-T-T (6)——Condensation was performed as shown in Table I and the reaction mixture was worked up as described above. After alkali treatment, the mixture was applied to a column (3×35 cm) of DEAE-cellulose (bicarbonate) equilibrated with 0.05 M triethylammonium bicarbonate in 30% ethanol. Elution was performed with a linear gradient of the same salt (0.05 M—0.4 M, total 4 l). Fractions of 14.5 ml were collected every 10 min. The product (710 A_{280}) was obtained in fractions 159—223 and was precipitated with ether from its solution in pyridine.

G-A-A-T-T-C (8)——The hexanucleotide (7) was synthesized as summarized in Table I and separated by chromatography on a column (3.0×45 cm) of DEAE-cellulose (bicarbonate) after aqueous pyridine treatment. Elution was performed with a linear gradient of triethylammonium bicarbonate (0.01—0.1 M, total 2 l) and then with another gradient of the same salt (0.15—0.40, total 6 l). Fractions of 18 ml were collected every 10 min and fractions 246—365 were combined. Debenzoylation of dbzC and dbzA was performed by treatment with concentrated ammonia at room temperature for 2 days and then the mixture was treated with 1:1 *n*-butylamine–methanol (30 ml) at room temperature for 2 days. Volatile materials were removed and the residue was treated with 80% acetic acid at room temperature for 45 min. Acetic acid was removed by evaporation, and the residue was dissolved in 0.2 M triethylammonium bicarbonate (10 ml), washed with ethyl acetate and then concentrated. One half of mixture was dissolved in 7 M urea (3 ml) and applied to a column of DEAE-cellulose (chloride). The conditions for chromatography are shown in Fig. 1. The hexamer was desalted by absorption on DEAE-cellulose (bicarbonate) (10 ml) and eluted with 1 M triethylammonium bicarbonate after removing chloride ions with 0.01 M of the same buffer. The yield was 125 A_{260} units. The hexanucleotide was stored in 10 mM Tris-HCl (pH 7.5)–0.1 mM EDTA at −20°. An aliquot (0.125 A_{260}, 1.83 mmol) was labeled by phosphorylation and characterized by two-dimensional homochromatography after partial digestion with venom phosphodiesterase.

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