Expression in *Escherichia coli* of chemically synthesized gene for the human immune interferon

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ABSTRACT

A 454 base pair fragment of double stranded DNA consisting of a gene for a human immune interferon (hIFN-γ), initiation and termination signals plus appropriate restriction endonuclease sites, was totally synthesized. The synthesis involved preparation of 62 oligodeoxyribonucleotides by rapid, solid phase procedures, and enzymatic ligation of the oligonucleotides. This synthetic gene was expressed in E. coli under the control of the lac UV5 promoter. The product has antiviral activity which was acid labile and completely neutralized by antiserum to hIFN-γ but not by antiserum to hIFN-α or hIFN-β. Molecular weight of hIFN-γ produced by E. coli was estimated to be about 32,000 and 17,000 by gel filtration and SDS-polyacrylamide gel electrophoresis respectively.

INTRODUCTION

Among interferons (IFNs), immune interferon (IFN-γ) was indicated to have a better potential use in the treatment of neoplasia. The antiproliferative effect of IFN-γ on transformed cells has been reported to be 10 to 100 times greater than that of IFN-α or IFN-β[1]. However, this comparison could be completely irrelevant since the amounts of IFNs used were chosen based not on their absolute amounts but on their antiviral units due to lack of enough purified IFN-γ. The difficulty of obtaining large amounts necessary for the investigation of the efficacy of IFNs from human cells has been a major cause of the ambiguity surrounding them.

The recent progress of genetic engineering has changed this situation dramatically by opening the way to produce large amounts of several IFN-αs[2-4] and IFN-β[5,6] in bacterial cells, and has now made possible the purification of large quantities of these proteins in biologically active form. However, IFN-γ has not been investigated as much.

Recently, c-DNA clones prepared from hIFN-γ mRNA were obtained and amino acid sequence of the hIFN-γ was deduced from the nucleotide sequence of the c-DNA[7,8]. On the basis of the amino acid sequence of the hIFN-γ (146 amino acid residues) , we designed a DNA fragment which would encode the same protein and be amenable to total chemical synthesis (Fig.1). In the case of hIFN-α_1, the total chemical synthesis of the gene was already reported by Edege et al.[9].

We synthesized the 62 different oligodeoxyribonucleotides by improved solid phase phosphotriester method which enabled us to reduce the time of the synthesis. These DNA fragments were separately purified and 5' phosphorylated, then sequentially ligated to construct a 454 base pair (bp) fragment. After insertion into an expression plasmid and cloning in E. coli, the synthetic gene was characterized by restriction enzyme digestion and total sequence determination. Preliminary result on biological activity of the protein expressed in E. coli and molecular nature of the protein is also described.

Design of the synthetic gene

We designed the gene of hIFN-γ with the following consideration. a) According to the recent studies by Grantham et al.[10-12], comparison of the mRNA sequences of highly expressed and weakly expressed genes in bacteria suggested that these genes have different selection of codons reflecting different degrees of their expression. We have attempted to use codons which are used in highly expressed genes as far as possible. b) The unique restriction endonuclease recognition sequences were introduced into the coding region of hIFN-γ gene for further manipulation of the gene. c) In order to facilitate the insertion into plasmid DNA, the 5'-ends of this gene were designed to carry single-stranded cohesive termini for the EcoRI and SalI restriction endonucleases. d) In addition to the 146 hIFN-γ codons, a methionine codon (ATG) preceding the normal N-terminal amino acid of the polypeptide and a nonsence codon (TAA) after its C-terminal codon were built into the nucleotide sequence of the gene.

The following view points guided the division of the hIFN-γ gene into the 62 oligonucleotide fragments. a) Nucleotide overlaps of at least six base pairs on each of the opposing fragments of the complementary strand were chosen for proper alignment during enzymatic ligation. b) We avoided the oligonucleotides whose 3'-terminal base sequence was adenine, since depurination[13] of adenosine at that position can not be prevented in our synthetic procedure.

In order to construct total hIFN-γ gene with the 62 chemically synthesized oligonucleotides using T4 DNA ligase, we divided the gene into six groups as shown in Fig.3a which were then ligated into larger fragments I and II before constructing the complete gene. To avoid the possibility of wrong joinings which would result from imperfect Watson-Crick structures or self-

```
                 1                                            10                                           20
EcoRI      Met Cys Tyr Cys Gln Asp Pro Tyr Val Lys Glu Ala Glu Asn Leu Lys Lys Tyr Phe Asn Ala
       <-- 1 --><-- 3 --><-- 5 --><-- 7 --><-- 9 -->
5'-AATTCATGTGCTACTGCCAGGACCCATACGTGAAGGAAGCTGAAAACCTGAAGAAATACTTCAACGCT-3'
3'-    GTACACGATGACGGTCCTGGGTATGCACTTCCTTCGACTTTTGGACTTCTTTATGAA       -5'
       <-- 2 --><-- 4 --><-- 6 --><-- 8 -->

                                          30
      Gly His Ser Asp Val Ala Asp Asn Gly Thr Leu Phe Leu Gly Ile Leu Lys Asn Trp
       <-- 11 --><-- 13 --><-- 15 --><-- 17 -->
5'-       GGTCATTCTGACGTTGCTGACAACGGTACTCTGTTCCTGGGTATCCTGAAAAACTG-3'
3'-GTTGCGACCAGTAAGACTGCAACGACTGTTGCCATGAGACAAGGACCCATAGGACT      -5'
   <-- 10 --><-- 12 --><-- 14 --><-- 16 -->

      40                                    50
      Lys Glu Glu Ser Asp Arg Lys Ile Met Gln Ser Gln Ile Val Ser Phe Tyr Phe Lys
       <-- 19 --><-- 21 --><-- 23 --><-- 25 -->
5'-       GAAAGAAGAATCTGACCGTAAAATCATGCAGTCTCAGATCGTTTCTTTCTACTTCAAG-3'
3'-TTTTGACCTTTCTTCTTAGACTGGCATTTTAGTACGTCAGAGTCTAGCAAAGAAAGA      -5'
   <-- 18 --><-- 20 --><-- 22 --><-- 24 -->

          60                                     70
      Leu Phe Lys Asn Phe Lys Asp Asp Gln Ser Ile Gln Lys Ser Val Glu Thr Ile Lys
       <-- 27 --><-- 29 --><-- 31 --><-- 33 -->
5'-       CTGTTCAAAAACTTCAAGGACGACCAGTCTATCCAGAAATCTGTTGAAACTATCAAG-3'
3'-TGAAGTTCGACAAGTTTTTGAAGTTCCTGCTGGTCAGATAGGTCTTTAGACAACTTT      -5'
   <-- 26 --><-- 28 --><-- 30 --><-- 32 -->

              80                                     90
      Glu Asp Met Asn Val Lys Phe Phe Asn Ser Asn Lys Lys Lys Arg Asp Asp Phe Glu Lys
       <-- 35 --><-- 37 --><-- 39 --><-- 41 -->
5'-       GAAGACATGAACGTTAAGTTCTTCAACTCTAACAAGAAAAAGCGTGACGACTTCGAAAAGC-3'
3'-GATAGTTCCTTCTGTACTTGCAATTCAAGAAGTTGAGATTGTTCTTTTTCGCACTGCTGAA      -5'
   <-- 34 --><-- 36 --><-- 38 --><-- 40 -->

              100                                   110
      Leu Thr Asn Tyr Ser Val Thr Asp Leu Asn Val Gln Arg Lys Ala Ile His Glu Leu Ile
       <-- 43 --><-- 45 --><-- 47 --><-- 49 -->
5'-       TTACTAACTACTCTGTTACTGACCTTAATGTACAGCGTAAAGCTATCCATGAACTGAT-3'
3'-GCTTTTCGAATGATTGATGAGACAATGACTGGAATTACATGTCGCATTTCGATAGGTA      -5'
   <-- 42 --><-- 44 --><-- 46 --><-- 48 -->

              120                                    130
      Gln Val Met Ala Glu Leu Ser Pro Ala Ala Lys Thr Gly Lys Arg Lys Arg Ser Gln Met
       <-- 51 --><-- 53 --><-- 55 --><-- 57 -->
5'-       CCAGGTTATGGCTGAACTGTCCCCGGCTGCTAAAACTGGTAAGCGTAAAAGATCTCAGATG-3'
3'-CTTGACTAGGTCCAATACCGACTTGACAGGGGCCGACGATTTTGACCATTCGCATTTTCTA      -5'
   <-- 50 --><-- 52 --><-- 54 --><-- 56 -->

              140                  146
      Leu Phe Arg Gly Arg Arg Ala Ser Gln Stp   SalI
       <-- 59 --><-- 61 -->
5'-       CTGTTCCGTGGTCGTCGTGCTTCTCAGTAAG      -3'
3'-GAGTCTACGACAAGGCACCAGCAGCACGAAGAGTCATTCAGCT-5'
   <-- 58 --><-- 60 --><-- 62 -->
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Fig.1 Nucleotide and amino acid sequence of the synthetic human immune interferon gene

Individual oligodeoxyribonucleotides (1-62) are arrowed.

Numbers above each line refer to amino acid position.

complementality, the fragments of each group were scanned with a computer program designed to detect complementality among fragments.

When we found cross-hybridizing sequences, we redesigned the sequences within the constraints of genetic code to construct the final sequence of hIFN-γ gene as shown in Fig.1.

MATERIALS AND METHODS

Enzymes and reagents

Restriction endonucleases ClaI and AvaII were obtained from Boehringer and Bethesda Research Laboratories, respectively. All other restriction enzymes, T4 polynucleotide kinase and T4 DNA polymerase were purchased from Takara Shuzo. T4 DNA ligase was from Boehringer. The following reaction buffers were used; kination buffer, 70 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$; ligation buffer, 20 mM Tris-HCl (pH 7.6)/10 mM $MgCl_2$/0.4 mM ATP/10 mM DTT; T4 DNA polymerase buffer, 100 mM Tris-HCl (pH 8.8)/10 mM $MgCl_2$/25 mM $(NH_4)_2SO_4$/10 µM EDTA/BSA (50 µg/ml); TA buffer[14], 33 mM Tris-HCl (pH 7.6)/66 mM potassium acetate/10 mM magnesium acetate/0.5 mM DTT.

Synthesis and purification of oligodeoxyribonucleotides

The 62 oligodeoxyribonucleotides were synthesized by a solid phase procedure on the 1 % cross-linked polystyrene with divinylbenzene[15]. All reactions were manually carried out in a round-bottomed flask, equipped with a sintered-grass filter and a two-way stopcock at the bottom. Functionalized polystyrene resin (200 mg), substituted with the appropriate 5'-O-dimethoxy-trityl-2'-deoxynucleoside via 3'-O-succinamido linkage[16] (25 µmol total functionality) was used.

Each oligonucleotide assembly involved addition of protected dinucleotide[17] (or trinucleotide) units by following cycle.

The resin was washed with chloroform (X 2), 10% trichloroacetic acid[18] in chloroform(3 X 15s) to remove dimethoxytrityl group, chloroform (X 2), pyridine (X 2). Each washing operation involved a solvent volume of 15ml. A mixture of the 5'-hydroxyl nucleoside attached resin and appropriately protected dinucleotide (4 equivalents) or trinucleotide block (3 equivalents) was co-evaporated with dry pyridine three times. To this residue were added the coupling reagent, mesitylenesulfonyl tetrazolide[19] (40 equivalents), and dry pyridine (4 ml) and the mixture was shaken at room temperature for 2h. After the reaction, the mixture was filtered, and the resin was washed with pyridine (2 X 10 ml). The resin was treated with acetic anhydride in pyridine (10% solution, 15 ml) under shaking for 1 h to mask the unreacted 5'-hydroxyl group. The reaction mixture was filtered and the resin was washed with pyridine (2 X 10 ml). After the cycle was repeated with the appropriate di- or trinucleotide block units until the required sequence was obtained, each oligonucleotide was cleaved from the solid phase (50 mg of oligomersubstituted resin) and all protecting groups were removed by the published procedure[20].

The completely deprotected oligonucleotide was purified by HPLC on Partisil 10-SAX[21,22]. The column was eluted at a flow rate of 2 ml/min with a gradient from 1 mM to 0.5 M potassium phosphate, pH 6.54 in 20 % acetonitrile, over a period of 33 min at 55°C. The desired peak was collected, desalted by dialysis, and lyophilized.

The purity and nucleotide sequence of each fragment was confirmed by two-dimentional sequence analysis[23] after labeling of the oligomers with [γ-^{32}P] ATP in the presence of T4 polynucleotide kinase.

Ligation of chemically synthesized oligonucleotides

62 synthetic oligomers were built into the IFN-γ gene by the following three-step reactions catalyzed by T4 DNA ligase.

Step 1. The chemically synthesized oligonucleotides were separately phosphorylated by T4 polynucleotide kinase at their 5'-ends. One nmol each of oligonucleotide was phosphorylated using T4 polynucleotide kinase and 5 μCi [γ-^{32}P] ATP (5,100 Ci/mmol), as described previously[24].

Six separate T4 ligase-catalysed joining reactions were performed to give fragments A to F. For the construction of each fragment, the terminal oligonucleotides were not phosphorylated. For example, fragment A was constructed by combining each of 500 pmol phosphorylated fragments 2 to 13 with 5'-OH fragments 1 and 14. The mixture was dialyzed against water for 16 h at 4°C and dried in vacuo. The mixture was dissolved in 14.5 μl of 20 mM Tris-HCl(pH 7.6) containing 10 mM $MgCl_2$, heated at 95°C for 2 min and cooled slowly to room temperature.

The ligation was carried out at 15°C for 16 h in 80 μl of ligation buffer using 30 units of T4 DNA ligase. The mixture was ethanol-precipitated and fractionated by electrophoresis on a 14 % polyacrylamide gel in 7 M urea. The band migrating at expected size was sliced from the gel and eluted. Fragments B to F were constructed similarly.

Step 2. After phosphorylation at 5'-ends, 20 pmol each of fragments A, B and C were mixed and ligated in 50 μl of ligation buffer using 8 units of T4 DNA ligase for 16 h at 15°C. Fragments D, E and F were ligated similarly. The ligated fragments were named fragment I and II respectively. The fragments were isolated by electrophoresis on a 8 % polyacrylamide gel in 7 M urea.

Step 3. Fragments I and II were re-phosphorylated and each 1 pmol was mixed and ligated as described above. After being treated with 20 units of nucleases EcoRI and SalI for 1 h at 37°C, the final product (454 bp) was isolated by electrophoresis on a 6 % polyacrylamide gel in 7 M urea.

Construction of recombinant plasmids

DNA(1.5 µg) of pBR322 was digested for 60 min at 37°C with EcoRI (3 units) and ClaI (3 units) in 20 µl of TA buffer. After the nucleases were inactivated by heating at 65°C for 30 min, the digests were recovered by ethanol precipitation. The DNA was resuspended in 40 µl of T4 DNA polymerase buffer and heated at 65°C for 10 min. After cooling to 0°C, 100 mM 2-mercaptoethanol (5 µl), 4 mM dNTP (5 µl each) and T4 DNA polymerase (1 unit) were added. The reaction was carried out for 60 min at 37°C and the DNA was precipitated with ethanol.

The plasmid pKO601 was constructed by inserting a 95 bp AluI fragment of lac UV5 promoter/operator region into the 'filled-in' EcoRI cleaved pBR322 (unpublished results). Plasmid pKO601 DNA (80 µg) was digested with EcoRI (150 units) and the fragment containing the lac UV5 promoter/operator region was recovered by electroelution from a 10 % polyacrylamide gel and ethanol precipitation. The DNA was resuspended in a 40 µl T4 DNA polymerase buffer and the solution was heated for 10 min at 65°C. After cooling to 0°C, 100 mM 2-mercaptoethanol (5 µl), 4 mM dNTP (5 µl each) and T4 DNA polymerase (10 units) were added. The reaction was carried out at 37°C for 60 min and the DNA was ethanol-precipitated. The 'filled-in' EcoRI/ClaI cleaved pBR322 DNA (0.8 µg) was blunt-end ligated to the 103 bp 'filled-in' EcoRI fragment (0.8 µg) containing lac UV5 promoter/operator region with T4 DNA ligase (2.5 units) at 16°C for 20 h.

The ligated mixture was transformed to E. coli strain WA802[24] and ampicillin resistant transformants were selected on M9S-X-gal (100 µg/ml) plate[25] containing the antibiotic (40 µg/ml). Ten independent blue transformants were screened for tetracycline resistance on a nutrient agar plate containing the antibiotic (10 µg/ml). Plasmid DNA, isolated from independent tetracycline resistant transformants, was screened by digestion with EcoRI and HpaII. Two out of four clones tested were shown to carry the fragment in the desired orientation. One of these plasmids was named pKO703 and was used for the subsequent cloning of the synthetic interferon gene.

DNA (15 µg) of plasmid pKO703 was digested with EcoRI and SalI (10 units each) in 100 mM Tris-HCl(pH 7.3), 5 mM $MgCl_2$ and 50 mM NaCl for 60 min at 37°C. The purified EcoRI-SalI 3.8 K bp vector fragment was ligated to the synthetic hIFN-γ gene (29 ng) with T4 DNA ligase (3 units) in 30 µl of ligation buffer for 20 h at 16°C. The ligated mixture was transformed to E.

coli strain WA802. Transformants which were ampicillin resistant and showed deep blue coloration on M9S-X-gal plate containing the antibiotic (40 µg/ml) were examined as to tetracycline sensitivity.

Preparation of bacterial extracts and IFN bioassay

E. coli strains were cultured overnight at 37°C in 2 ml of M9S-glycerol medium (NaCl 5 g, NH_4Cl 1 g, Na_2HPO_4 5.94 g, KH_2PO_4 3 g, $MgSO_4.7H_2O$ 0.2 g, glycerol 2.5 g, casamino acids 2 g, in one liter of water). The culture was inoculated into 5 ml of M9S-glycerol-ampicillin (40 µl/ml) medium so as to make A_{660} to 0.1 and further incubated at 37°C. When A_{660} reached 0.8, isopropyl thiogalactoside (IPTG) was added to the final concentration of 1 mM and the culture was continued for 2.5 h. Cells harvested by centrifugation were suspended in 0.5 ml of PBS containing egg white lysozyme (1 mg/ml). After standing for 40 min on ice, cells were lysed by sonication, centrifuged for 30 min at 25,000 rpm at 4 °C and the supernatant was submitted to IFN assay by the inhibition of the cytopathic effects of sindvis virus on FL amnion cells[26].

RESULTS

Synthesis of oligodeoxyribonucleotides

The basic phosphotriester approach to synthesize oligonucleotides on solid supports has been reported by several investigators[27-29]. In employing the solid phase method, we were most concerned with the following two aspects. The method should be very simple, rapid and reliable. The final deprotected oligomers should be easily purifiable.

Figure 2a shows our synthetic approach. The essential features of this method were a) elongation of an oligonucleotide chain by coupling appropriate di- or trinucleotide unit consecutively on the polystyrene resin using a phosphotriester approach[15], b) the use of trichloroacetic acid to remove the dimethoxytrityl group from the oligonucleotides bound on the support so as to reduce depurination[18] during chain assembly and c) the use of HPLC on a Partisil 10-SAX ion-exchange column[21,22] with phosphate gradients in a 20 % acetonitrile system at 55°C to improve the speed and the resolution during the purification of desired products after removal of all protecting groups (Fig.2b).

In our hands, this chromatography system employing acetonitrile instead of ethanol gave pure material in most cases without further purification, as judged not only by sizing on 20 % polyacrylamide gels in 7M urea[30] but also by two-dimensional sequence analysis[23] (for example, see Fig.2c and 2d). The

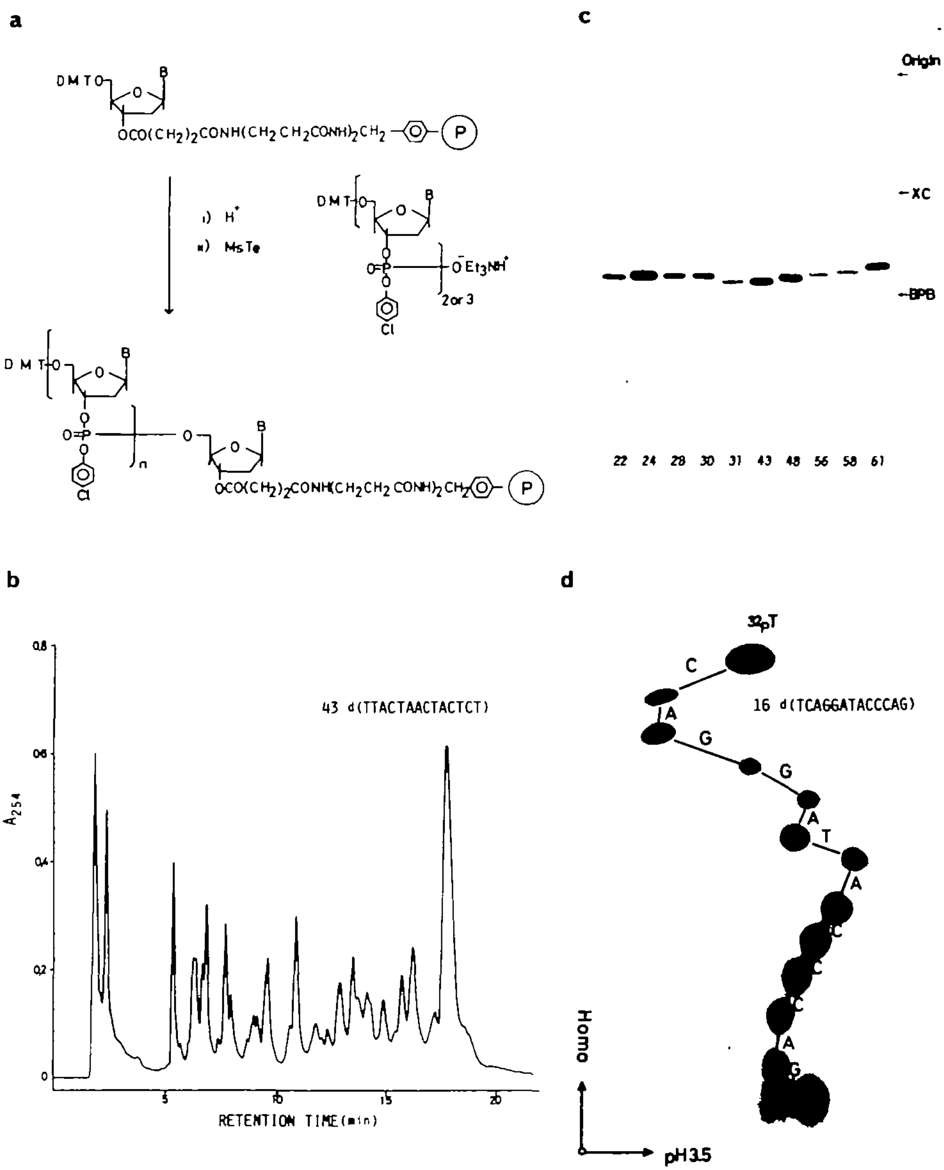

Fig.2 Synthesis and purification of oligodeoxyribonucleotides.
(a) Synthesis strategy
(b) HPLC profiles of the fragment 43 on Partisil 10-SAX.
(c) Polyacrylamide gel electrophoresis of the synthetic fragments.
(d) Two-dimensional sequence analysis of the fragments 16.

oligomers 35 and 56, however, could not be purified by this method and further resolution by reverse-phase HPLC on a μ-Bondapack C_{18} column[28] was necessary to get pure materials.

Using the improved chemical synthesis of oligodeoxyribonucleotides by solid phase method, together with the efficient purification of the unmasked oligomers, we synthesized the 62 different oligodeoxyribonucleotide fragments with chain length of 11-16 residues in two months.

Construction of the synthetic hIFN-γ gene

As shown in Fig.3a, ^{32}P-labeled oligomers were built into the IFN-γ gene by a three-step reaction catalyzed by T4 DNA ligase using the complementality of overlapping fragments to ensure proper ordering.

Six groups of oligomers (A-F), which consisted of 8 to 14 fragments, were initialy ligated separately. Preliminary experiments using all phosphorylated fragments showed that the ligation mixture of B to E checked by electrophoresis on a polyacrylamide gel gave several higher molecular weight products than desired (data not shown). This seems to indicate that wrong joinings occured at the 5' terminus of the strand. Therefore, during the first step ligation, the terminal fragments of each group were not phosphorylated. Ligated products of the expected size were isolated by a preparative polyacrylamide gel electrophoresis in denaturing condition, elution and ethanol precipitation. An aliquot of each purified fragment was examined by 8 % polyacrylamide gel electrophoresis in 7 M urea (Fig. 3b).

Before the second step ligation, the terminal 5'-hydroxyl groups of the six blocks were phosphorylated enzymatically with ATP immediately before use. Without annealing, aliquots of the six blocks were combined and ligated with T4 DNA ligase to give the two blocks I and II as shown in Fig. 3c.

A similar procedure, with the following exception, was used to assemble the total hIFN-γ gene. The phosphorylated fragments I and II were ligated and before purification by gel electrophoresis, the reaction mixture was treated with restriction endonucleases EcoRI and SalI. The band migrating at 454 bp was sliced from the 6 % polyacrylamide gel in 7M urea and eluted (Fig. 3d).

Molecular cloning of the synthetic IFN-γ gene

To express hIFN-γ directly in *E. coli*, the plasmid pKO703 having a *lac* UV5 promoter region was constructed as follows.

The plasmid pBR322 was digested with EcoRI and ClaI, and the resulting single stranded ends were filled in using T4 DNA polymerase. The large fragment, which contained whole ampicillin resistance (Ap^r) gene and all of the tetracycline resistance (Tc^r) structual gene but the Tc^r promoter, was

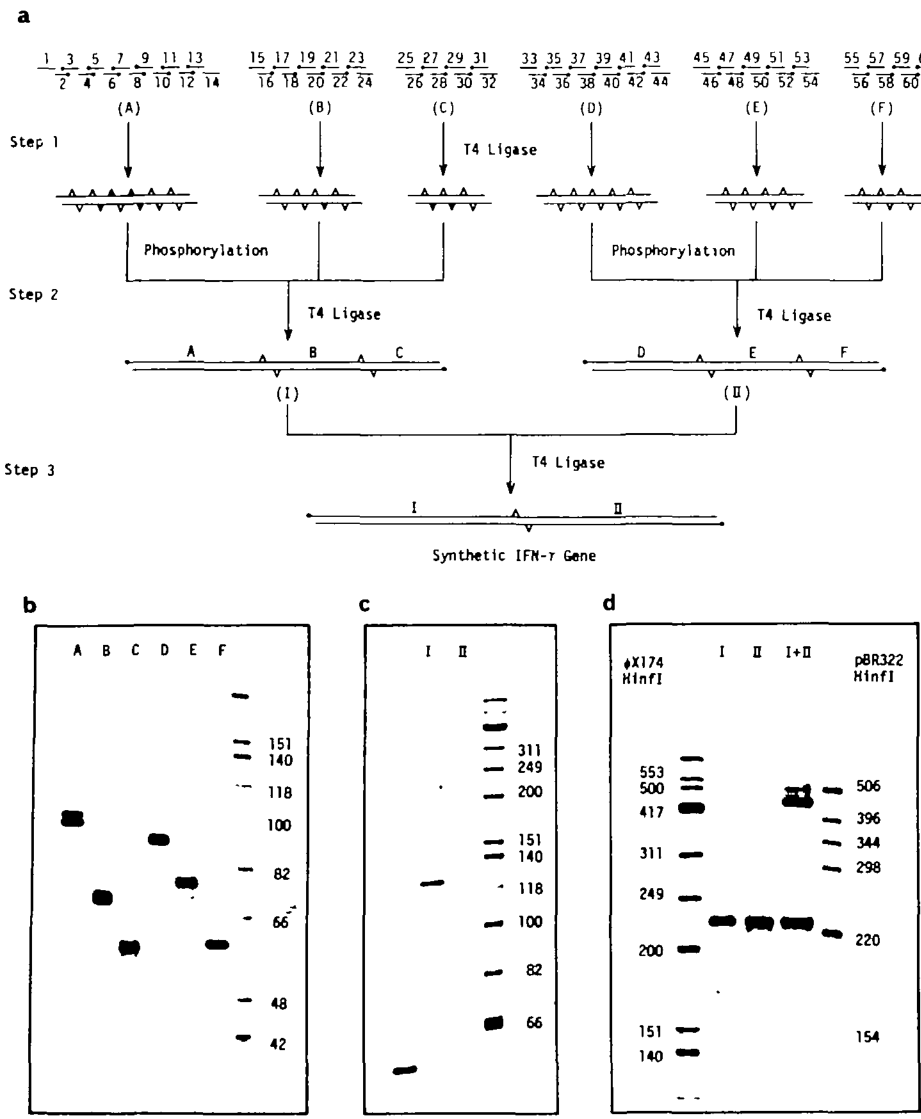

Fig.3 Ligation of the chemically synthesized oligonucleotides to construct the hIFN-γ gene.
(a) Ligation strategy. Dots on the ends of oligonucleotides indicate the positions of 5'-terminal phosphate groups.
(b) Analysis of fragments A to F isolated from the gel (14 % polyacrylamide/7 M urea).
(c) Analysis of the ligated products in the step 2 reactions .
Lane I: reaction mixture of fragment A to C.
Lane II: reaction mixture of fragment D to F.
(d) Analysis of the ligated products in the step 3 reaction.

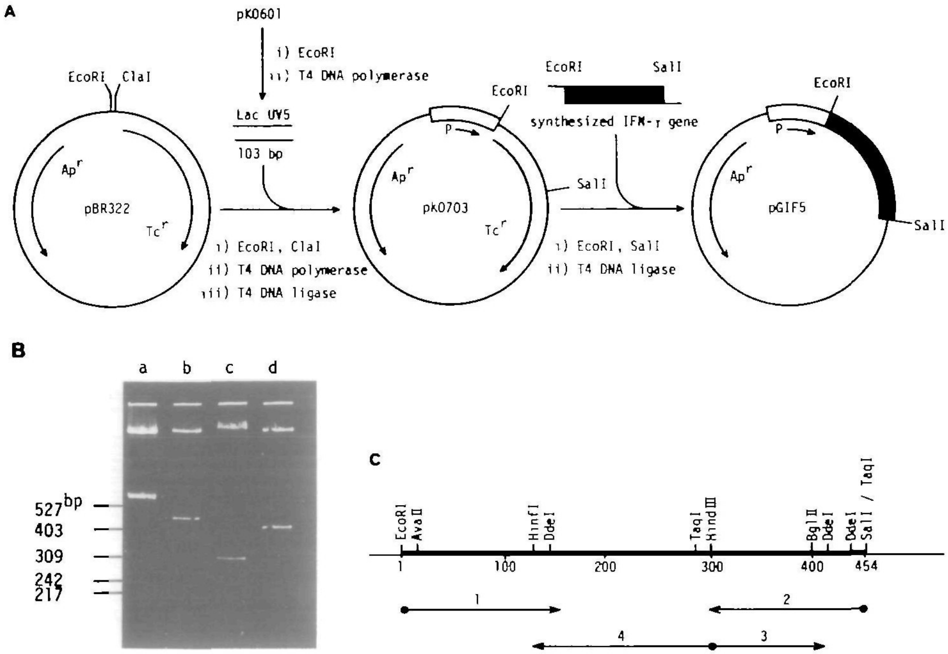

Fig.4 Construction of recombinant plasmids

A. Construction of recombinant pGIF plasmids.

B. Structure of the plasmid containing the synthetic gene. Restriction endonuclease-digested plasmid pGIF5 was analyzed by electrophoresis through 5 % polyacrylamide gel. EcoRI and SalI double digests of pKO703 (a) and pGIF5(b); EcoRI and HindIII double digests of pGIF5(c); EcoRI and BglII double digests of pGIF5(d).

C. Strategy for sequence determination of the synthetic interferon gene. Top line shows the positions of relevant restriction endonuclease cleavage sites within the synthetic oligonucleotide insert (heavy line) of the recombinant plasmid pGIF5. The nucleotide sequence was determined by use of the Maxam-Gilbert method(41). The arrows (1-4) indicate the direction and extent of the sequence determined from each labeling position.

blunt-end ligated to the 'filled-in' EcoRI fragment which contained lac UV5 promoter region[31]. The construction of this plasmid, as shown in Fig.4A, was arranged so that insertion of the EcoRI fragment should result in the regeneration of one EcoRI recognition sequence. The tetracycline resistant transformants were selected from the ampicilin resistant and β-galactosidase constitutive transformants. The tetracycline resistance showed that the EcoRI fragment on the plasmid was in desired orientation, that is, lac UV5 transcription going into the Tc^r gene. Plasmids prepared from Tc^r transformants were further submitted to restriction analysis and one plasmid, pKO703, was selected and used in the construction of plasmid pGIFs.

As is clear from Fig.4A, the synthetic IFN-γ gene introduced between the EcoRI and SalI cleavage sites of the pKO703 vector plasmid should be expressed under the control of the lac UV5 promoter.

Recombinant plasmids of this type were constructed by ligation of purified EcoRI-SalI-cut pKO703 vector DNA to the synthetic gene. The ligation mixture was used for transformation of *E. coli* WA802. Twenty ampicillin resistant transformants were screened for tetracycline sensitivity and 5 clones (WA802/pGIF 1 to 5) were selected for more detailed analysis.

Inspection of the designed nucleotide sequence of the 454 bp synthetic gene would indicate that the d(AAGCTT) sequence which was cut by endonuclease HindIII was present at amino acid residues 97-98 and the d(AGATCT) sequence which was cut by the endonuclease BglII was present at amino acid residues 134-135. Therefore, cleavage of these plasmids (pGIF1 to pGIF5) by EcoRI/HindIII and EcoRI/BglII endonucleases should yield a 301 bp and a 411 bp fragments respectively.

Examination by 5 % polyacrylamide gel electrophoresis of the DNA fragments produced on treatment of the five selected pGIF1-5 plasmid DNAs with these endonucleases showed that pGIF4 and pGIF5 produced these predicted fragments. Figure 4B shows these digestion patterns of the recombinant plasmid, pGIF5.

To confirm the correct interferon gene sequence present in these clones, the 454 bp EcoRI/SalI fragment from the plasmid, pGIF5, was subjected to complete nucleotide sequence determination according to the strategy shown in Fig. 4C. The nucleotide sequence so determined was in complete agreement with the designed sequence shown in Fig. 1.

Expression of the synthetic IFN-γ gene in E. coli and the characterization of the products

In order to determine whether an IFN-like polypeptide is produced, we assayed lysates of *E. coli* cells carrying either pGIF5 or pKO703 plasmid for the production of an antiviral activity. The results are presented in Table 1. Lysates from cultures of WA802/pGIF5 exhibited definite antiviral activity, whereas no activity was detected in extracts of WA802/pKO703. In the case of the WA802/pGIF5, high level of antiviral activity was observed even in the absence of a lac operon inducer, IPTG. This result might be due to gene dosage effects[32].

The antiviral activity was neutralized by antiserum to hIFN-γ, but not by

Table 1 Expression of hIFN-γ gene in E.coli

	Induction by IPTG	Treatment	IFN activity units/ml lysate
Exp 1			
WA802/pK0703	-	-	0
	+	-	0
WA802/pGIF5	-	-	281
	+	-	486
Exp 2			
WA802/pGIF5	+	-	850
	+	+Anti-α*	850
	+	+Anti-β*	850
	+	+Anti-γ*	6
	+	pH 2**	6

* Supernatant was treated with anti-hIFN-α, -β or -γ antiserum for 30 min at 37°C and overnight at 4°C before centrifugation and submission to IFN assay. The amounts of the serum used were enough to neutralize natural human IFNs which possessed similar antiviral activity.

** Supernatant was kept at 4°C overnight after adjusting to pH 2 by addition of 1N HCl. After dialysis against PBS (pH 7.4), the antiviral activity was assayed.

antiserum to hIFN-α or hIFN-β. Furthermore, the complete loss of activity was observed when lysate was treated at pH 2 [33]. Therefore, the polypeptide encoded by the synthetic gene has the same immunological and biochemical properties as natural hIFN-γ.

The molecular weight of the synthetic gene product was shown to be about 17,000 by SDS polyacrylamide gel electrophoresis of ^{35}S-Met-labeled proteins synthesized in minicells[34] prepared from pGIF5 transformed E. coli strain P678-54 (Fig.5A). On the other hand, when extracts of the induced WA802/pGIF5 was fractionated by gel filtration chromatography, the antiviral activity was found to elute almost as a single peak between ovalbumin and chymotrypsinogen A (Fig.5B), and the molecular weight was estimated to be 32,000. These results suggested that a dimer form protein was produced in E. coli, although the possibility remained that the protein was associated with some of the E. coli components[7,37].

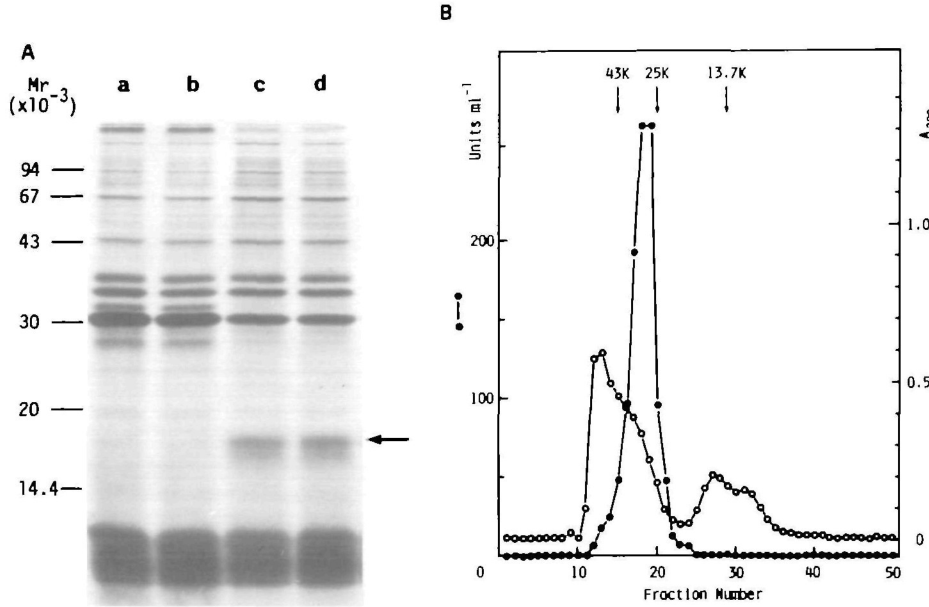

Fig.5 Molecular weight estimation of the IFN-γ gene products
A. Autoradiography of minicell products. Minicells were prepared from E. coli P678-54/pKO703 and P678-54/pGIF5 grown in L-broth (pH 7.4) at 37°C. The minicells were isolated by the use of discontinuous sucrose density gradient (20%, 15% and 10% w/v) and were suspended in M9 minimal salt medium to 8 X 10^8 minicells/ml. After vigorous shaking for 30 min at 37°C, 100 µCi of ^{35}S -methionine (1,170 Ci/mmol) and IPTG were added at 1 mM to the suspension. Following incubation for 30 min at 37°C, unlabeled methionine was added to the final concentration of 100 µg/ml. Throughly washed cells were treated with SDS sample buffer and subjected to electrophoresis in a 13 % polyacrylamide-SDS gel by the method of Laemmli(38) and the autoradiogram was developed. a, P678-54/pKO703; b, P678-54/pKO703 induced with 1 mM IPTG; c, P678-54/pGIF5; d, P678-54/pGIF5 induced with IPTG.Arrow refers to the location of the IFN-γ gene product.
B. Gel filtration chromatography of the lysate of E. coli WA802/pGIF5. High speed centrifuge supernatant (0.5 ml) was applied to an Ultrogel AcA54 (LKB) column (0.5 X 50 cm) equilibrated with 0.15 M NaCl in 20 mM phosphate buffer, pH 7.4. The column was developed with the same buffer at a flow rate of 8 ml/h. Fractions of 0.7 ml were collected. IFN activity (●——●) was measured as in Table 1. The recovery was about 70 % of the input. Arrows indicate the peak position of ovalbumin (MW 43,000), chymotrypsinogen A (MW 25,000) and RNase A (MW 13,700) used as size markers in calibration of the column. o——o; A_{280}.

DISCUSSION

As discussed in detail by Edge et al.[9], the synthetic gene will serve as a useful tool in the study of the regulation of the gene expression in different organisms, and also the study of the structure-activity analysis of

Table 2. Codon usage in synthetic (and natural) hIFN-γ

	U	C	A	G	
U	Phe 0 (6)	Ser 10 (0)	Tyr 0 (3)	Cys 0 (1)	U
	10 (4)	1 (2)	5 (2)	2 (1)	C
	Leu 0 (1)	0 (1)	Ocher 1 (1)	Opal —	A
	0 (2)	0 (2)	Amber —	Trp 1 (1)	G
C	Leu 2 (3)	Pro 0 (0)	His 2 (2)	Arg 7 (0)	U
	0 (1)	0 (0)	0 (0)	0 (1)	C
	0 (0)	1 (2)	Gln 0 (4)	0 (4)	A
	8 (3)	1 (0)	9 (5)	0 (0)	G
A	Ile 0 (2)	Thr 5 (3)	Asn 1 (7)	Ser 0 (3)	U
	7 (3)	0 (1)	9 (3)	0 (3)	C
	0 (2)	0 (1)	Lys 10 (12)	Arg 1 (2)	A
	Met 5 (4)	0 (0)	10 (8)	0 (1)	G
G	Val 6 (0)	Ala 8 (2)	Asp 0 (4)	Gly 5 (2)	U
	0 (3)	0 (0)	10 (6)	0 (1)	C
	1 (3)	0 (5)	Glu 9 (6)	0 (1)	A
	1 (2)	0 (1)	0 (3)	0 (1)	G

( ) : natural

Codon number = 146 + 2 (including initiation and termination codons)

proteins. Rapid synthesis of genes is essential for these studies to be feasible.

Using improved solid phase phosphotriester approach, we synthesized 62 different oligodeoxyribonucleotide fragments in short time and enzymatically ligated them into a 454 bp DNA which encodes for translation initiation and termination signals and putative hIFN-γ protein moiety. We have demonstrated expression of this chemically synthesized hIFN-γ gene in E. coli. The synthetic gene product has antiviral activity whose characteristics are similar to those of natural human IFN-γ, namely, acid lability and neutralization by anti-hIFN-γ antiserum but not by antisera against IFN-α and IFN-β.

The result of the gel filtration chromatography of the lysate of E. coli WA802/pGIF5 indicated that the synthetic IFN-γ gene product was in a dimer form, although the possibility remained that it was associated with other molecules of E. coli. It is noteworthy in this regard that the natural hIFN-γ produced by human lymphocytes in culture was also suggested to be an aggregate, possibly a dimer[35-37].

Although we chose codons which are used in genes highly expressed in *E. coli*(Table 2), the expression attained was rather low. We could not see any Coomassie blue stained[38] band corresponding to the gene product on SDS-polyacrylamide gel electrophoresis of the total proteins of the induced WA802/pGIF5 (data not shown). One explanation for this is that the Shine-Dalgano sequence[39] of the *lac* UV5 promoter we used and the sequence around the initiation codon is not in favour of efficient translation of the synthetic IFN-γ gene.

The supporting evidence for this interpretation is that, when we constructed a chimeric gene by fusing the synthetic hIFN-γ gene to the C-terminal proximate EcoRI site[40] of alkaline phosphatase structural gene, amounts of the chimeric protein expressed under the control of the alkaline phosphatase promoter reached almost 15 % of the total proteins of the *E. coli* (unpublished results).

Studies to change the sequence between the SD sequence and the ATG codon and to use other promoters for better direct expression of the gene are now in progress.

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