Volume 6 Number 4 April 1979 Nucleic Acids Research

Synthesis of human insulin gene. Part I. Development of reversed-phase chromatography in the modified triester method. Its application in the rapid and efficient synthesis of eight deoxyribo-oligonucleotides fragments constituting human insulin A DNA

Hansen M.Hsiung, Roland Brousseau, Joseph Michniewicz and Saran A.Narang

Division of Biological Sciences[1], National Research Council of Canada, Ottawa, K1A 0R6, Canada

Received 15 January 1979

ABSTRACT

Preparative reversed-phase thin layer chromatography on silanized silica-gel (RP-2 and RP-18) has been developed to purify triester deoxyribooligonucleotides prepared by the modified triester method. The effectiveness of this technique has been demonstrated in the rapid synthesis of eight pure deoxyribooligonucleotides constituting the sequence of human insulin A DNA. The sequence of each of the deoxyribooligo-nucleotides was confirmed by the two-dimensional mobility-shift method of finger-printing.

INTRODUCTION

One of the most formidable tasks in the synthesis of DNA with defined sequences of bases has been the unambiguous chemical synthesis of short deoxyribooligonucleotides. During the last five years, our laboratory has been involved in improving the triester method[2] of synthesis as an alternative to the diester approach[3]. This resulted in the development of various new and novel features in the triester approach such as (i) modified triester method[4]; (ii) improved phosphorylating reagent[5]; (iii) coupling reagents[6]; and (iv) deblocking reagent[7]. These have played a major role in establishing the modified triester approach as an efficient method of polynucleotide synthesis in terms of simplicity, speed and yield. As a result, the syntheses of various biologically important sequences such as lac operator-DNA[8], linker molecules containing restriction sites of the endonucleases[9] used for cloning, somatostatin[10] gene and tridecadeoxyoligonucleotide inhibiting the growth of Rous Sarcoma Virus[11] were achieved in the last couple of years. In spite of

these achievements, an efficient method of purifying the triester oligonucleotides was still lacking. Invariably, it had been found to be quite difficult to resolve triester product from starting material containing purine bases (especially guanine) by conventional silica-gel chromatography. In this paper, we now wish to report the development of preparative reversed-phase chromatography on C_2-, C_8-, and C_{18}-bonded phase which has been found to resolve most of the deoxyribooligonucleotides containing guanine base. The effectiveness of this technique has been demonstrated by the efficient and rapid synthesis of eight fragments constituting the sequence of human insulin A DNA as shown in Figure 2. (I) GGCATTGTGGAGCAG (15-mer); (II) TGCTGCACCAGCATCTG (17-mer); (III) CTCCCTCTACC (11-mer); (VIII) GGAGAACTAC (10-mer); (VII) TGCAGCACTGCT (12-mer); (IV) AGGGAGCAGAT (11-mer); (V) TCTCCAGTTGGTAG (14-mer); (VI) GTTGCAGTAGT (11-mer). Each of the oligonucleotides was well-characterized by two-dimensional mobility-shift method and their finger-printings are shown in Figure 5. The enzymatic joining of these, repair to form a duplex, cloning and its expression studies will be published elsewhere.

The DNA sequence for human insulin chain-A was derived from the known sequence of the gene for rat insulin[12] chain-A by replacing the codon of aspartic acid number 69 (GAU) by (GAG) of Glu giving the sequence of mRNA and the H and L strands of DNA as shown in Figure 1. The present synthetic project was initiated with two objectives in mind: (i) to improve triester synthetic methodology for the synthesis of mammalian genes which are generally rich in guanine and cytidine; (ii) to use synthetic gene as a probe to develop and synthesize various initiation control regions for the expression of mammalian genes in bacteria and in yeast systems.

RESULTS AND DISCUSSION

General Plans for the Synthesis of Insulin Chain A DNA Duplex

We planned to synthesize only the nucleotide sequence 1-43 of H-strand and 1-35 of the L-strand (Figure 2) and then to use the primer-extension repair-synthesis[13] to complete the 63-

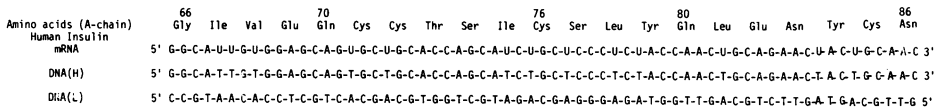

Figure 1. Amino acid sequence of human insulin A chain and the corresponding mRNA and DNA sequence. The designation of the H- and L-strands of DNA are arbitrary.

nucleotide long duplex. In this approach we minimize the rather time-consuming efforts for the chemical synthesis of the entire two strands. The overlapping of 15-nucleotide between the H- and L-strands is more than sufficient to allow efficient repair-synthesis by AMV reverse transcriptase[13] at 37°. Repair synthesis will convert the structure shown in Figure 2 into a completely double-stranded 63-nucleotide long duplex DNA representing the entire coding region for the chain A. This will be reported in the subsequent paper.

Fractionation of Protected Oligonucleotides Containing Triester Groups by Reversed-Phase Chromatography

During our studies on modified triester approach for the last five years, we generally observed that the pure, fully protected product was difficult to separate quantitatively by conventional silica-gel chromatography from the unreacted starting component containing 5'-hydroxyl group, especially in the case of sequences containing several guanine bases. Previously[7], we attempted to remove the hydroxyl component by converting to a phosphodiester group by treatment with β-cyanoethyl phosphate in the presence of benzenesulfonyl triazole. In the presence studies, we found that tlc on silanized silica-gel (RP-2) and KC_{18} (RP-18) plate in acetone-water solvent gave excellent separation between components containing trityl- and hydroxyl group and also between

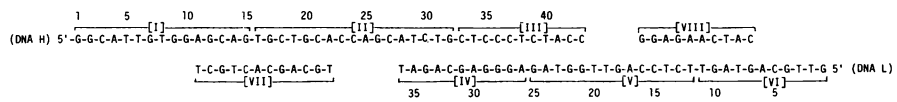

Figure 2. Deoxyoligonucleotide sequences (I-VIII) synthesized chemically - partial sequence coding for the A chain of human insulin.

those differing in their sizes. A typical example is a fractionation on RP-2 tlc plate of a complex mixture formed during preparation of protected CA as compared to fractionation on silica-gel as is shown in Figure 3A and B. Similarly, purification of GGCA could not be achieved on silica-gel as determined after complete deblocking and tlc on PEI-plate whereas purification by tlc on

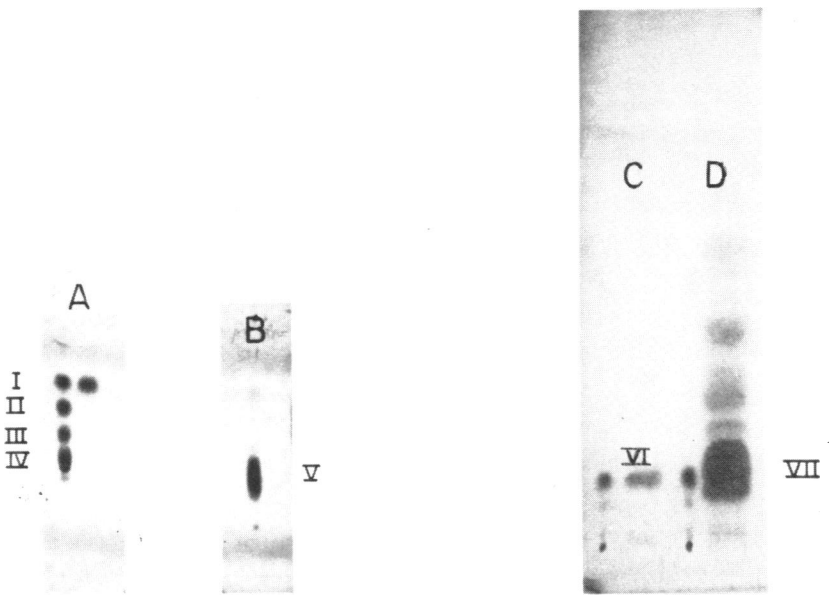

Figure 3. A = Resolution by reversed-phase tlc on silanized silica-gel (RP-2) in acetone-water (30%) of a reaction mixture containing the following components, I = HO-dbzC-OBz; II = HO-dbzA+bzC-OBz; III = $(MeO)_2$TrdbzA-ClPh; IV = $(MeO)_2$TrdbzA+bzC-OBz.
B = Resolution on silica-gel tlc in chloroform-methanol (5%) of the same reaction mixture as in A.
C = PEI-tlc of deblocked tetranucleotide G-G-C-A-P-ClPh after purification of the fully protected tetranucleotide on reserved-phased tlc on RP-2 in acetone-water (25%).
D = PEI-tlc of deblocked tetranucleotide G-G-C-A-P-ClPh after the purification of the fully protected tetranucleotide on silica-gel tlc in chloroform-methanol (10%).

reverse-phase (RP-2) gave pure GGCA as analyzed on PEI-plate shown in Figure 3C. These results indicated that reversed-phase chromatography of triester oligonucleotide could yield pure product at each step of synthesis.

General Method of Synthesis and Purification of Fully Protected Oligonucleotides

The preparation of five commonly occurring dinucleotides such as CA, CT, TG, AG and GG as found in insulin chain A DNA sequence (see Figure 1) was carried out by condensing 5'-dimethoxytrityl mononucleotide (1.2-1.5 molar equivalent) with 5'-hydroxyl mononucleotide in the presence of mesitylenesulfonyl tetrazole. The progress of the reaction was monitored by two different tlc systems: (i) appearance of faster-moving trityl positive spot on silica-gel tlc in chloroform-methanol solvent, and (ii) appearance of slower-moving spot on reversed-phase C_2- and C_{18}-bonded phase tlc acetone-water (20-30%) solvent. Medium-pressure liquid chromatography of crude fully protected CT on silica-gel gave pure product in 75% yield whereas the isolated yields of CA, TG, AG and GG were quite low (30-35%). The poor yield may be due to the degradation of the product perhaps by liberation of trityl or cyanoethyl protecting groups during chromatography stage. In such cases reversed-phase medium-pressure liquid chromatography was found to give better separation with very little degradation. This may be due to the masking of the active sites on silica-gel during silanization.

In each coupling reaction, three types of side products were encountered: (i) unreacted 5'-hydroxyl component; (ii) component sulfonated on the 5'-hydroxyl; (iii) tritylated component containing 3'-fully protected phosphate. These were characterized on PEI-tlc after completely deblocking followed by sequence analysis. The presence of (iii) fully protected tritylated starting material is most intriguing. It might have been reformed from acrylonitrile hydrolysis to cyanoethanol in the presence of some atmospheric moisture during coupling step which on recondensation with the activated 3'-phosphate diester group generated the starting material. This difficulty was overcome by removal of any excess acrylonitrile by triturating the foam with anhydrous ether before each coupling reaction.

The synthesis of seven commonly occurring trinucleotide sequences found in insulin chain A DNA (Figure 1) can be derived from the three dinucleotides. For example fully protected CA yielded four trinucleotides, CAG, CAT, CAC and CAA; AG yielded AGC, AGT and TG yielded TGC and TGG. In our experience, it is much more economical to synthesize large amounts of the 16 possible dinucleotide units instead of the 64 possible trinucleotides to be used in the synthesis of long sequences of polynucleotides.

Chemical Coupling of Longer Protected Oligonucleotides and their Purification

The efficiency of coupling reaction between the oligonucleotide fragments is particularly dependent upon the nature of bases at the junction. For example, the coupling of fragments through two guanines is less efficient than between thymidine. We observed that the efficiency of coupling between pyrimidine to pyrimidine > pyrimidine to purine > purine-purine. The fragments rich in purine invariably gave poor yield in coupling reactions. The synthesis of pentadecamer (I) was carried out by condensing equimolar amounts of octamer GGCATTGT with heptamer GGAGCAG in the presence of mesitylenesulfonyl tetrazole for 4 hr in 40% yield. The pure fully protected pentadecamer I was isolated by preparative tlc on reversed-phase plate (RP-2) in acetone-water (25%). The heptadecamer (II) was prepared in 37% yield by coupling equimolar amounts of octamer with nonamer. Extending the coupling time more than 4 hr was found to cleave the internucleotide phosphotriester linkages as checked by sequence analysis and therefore should be avoided.

The scheme for the synthesis of pentadecamer is outlined in Fig. 4. The reaction conditions and yields of the oligonucleotides are documented in Table I.

Complete Removal of the Protecting Groups and Isolation of Deoxyribooligonucleotides Containing 3'-5' Phosphodiester Linkages

Each of the intermediate oligomers was deblocked completely by treatment with (i) benzenesulfonic acid in chloroform/methanol and then with (ii) concentrated ammonia for 4 to 6 hr at 50° as described previously[7]. The diester compounds were isolated

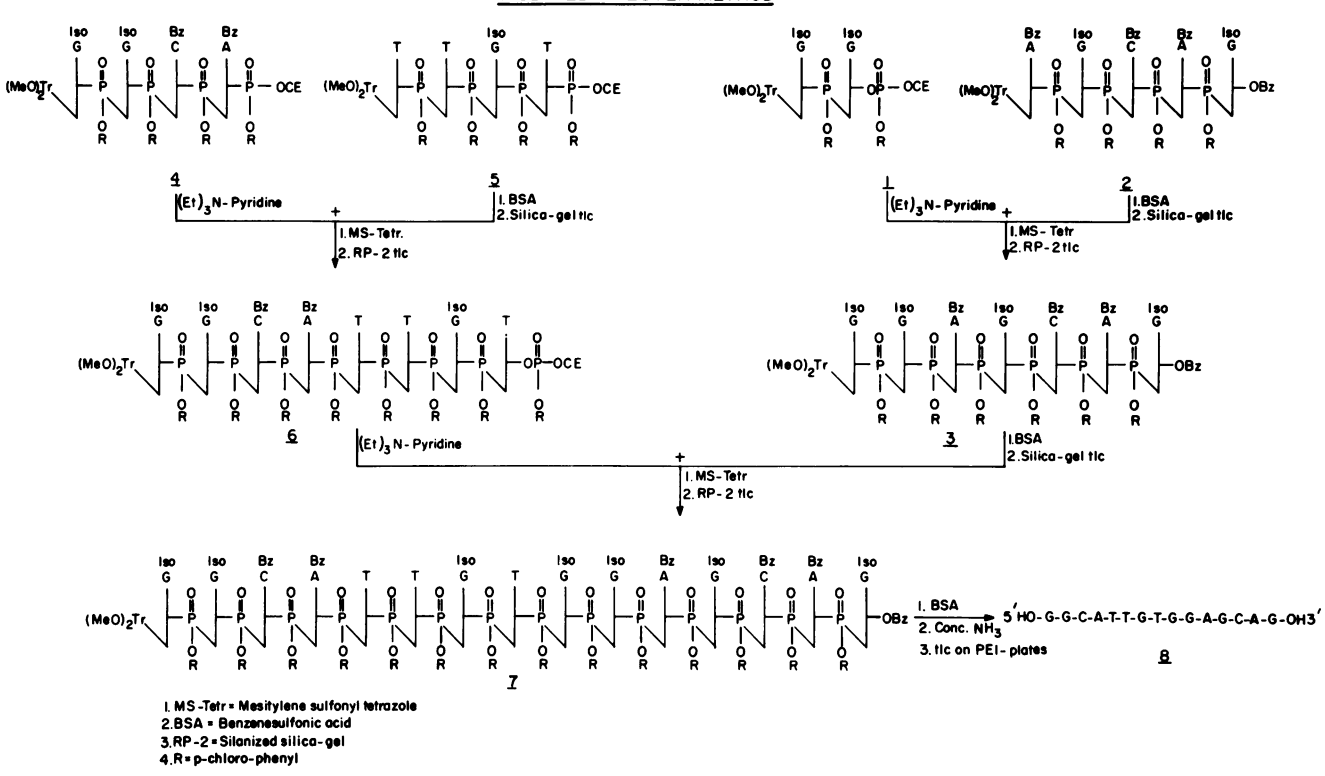

Figure 4. Chemical synthesis of pentadecaoligonucleotide by the modified triester method.

Table I. Reaction conditions and yields of deoxyribooligonucleotide synthesis constituting human insulin chain A gene

5'-Protected[a] component (μmole)	5'-Hydroxyl component (μmole)	Condensing reagent (μmole)	Reaction time (hr)	Product (isolated yield %)	Deblocked product
		(I) 5' OH-G-G-C-A-T-T-G-T-G-G-A-G-C-A-G-OH 3' (15-mer)			
$[(MeO)_2Tr]$dIsoG‡IsoG‡bzC‡bzA-ClPh (11)	dT‡T‡IsoG‡T-ClPh (14)	80	3	$[(MeO)_2Tr]$dIsoG‡IsoG‡bzC‡bzA‡T‡T‡IsoG‡T-ClPh (30)	
[(MeO)Tr]dIsoG‡IsoG-ClPh (57)	dbzA‡IsoG‡bzC‡bzA‡IsoG-OBz (40)	300	2	$[(MeO)_2Tr]$dIsoG‡IsoG‡bzA‡IsoG‡bzC‡bzA‡IsoG-OBz (44)	
$[(MeO)_2Tr]$dIsoG‡IsoG‡bzC‡bzA‡T‡T‡IsoG‡T-ClPh (5)	dIsoG‡IsoG‡bzA‡IsoG‡bzC‡bzA‡IsoG-OBz (3)	40	4	$[(MeO)_2Tr]$dIsoG‡IsoG‡bzC‡bzA‡T‡T‡IsoG‡T‡IsoG‡IsoG‡bzA‡IsoG‡bzC‡bzA‡IsoG-OBz (40)	G-G-C-A-T-T-G-T-G-G-A-G-C-A-G (I)
		(II) 5' OH-T-G-C-T-G-C-A-C-C-A-G-C-A-T-C-T-G-OH 3' (17-mer)			
$[(MeO)_2Tr]$dT‡IsoG‡bzC‡T‡IsoG-ClPh (80)	dIsobzC‡bzA‡bzC-ClPh (75)	250	3	$[(MeO)_2Tr]$dT‡IsoG‡bzC‡T‡IsoG‡bzC‡bzA‡bzC-ClPh (32)	
$[(MeO)_2Tr]$dbzC‡bzA‡IsoG‡bzC‡bzA‡T-ClPh (110)	dbzC‡T‡IsoG-OBz (110)	500	4	[(MeO)Tr]dbzC‡bzA‡IsoG‡bzC‡bzA‡T‡bzC‡T‡IsoG-OBz (36)	
$[(MeO)_2Tr]$dT‡IsoG‡bzC‡T‡IsoG‡bzC‡bzA‡bzC-ClPh (10)	dbzC‡bzA‡IsoG‡bzC‡bzA‡T‡bzC‡T‡IsoG-OBz (10)	(125)	4	$[(Meo)_2Tr]$dT‡IsoG‡bzC‡T‡IsoG‡bzC‡bzA‡bzC‡bzC‡bzA‡IsoG‡bzC‡bzA‡T‡bzC‡T‡IsoG-OBz (37)	T-G-C-T-G-C-A-C-G-A-G-C-A-T-C-T-G (II)
		(III) 5' OH-C-T-C-C-C-T-C-T-A-C-C-OH 3' (11-mer)			
$[(MeO)_2Tr]$dbzC‡T‡bzC‡bzC-ClPh (75)	dbzC‡T‡bzC-ClPh (63)	300	2	$[(MeO)_2Tr]$dbzC‡T‡bzC‡bzC‡bzC‡T‡bzC-ClPh (57)	
$[(MeO)_2Tr]$dbzC‡T‡bzC‡bzC‡bzC‡T‡bzC-ClPh (35)	dT‡bzA‡bzC‡C-OBz (36)	140	2	$[(MeO)_2Tr]$dbzC‡T‡bzC‡bzC‡bzC‡T‡bzC‡T‡bzA‡bzC‡bzC-OBz (16)	C-T-C-C-C-T-C-T-A-C-C (III)

5'-Protected[a] component (μmole)	5'-Hydroxyl component (μmole)	Condensing reagent (μmole)	Reaction time (hr)	Product (isolated yield %)	Deblocked product
		(IV) 5' OH-A-G-G-G-A-G-C-A-G-A-T-OH 3' (11-mer)			
[$(MeO)_2$Tr]dbzA∓IsoG∓ IsoG∓IsoG-ClPh (12)	dbzA∓IsoG∓bzC∓bzA∓ IsoG∓bzA∓T-OBz (6)	80	3	[$(MeO)_2$Tr]dbzA∓IsoG∓ IsoG∓IsoG∓bzA∓IsoG∓ bzC∓bzA∓IsoG∓bzA∓ T-OBz (75)	A-G-G-G-A-G-C-A-C-A-T (IV)
		(V) 5' OH-T-C-T-C-C-A-G-T-T-G-G-T-A-G-OH 3' (14-mer)			
[$(MeO)_2$Tr]dT∓bzC∓T∓bzC∓ bzC∓bzA∓IsoG∓T-ClPh (10)	dT∓IsoG∓IsoG∓T∓bzA∓ IsoG-OBz (7)	50	3	[$(MeO)_2$Tr]dT∓bzC∓T∓bzC∓ bzC∓bzA∓IsoG∓T∓T∓ IsoG∓IsoG∓T∓bzA∓IsoG-OBz (34)	T-C-T-C-C-A-G-T-T-G-G-T-A-G (V)
		(VI) 5' OH-G-T-T-G-C-A-G-T-A-G-T-OH 3' (11-mer)			
[$(MeO)_2$Tr]dIsoG∓T∓T∓ IsoG-ClPh (20)	dbzC∓bzA∓IsoG∓T∓bzA∓ IsoG∓T-OBz (5)	80	2.5	[$(MeO)_2$Tr]dIsoG∓T∓T∓ IsoG∓bzC∓bzA∓IsoG∓ T∓bzA∓IsoG∓T-OBz (40)	G-T-T-G-C-A-G-T-A-G-T (VI)
		(VII) 5' OH-T-G-C-A-G-C-A-C-T-G-C-T-OH 3' (12-mer)			
[$(MeO)_2$Tr]dT∓IsoG∓ bzC∓bzA∓IsoG-ClPh (19)	dbzC∓bzA∓bzC∓T∓IsoG∓ bzC∓T-OBz (6)	100	4	[$(MeO)_2$Tr]dT∓IsoG∓bzC∓ bzA∓IsoG∓bzC∓bzA∓ bzC∓T∓IsoG∓bzC∓T-OBz (31)	T-G-C-A-G-C-A-C-T-G-C-T (VII)
		(VIII) 5'-OH-G-G-A-G-A-A-C-T-A-C-OH 3' (10-mer)			
[$(MeO)_2$Tr]dIsoG∓IsoG∓ bzA∓IsoG-ClPh (20)	dbzA∓bzA∓bzC∓T∓bzA∓ bzC-OBz (20)	100	2	[$(MeO)_2$Tr]dIsoG∓IsoG∓ bzA∓IsoG∓bzA∓bzA∓ bzC∓T∓bzA∓bzC-OBz (40)	G-G-A-G-A-A-C-T-A-C (VIII)

[a]Abbreviations are as suggested by IUPAC-IUB Biochemistry 9, 4022 (1970). A phosphotriester linkage is represented by hyphen and phosphodiester linkage is represented by (∓) symbol. Each internal internucleotidic phosphate is protected with β-chlorophenyl group (ClPh).

by two procedures: (i) Oligonucleotides up to pentadecamers in size were purified by tlc PEI-plates using lithium chloride - 7M urea solvent at 60°. (ii) Some of the oligomers higher than decamer size were fractionated by applying about 50-100 A_{260} on 10 cm band on (20 cm x 40 cm) slab for preparative gel-electrophoresis in 20% gel at pH 7.5. The ultraviolet absorbing bonds were made visible by placing the gel slab on silica-gel tlc plate containing fluorescent indicator covered with saran wrap under the UV light.

Sequence Analysis

Each of the oligomers was labelled using ATP-[γ P^{32}] and polynucleotide kinase and the labelled compound was sequenced by the mobility-shift method[14]. The finger-printing pattern of the eight oligomers are shown in Figure 5.

The sequence analysis of oligomer containing 3'-terminal-p-chlorophenyl phosphate group was carried by first digesting with pancreatic DNase followed by snake venom phosphodiesterase[15]. The finger-printing pattern of P^{32}GGCATTG-P-ClPh is also given in Figure 5.

Concluding Remarks

With the development of modified triester method of synthesis, arylsulfonyl tetrazoles as coupling reagents, reversed-phase chromatography for the purification of fully protected deoxyribooligonucleotides, the synthesis of a gene has become practical within a reasonable period of time.

EXPERIMENTAL SECTION

General Methods and Materials

Silanized silica-gel 60 F-254 tlc plates (RP-2) .25 mm thickness (E.M. Brinkman); KC_{18} reversed-phase tlc plates with fluorescent indicator. 200μ thickness (Whatman); Lichroprep RP-8 (40-63 μm) Size B (Merck, Brinkman); Bondapak C_{18}/Parasil B (Waters columns), FMI laboratory pump; silica-gel 60 silanized (E.M. Brinkman) were purchased commercially. The fully protected mononucleotides were prepared as described previously[7] and purified on Waters Prep LC/system 500 preparative liquid chromatographic system.

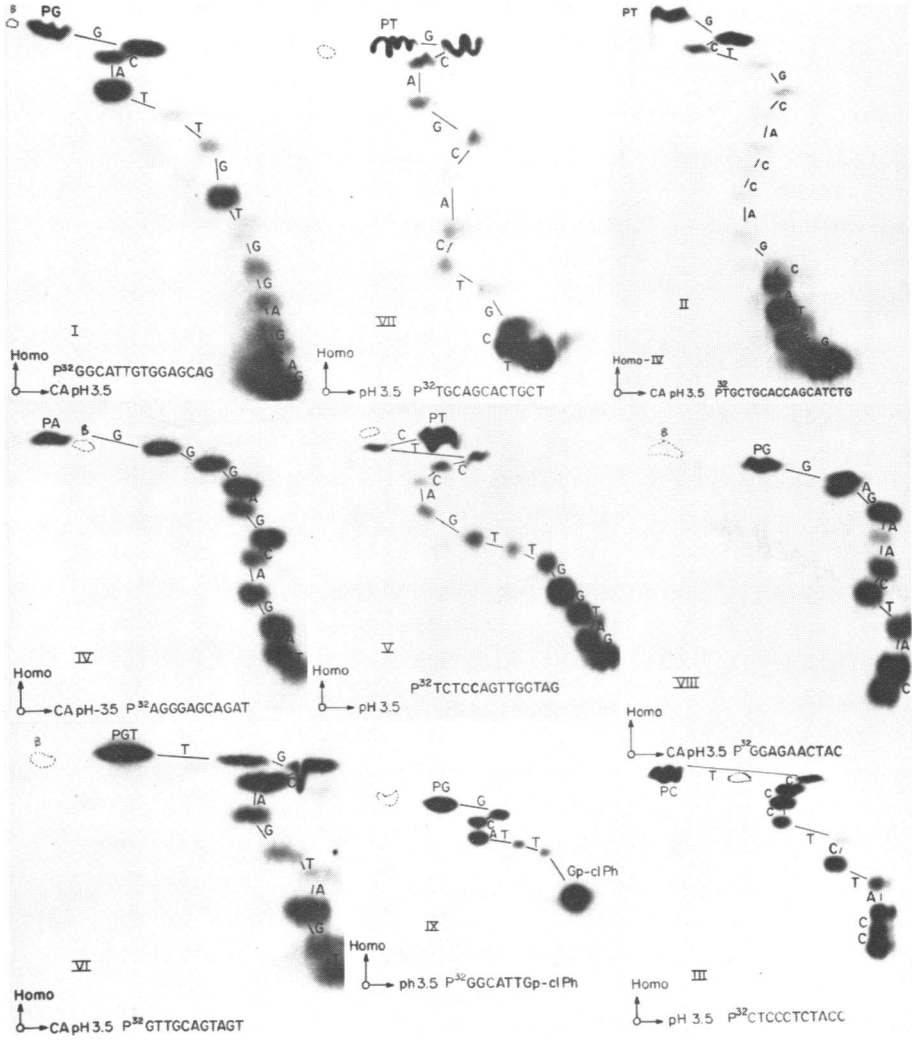

Figure 5. Two dimensional chromatography fingerprint of synthetic deoxyoligonucleotides after partial phosphodiesterase digestion of (I) 5'd(^{32}PG-G-C-A-T-T-G-T-G-G-A-G-C-A-G); (II) d(^{32}PT-G-C-T-G-C-A-C-C-A-G-C-A-T-C-T-G); (III) d(^{32}PC-T-C-C-C-T-C-T-A-C-C); (VIII) d(^{32}P-G-G-A-G-A-A-C-T-A-C); (VII) d(^{32}PT-G-C-A-G-C-A-C-T-G-C-T); (IV) d(^{32}PA-G-G-G-A-G-C-A-G-A-T); (V) d(^{32}PT-C-T-C-C-A-G-T-T-G-G-T-A-G); (VI) d(^{32}PG-T-T-G-C-A-G-T-A-G-T); (IX) d(^{32}PG-G-C-A-T-T-G-P-ClPh). The first dimension is electrophoresis on cellulose acetate strip at pH 3.5, and the second dimension is homochromatography on 20 x 20 cm DEAE-cellulose tlc plate. B represents the location of the xylene cyanol FF dye marker.

Solvent Systems

Triester oligonucleotides were analyzed on (i) silica-gel F-254 tlc plates in solvent A, chloroform-methanol (1-15%) containing 2% formamide; and (ii) silanized silica-gel F-254 (RP-2) or KC_{18} reversed-phase (RP-18) tlc plates in solvent B, acetone-water (20-30% v/v). The medium pressure reversed-phase column was run in acetonitrile-water (20-30%) solvent.

General Method for the Synthesis of Fully Protected Di-, Tri-nucleotides

Fully protected mono- or dinucleotide (A) was first dried by co-evaporation of anhydrous pyridine (2 x 10 ml) and then dissolved in anhydrous pyridine (10-20 ml per mM of the nucleotide component). Excess anhydrous triethylamine[16] (at least 10 molar equivalent) was added and this reaction mixture was kept at room temperature and monitored by two tlc systems (i) appearance of the trityl positive spot at the origin on silica-gel plate in chloroform-methanol (10%) and (ii) movement of the trityl positive on RP-2 or RP-18 tlc at the solvent front in acetone-water (20-30%) solvent system. After 2 to 3 hr, the reaction was complete and the mixture was evaporated to a foam to remove excess of triethylamine and acrylonitrile liberated during the deblocking reaction. The foamy material was then washed with anhydrous ether (2 x 5 ml) and mixed with 5'-hydroxyl mononucleotide (B) in dry pyridine and evaporated to dryness under reduced pressure.

The syrupy residue was redissolved in dry pyridine (5 ml per 1 mM of the nucleotidic components), followed by the addition of mesitylenesulfonyl tetrazole (3 to 5 molar equivalents). The coupling reaction was over in less than 2 hr (as judged by two tlc systems such as (i) appearance of faster moving trityl-positive component on silica-gel tlc in chloroform-methanol (10%) solvent and (ii) appearance of slower moving trityl-positive component on RP-2 or RP-18 tlc plates in acetone-water (20, 25, 30% v/v). The reaction mixture was then decomposed with cold distilled water (5 ml) and the resultant solution was evaporated to a gum in vacuo. The gum was dissolved in ice-cold chloroform (50 ml) followed by washing with 5% sodium bicarbonate (2 x 25 ml) and water (1 x 25 ml). The organic layer was dried over sodium sulfate. The crude reaction mixture was dissolved in 1 ml of

acetonitrile-water (20% v/v) and purified by medium pressure liquid chromatography reversed-phase column (RP-8). The fraction containing the desired product was concentrated in vacuo till it became turbid. It was next extracted with chloroform (3 x 250 ml) which was washed once with water. On drying the chloroform and then on evaporating, the product was obtained as white foamy material in 60-80% yield.

General Method of Coupling Longer Oligonucleotides and their Purification

The fragment containing the 5'-terminus sequence was decyanoethylated with anhydrous pyridine and triethylamine whereas fragment containing the 3'-terminus sequence was detritylated with benzenesulfonic acid and purified by preparative tlc on silica-gel in chloroform-methanol (10%) as described earlier[7]. The coupling reaction was carried out in anhydrous pyridine with mesitylene-sulfonyl tetrazole (3 to 5 molar equivalents) for 2 to 4 hr. After the usual work up as described above, the organic layer was passed through Whatman phase separating filter paper[17] to remove water[18]. The dried crude mixture was fractionated by preparative tlc (30 mg/plate) on RP-2 or RP-18 (20 x 20 cm) in acetone-water (20-30% v/v) solvent. The desired band was eluted with chloroform-methanol (20% v/v) (60 ml). The reaction conditions and yields for the coupling reaction are given in Table 1.

General Method of Deblocking and Isolation of Oligomers Containing 3'-5' Phosphodiester Linkages

The complete deblocking of each fragment was carried out by treatment with benzenesulfonic acid in chloroform/methanol to remove dimethoxytrityl group followed by concentrated ammonia to remove all N-benzoyl and p-chlorophenyl groups as described previously[7]. The oligomers up to fifteen bases long were purified by PEI-tlc plate at 60°C using appropriate concentration of lithium chloride pH 7.1 containing 7 M urea[7]. Fractionation of oligomers higher than decamer was achieved by the preparative gel-electrophoresis on (20 cm x 40 cm) slab as described below.

Preparative Gel-Electrophoresis of Non-radioactive Deoxyribo-oligonucleotides Containing 3'-5' Phosphodiester Linkages

A sample of oligonucleotide (~50-100 A_{260}) in 100 µl of 7 M urea was applied in a lot 10 cm wide and 1 cm deep on a slab 20 x

40 cm containing 20% (wt/vol) acrylamide 10.7% (wt/vol) methylene bisacrylamide/7 M urea/50 mM Tris-bonate, pH 7.5/1 mM EDTA/3 mM ammonium sulfate. A mixture of dye markers bromophenol blue and xylene cyanol was applied in a well 1 cm deep and 1.3 cm wide on each side of the 10 cm band. Electrophoresis was carried out at 800 V/25 mA for 5 hr.

The bands on the gel were visualized under UV light by transferring on two (20 x 20 cm) silica-gel tlc plates containing fluorescent indicator covered with saran wrap. The desired band was sliced, homogenized and eluted with 0.25 M triethylammonium bicarbonate (3 x 5 ml) and then passed through a plastic pipette containing 0.5 ml of DE-50 cellulose supported on a cotton plug. It was first washed with water and the desired compound was eluted with 2 M triethylammonium bicarbonate pH 9.0 in about 70% recovery.

Sequence Determination of Synthetic Oligonucleotides

The sequence of bases in each chemically synthesized oligomer was checked by two-dimensional mobility-shift method[14] with some modification as described below.

(I) Oligomer Containing 3'-Hydroxyl End

In the case of those sequences which contain a pyrimidine base among a sequence of purine near 3'-end, it was found important to take out aliquots at shorter intervals in the beginning of the enzymatic reaction. For example, in the case of 14-mer, P^{32}TCTCCAGTTGGTAG; aliquots were taken at 0, 2, 4, 6, 10, 20, 30, 45 and 60 min. If the aliquots were taken at 0, 5, 10, 20, 30, 35 and 60 min, the thymidine at third position from 3'-end was missing in the x-ray autoradiograph.

(II) Oligomers Containing 5'-P-Chlorophenyl Phosphate Group

The 5'-labelled oligonucleotide was digested at 37° in a 10 µl solution of 5 mM $MgCl_2$, 10 mM Tris (pH 7.6) and 10 µg calf thymus DNA and 1 µl of pancreatic DNase (2 mg/ml stock, 1µ = 2 µg). Three microlitre samples were withdrawn after 3 min, 10 min and 60 min into a siliconized tube and kept at 90°C for 3 min (each aliquot). To the combined heated sample (11 µl) was added 2 µl of venom phosphodiesterase (1 mg/ml) and 2 µl of 300 mM triethylammonium bicarbonate (pH 9.2); this was incubated at 37°C and after 3 min, 30 min and 60 min samples of 4 µl were transferred to another tube and heated each time at 90°C for 3 min. Finally

all the samples were combined and dried in a desiccator, dissolved in 2 µl of water and subjected to two-dimensional chromatography by the standard procedure.

REFERENCES

1 NRCC No. 17216.
2 Letsinger, R.L. and Ogilvie, K.K. (1969) J. Amer. Chem. Soc. 91, 3350; Arentzen, R. and Reese, C.B. (1977) J. Chem. Soc. Perkin I, 445; Eckstein, F. and Rizk, I. (1969) Chem. Ber. 102, 2362; Neilson, T. and Werstink, E.S. (1974) J. Amer. Chem. Soc. 96, 2362.
3 Khorana, H.G., Agarwal, K.L., Bresmer, P., Büchi, H., Caruthers, M.H., Cashion, P.J., Fridkin, M., Jay, E., Kleppe, K., Kleppe, R., Kumar, A., Loewen, P.C., Miller, R.C., Minamoto, K., Panet, A., RajBhandary, U.L., Ramamoorthy, B., Sekiya, T., Takeya, T. and van de Sande, J.H. (1976) J. Biol. Chem. 251, 565.
4 Itakura, K., Bahl, C.P., Katagiri, N., Michniewicz, J., Narang, S.A., Wightman, R.H. (1973) Cand. J. Chem. 51, 3649; Catlin, T.C. and Cramer, F. (1973) J. Org. Chem. 38, 245.
5 Katagiri, N., Itakura, K. and Narang, S.A. (1975) J. Amer. Chem. Soc. 97, 7332.
6 Stawinski, J., Hozumi, T. and Narang, S.A. (1976) Cand. J. Chem. 54, 670.
7 Stawinski, J., Hozumi, T., Narang, S.A. Bahl, C.P. and Wu, R. (1977) Nucleic Acid Res. 4, 353.
8 Bahl, C.P., Wu, R., Itakura, K., Katagiri, N. and Narang, S.A. (1976) Proc. Natl. Acad. Sci. USA 73, 91.
9 Bahl, C.P., Marians, K.J., Wu, R., Stawinski, J. and Narang, S.A. (1976) 1, 81.
10 Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heynekar, H.L., Bolivar, F. and Boyer, H.W. (1977) Science, 198, 1056.
11 Zamecnik, P.C. and Stephenson, M.L. (1978) Proc. Natl. Acad. Sci. USA 75, 280.
12 Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W.J. and Goodman, H.W. (1977) Science 196, 1313.
13 Bahl, C.P., Wu, R., Stawinski, J. and Narang, S.A. (1977) Proc. Natl. Acad. Sci. USA 74, 966.
14 Tu, C.D., Jay, E., Bahl, C.P. and Wu, R. (1976) Anal. Biochem. 74, 73.
15 Personal communication with Professor Ray Wu, Cornell University, Ithaca, N.Y.
16 Sood, A.K. and Narang, S.A. (1977) Nucleic Acid Res. 4, 2757; Adamiak, R.W., Barciszewska, M.Z., Biala, E., Grzéskowiak, K., Kierzek, R., Kraszewski, A., Markiewicz, W.T. and Wiewiorwski, M. (1976) Nucleic Acid Res. 3, 3397.
17 Personal communication with Dr. J. Hackman, Collaborative Research Inc., Waltham, Mass.
18 The drying of chloroform solution containing higher oligonucleotides on inorganic drying reagents was avoided because of the considerable loss through absorption on the usual drying agents.