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Synthesis and Expression of a Gene for Human Tumor Necrosis Factor

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A gene for human tumor necrosis factor has been synthesized by joining 60 oligodeoxyribonucleotides of 13 to 20 residues using deoxyribonucleic acid (DNA) ligase. These oligodeoxyribonucleotides were prepared by the solid-phase phosphotriester method. The gene (483 base pairs) was designed to carry a *Cla* I site plus a start codon at the N-terminal end and stop codons plus a *Sal* I site at the C-terminal end. The gene was expressed in *Escherichia coli* under the control of the tryptophan promoter of *E. coli*, and the expressed product was purified and tested for cytotoxic activity.

Keywords——oligodeoxyribonucleotide; phosphotriester method; DNA ligase; tryptophan promoter; peptide gene; anion-exchange chromatography; cytotoxic peptide

Tumor necrosis factor (TNF) was found originally in sera of bacillus Calmette-Guerin (BCG)-infected mice as a macrophage-derived factor.[1] It is cytotoxic to tumor cells and less toxic or not toxic to normal cells *in vitro*. TNF has been isolated from serum of other animals and no species specificity has been observed for the TNF activities isolated from rabbits, mice, rats and human.[2-4] Cloning and expression of the complementary deoxyribonucleic acid (cDNA) for human TNF (hTNF) have been reported and the deduced amino acid sequences of hTNF and murine TNF have been compared.[3] We wished to produce a large amount of hTNF and to crystallize it for structural studies by X-ray crystallography. In this paper we describe the design and synthesis of an hTNF gene. A new, efficient method to construct an expression plasmid is described, together with the isolation of the expressed product.

Materials and Methods

Synthesis of Oligonucleotides——Oligodeoxyribonucleotides (U1—U30, L1—L30 in Fig. 1) were prepared by the phosphotriester method using the procedures described previously[5,6] with some modifications, as follows. Smaller amounts of nucleoside resins (2.5 μmol), and dinucleotides (15 mg, *ca.* 10 μmol) were used for the synthesis. Decyanoethylation of fully protected dinucleotides was performed in a mixture of *tert*-butylamine and pyridine.[7]

Phosphorylation and Ligation——Enzymes and coenzymes were obtained and used as described previously.[5] G-Rich fragments (L2, L20 and U23) were 5′-phosphorylated with polynucleotide kinase using conditions for blunt ends.[8] Methods for isolation and characterization of ligation products were as described.[5]

Transformation and Colony Hybridization——Transformation of *Escherichia coli* HB101 (gift of Dr. K. Matsubara, Osaka University) was carried out with plasmids according to Morrison's procedure[9] and positive colonies were screened by colony hybridization.[10] Labeled oligonucleotide probes (20 pmol, 1 μCi/pmol) were also used for hybridization with plasmids on agarose gel.[11,12]

Construction of Plasmids and Expression of the Gene——Oligodeoxyribonucleotides (*ca.* 1.5 nmol) were 5′-phosphorylated by polynucleotide kinase plus adenosine triphosphate (ATP) and joined by T4 DNA ligase as shown

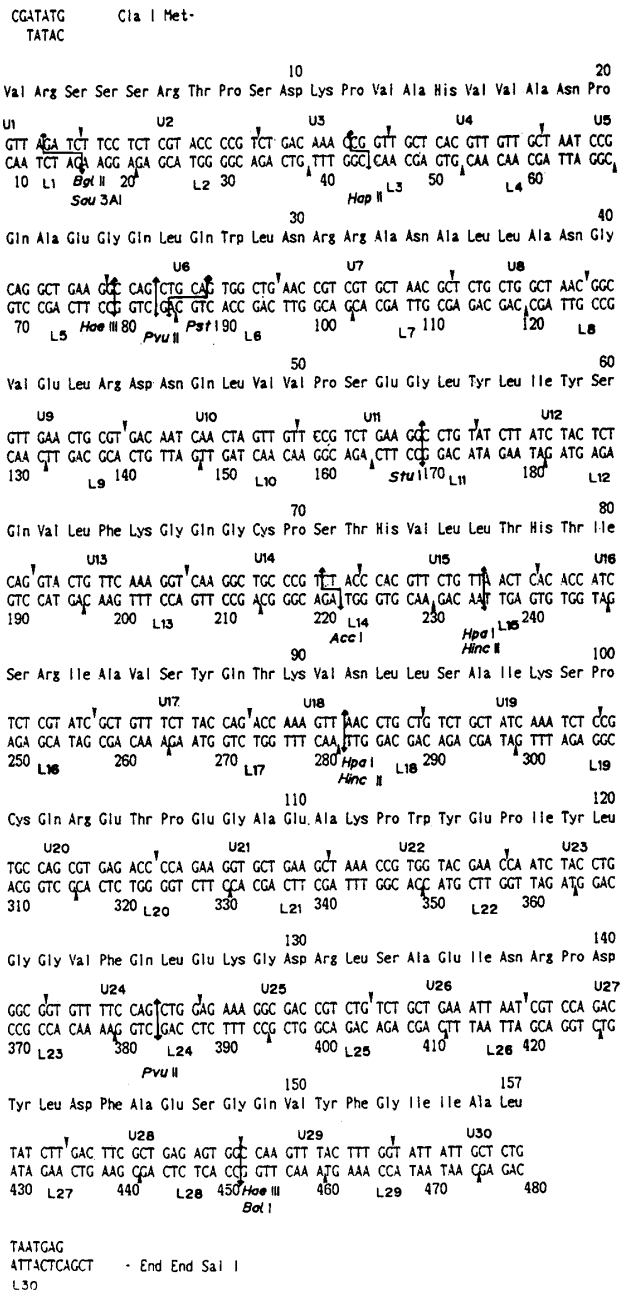

Fig. 1. Amino Acid Sequence of hTNF and Nucleotide Sequence of a Synthetic Gene for hTNF

Individual oligonucleotides (U1—U30, L1—L30) are separated by arrowheads. The flanking sequence at the N-terminal part of the gene corresponds to the recognition sequence of *Cla* I and the codon for methionine. The flanking sequence at the C-terminal part of the gene corresponds to two stop codons and recognition sequence of *Sal* I.

in Figs. 2 and 3. Segments I—VI (4 μg, *ca.* 80 pmol each) were treated with T4 DNA ligase (6 units) at 15°C for 5 h at the concentration of 1.5 μM. The product was precipitated with ethanol and isolated by 10% polyacrylamide gel electrophoresis (PAGE) (*ca.* 20 pmol). The larger segments (I—II and III—VI, Fig. 3) were obtained (*ca.* 20 pmol) by the same procedure using 5% PAGE. Each segment, I—II or III—VI, in 1 μM solution was 5′-phosphorylated by using polynucleotide kinase (5.5 units) and ATP (20 nmol) at 37°C for 2 h. pGH-L9 (10 μg, 3.5 pmol) was treated with *Cla* I (21 units) at 37 °C for 7.5 h and then treated with *Sal* I (24 units) at 37 °C for 12 h at a substrate concentration of 14 nM. The products were precipitated with ethanol and the larger fragment was isolated by 1% low-melting-temperature agarose gel electrophoresis. The product (3 μg, *ca.* 1 pmol at 7 μM) was annealed with segment III—VI (5.33 pmol) at 65 °C for 1 h and kept at room temperature for 1.5 h. The mixture was treated with DNA ligase (18 units) at 15 °C for 14 h then cleaved with *Cla* I (21 units) at 37 °C for 3.5 h (Fig. 3). The product (0.7 pmol) was isolated by 1% low-melting-temperature agarose gel electrophoresis and an aliquot (0.6 μg, 0.21 pmol) was annealed with segment I—II (1.2 pmol) as described above. phTNF was obtained by treating the mixture (at 1.4 nM) with DNA ligase (3 units) at 15 °C for 13 h. *E. coli* HB101 was transformed with phTNF and transformants were screened by colony hybridization[10] and plasmid mini-preparation.[8]

Gene expression under the tryptophan promoter was carried out as follows: phTNF/HB101 was incubated in LB medium containing Amp. (40 μg/ml) at 37 °C for 8—10 h and 0.8 ml of this culture was inoculated with M9-0.2%

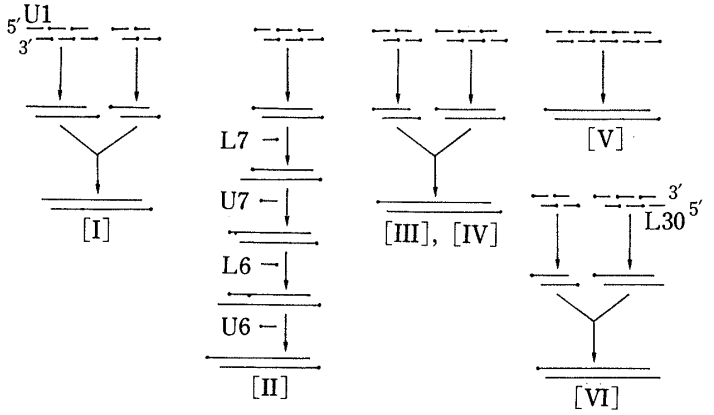

Fig. 2. Preparation of Gene Segments I—VI for the Gene by Ligation of Chemically Synthesized Oligodeoxyribonucleotides

The segments are composed of the following oligonucleotides; I, U1—U5 and L1—L5; II, U6—U10 and L6—L10; III, U11—U15 and L11—L15; IV, U16—U20 and L16—L20; V, U21—U25 and L21—L25; VI, U26—U30 and L26—L30. Terminal dots indicate the 5′-phosphate.

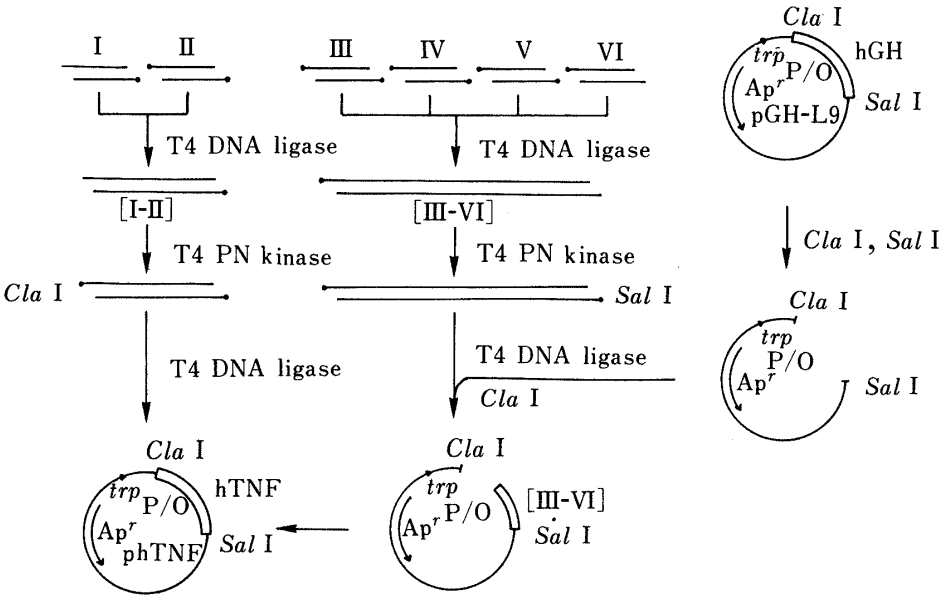

Fig. 3. Construction of phTNF

Segments I—IV obtained as shown in Fig. 2 were further joined to yield two duplexes (I—II and III—VI). The longer duplex was first ligated to the *Sal* I site of pGH-L9, which had been treated with *Cla* I and *Sal* I. The smaller synthetic duplex (I—II) was then joined to the uncircularized plasmid to yield phTNF.

PN kinase, polynucleotide kinase.

casamino acid (M9CA) medium (containing Amp. 40 μg/ml) (40 ml) at 37 °C for 10—16 h. A 20 ml aliquot of this preculture was inoculated with fresh M9CA medium (1 l) and shaken vigorously at 37 °C for 1 h, and then 3-indoleacrylic acid (IAA, 20 μg/ml), an inducer of tryptophan promoter, was added. After a further 20 h cultivation at 37 °C, the induced protein was found to amount to 5% of the total cellular proteins.

Purification and Activity of hTNF——phTNF/HB101 (1 l culture) was harvested, and the cells (2.0 g) were washed with 50 mM Tris–HCl (pH 8.0) containing 30 mM NaCl and suspended in the same buffer (20 ml). Lysozyme (final concentration 1 mg/ml) was added at 0 °C and kept for 30 min. Freezing and thawing were repeated 5 times, and the mixture was centrifuged at 7500 × *g* for 20 min and at 17000 × *g* for 20 min. The supernatant was centrifuged at 100000 × *g* for 1 h to remove trace amounts of precipitates and applied to a column (3.2 × 9.0 cm) of DEAE-cellulose. The product was eluted with a gradient of NaCl (0—0.2 M) at a flow rate of 0.5 ml/min, and analyzed by PAGE in the presence of sodium dodecyl sulfate (SDS–PAGE) using markers (ovalbumin, α-chymotrypsinogen, β-lactoglo-

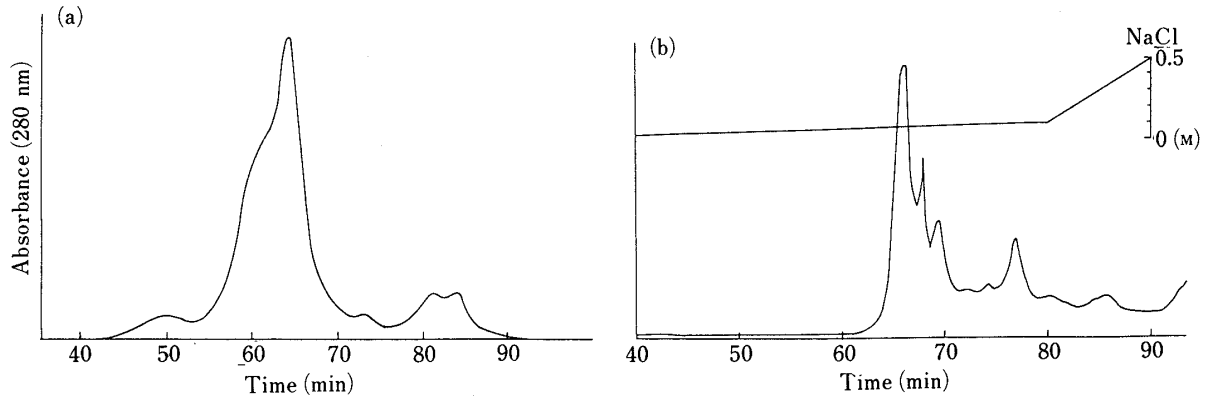

Fig. 4. a) High-Pressure Gel Filtration of the Expressed Protein on a Column (7.5 × 600 mm) of TSK-Gel G3000SW (Toyo-Soda Co.) in 50 mM Sodium Phosphate (pH 7.0)

The flow rate was 0.3 ml/min. Fractions (55—71) were collected.

b) Anion-Exchange HPLC of the Product from Gel Filtration

The sample was dialyzed against 2 l of 20 mM Tris–HCl (pH 7.5) and applied to a column (4.6 × 250 mm) of TSK-Gel DEAE 2SW (Toyo Soda Co.). Fractions (63—67) were collected.

bulin, lysozyme and bovine trypsin inhibitor) obtained from BRL Inc. Collected fractions were concentrated by ultrafiltration using a Diaflo membrane (Amicon YM10).

High-performance liquid chromatography (HPLC) was performed for further purification as shown in Fig. 4. The biological activity of the purified hTNF was tested by measuring cytotoxic activity against mouse L-M cells (ATCC, CCL1.2) as described.[13]

Results and Discussion

Design and Synthesis of hTNF Gene Fragments

The synthetic gene for hTNF was designed according to the amino acid sequence deduced from the cDNA,[3] and optimal codons for *E. coli*[14] were used (Fig. 1). Moreover several unique restriction sites were incorporated for cassette mutagenesis (Fig. 1). The gene was constructed so as to be expressed under the control of the tryptophan promoter of *E. coli*, which was used in plasmid pGH-L9 for the expression of a synthetic human growth hormone gene.[5] Recognition sequences for *Cla* I and *Sal* I were attached to the 5′ and 3′ ends of the hTNF gene, respectively. Homologous and invert-repeat sequences within 100 base pairs were removed by using alternative codons to avoid mispairing during ligation. Figure 1 shows the amino acid sequence for hTNF and the base sequence for the synthetic gene. The gene with 483 base pairs (bp) was divided into 60 oligonucleotides (U1—U30 and L1—L30 in Fig. 1) having chain lengths of 13—20. The solid-phase phosphotriester method[5] was used for the synthesis of these oligonucleotides. In this procedure we made two modifications to shorten the time required for the synthesis. First, the amount of reaction resin and dinucleotide blocks were reduced to 2.5 and 10 μmol, respectively. The volume of solvents for washing was correspondingly reduced to 1 ml and the resin was washed twice. The average time required for one cycle could be reduced from 40 min (40 °C) to 30 min (room temperature). Secondly, oligonucleotides were removed from the support by treatment with N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridin-2-aldoximate[15] and the solution was directly treated *in situ* with concentrated ammonia at 55 °C for 2 h to yield 5′-dimethoxytritylated oligonucleotides. Thus, the time required for partial deblocking could be shortened from more than 20 to 2.5 h, and this treatment was found to cause no serious side reactions compared with the previous treatments at 30 °C, as examined by reversed-phase and anion-exchange chromatographies[6] for purification of products with or without the 5′-dimethoxytrityl group.

Construction and Expression of the hTNF Gene

The 60 oligonucleotide fragments obtained were phosphorylated (except U1 and L30) by polynucleotide kinase plus ATP and were joined by T4 DNA ligase as shown in Fig. 2. Segments I—VI were formed in two steps, except II and V. Segment II was started by ligation of 6 fragments followed by the subsequent addition of L7, U7, L6 and U6 and segment V was prepared by ligating 10 oligonucleotides at once.

Usually a synthetic gene is constructed from segments, such as I, II, III ... *etc.*, but this strategy requires a lot of segments to take account of the ligation and isolation yields at each step. To overcome this defect, larger segments I—II and III—VI were made at first in this case, and they were joined to the expression vector, pGH-L9[5] in two steps (Fig. 3). Segment III—VI was assembled and joined to the longer fragment of *Cla* I-*Sal* I digest of pGH-L9. Plasmids phTNF containing the total gene were formed by joining segment I—II and used to transform *E. coli* HB101. The desired recombinant was selected at first by hybridization with ^{32}P-labeled oligonucleotides L10 and L26 used for gene synthesis, as shown in Fig. 1. Plasmids from positive colonies to both probes were analyzed by *Cla* I plus *Sal* I and *Bgl* II plus *Sal* I digestion. The base sequence of the gene in the plasmid was confirmed by the dideoxy method[16] by using synthetic deoxyoligomer (Fig. 1) as the primer under denaturing conditions.

The incorporated gene was expressed by induction with IAA and the amount of the induced protein was estimated by SDS–PAGE. The relative molecular weight was estimated as 1.7×10^4 (Fig. 5) which is consistent with that predicted from the amino acid sequence. Conditions to obtain maximum expression of hTNF in *E. coli* were examined: 20 μg/ml IAA was used for induction of tryptophan promoter, and a 20 h cultivation time was employed. The cytotoxic activity of crude extract against mouse L-M cells was estimated as 2.6×10^4 units/ml of culture.

Isolation and Biological Activity of the Product

The induced protein was isolated by anion-exchange chromatography initially, and

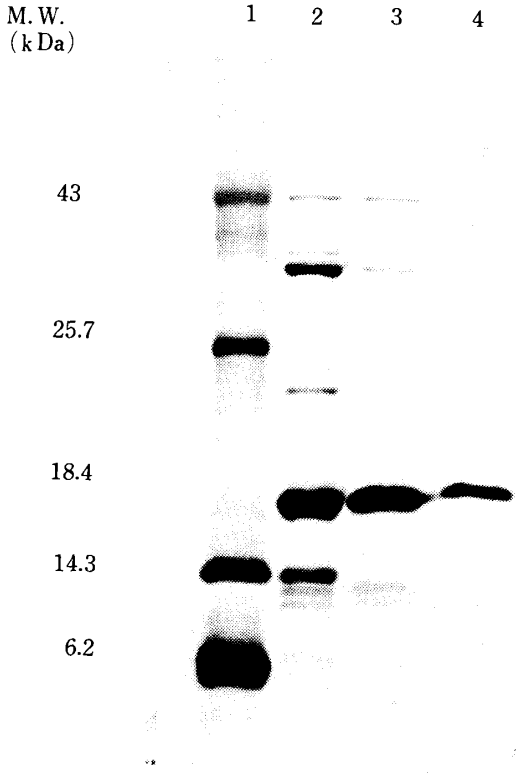

Fig. 5. PAGE of hTNF in the Presence of SDS

1, marker; 2, after anion-exchange chromatography; 3, after high-pressure gel-filtration; 4, after anion-exchange HPLC.

purified by HPLCs. Figure 4a shows an elution profile of hTNF on gel-filtration; hTNF was eluted in the main peak as judged by SDS–PAGE. The recovered fractions were further purified by anion-exchange HPLC as shown in Fig. 4b. The product at various stages of purification was analyzed by SDS–PAGE (Fig. 5). Finally 1.5 mg of recombinant hTNF was isolated as a single band on SDS–PAGE (Fig. 5, lane 4), from 1 l of culture. The amino acid sequence of the purified hTNF was determined by using a gas-phase sequencer, and methionine was detected as the N-terminal residue. The recombinant human growth hormone produced in the same system also possessed methionine at its N-terminus.[5]

The cytotoxic activity of the purified recombinant hTNF was tested against mouse L-M cells and found to be 8.0×10^5 units/mg. This value is lower than that of previously reported hTNF,[13] from which the N-terminal two amino acids, valine and arginine, had been deleted. However, it has been reported that the existence of these two amino acids reduces the cytotoxic activity.[17] Actually the activity of our recombinant hTNF against mouse L-929 cells was of the same order as that reported previously[18] (data not shown).

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References

1) E. A. Carswell, L. J. Old, R. L. Kassel, S. Green, N. Fiore and B. Williamson, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3666 (1975).
2) G. A. Currie and C. Basham, *J. Exp. Med.*, **142**, 1600 (1975).
3) D. Pennica, G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal and D. V. Goeddel, *Nature* (London), **312**, 724 (1984).
4) W. P. Reed and Z. J. Lucas, *J. Immunol.*, **115**, 395 (1975).
5) M. Ikehara, E. Ohtsuka, T. Tokunaga, Y. Taniyama, S. Iwai, K. Kitano, S. Miyamoto, T. Ohgi, Y. Sakuragawa, K. Fujiyama, T. Ikari, M. Kobayashi, T. Miyake, S. Shibahara, A. Ono, T. Ueda, T. Tanaka, H. Baba, T. Miki, A. Sakurai, T. Ohishi, O. Chisaka and K. Matsubara, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 5956 (1984).
6) E. Ohtsuka, S. Iwai, T. Tokunaga and M. Ikehara, *Chem. Pharm. Bull.*, **33**, 3153 (1985).
7) H. Hsiung, S. Inouye, J. West, B. Sturm and M. Inouye, *Nucleic Acids Res.*, **11**, 3227 (1983).
8) T. Maniatis, E. F. Fritsch and J. Sambrook, "Molecular Cloning," Cold Spring Harbor Laboratory, Cold Spring Harbor, 1982, p. 124.
9) D. A. Morrison, *Methods Enzymol.*, **68**, 326 (1979).
10) D. Hanahan and M. Meselson, *Gene*, **10**, 63 (1980).
11) M. Botchan, W. Topp and J. Sambrook, *Cell*, **9**, 269 (1976).
12) A. J. Jeffreys and R. A. Flavell, *Cell*, **12**, 429 (1977).
13) M. Yamada, Y. Furutani, M. Notake, J. Yamagishi, M. Yamayoshi, T. Fukui, H. Nomura, M. Komiya, J. Kawashima, K. Nakano, Y. Sohmura and S. Nakamura, *J. Biotechnol.*, **3**, 141 (1985).
14) T. Ikemura, *J. Mol. Biol.*, **146**, 1 (1981).
15) C. B. Reese, R. Titmas and L. Yau, *Tetrahedron Lett.*, **1978**, 2727.
16) F. Sanger, S. Nicklen and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463 (1977).
17) J. Yamagishi, R. Furuta, H. Kotani, S. Kawata, H. Kawashima, M. Yamayoshi, T. Fukui, Y. Sohmura, M. Yamada and S. Nakamura, Abstracts of Papers, 5th International Lymphokine Workshop, Clear Water Beach, Florida, January 1987, p. 1439.
18) B. B. Aggarwal, W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel and R. C. Harkins, *J. Biol. Chem.*, **260**, 2345.