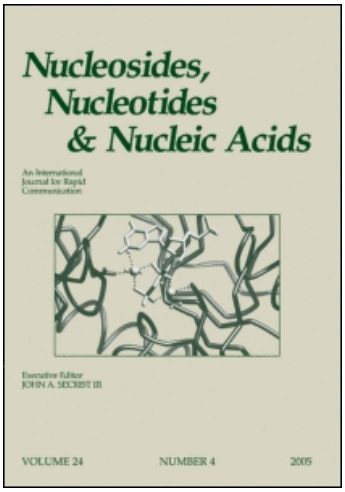

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RESTRICTED JOINING OF A DECADEOXYRIBONUCLEOTIDE FOR PREPARATION OF AN EICOSADEOXYRIBONUCLEOTIDE DUPLEX CONTAINING RECOGNITION SITES FOR RESTRICTION ENZYMES

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Abstract. A partially self-complementally decadeoxyribonucleotide, d(pCGACCCGGGT) has been 3'-blocked with the acetyl group and joined to d(CGACCCGGGT) to yield an eicosadeoxyribonucleotide duplex which contained recognition sites for six restriction enzymes.

INTRODUCTION

Restriction endonucleases[1] have been used extensively in gene manipulations and so called oligonucleotide linkers which contain recognition sequences for restriction endonucleases have been synthesized chemically.[2] Oligonucleotides including modified nucleotides have also been synthesized and used to investigate mode of action of these enzymes.[3] We have previously prepared a partially self-complementary decanucleotide d(CGACCCGGGT) and studied its physico-chemical properties.[2d] In this paper we report a restricted joining reaction by DNA ligase[4] for the preparation of oligonucleotide linkers. By doubling the size of the self-complementally decadeoxyribonucleotide containing recognition sequences for Hpa II, Ava I and Sma I, new recognition sites for Sal I and Tag I and Hind II can be generated as shown in Fig 1. The eicosanucleotide duplex served as a substrate for the six endonucleases.

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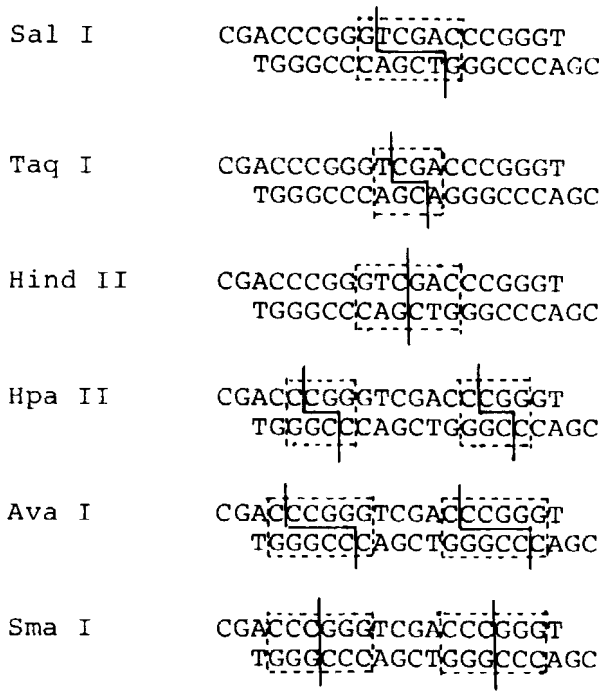

FIG.1
Cleavage sites for restriction endonucleases in the eicosamer

RESULTS AND DISCUSSION

Joining of the 3'-O-acetyloligonucleotide d[pCGACCCGGGT(OAc)] to CGACCCGGGT

The chemically synthesized decanucleotide d(CGACCCGGGT) was 5'-phosphorylated with polynucleotide kinase and ATP. The 3'-end of the decanucleotide was blocked by selective acetylation with acetic anhydride at neutral pH.[6] The product was isolated by reversed phase high pressure liquid chromatography(HPLC) as shown in Fig. 2 and used for joining to d(CGACCCGGGT) to yield a self-complementally eicosanucleotide. The DNA ligase reaction was analyzed by gel electrophoresis in the presence of formamide. In the presence of 7 M urea minor bands due to

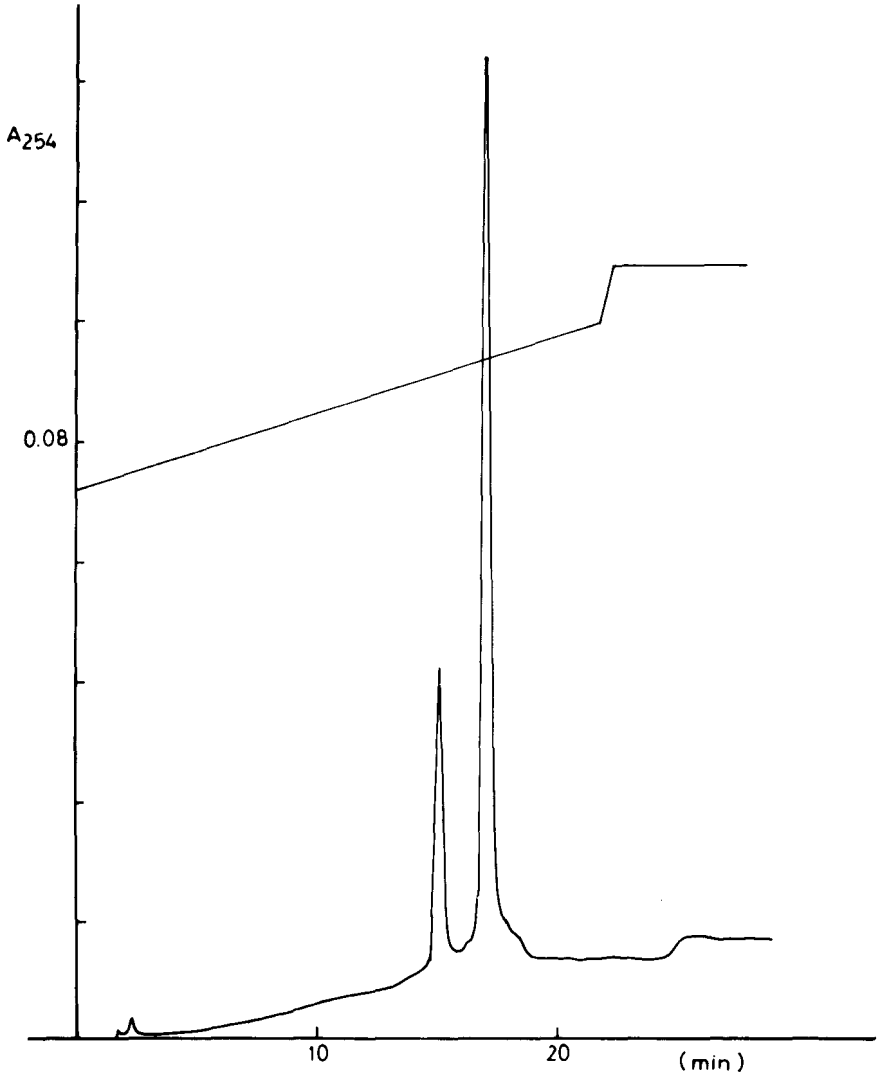

FIG.2
Reversed phase HPLC of d(pCGACCCGGGT-OAc) on C-18 silica gel (TSK gel ODS 120A, 0.46 x 250 mm) in 0.1 M triethylammonium acetate (pH 7.0) with a gradient of acetonitrile (5-25 %) for 30 min. The flow rate was 2 ml/min. The main peak contained the acetylated product which was separated from the unchanged decanucleotide.

aggregation were observed. The structure of the eicosamer was confirmed by mobility shift analysis[7] (Fig. 3) and nearest neighbor analysis.[2e] These results indicated that the 3'-acetyl group could survive during the DNA ligase reaction and prevent multiple joining. For comparison, the 5'-phosphorylated decamer was used in the above reaction without 3'-blocking. The eicosamer was obtained in a yield of one fifth with respect to the above result.

Cleavage of the duplexes with restriction enzymes

Six restriction enzymes shown in Fig. 1 were used to cleave the eicosamer duplex under the conditions shown in Table 1. The 5'-labeled eicosamer was recognized and cleaved by these six enzymes as judged by homochromatography.[8] The result of hydrolysis with Taq I is shown in Fig. 4. Taq I cleaved the eicosamer more rapidly at 65°C than at 37°C. The eicosamer was digested by Sal I rather slowly and by Hind II the cleavage was slower at 25°C than at 37°C (Fig. 5). Since Hpa II, Sma I and Ava I recognize the decamer, reactions with the decamer and eicosamer were compared. The decamer was cleaved by these enzymes. However, a doubled amount of Ava I was required compared to the amount used for the eicosamer. It was found that the two recognition sites existing in the eicosamer were cleaved in almost the same time. An example is shown in Fig. 6, in which cleaved products by Sma I are indicated.

CONCLUSION

Selective 3'-acetylation was utilized for a donor strand in the DNA ligase reaction of a partially self-complementary decanucleotide to yield the double-sized product exclusively. The method provides a convenient procedure to control reactions involving the 3'-hydroxy end of oligodeoxyribo-nucleotides, although the acetyl group cannot survive at alkaline pH. The present method allowed the synthesis of an eicosanucleotide containing

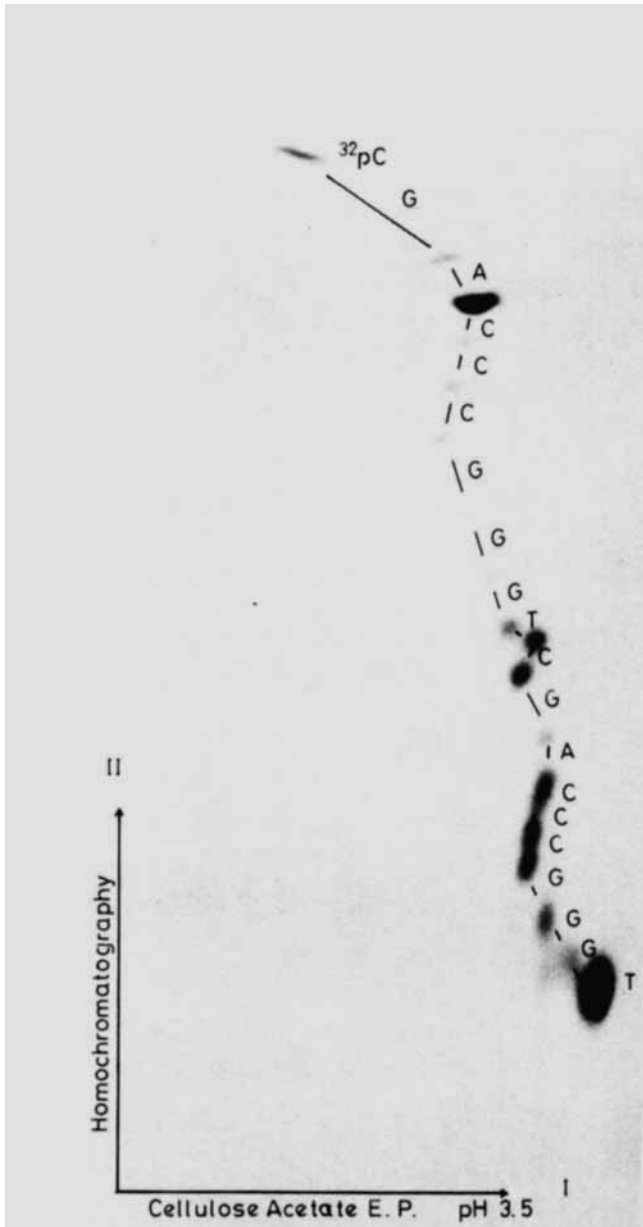

FIG.3
Mobility shift analysis of the joined product d(CGACCCGGGTCGACCCGGGT). The 5'-labeled eicosamer was partially digested by nuclease P1.

TABLE 1

	Tris-HCl(pH) mM	$MgCl_2$ mM	NaCl mM	β-EtSH mM	Other
Sal I	10 (7.5)	7	150	7	0.2mM EDTA 0.1 % BSA
Taq I	10 (7.5)	10	100	10	
Hind II	10 (8.0)	7	60	7	
Hpa II	10 (7.5)	7		7	
Ava I	6 (8.0)	12	60	6	
Sma I	10 (8.0)	7		7	20mM KCl

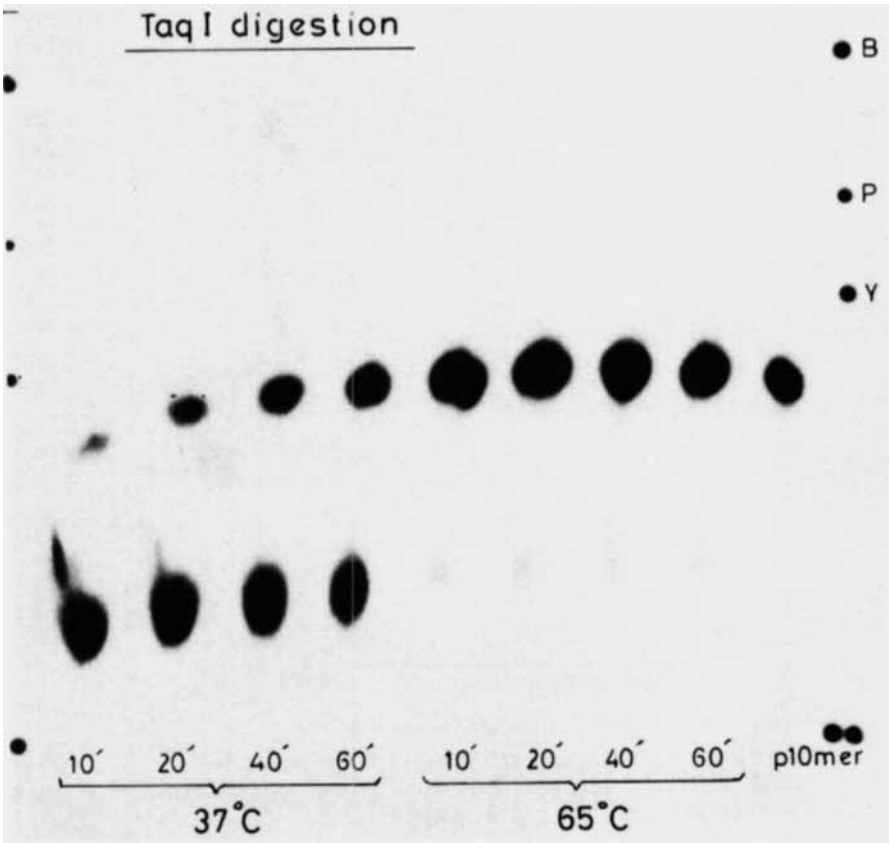

FIG.4
Homochromatography of the products in the reaction of the 5'-labeled eicosamer (0.4 μM) by TaqI. The decamer was used us a marker.

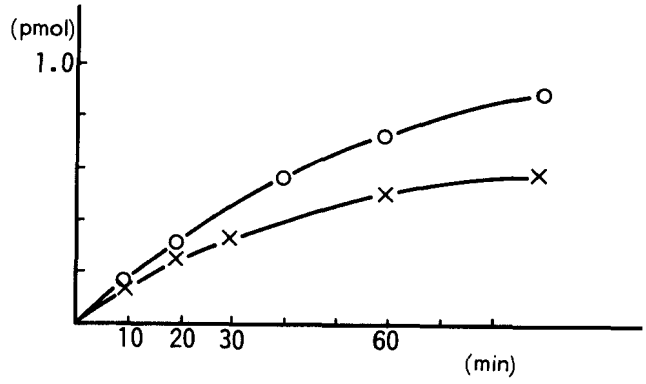

FIG.5
Hydrolysis of the eicosamer (0.4 μM) by Hind II at 37°C (o—o) and 25°C(x—x).

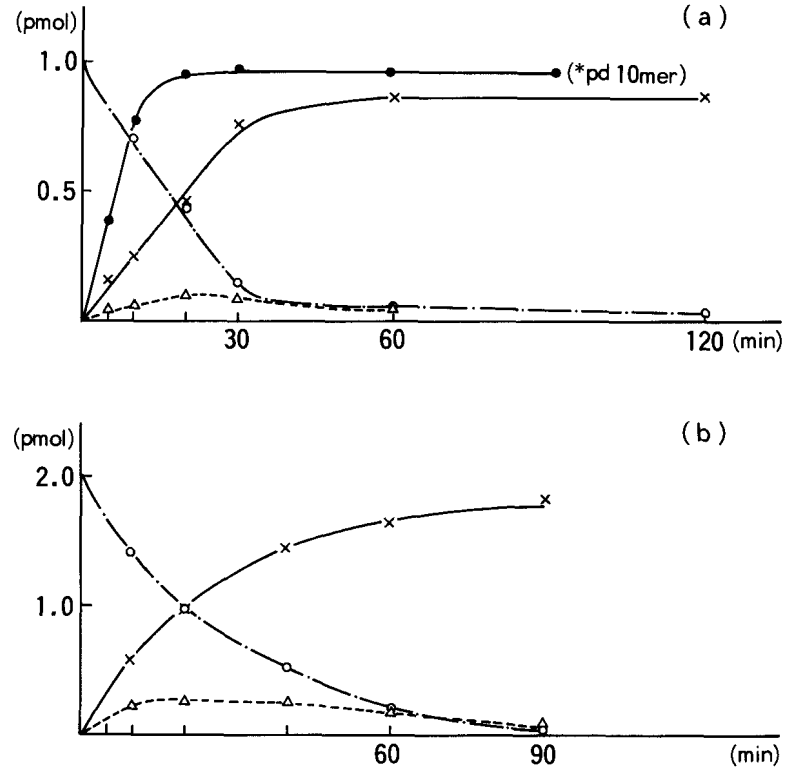

FIG.6
Hydrolysis of the decamer (0.4 μM) and eicosamer (0.4 μM) by Sma I at 37°C (a) and 25°C (b). o-—-o; the 5'-labeled eicosamer; Δ--Δ ; the hexadecamer; x——x; the hexamer.

recognition sites for restriction enzymes and provided substrates for size-dependent cleavage studies of some restriction enzymes.

EXPERIMENTAL

General Methods

Homochromatography and electrophoresis

Homochromatography was performed by using Homo-mix I.[8b] Radioactive nucleotides were recovered and counted. Paper electrophoresis was performed at pH 3.5 with 0.2 M morpholinium acetate. Gel electrophoresis was carried out on polyacrylamide (10-20 %) in the presence of 7 M urea or formamide. Formamide (100 ml) was treated with ambient resin (Biorad AG 501-X8, 5g) for 2 hr. Acrylamide (20%) and 20 M formamide in 20 mM phosphate buffer (pH 7.5) and 1 mM EDTA were treated with ammonium persulfate and TEMED.

Enzymes

Polynucleotide kinase, T4 DNA ligase and restriction enzymes were obtained from Takara Shuzo Co. except that Ava I was from Bio Rad Co. Nuclease P1 was from Yamasa Shoyu.

5'-Phosphorylation with Polynucleotide kinase

The decanucleotide CGACCCGGGT (1 nmol) was phosphorylated by polynucleotide kinase (1 unit) with ATP (1 nmol) in 50 mM Tris-HCl (pH 9.6), 10 mM $MgCl_2$, 10 mM DTT, 2 mM Spermine, 100 mM KCl (5 μl) at 37°C for 1.5 hr, and the product was separated by gel filtration on a column (0.7 x 40 cm) of Sephadex G-50 in 50 mM triethylammonium bicarbonate.

Selective acetylation of the 3'-hydroxy group of oligonuclotides

d(pCGACCCGGGT) (10 A_{260} units) was dissolved in 10 mM phosphate buffer (pH 7.0) and acetic anhydride (1.5 mmol, 140 μl) was added during 15 min at pH 7.0 by adjusting with 4 N NaOH using a microelectrode. The solution was dialyzed against water three times and the 3'-acetylated product was isolated by reversed phase chromatography using the conditions shown in Fig. 3.

Joining of decamers by DNA ligase

The decanucleotides (1 A_{260}, ca. 10 nmol each) were treated with T4 DNA ligase (2400 units) in a mixture (0.6 ml) of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT and 70 μM ATP at 25° C for 20 hr. The mixture was treated with phenol and applied to 20% polyacrylamide gel electrophoresis in the presence of 20 M formamide. The product was isolated by electroelution and desalted by gel filtration. The isolated yield was 0.28 A_{260} unit (ca. 14%).

Nearest neighbor analysis

The eicosanucleotide which was obtained by ligation of [5'-^{32}P]-d(pCGACCCGGGT)OAc with d(CGACCCGGGGT) was digested with micrococcal nuclease (15 units) in 50 mM Tris-HCl (pH 8.9) and 5 mM $CaCl_2$ for 30 min at 37°C. The mixture was then treated with spleen phosphodiesterase (0.17 unit) in 75 mM ammonium acetate and potassium phosphate (pH 6.3) at 37°C for 45 min. [5'-^{32}P]pT was identified by paper electrophoresis.

Reactions with restriction endonucleases

The duplexes (1-10 pmoles) were labeled by polynucleotide kinase and [γ-^{32}P]ATP. Cleavage reactions were performed at 37° C unless otherwise specified in a total volume of 10 μl under the conditions shown in Table 1. Digested products were applied to homochromatography and radioactivities were counted by Cerenkov's method.

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