Synthesis of human fibroblast interferon by E. coli

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ABSTRACT

A cDNA library was constructed using mRNA from human fibroblasts induced with poly(I):poly(C). A bacterial clone containing fibroblast interferon cDNA sequences was identified by hybridization to a cDNA probe synthesized using deoxyoligonucleotide primers which hybridize to fibroblast interferon mRNA specifically. Expression plasmids were constructed which permitted the synthesis in E. coli of 8 X 10^7 units of human fibroblast interferon per liter of culture. The bacterially produced fibroblast interferon is indistinguishable from authentic human fibroblast interferon by several criteria.

INTRODUCTION

Human fibroblast interferon (FIF) is an antiviral protein which also exhibits a wide range of other biological activities (see ref. 1 for review). It has been purified to homogeneity as a single polypeptide of 19,000-20,000 molecular weight having a specific activity of 2 to 10 X 10^8 units/mg (2,3). The sequence of the 13 NH_2-terminal amino acids of FIF has been determined (4). Houghton et al. (5) have used synthetic deoxyoligonucleotides (predicted from this amino acid sequence) to determine the sequence of the 276 5'-terminal nucleotides of FIF mRNA. Taniguchi et al. (6) and Derynck et al. (7) have recently employed RNA selection procedures to identify cloned cDNA copies of FIF mRNA in E. coli.

We have used a battery of synthetic DNA primers designed from the published amino acid sequence data to identify bacterial clones containing FIF cDNA sequences. We also report the construction of a series of plasmids which direct the high level synthesis in E. coli of a polypeptide with the properties of authentic human fibroblast interferon.

MATERIALS AND METHODS.

General methods. Restriction enzymes were purchased from New England Biolabs and used as directed. Plasmid DNA was prepared by a standard cleared lysate procedure (8) and purified by column chromatography on Biogel A-50M (Bio-Rad). DNA sequencing was performed using the method of Maxam and Gilbert (9). DNA restriction fragments were isolated from polyacrylamide gels by electroelution. DNA fragments were radiolabeled for use as hybridization probes by the random calf thymus DNA priming procedure of Taylor et al. (10). In situ colony hybridizations were performed by the Grunstein-Hogness procedure (11).

Chemical synthesis of deoxyoligonucleotides. The deoxyoligonucleotides were synthesized by the modified phosphotriester method in solution (12), using trideoxynucleotides as building blocks (13). The materials and general procedures were similar to those described recently (14). The six pools of primers (Fib 1-6) containing four dodecanucleotides each were obtained by separately coupling two hexamer pools (of two different 5'-terminal sequences each) with three different hexamer pools (of two different 3'-terminal sequences each).

Induction of fibroblasts. Human fibroblasts (cell line GM-2504A) were grown as described previously (15). Growth medium (Eagle's minimal essential medium containing 10% fetal calf serum) was removed from roller bottles (Corning, 850 cm^2) and replaced with 50 ml growth medium containing 50 µg/ml of poly(I):poly(C) (PL Biochemicals) and 10 µg/ml cycloheximide. This induction medium was removed after 4 hours at 37°C and cell monolayers were washed with PBS (0.14M NaCl, 3mM KCl, 1.5 mM KH_2PO_4, 8mM Na_2HPO_4). Each bottle was incubated at 37°C with 10 ml of a trypsin - EDTA solution (Gibco #610-5305) until cells were detached, and fetal calf serum was added to a concentration of 10% . Cells were spun for 15 minutes at 500 x g and pellets were resuspended in PBS, pooled, and resedimented. Cells were frozen in liquid nitrogen. Approximately 0.17g of cells were obtained per roller bottle.

Preparation and assay of interferon mRNA. Poly(A)-containing mRNA was prepared from human fibroblasts by phenol extraction and oligo(dT)-cellulose chromatography as described elsewhere (16). The poly (A) containing RNA was enriched for interferon mRNA by centrifugation on a linear 5% to 20% (w/v) sucrose gradient. The RNA samples were heated to 80°C for 2 minutes, rapidly cooled, layered over the gradient, and centrifuged for 20 hours at 30,000 rpm at 4°C in a Beckman SW-40 rotor.

Fractions were collected, ethanol precipitated, and dissolved in H_2O.

One microgram samples of mRNA were injected into Xenopus laevis oocytes as described previously (17,18). The injected oocytes were incubated 24 hours at 21°C, homogenized, and centrifuged for 5 minutes at 10,000 x g. The interferon in the supernatant was determined by the cytopathic effect (CPE) inhibition assay (1) using Sindbis virus and human diploid (WISH) cells. Interferon titers of 1,000 to 6,000 units recovered (NIH reference standard) per microgram of RNA injected were routinely obtained for the 12S species of mRNA.

Synthesis and cloning of cDNA. Single stranded cDNA was prepared in 100 µl reactions containing 5 µg of 12S fraction mRNA, 20 mM Tris-HCl (pH 8.3), 20 mM KCl, 8mM $MgCl_2$, 30 mM β-mercaptoethanol, 100 µCi of (α^{32}P)dCTP (Amersham) and 1mM dATP, dCTP, dGTP, dTTP. The primer was the synthetic Hind III decamer dCCAAGCTTGG (19), which had been extended at the 3' terminus with about 20 to 30 deoxythymidine residues using terminal deoxynucleotidyl transferase (20). 100 units of AMV reverse transcriptase (gift of Dr. J. Beard) were added and the reaction mixture was incubated at 42°C for 30 minutes. The second strand DNA synthesis was carried out as described previously (21). The double stranded cDNA was treated with 1200 units of S1 nuclease (Miles Laboratories) for 2 hours at 37°C in 25 mM sodium acetate (pH 4.5), 1mM $ZnCl_2$, 0.3M NaCl. After phenol extraction the mixture was separated electrophoretically on an 8% polyacrylamide gel. cDNA (~ 0.5 µg) ranging from 550 to 1500 base pairs in size was recovered by electroelution. A 20 ng aliquot was extended with deoxyC residues using terminal deoxynucleotidyl transferase (20), and annealed wih 100 ng of pBR322 which had been cleaved with Pst I and tailed with deoxyG residues (20). The annealed mixture was used to transform E. coli K-12 strain 294 (22) by a published procedure (23).

Preparation of induced and uninduced ^{32}P-cDNA probes. 5 µg of 12S mRNA were combined with either 2 µg of oligo $(dT)_{12-18}$ (Collaborative Research) or 5 µg of each synthetic primer pool (Fib 1 to Fib 6) in 60 µl of 10mM Tris-HCl (pH 8), 1 mM EDTA. The mixtures were boiled 3 minutes, and quenched on ice. 60 µl of 40 mM Tris-HCl (pH 8.3), 40 mM KCl, 16mM $MgCl_2$, 60 mM β-mercaptoethanol, 1 mM dATP, dGTP, dTTP and 5 x 10^{-7}M (α-^{32}P) dCTP (Amersham, 2,000 - 3,000 Ci/mmole) was added to each template-primer mix at 0°C. After the addition of 100 units of AMV reverse transcriptase, the reactions were incubated at 42°C for 30 minutes and purified by passage over 10 ml Sephadex G-50 columns. The products were treated with

0.3N NaOH for 30 minutes at 70°C, neutralized, and ethanol precipitated.

The ^{32}P-cDNAs were combined with 100µg of poly(A) mRNA from uninduced fibroblasts in 50 µl of 0.4M sodium phosphate (pH6.8), 0.1% SDS. The mixtures were heated at 98°C for 5 minutes and allowed to anneal 15 hours at 45°C. The DNA-RNA hybrids (containing uninduced cDNA sequences) were separated from single-stranded DNA (induced cDNA sequences) by chromatography on hydroxyapatite as described by Galau et al. (24). The DNA-RNA hybrids were treated with alkali to remove RNA.

Screening of recombinant plasmids with ^{32}P-cDNA probes. Approximately 1 µg samples of plasmid DNA were prepared from individual transformants by a published procedure (25). The DNA samples were linearized by digestion with Eco RI, denatured in alkali, and applied to each of three nitrocellulose filters (Schleicher and Schuell, BA85) by the dot hybridization procedure (26). The filters were hybridized with the ^{32}P-cDNA probes for 16 hours at 42°C in 50% formamide, 10x Denhardt's solution (27), 6xSSC, 40 mM Tris-HCl (pH 7.5), 2mM EDTA, 40 µg/ml yeast RNA. Filters were washed with 0.1xSSC, 0.1% SDS twice for 30' at 42°C, dried, and exposed to Kodak XR-2 x-ray film using Dupont Lightning-Plus intensifying screens at -80°C.

Construction of plasmids for direct expression of FIF. The synthetic primers I (dATGAGCTACAAC) and II (dCATGAGCTACAAC) were phosphorylated using T4 polynucleotide kinase and (γ-^{32}P)ATP (Amersham) to a specific activity of 700 Ci/mmole as described previously (28). Primer repair reactions were performed as follows: 250 pmoles of the ^{32}P-primers were combined with 8 µg (10 pmole) of a 1200 bp Hha I restriction fragment containing the FIF cDNA sequence. The mixture was ethanol precipitated, resuspended in 50 µl H_2O, boiled 3 minutes, quenched in a dry ice-ethanol bath, and combined with a 50 µl solution of 20mM Tris-HCl (pH 7.5), 14 mM $MgCl_2$, 120 mM NaCl, 0.5 mM dATP, dCTP, dGTP, dTTP at 0°C. 10 units of DNA polymerase I Klenow fragment (Boehringer-Mannheim) were added and the mixture was incubated at 37°C for 4 1/2 hours. Following extraction with phenol/$CHCl_3$ and restriction with Pst I, the desired product was purified on a 6% polyacrylamide gel. Subsequent ligations were done at room temperature (cohesive termini) or 4°C (blunt ends) using previously detailed conditions (21,28). The plasmids pHKY2 and pHKY10 (H. Heyneker, D. Kleid, D. Yansura; unpublished results) were gifts from Dr. D. Kleid. The plasmid pGH6 has been described previously (21).

Assay for interferon expression in E. coli. Bacterial extracts were prepared for IF assay as follows: One ml cultures were grown overnight in LB (29) containing 5 μg/ml tetracycline, then diluted into 25 ml of M9 medium (29) containing 0.2% glucose, 0.5% casamino acids and 5 μg/ml tetracycline. 10 ml samples were harvested by centrifugation when A_{550} reached 1.0. The cell pellets were quickly frozen in a dry ice-ethanol bath and cleared lysates were prepared as described by Clewell (8). Interferon activity in the supernatants was determined by comparison with NIH FIF standards using cytopathic effect (CPE) inhibition assays as reviewed previously (1). Two different assays were used: (a) WISH (human amnion) cells were seeded in microtiter dishes. Samples were added 16 to 20 hours later and diluted by serial 2-fold dilution. Sindbis virus was added after at least 3 hours of incubation. Plates were stained 20 to 24 hours later with crystal violet. (b) MDBK (bovine kidney) cell line was seeded simultaneously with 2-fold dilutions of samples. Vesicular stomatitis virus was added after 2 to 3 hours incubation and plates were stained with crystal violet 16 to 18 hours later. To test pH 2 stability bacterial extracts and standards were diluted in minimal essential medium to a concentration of 1000 units/ml. One ml aliquots were adjusted to pH 2 with 1N HCl, incubated at 4°C for 16 hours, and neutralized by addition of NaOH. IF activity was determined by the CPE inhibition assay using human amnion cells. To establish antigenic identity 25 μl aliquots of the 1000 U/ml interferon samples (untreated) were incubated with 25 μl of rabbit antihuman leukocyte interferon for 60' at 37°C, centrifuged at 12,000 x g for 5 minutes and the supernatant assayed. Fibroblast and leukocyte interferon standards were obtained from the National Institutes of Health. Rabbit antihuman leukocyte interferon was obtained from the National Institute of Allergy and Infectious Diseases.

RESULTS

Chemical synthesis of primer pools complementary to FIF mRNA. The amino-terminal protein sequence of human fibroblast interferon (4) permitted us to deduce the 24 possible mRNA sequences which could code for the first four amino acids. The 24 complementary deoxyoligonucleotides were synthesized in 6 pools of 4 dodecamers each (Figure 1).

The six pools of 4 deoxyoligonucleotides each were synthesized by a modified phosphotriester method that has been used previously for the

Protein	1 2 3 4 Met-Ser-Tyr-Asn-	
mRNA	(5') AUG-UC$^{G}_{A}_{C}$-UA$^{U}_{C}$-AA$^{U}_{C}$	(16 combinations)
	(5') AUG-AG$^{U}_{C}$-UA$^{U}_{C}$-AA$^{C}_{U}$	(8 combinations)
Complementary DNA primers	ATT-$^{A}_{G}$TA-$^{T}_{C}$GA-CAT	Pool 1
	ATT-$^{A}_{G}$TA-$^{A}_{G}$GA-CAT	Pool 2
	ATT-$^{A}_{G}$TA-$^{A}_{G}$CT-CAT	Pool 3
	GTT-$^{A}_{G}$TA-$^{T}_{C}$GA-CAT	Pool 4
	GTT-$^{A}_{G}$TA-$^{A}_{G}$GA-CAT	Pool 5
	GTT-$^{A}_{G}$TA-$^{A}_{G}$CT-CAT	Pool 6

Figure 1. The synthetic deoxyoligonucleotides designed to prime cDNA synthesis from FIF mRNA. The 24 possible mRNA sequences which could code for the first four amino acids of FIF (4) are shown. The 24 deoxyoligonucleotides, synthesized as 6 pools of 4 dodecamers, are complementary to the mRNA sequences.

rapid synthesis of oligonucleotides in solution (12) and on solid phase (14). The basic strategy involved reacting two different 3'-blocked trimers with an excess of a single 5'-protected trimer to yield a pool of two hexamers, each represented equally. The coupling of two pools, each containing two hexamers, then resulted in a pool of four dodecamers.

Identification of FIF cDNA clones. Using 12S mRNA from induced human fibroblasts (1,000 units IF activity per μg in oocyte assay), double stranded cDNA was prepared and inserted into pBR322 at the Pst I site by the standard dG:dC tailing method (20). A fibroblast cDNA library consisting of 30,000 ampicillin-sensitive, tetracycline-resistant transformants of E. coli 294 was obtained from 20 ng of cDNA ranging in size from 550 to 1300 base pairs. Plasmid DNA was prepared from 600 of the transformants and applied to 3 sets of nitrocellulose filters as described in Materials and Methods.

The approach followed in the identification of hybrid plasmids containing fibroblast interferon cDNA sequences was similar to that used to identify human leukocyte interferon recombinant plasmids (30).

Radiolabeled cDNA hybridization probes were prepared using either the 24 synthetic dodecamers or oligo(dT)$_{12-18}$ as primers and 12S RNA from induced fibroblasts (5000 units/μg in oocytes) as template. The ^{32}P-cDNAs (specific activity >5 x 10^8 cpm/μg) obtained were hybridized to a large excess of mRNA isolated from uninduced human fibroblasts, and the mRNA-cDNA hybrids were separated from unreacted cDNA by hydroxyapatite chromatography (24). The single stranded cDNA fractions should be enriched for sequences which are present in induced fibroblasts but absent in uninduced cells, and the mRNA-cDNA hybrids should represent sequences common to both induced and uninduced cells. Approximately 4 x 10^6 cpm of single stranded cDNA (hybridization probe A) and 8 x 10^6 cpm of cDNA-mRNA hybrids were obtained using oligo(dT)$_{12-18}$ primed cDNA; 1.5 x 10^6 cpm of single stranded (hybridization probe B) and 1.5 x 10^6 cpm of hybrids were obtained from cDNA primed using synthetic dodecamer pools Fib 1-6. The cDNA-mRNA hybrids from both fractionations were combined, the RNA hydrolyzed by treatment with alkali, and the ^{32}P-cDNA used as hybridization probe C. Many of the 600 plasmid samples hybridized with both probes A and C, indicating that the hybridization reactions between uninduced mRNA and ^{32}P-cDNA (prior to the hydroxyapatite fractionation step) had not gone to completion. However, only one of the 600 plasmids (pF526) hybridized strongly with the specifically primed, induced cDNA probe B (Figure 2). Plasmid pF526 also hybridized with the total oligo(dT)$_{12-18}$ primed, induced cDNA probe A, and failed to give detectable hybridization to the combined uninduced probe C.

Pst I digestion of pF526 showed the cloned cDNA insert to be about 550 base pairs long, probably too short to contain the entire coding region for a protein the size of fibroblast interferon. Therefore, a ^{32}P-labeled DNA probe was prepared from this Pst I fragment by random priming with calf thymus DNA (10). This probe was used to screen 2000 individual colonies from a newly constructed fibroblast cDNA library (the new cDNA library was prepared using 12S mRNA from induced fibroblasts having a titer of 6,000 units/ml in the oocyte assay system). Sixteen clones hybridized to the probe. Plasmids prepared from the majority of these released two fragments when cleaved with Pst I, indicating that the cDNA contained an internal Pst I site. Clone pFIF3 contained the largest cDNA insert, about 800 base pairs. The DNA sequence of the insert was determined by the Maxam-Gilbert procedure (9) and is shown in Figure 3.

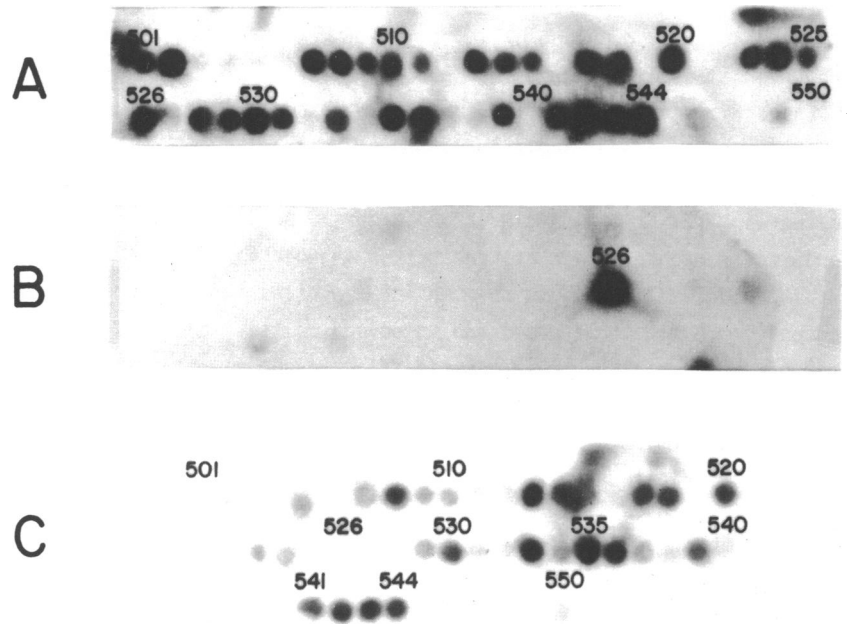

Figure 2. Hybridization of cDNA recombinant plasmids pF501-550 with ^{32}P- cDNA hybridization probes A, B and C (see text).

The amino acid sequence of human fibroblast interferon predicted from the nucleotide sequence is identical to that reported recently by Taniguchi et al. (31) and by Derynck et al. (7) from DNA sequencing of FIF cDNA clones. A precursor or signal peptide of 21 amino acids is followed by a mature interferon polypeptide of 166 amino acids, a stretch of 196 3'-untranslated nucleotides and a poly(A) tail. The NH_2-terminal 20 amino acids of mature FIF have now been directly determined by protein microsequencing (4,32) and are the same as those predicted from the DNA sequence.

Direct expression of fibroblast interferon. To express high levels of mature fibroblast interferon in E. coli, initiation of protein synthesis must occur at the ATG codon of the mature polypeptide (amino acid 1) rather than at the ATG of the signal peptide (amino acid S1) (Figure 3).

Our approach to removing the signal peptide coding regions from pFIF3 is depicted in Figure 4. A 1200 bp DNA fragment which contained the entire cDNA insert was isolated from a polyacrylamide gel after digesting

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5'
S1                                  S10                                         S20 S21
met thr asn lys cys leu leu gln ile ala leu leu leu cys phe ser thr thr ala leu ser MET SER TYR ASN
ATG ACC AAC AAG TGT CTC CTC CAA ATT GCT CTC CTG TTG TGC TTC TCC ACT ACA GCT CTT TCC ATG AGC TAC AAC
                                                                  50

                    10                                  20
LEU LEU GLY PHE LEU GLN ARG SER SER ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU
TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA
                              100                                                           150

30                                  40                                          50
TYR CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU GLN GLN PHE GLN LYS GLU ASP
TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC
                                                        200

                    60                                  70
ALA ALA LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP
GCC GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG
                          250                                                           300

80                                  90                                          100
ASN GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU
AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA
                                                    350

                    110                                 120
LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE
AAA CTG GAG AAA GAA GAT TTT ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT
                        400                                                             450

130                                 140                                         150
LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE
CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT
                                                    500

                    160                 166
TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN END
TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC TGA AGATCTCCTAGCCTGTCCCTCTGGGACTGGACAATTGCTTCAAGCA
                          550                                                           600

TTCTTCAACCAGCAGATGCTGTTTAAGTGACTGATGGCTAATGTACTGCAAATGAAAGGACACTAGAAGATTTTGAAATTTTTATTAAATTATGAGTT
                                    650                                                         700

ATTTTTATTTATTTAAATTTTATTTTGGAAAATAAATTATTTTTGGTGCAAAA
                                    750
                                                    3'
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Figure 3. Nucleotide and predicted amino acid sequences of pFIF3. Numbers above each line refer to amino acid position (S refers to signal peptide) and numbers below each line refer to nucleotide position.

pFIF3 with Hha I. Two separate synthetic deoxyoligonucleotide primers, dATGAGCTACAAC(I) and dCATGAGCTACAAC(II), were prepared. Both primers contain the coding sequence for the first four amino acids of mature fibroblast interferon; primer II has an additional C at the 5'-terminus. Primer repair reactions and subsequent ligations were carried out separately for primers I and II, and gave nearly identical results. Therefore, only reactions using primer I are discussed in detail here. The primers were 5'-radiolabeled using (γ-^{32}P)ATP and T4 polynucleotide kinase, combined with the 1200 bp Hha I DNA fragment and the mixture denatured by boiling. Following hybridization of the primer to the denatured Hha I DNA fragment, E. coli DNA polymerase I Klenow fragment (33) was used to catalyze the repair synthesis of the plus (top) strand (Figure 4). In addition, the

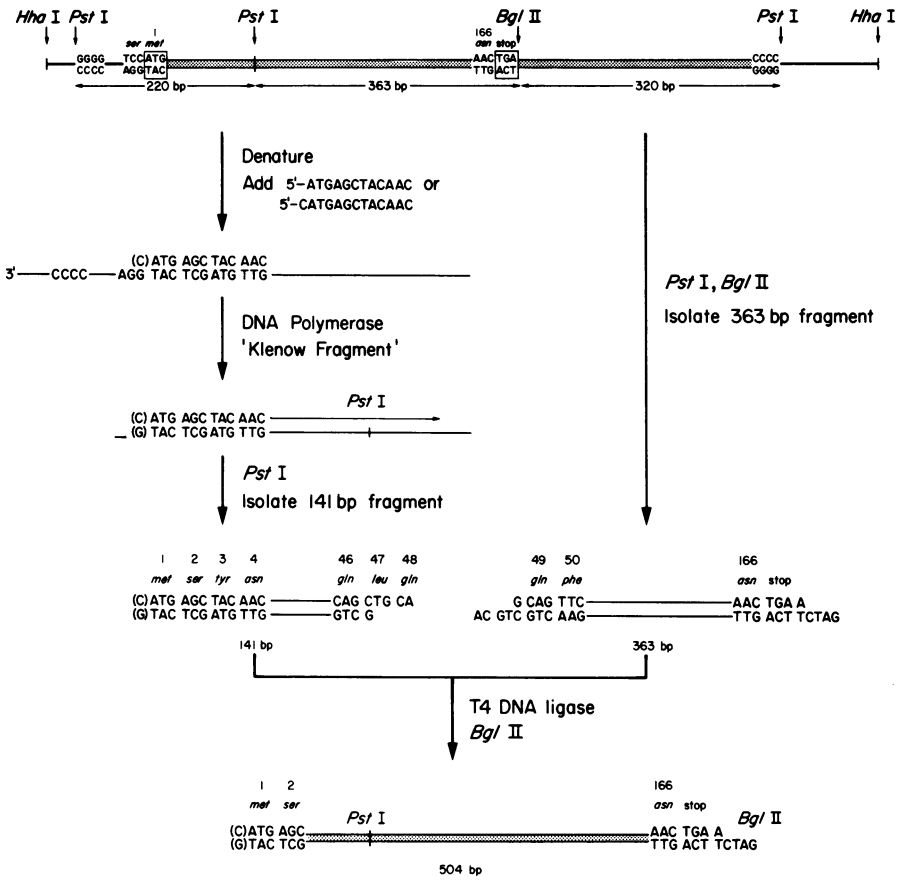

Figure 4. Assembly of gene coding for mature human fibroblast interferon starting with the 1200 base pair Hha I fragment from pFIF 3.

associated 3'→5' exonuclease activity of the Klenow fragment removed the 3'-protruding end from the minus (bottom) strand, leaving a flush end. Analysis of samples of the reaction mixture by polyacrylamide gel electrophoresis indicated that the repair synthesis did not go to completion, but stopped at several discrete sites. Therefore, the entire reaction mixture was treated with Pst I and the desired 141 bp fragment (180,000 Cerenkov cpm; ~0.3 pmole) was purified by polyacrylamide gel electrophoresis (Figure 5). Ligation of this fragment to 1 µg (~4 pmole) of the 363 bp Pst I-Bgl II fragment isolated from pFIF3 (Fig. 4),

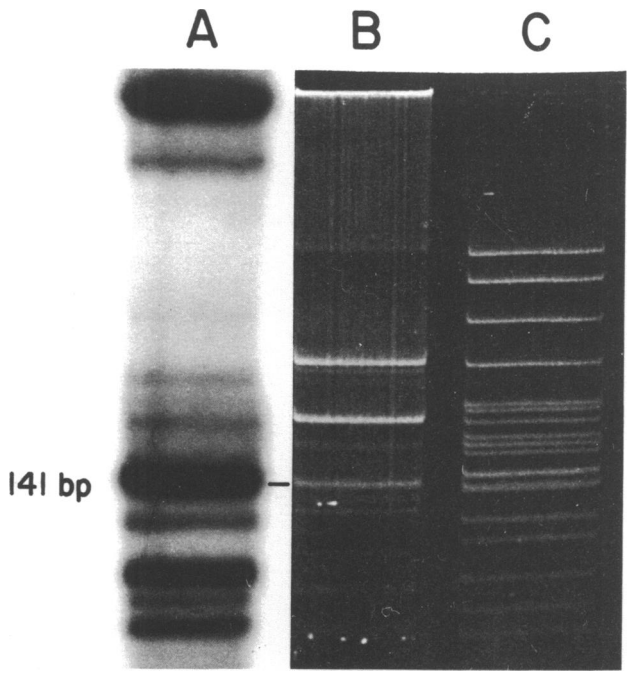

Figure 5. Polyacrylamide gel electrophoresis a of Pst I digest of the primer repair reaction mixture. The synthetic deoxyoligonucleotide ^{32}p-dATGAGCTACAAC was used as primer and the denatured 1200 base pair Hha I fragment from pFIF3 was used as template for the DNA Polymerase (Klenow fragment) catalyzed repair reaction (see Figure 3 and text). The mixture was digested with Pst I and electrophoresed on a 6% polyacrylamide gel. Lane A, autoradiograph of reaction mixture; Lane B, ethidium bromide stained gel of reaction mixture; Lane C ethidium bromide staining of pBR322 (HpaII digestion) size markers.

followed by Bgl II digestion, yielded 50,000 Cerenkov cpm (~0.1 pmole, ~30 ng) of the 504 bp DNA fragment containing the entire coding sequence for mature fibroblast interferon. The same reactions using primer II gave 83,000 cpm (~0.15 pmole, ~50 ng) of 505 bp product.

The construction of plasmids which direct the synthesis of human fibroblast interferon is outlined in Figure 6. Separate expression plasmids were constructed which placed FIF synthesis under the control of the E. coli lac or trp promoter-operator systems. Both of these systems have proven useful for the direct expression of eukaryotic genes in

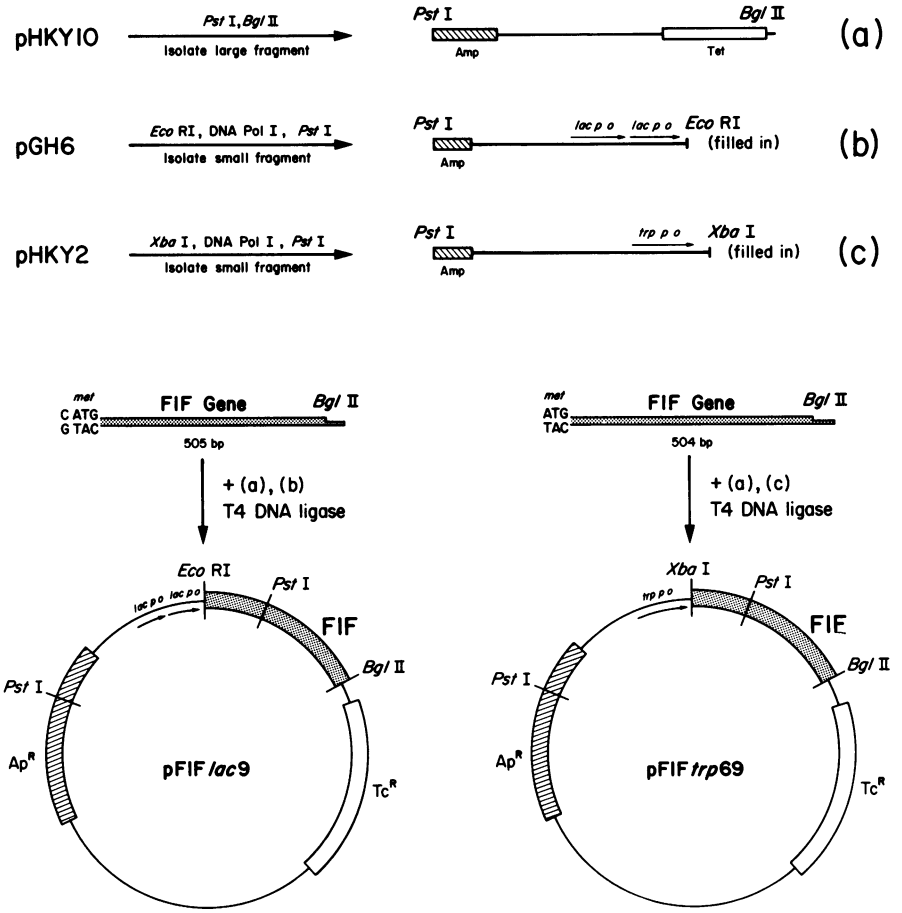

Figure 6. Construction of plasmids for the bacterial expression of human fibroblast interferon.

E. coli: human growth hormone has been efficiently synthesized using the lac system (21) and human leukocyte interferon has been produced at high levels using the trp system (30).

The plasmid pHKY10 is a derivative of pBR322 which contains a Bgl II site between the tetracycline resistance (Tc^R) promoter and structural gene (H. Heyneker, D. Yansura; unpublished results). The large DNA fragment isolated after digesting pHKY10 with Pst I and Bgl II therefore contains part of the ampicillin resistance (Ap^R) gene and all of the

Tc^R structural gene, but lacks the Tc^R promoter (Fig 6). The plasmid pGH6 (21) was digested with Eco RI, the resulting single stranded ends were filled in with DNA polymerase I, and the plasmid was cleaved with Pst I. The small fragment, containing part of the Ap^R gene, a double lac promoter and lac ribosome binding site, but lacking an ATG initiation triplet was isolated. A similar trp promoter fragment, containing the trp leader ribosome binding site, but lacking an ATG sequence (30), was isolated from pHKY2 (see Figure 6).

The expression plasmids were assembled via three part ligation reactions as shown in Figure 6. 15 ng (~.05 pmole) of the assembled FIF gene (504 or 505 bp), 0.5 µg (~0.2 pmole) of the large Pst I - Bgl II fragment of pHKY10 and 0.2 µg (~0.3 pmole) of the appropriate promoter fragment were ligated and the mixture used to transform E. coli 294 (22). Plasmid DNA was prepared from individual transformants and analyzed by restriction mapping. Correct joining of the assembled gene to the promoter fragment should restore the Eco RI (lac) or the Xba I (trp) recognition sequences. The majority of the plasmids gave the expected restriction enzyme digestion patterns. Individual clones (12 containing the trp promoter and 12 containing the lac promoter) were grown and extracts prepared for interferon assay as described in Materials and Methods.

When assayed on human amnion (WISH) cells for antiviral activity by the CPE inhibition assay (1) five of the trp transformants were positive (each approximately equivalent); eleven of the lac transformants gave equivalent IF activities. Therefore, one transformant from each series (pFIFlac9 and pFIFtrp69) was selected for further study (Table 1). DNA sequence analysis demonstrated that the desired attachment of promoter to FIF structural gene had occurred in both cases.

The amounts of fibroblast interferon produced by pFIFlac9 and pFIFtrp69 are shown in Table 1. The trp promoter gave a FIF expression level measurably higher than did the lac promoter; this finding is consistent with results we have obtained for expression of human growth hormone and human leukocyte interferon in E. coli (unpublished results). In an attempt to further increase FIF expression levels, pFIFtrp69 was cleaved with Eco RI and two 300 base pair Eco RI fragments containing the trp promoter (30) were inserted. The resulting plasmid, pFIF$trp^3$69, contains three successive trp promoters which read toward the FIF gene. The amount of FIF synthesized by E. coli 294/pFIF $trp^3$69 is 4-5 times

Table 1. Interferon activity in extracts of E. coli

E. coli 294 transformed by:	Cell density (cells/ml)	IF Activity (units/l culture)	FIF molecules per cell
pBR322	3.5×10^8	-	-
pFIFlac9	3.5×10^8	9.0×10^6	2,250
pFIFtrp69	3.5×10^8	1.8×10^7	4,500
pFIFtrp^{3}69	3.5×10^8	8.1×10^7	20,200

Cells were grown and extracts prepared as described in Materials and Methods. The human amnion (WISH) cell line was used for the CPE inhibition assay (1). Activities given are the average for three independent experiments. To determine the number of IF molecules per cell a FIF specific activity of 4×10^8 units/mg was used (2).

that produced by pFIF trp 69 (Table 1). This is apparently due to the derepression of the trp promoter which occurs when trp repressor levels are titrated by the multiple copies of the trp operator (H. de Boer, unpublished results).

The FIF produced by E. coli 294/pFIFtrp69 behaves like authentic human FIF. As shown in Table 2, its antiviral activity is about 30 times greater on human cells than bovine cells. In addition, the bacterially produced FIF is stable to treatment at pH 2 overnight and is not neutralized by rabbit antihuman leukocyte interferon antibodies (Table 3).

DISCUSSION

We have designed and synthesized the 24 deoxyoligonucleotides which

Table 2. Interferon activities measured on different cell types

	Interferon Activity (units/ml)		
Cells	LeIF	FIF	294/pFIFtrp69 extract
Human amnion	20,000	10,000	1280
Bovine kidney	13,000	400	40

LeIF and FIF were NIH standard solutions having 20,000 units/ml and 10,000 units/ml respectively. Assays were performed as described in Materials and Methods.

Table 3. Comparison of activities of extracts from E. coli 294/pFIFtrp69 with standard human leukocyte and fibroblast interferons

	Interferon Activity (units/ml)		
	LeIF	FIF	294/pFIFtrp69
untreated	1000	1000	1000
pH2	1000	1000	1000
rabbit antihuman LeIF antibodies	<16	1000	1000

Experimental procedures described in Materials and methods. Assayed by CPE inhibition using WISH cells/Sindbis virus.

are complementary to all possible coding sequences for the first four amino acids of human fibroblast interferon. A radiolabeled cDNA hybridization probe was prepared by using 12S poly (A) mRNA from induced fibroblasts as template and the synthetic deoxyoligonucleotides as primers. This probe was used to identify a clone (pF526) containing a partial length FIF cDNA insert from a library prepared from polyI-polyC induced human fibroblasts. The cDNA insert of pF526 was then used as a hybridization probe to identify a cDNA recombinant plasmid (pFIF3) containing the entire FIF coding sequence.

Subsequent DNA sequence analysis of pF526 showed that the cDNA insert begins with the nucleotides encoding amino acid 52 of FIF and extends through the 3' end of FIF mRNA. Therefore, the FIF specific cDNA hybridization probe, which should extend from the nucleotides encoding amino acid 4 into the FIF signal sequence and 5'-untranslated regions, would not be expected to detect this clone. However, examination of the sequence of pF526 (and pFIF3) reveals a region of sequence in the 3'-untranslated region (nucleotides 702-713 in Figure 3) which could form 10 base pairs with the synthetic dodecamer dATTATAACTCAT found in pool 3 (see Figure 1). The two mismatches would both be T/T pairs which occur at the 5' end of the primer, away from the site of nucleotide addition by reverse transcriptase. We postulate that the chimeric plasmid pF526 was identified by hybridization to cDNA primed from this site in the 3'-untranslated region of FIF mRNA. Because of the large number of different oligonucleotide sequences represented in pools 1-6 it is likely

that priming of cDNA synthesis could have occured on additional, non-interferon mRNAs. The separation of the cDNA hybridization probes into "induced" and "uninduced" fractions by hydroxyapatite chromatography was therefore important for the identification of a FIF cDNA clone.

The method described here for identifying human fibroblast interferon cDNA clones should be generally applicable for identification of chimeric plasmids containing inducible cDNA sequences. We have used a similar approach to identify cloned double-stranded cDNA copies of human leukocyte interferon mRNA (30). Houghton et al. (5), employing a variation of this approach, predicted and synthesized two deoxyoligonucleotides, one of which primed FIF cDNA synthesis specifically. Using this primer they were able to sequence the 5' end of FIF mRNA.

The amino acid sequence of human fibroblast interferon as deduced from the DNA sequence of pFIF3 is identical to that deduced previously from the sequence of cDNA clones (7,31) and from direct sequencing of FIF mRNA (5). Recent experiments indicate there is only a single human fibroblast interferon gene (R. Lawn, unpublished results). In contrast, there are at least six distinct human leukocyte interferon genes (30). In our human fibroblast cDNA library constructed with our best mRNA preparation, approximately one out of every 125 recombinant plasmids contained FIF cDNA sequences. We have also recently screened our human leukocyte cDNA library (34), prepared using 12S poly (A) mRNA from the cell line KG-1 (33) for FIF cDNA sequences. Surprisingly, 8 of the 1600 clones screened hybridized strongly with a probe prepared from pFIF3. The cDNA insert from one of these clones was sequenced and also found to code for the same amino acid sequence as pFIF3.

To express mature human FIF interferon directly in E. coli, we constructed a series of plasmids which placed the synthesis of the 166 amino acid polypeptide under trp promoter or lac promoter control. These constructions utilized synthetic deoxyoligonucleotides which primed the synthesis of double-stranded FIF DNA beginning precisely with the coding sequence of mature FIF. Human FIF produced by E. coli harboring these expression plasmids behaves like authentic FIF when its antiviral activity is compared on human amnion cells and bovine kidney cells; it is also stable to treatment at pH 2 and is not neutralized by rabbit anti-human leukocyte interferon antibodies. The amount of human fibroblast interferon synthesized by E. coli (8×10^7 units/liter of culture at $A_{550}=1$) is comparable to the expression level we have obtained for human leukocyte

interferon under similar conditions (3). This corresponds to about 200 μg of FIF per liter if a specific activity of $4x10^8$ units/mg (2) is assumed.

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