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Deoxyribonucleic Acids (DNA) and Related Compounds. XIII.[1] Synthesis of DNA Duplexes Containing a Ribosome Binding Site

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To investigate the efficiency of expression of synthetic genes, deoxyribonucleic acid (DNA) duplexes (33—37 base pairs) containing the ribosome binding site (S.D. sequence) for *trp*L and appropriate termini were synthesized. This involved chemical synthesis of 8 oligonucleotides with chain lengths of 16—19 by the solid-phase phosphotriester method followed by enzymatic joining with DNA ligase. The duplexes were designed to replace a restriction fragment from a plasmid containing the *trp* promoter and a synthetic human growth hormone gene. The S.D. sequence in these duplexes was located upstream from a *Cla* I site to make a distance of 9, 11 or 13 bases from the initiation signal (ATG).

Keywords——solid-phase phosphotriester method; restriction sequence; Shine–Dalgarno sequence; tryptophan promoter; high pressure liquid chromatography; thin-layer chromatography

Expression in *E. coli* of synthetic genes for various peptides has been performed by using plasmids containing regulatory regions from *E. coli* genes.[3] Regulatory regions include promoters which are binding sites for ribonucleic acid (RNA) polymerase, and genes for the ribosome binding sites, Shine–Dalgarno (S.D.) sequence.[4] The distance between the S.D. sequence and the initiation codon (AUG) varies between 7—12 nucleotides and this may affect the efficiency of expression of genes at the translation level.[5] In this paper we report a synthesis of three deoxyribonucleic acid (DNA) duplexes with chain lengths of 33 to 37 base pairs (bp) containing the S.D. sequence for *E. coli trp*L, by enzymatic ligation of oligonucleotides (A—D in Fig. 1) synthesized by the phosphotriester method on a polymer support. These duplexes were used to compare the efficiency of expression of a human growth hormone (HGH) gene by insertion in a plasmid pHGH-1.[6] Figure 1 shows the structures of 8 deoxyoligonucleotides which compose the three duplexes with restriction sequences at both ends as well as initiation codons.

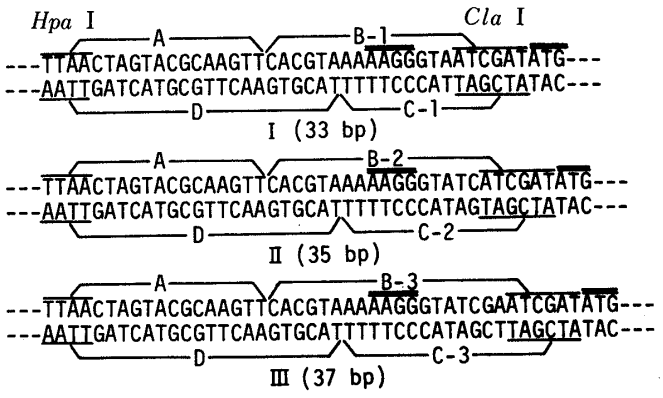


Fig. 1. Structures of the Duplex (I—III) and Restriction Sequences

Deoxyoligonucleotides (A—D) were joined by treatment with T4 DNA ligase as indicated by lines.

Experimental

General Methods——Thin-layer chromatography (TLC) and reversed phase TLC (RTLC) were performed on plates of Kieselgel $60F_{254}$ (Merck) in chloroform–methanol (10 : 1, v/v) and Kieselgel $60F_{254}$ silanisiert (Merck) in acetone–20 mM tirethylammonium acetate (55 : 45), respectively. The purity of protected dinucleotides was examined by using a scanner (Shimadzu C5-900). For column chromatography, Kieselgel 60H (Merck) in chloroform–methanol and alkylated silica gel (C-18 Silica gel, 35—105 μ, Waters) in acetone–0.1% aqueous pyridine were used. Deprotected oligonucleotides were purified by reversed phase chromatography on a column (0.7 × 15 cm, Bio-Rad) of C-18 Silica gel equipped with a Gilson Miniplus 2 monitor and FC-80K fractionator.

High pressure liquid chromatography (HPLC) was performed on an Altex 322 MP chromatograph system or a Gilson MS-3 using columns of TSK-Gel LS-410 AK, (4.0 i.d. × 250 mm, Toyo Soda Co.) or YMC Pack A-312 (0.1 i.d. × 150 mm, Yamamura Chemical Co.) for reversed phase HPLC. Solvents used: A, 0.1 M triethylammonium acetate (5% acetonitrile); B, 0.1 M triethylammonium acetate (25% acetonitrile). For anion-exchange HPLC, a column (4.0 i.d. × 250 mm) of diethylaminoethyl (DEAE) silica gel (TSK Gel IEX 540K, Toyo Soda Co.) was used in solvent A, 20% aqueous acetonitrile and solvent B, 20% acetonitrile in 1 M ammonium formate. Mobility shift analysis[7] was performed after 5′-phosphorylation of oligonucleotides with polynucleotide kinase.[8]

Polynucleotide kinase, T4 DNA ligase, *Hpa* I, and bacterial alkaline phosphatase were obtained from Takara Shuzo Co.

Other general methods for characterization of oligonucleotides were as described previously.[6,9,10]

Preparation of *o*-Chlorophenyl Phosphoditriazolide——1,2,4-Triazole (6.38 g, 92.4 mmol) was coevaporated with pyridine and dissolved in dioxane (84 ml). *o*-Chlorophenyl phosphodichloridate (4.6 ml, 28 mmol) was added to the mixture and triethylamine (8.20 ml, 58.8 mmol) was added with cooling. The mixture was stirred at room temperature for 1 h and the filtered solution was stored at −20 °C.

Preparation of Dinucleotides——5′-*O*-Dimethoxytrityl-*N*-protected nucleosides (**1**)[11] were phosphorylated with *o*-chlorophenyl phosphoroditriazolide[12] to give *β*-cyanoethyl derivatives (**3**) and 5′-*O*-dimethoxytrityl-*N*-acyldeoxynucleoside 3′-(*o*-chlorophenyl)phosphates (**4**) *via* active intermediates (**2**) and these mononucleotides were condensed as described[12] using 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT).[13] As an example, protected d(TpAp) was synthesized and purified by the following procedures. Compound **1** (B, thymin-1-yl) (2.72 g, 5 mmol) was dried by coevaporation of pyridine and treated with the above phosphorylating reagent (21 ml, 7 mmol) for 30 min with stirring. Aqueous pyridine (30%) was added to the mixture and the nucleotide (**4**) was extracted with chloroform then washed 3 times with 0.1 M triethylammonium bicarbonate. The solution was concentrated and **3** (B, *N*-benzoyladenin-9-yl) (2.40 g, 4 mmol) was added. The mixture was dried by coevaporation of pyridine twice and treated with MSNT (2.96 g, 10 mmol) for 20 min in pyridine (12 ml). The reaction was examined by TLC and halted at the appropriate time by addition of 30% pyridine. The dinucleotide (**5**) was extracted with chloroform, washed 3 times with water, concentrated and applied to a column (6.5 i.d. × 5.8 cm) of silica gel (60H, 70 g). The product was eluted with chloroform containing 3—4% methanol and precipitated with pentane. The yield was 4.99 g, 3.79 mmol, 95%. The cyanoethyl group was removed by dissolving **5** (1 g) in pyridine–triethylamine (3 : 1, v/v, 8 ml) followed by treatment with water (2 ml) for 5 min. The solvents were removed and the residue was dried by coevaporation of pyridine. Compound **6** was precipitated with pentane–ether (1 : 1) from its solution in chloroform.

Synthesis of Deoxyoligonucleotides——The deoxynucleoside resin was prepared from aminomethylated (0.14 mmol/g) polystyrene (1% cross linked, Peptide Institute Inc.) and 3′-succinyldeoxynucleosides.[14] For each oligonucleotide, 5 μmol of nucleoside resin was used as the starting material. Procedures for elongation of the chain were essentially the same as reported.[6] Eight different deoxyoligonucleotides (A, B-1, B-2, B-3, C-1, C-2, C-3 and D) were synthesized (*cf.* Tables I and II).

Preparation of Duplexes (I—III)——Oligonucleotides A (0.03 A_{260}), B-1 (0.01 A_{260}) and D (0.03 A_{260}) were phosphorylated with [γ-^{32}P] adenosine triphosphate (ATP) (150 pmol, *ca.* 1 μCi) using polynucleotide kinase (2 U)[8] in 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, and 1 mM spermine (total volume, 10 μl) at 37 °C for 45 min. The mixture was treated with ATP (10 mmol) at 37 °C for 45 min, then heated at 90 °C for 3 min. For the preparation of I, fragment C-1 (0.01 A_{260}, *ca.* 75 pmol) and the oligonucleotides were annealed in 66 mM Tris–HCl (pH 7.6) and 6.6 mM $MgCl_2$ (total 27.5 μl) by heating to 75 °C followed by cooling to room temperature in a water bath during 1 h. The mixture was cooled to 15 °C then DNA ligase (0.12 U) and 0.2 M *β*-mercaptoethanol (1.5 μl) were added and the whole was incubated at 15 °C for 15 h. The enzyme was inactivated by heating at 65 °C for 5 min and the joined products were treated with *Hpa*I (30 U) in the presence of 100 mM NaCl at 37 °C for 5 h. The mixture was treated with phenol–chloroform (1 : 1) and washed with ether, then the duplex was precipitated with ethanol. The product (*ca.* 50 pmol) was isolated by 15% polyacrylamide gel electrophoresis and phosphorylated with [γ-^{32}P]ATP (50 pmol, *ca.* 0.2 μCi) using polynucleotide kinase (4 U) by the procedure described for the phosphorylation of A, B-1 and D.

Results

Deoxyoligonucleotides (A—D) (Fig. 1) were synthesized by the phosphotriester method

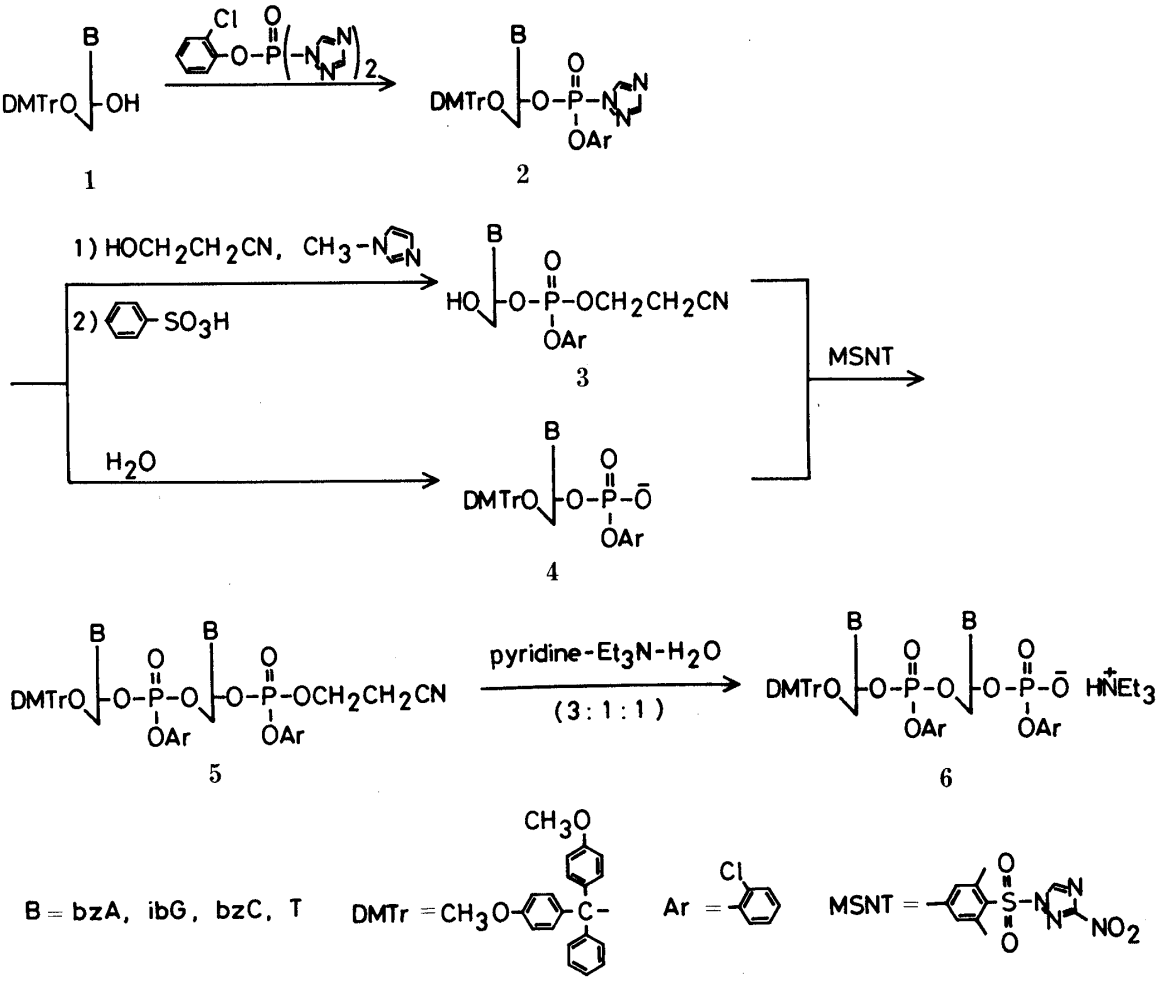
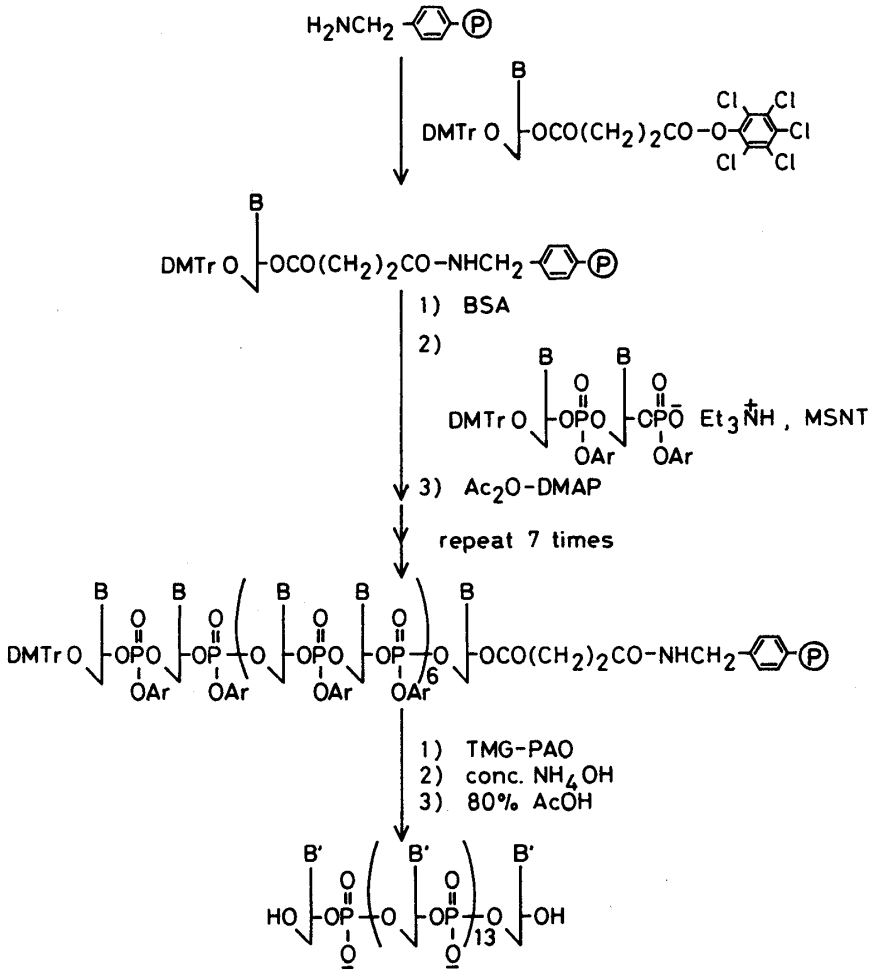


Chart 1

TABLE I. Polymer Support Synthesis of Deoxyoligonucleotides

Step	Solvent or reagent	Amount	Reaction time	Number of operation
1	CH_2Cl_2–MeOH (7 : 3)	2 ml	0.1 min	3
2	2% BSA in CH_2Cl_2–MeOH	2 ml	1 min	1
3	CH_2Cl_2–MeOH (7 : 3)	2 ml	0.1 min	1
4	2% BSA in CH_2Cl_2–MeOH (7 : 3)	2 ml	1 min	1
5	CH_2Cl_2–MeOH (7 : 3)	2 ml	0.1 min	2
6	Pyridine	2 ml	0.1 min	3
7	Pyridine	0.3 ml	Coevaporation	1
8	Dimer block	200 mg/0.3 ml	Coevaporation	1
9	MSNT in pyridine	200 mg/0.3 ml	20 min (40 °C)	1
10	Pyridine	2 ml	0.1 min	2
11	0.1 M DMAP in pyridine	1.8 ml	3 min	1
	Ac_2O	0.2 ml		
12	Pyridine	2 ml	0.1 min	3

BSA = benzenesulfonic acid; MSNT = 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole; DMAP = 4-dimethylaminopyridine.



Ⓟ = polystyrene copolymer with 1% divinylbenzene
B = bzA, ibG, bzC, T
B′ = A, G, C, T
DMTr = 4,4′-dimethoxytrityl
Ar = *o*-chlorophenyl
BSA = 2% benzenesulfonic acid in CH_2Cl_2–MeOH (7 : 3)
MSNT = 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole
DMAP = 0.1 M 4-dimethylaminopyridine in pyridine
TMG-PAO = 0.5 M N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridine-2-aldoximate in dioxane–H_2O (9 : 1)

Chart 2

on a polymer support using protected dinucleotides which had been prepared by the reported procedure[12] with some modifications. Schemes for the reactions are shown in Chart 1. The 3′-terminal nucleosides were linked to aminomethylene polystyrene with succinyl residues.[14] Schemes for the synthesis of deoxyoligonucleotides are shown in Chart 2. A similar procedure has been used for the synthesis of gene fragments for human growth hormone.[6] Some modifications in the polymer support synthesis are included in Table I which shows the operations for one cycle of elongation. In step 9, the reaction time for condensation was shortened to 20 min by heating at 40 °C, and the step II, 3 min for acetylation was found to be sufficient. Methods for purification were also modified to shorten the time required for isolation without decreasing the purity of the product. The procedure involved reversed-phase chromatography of 5′-dimethoxytritylated oligonucleotides at low pressure followed by

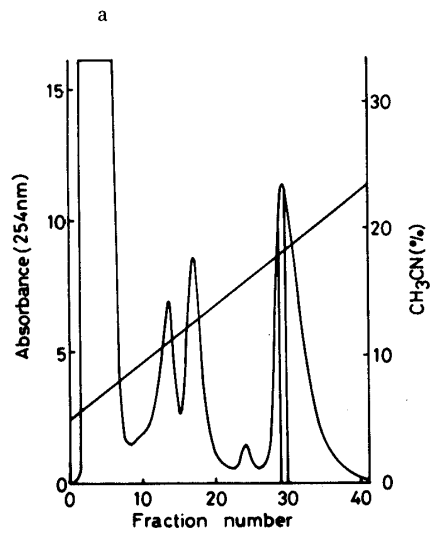


Fig. 2a. Reversed Phase Chromatography of 5′-Dimethoxytrityl 17 mer (B-1) on a Column (0.7 × 12 cm) of C-18 Silica Gel (35—105 μ, Waters) Using a Linear Gradient of Acetonitrile (5—35%) in 50 mM Triethylammonium Acetate (Total, 200 ml)

Fractions of 3 ml were collected every 4 min.

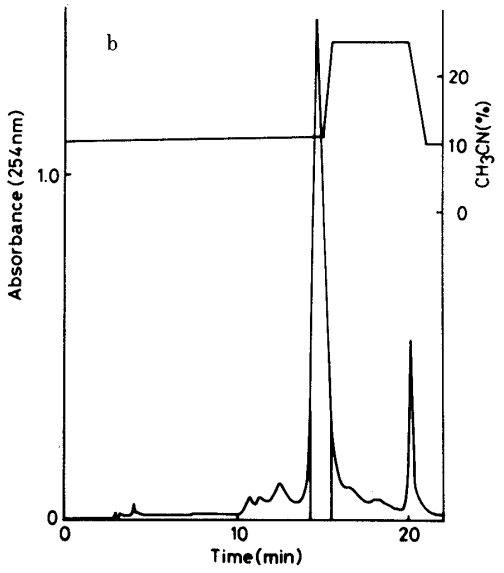


Fig. 2b. Reversed Phase HPLC of the Dedimethoxytritylated 17 mer Obtained from the Last Peak in Fig. 2a on a Column (6 × 150 mn) of C-18 Silica Gel (YMC Pack A-312) Using a Gradient of Acetonitrile in 0.1 M Triethylammonium Acetate

The flow rate was 1 ml/min.

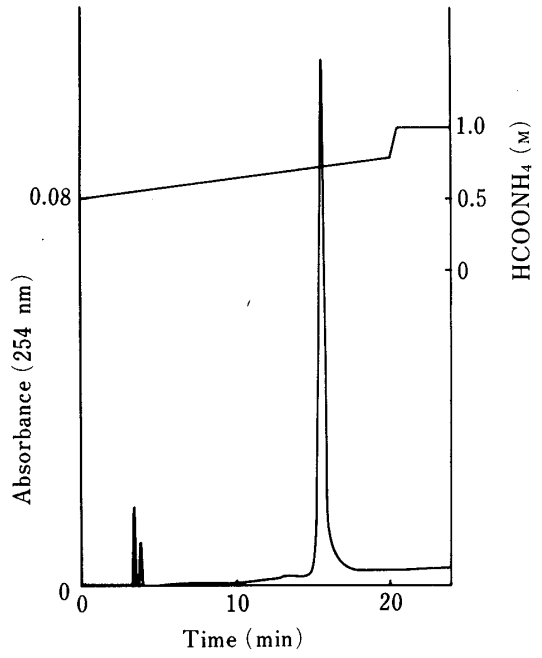


Fig. 3. Analysis of the 17 mer (B-1) by Anion-Exchange HPLC on a Column of DEAE-Silica Gel (TSK-gel IEX-540K) with a Gradient of Ammonium Formate in 20% Acetonitrile

The flow rate was 1 ml/min.

reversed-phase chromatography of completely deblocked products. As an example, Fig. 2a shows an elution profile for low-pressure chromatography of a 17-mer (B-1) on C-18 Silica gel. The main fraction from the last peak was treated with 80% acetic acid and analyzed by reversed phase HPLC. The product was fractionated as shown in Fig. 2b under the conditions used for the analytical chromatography, and then analyzed by anion-exchange chromatography using formate ion as shown in Fig. 3. If impurities were detected, products were purified by this method and desalted by gel filtration. The purity of the products was further confirmed

TABLE II. Yields and Retention Times of Deoxyoligonucleotides

Reagent number	Sequence	Chain length	Overall yield (%)	Average yield (%)	Retention times (min) Reversed-phase[a]	Ion-exchange[b]
A	AACTAGTACGCAAGTT	16	50	91	11.4	15.5
B-1	CACGTAAAAAGGGTAAT	17	66	94	10.2	15.6
B-2	CACGTAAAAAGGGTATCAT	19	48	91	10.6	17.3
B-3	CACGTAAAAAGGGTATCGAAT	21	18	82	10.5	18.0
C-1	CGATTACCCTTT	13	62	91	10.3	14.0
C-2	CGATGATACCCTTT	15	59	92	10.2	15.0
C-3	CGATTCGATACCCTTT	17	60	93	10.8	16.6
D	TACGTGAACTTGCGTACTAGTT	22	19	85	14.3	17.6

a) A column (4 × 250 nm) of TSK-GEL LS-410AK was used. Elution was performed with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate. The gradient was 25—75% of buffer B (25% acetonitrile) in buffer A (5% acetonitrile) in 20 min at a flow rate of 2 ml/min. *b*) The conditions were as described in Fig. 3.

by mobility shift analysis.[7] Table II summarizes the yields and retention times of the oligonucleotides.

For the preparation of duplexes I—III by enzymatic joining, the donor strands (B-1, B-2, B-3, and D) and acceptor strands at the fresh end (A) were 5′-phosphorylated by treatment with polynucleotide kinase and [γ-^{32}P]ATP.[8] The four components for each combination were treated with DNA ligase and digested with the restriction enzyme *Hpa* I. Duplexes I—III were isolated by gel electrophoresis.

In conclusion, DNA duplexes can be synthesized efficiently by chemical and enzymatic procedures, especially when they have common regions. The present duplexes were used to compare the efficiency of expression of the HGH gene by insertion into a plasmid,[6] and *E. coli* clones harboring plasmids containing duplex I, with the S.D. sequence 9 bases upstream from the ATG, produced HGH most efficiently.

References

1) Paper XII: E. Ohtsuka, T. Ohgi, K. Kitano, T. Fukui, and M. Ikehara, *Chem. Pharm. Bull.*, **33**, 1849 (1985).
2) Press address: *Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan.*
3) *a*) K. Itakura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, *Science*, **198**, 1056 (1977); *b*) D. V. Goeddel, H. L. Heynecker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. H. Seeburg, *Nature* (London), **281**, 544 (1979); *c*) M. D. Edge, A. R. Greene, G. R. Heathcliffe, D. A. Meacock, W. Schuch, D. B. Scanlon, T. C. Atkinson, C. R. Newton, and A. F. Markham, *ibid.*, **292**, 756 (1981); *d*) M. Suzuki, S. Sumi, A. Hasegawa, T. Nishizawa, K. Miyoshi, S. Wakisawa, T. Miyake, and F. Misoka, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 2475 (1982); *e*) S. Tanaka, T. Oshima, K. Ohsue, T. Ono, S. Oikawa, I. Takano, T. Noguchi, K. Kangawa, N. Minamino, and H. Matsuo, *Nucleic Acids Res.*, **10**, 1741 (1982); *f*) H. A. de Boer, L. J. Comstock, and M. Vasser, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 21 (1983).
4) *a*) J. Shine and L. Dalgarno, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1342 (1974); *b*) J. A. Steiz and K. Jakes, *ibid.*, **72**, 4734 (1975).
5) *a*) T. Kurokawa, M. Seno, R. Sasada, Y. Ono, H. Onda, K. Igarashi, M. Kikuchi, Y. Sugino, and T. Honjo, *Nucleic Acids Res.*, **11**, 3077 (1983); *b*) M. D. Matteucci and H. L. Heynecker, *ibid.*, **11**, 3113 (1983).
6) M. Ikehara, E. Ohtsuka, T. Tokunaga, Y. Taniyama, S. Iwai, K. Kitano, S. Miyamoto, T. Ohgi, Y. Sakuragawa, K. Fukuyama, T. Ikari, M. Kobayashi, T. Miyake, S. Shibahara, A. Ono, T. Ueda, T. Tanaka, H. Baba, T. Miki, A. Sakurai, T. Oishi, O. Chisaka, and K. Matsubara, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 5956 (1984).
7) *a*) F. Sanger, J. E. Donelson, A. R. Carlson, H. Kössel, and D. Fischer, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 1209 (1973); *b*) M. Silberklang, A. M. Gillum, and U. L. RajBhandary, *Nucleic Acids Res.*, **4**, 4091 (1977).
8) C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.*, **54**, 158 (1965).

9) E. Ohtsuka, Y. Taniyama, R. Marumoto, H. Sato, H. Hirosaki, and M. Ikehara, *Nucleic Acids Res.*, **10**, 2597 (1982).
10) E. Ohtsuka, Y. Taniyama, S. Iwai, T. Yoshida, and M. Ikehara, *Chem. Pharm. Bull.*, **32**, 85 (1984).
11) *a*) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962); *b*) E. L. Brown, R. Belagaje, M. J. Ryan, and H. G. Khorana, *Methods Enzymol.*, **68**, 109 (1979).
12) C. Broka, T. Hozumi, R. Arentzen, and K. Itakura, *Nucleic Acids Res.*, **8**, 5461 (1980).
13) S. S. Jones, B. Rayner, C. B. Reese, A. Ubasawa, and M. Ubasawa, *Tetrahedron*, **36**, 3075 (1980).
14) H. Ito, Y. Ike, S. Ikuta, and K. Itakura, *Nucleic Acids Res.*, **10**, 1755 (1982).