Expression of human immune interferon cDNA in *E. coli* and monkey cells

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A cDNA sequence coding for human immune interferon (IFN-γ) has been identified in a cDNA library prepared from gel-fractionated IFN-γ mRNA. The DNA sequence codes for a polypeptide of 166 amino acids, 20 of which could constitute a signal peptide. The polypeptide produced through expression of this DNA sequence in Escherichia coli *or cultured monkey cells had properties characteristic of authentic human IFN-γ.*

INTERFERONS (IFNs) are secreted proteins which induce an antiviral state in their target cells, and which have immunomodulatory and antitumour properties[1]. On the basis of antigenicity and biological and chemical properties, human interferons have been grouped into three major classes: IFN-α (leukocyte), IFN-β (fibroblast) and IFN-γ (immune)[2]. Considerable information has accumulated on the structures and properties of the virus-induced acid-stable interferons (IFN-α and -β). These have been purified to homogeneity and partial amino acid sequences determined[3–7]. Analyses of cloned cDNA and gene sequences for IFN-β_1 (refs 8–10) and the IFN-α multigene family[11–16] have permitted the deduction of the complete amino acid sequences of many of these interferons. In addition, efficient synthesis of IFN-β_1 (ref. 10) and several IFN-αs[13,16] in *Escherichia coli* and IFN-α_1 in yeast[17] have now made possible the purification of large quantities of these proteins in biologically active form.

Much less data are available concerning the structure and properties of IFN-γ. IFN-γ is generally produced in cultures of lymphocytes exposed to various mitogenic stimuli, is acid labile and does not cross-react with antisera prepared against IFN-α or IFN-β (ref. 1). Molecular weights ranging from 35,000 to 70,000 have been reported for IFN-γ (refs 18–21). Recently, a quite extensive, but still partial, purification of IFN-γ has been reported[20], and IFN-γ mRNA has been isolated and translated in *Xenopus laevis* oocytes[22,23]. A broad range of biological activities have been attributed to IFN-γ, including potentiation of the antiviral activities of IFN-α and -β (ref. 24), and it differs from these interferons in its virus and cell specificities and in the antiviral mechanisms it induces[25]. However, *in vitro* studies performed with crude preparations suggest that the primary function of IFN-γ may be as an immunoregulatory agent[26]. The antiproliferative effect of IFN-γ on transformed cells has been reported to be 10–100-fold greater than that of IFN-α or -β (refs 25, 27), suggesting a potential use in the treatment of neoplasia. Indeed, murine IFN-γ preparations have been shown to have significant antitumour activity against mouse sarcomas[28]. However, the poor availability of purified IFN-γ has precluded the unambiguous determination of its physico-chemical and biological properties.

We describe here the first isolation and characterization of a recombinant plasmid containing a cDNA sequence coding for human IFN-γ. Expression of this sequence in *E. coli* and cultured monkey cells gives rise to a polypeptide having the properties of authentic human IFN-γ.

Construction and identification of bacterial clones containing induced cDNA sequences

Human peripheral blood lymphocytes (PBLs) from healthy donors were stimulated to produce IFN-γ by treatment with donors were collected 48 h after induction and used to prepare polyadenylated RNA[30,31] which was fractionated by electrophoresis through denaturing agarose gels[32,33]. Interferon mRNA activity was determined by injecting aliquots of each fraction into *X. laevis* oocytes[34–37] and after 24 h assaying the incubation medium for antiviral activity[36,37]. One peak of activity was consistently observed which co-migrated with 18S RNA (Fig. 1). Using different procedures for sucrose density gradient centrifugation, Wallace *et al.*[22] and Taniguchi *et al.*[23] have reported single peaks of IFN-γ mRNA activity sedimenting at 18S and 15S respectively. RNA from our most active 18S gel fraction (600 units ml^{-1} when 0.5 μg was injected into 15 oocytes at a concentration of 0.5 μg μl^{-1}) was used to prepare double-stranded cDNA by standard procedures[38,39]. The cDNA was fractionated according to size and material longer than 800 base pairs (bp) was extended with deoxy(C) residues, annealed to deoxy(G)-tailed *Pst*I-cleaved pBR322 and used to transform *E. coli* K-12 strain 294 (ref. 40) as described previously[13,19].

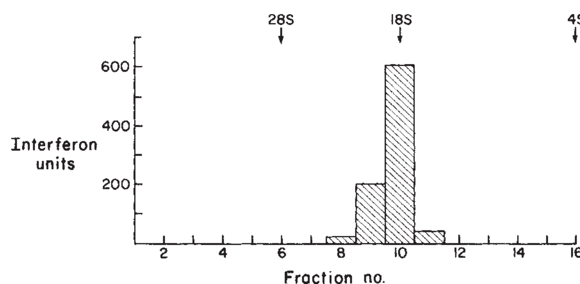

Fig. 1 Gel electrophoresis of induced lymphocyte polyadenylated RNA. Peripheral blood lymphocytes (PBLs) derived from individual human donors by lymphophoresis were purified by centrifugation on a Ficoll-Hypaque gradient and induced for IFN-γ production with staphlococcal enterotoxin B (1 μg ml^{-1}) and desacetylthymosin-α_1 (0.1 μg ml^{-1})[29]. Total RNA was extracted[30] from PBL cultures and poly(A)-containing RNA was purified by oligo(dT) cellulose chromatography[31]. 200 μg of mRNA was fractionated by electrophoresis through a denaturing agarose gel (1.75% agarose, 0.025 M sodium citrate (*p*H 3.8) and 6 M urea) for 7 h at 25 mA and 4 °C (refs 32, 33). The slab gel was sectioned with a razor blade and individual slices were melted at 70 °C and extracted twice with phenol and once with chloroform. Fractions were precipitated with ethanol and subsequently assayed for IFN-γ mRNA by injection into *X. laevis* oocytes[34–37] at a concentration of 0.5 μg μl^{-1}. After 24 h incubation at room temperature the antiviral activity of the translation products in the incubation medium was determined by the cytopathic effect (CPE) inhibition assay using vesicular stomatitis virus or encephalomyocarditis virus on WISH (human amnion) cells[1]. All IFN-γ units are expressed with reference to the NIH IFN-α standard (G-023-901-527), because no internationally accepted IFN-γ standard was available. In this assay system 0.2 units of the standard results in a 50% reduction in cytopathogenic effect. A single peak of activity was observed which co-migrated with 18S ribosomal RNA. The positions of ribosomal RNA markers which were electrophoresed in an adjacent lane and visualized by ethidium bromide staining are labelled above the activity profile. The activity seems to be due to IFN-γ, as no activity was observed when the same fractions were assayed on a bovine cell line (MDBK) which is

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We screened 8,300 bacterial clones from the cDNA library for the presence of specifically induced cDNA sequences by the following procedure. Two copies of the colony library were grown up on nitrocellulose filters and DNA was fixed to the filters for *in situ* colony hybridization[41,42]. One set of filters was hybridized with a ^{32}P-labelled cDNA probe prepared using the same 18S gel-fractionated mRNA (induced) used to prepare the colony library. The second set of filters was hybridized with ^{32}P-labelled cDNA prepared using 18S gel-fractionated poly(A) RNA isolated from unstimulated PBLs. The hybridization intensity of each colony with the two probes was compared, as IFN-γ sequences should only hybridize with the induced probe. 124 colonies hybridized with the induced probe but undetectably or weakly with the uninduced probe (see Fig. 2). Plasmid DNA was isolated from each of these colonies[43], bound to nitrocellulose filters[44] and hybridized with the same induced and uninduced probes. DNA from 22 colonies hybridized specifically with the induced probe in this rescreening.

The cDNA inserts from several of these 'induced' colonies were mapped using restriction endonucleases and found to contain common *Dde*I, *Hin*cII and *Rsa*I sites (Fig. 3*a*). A ^{32}P-labelled DNA probe was prepared[45] from one of the plasmids using two internal *Dde*I fragments (130 and 600 bp) and hybridized to DNA from the 124 colonies selected in the first round of screening. Only the 22 induced clones were found to hybridize with this probe, establishing their relatedness. Subsequent rescreening[41,42] of the cDNA library with this same probe revealed that approximately 1 in 120 *E. coli* clones contained related sequences. Digestion of plasmid DNA from these clones with *Pst*I showed that clone 69 contained the largest cDNA sequence (~1,250 bp).

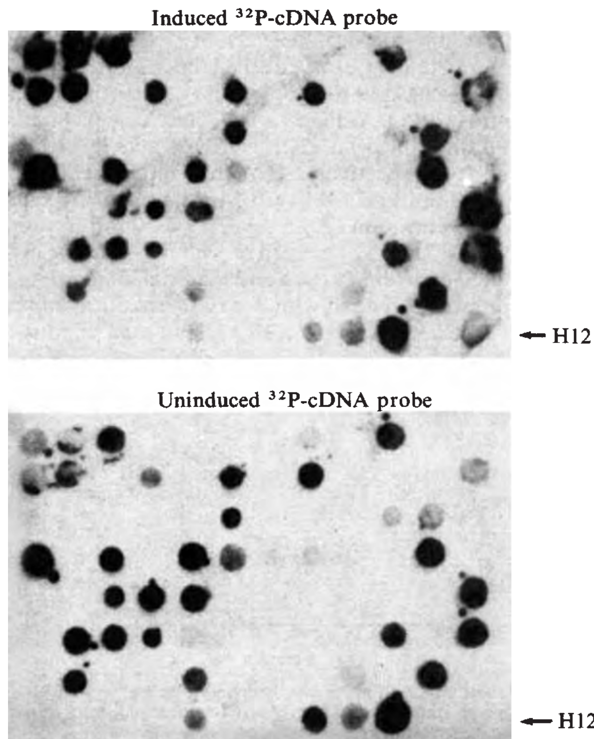

Fig. 2 Hybridization patterns of 96 colonies containing cloned cDNAs with induced and uninduced ^{32}P-labelled cDNA probes. Double-stranded cDNA was prepared by standard methods[13,38,39] using 3 μg of mRNA from gel fraction 10 (Fig. 1). The cDNA >800 bp (340 ng) was recovered by electroelution after fractionation on a 6% polyacrylamide gel. An aliquot (35 ng) of the cDNA was extended with deoxy(C) residues using terminal deoxynucleotidyl transferase[70], and annealed with 300 ng of the plasmid pBR322[71] which had been similarly tailed with deoxy(G) residues at the *Pst*I site. The annealed mixture was used to transform *E. coli* K-12 strain 294[40] and resultant tetracycline-resistant colonies were individually inoculated into wells of microtitre plates containing L broth[68] and 5 μg ml^{-1} tetracycline. Two copies of the cDNA library of 8,300 transformants were grown up on nitrocellulose filters and the DNA from each colony was fixed to the filter[41,42]. ^{32}P-labelled cDNA probes were prepared from 18S size gel-fractionated mRNA from induced and uninduced PBL cultures. Oligo(dT)[12-18] was used as a primer and reaction conditions have been previously described[13]. One set of 86 filters (96 colonies per filter) containing the entire cDNA library was hybridized[72] to 2×10^7 c.p.m. of the induced ^{32}P-cDNA. The duplicate set of filters was hybridized with 2×10^7 c.p.m. of uninduced ^{32}P-cDNA. After 16 h at 45 °C, filters were washed extensively[72] and exposed to X-ray film. The two filters shown here are representative of

Sequence analysis of the cDNA insert of plasmid p69

The complete nucleotide sequence of the *Pst*I insert from plasmid p69 was determined by the Maxam–Gilbert chemical procedure[46] and by the dideoxynucleotide chain termination method[47] after subcloning fragments into the M13 vector mp7 (ref. 48). The presence of 19 consecutive A residues corresponding to the 3′ poly(A) terminus of the mRNA permitted us to orient the 1,200 nucleotide long sequence as shown in Fig. 3*b*. As IFN-γ mRNA co-migrates with 18S ribosomal RNA (>2,000 nucleotides) on denaturing agarose gels, it was important to see whether the 1,200 bp insert of p69 represented a full-length copy of its respective mRNA. To measure the length of this mRNA the synthetic tridecanucleotide dTCGTTTCCGAGAG, complementary to nucleotides 98–110 of the p69 insert (Fig. 3*b*), was chemically synthesized[49], 5′ ^{32}P-labelled and used to prime the reverse transcription of induced lymphocyte poly(A) RNA into single-stranded ^{32}P-cDNA. The cDNA product was sized on a polyacrylamide–urea gel[46] and found to be 125–130 nucleotides long (unpublished results), indicating that the cDNA insert of p69 lacks only about 15–20 nucleotides of 5′ mRNA sequence. Induced poly(A) mRNA was also fractionated on a denaturing agarose gel[33,34], transferred to nitrocellulose paper[50] and hybridized with ^{32}P-labelled cDNA insert from p69. A single hybridizing band, with the same mobility as 18S rRNA, was observed. Therefore, the mRNA corresponding to the p69 cDNA insert co-migrates with IFN-γ mRNA activity.

The DNA sequence of the p69 cDNA insert contains a single large open reading frame, beginning with the ATG codon at nucleotides 110–112 from the 5′ end. This ATG is followed, 166 codons later, by a TAA termination triplet at nucleotides 608–610. This ATG is the first one encountered, consistent with the observation that the first ATG codon from the 5′ end of mRNA usually serves as the site of translation initiation in eukaryotes[51]. The 3′-untranslated region of 587 nucleotides contains the hexanucleotide AATAAA (position 1,173–1,178) which precedes the site of polyadenylation in many eukaryotic mRNAs[52]. The 20 N-terminal amino acids encoded by the cDNA insert of p69 may constitute a signal peptide which is involved in the secretion of the mature polypeptide from lymphocytes. This putative signal sequence is strongly hydrophobic, having features common to eukaryotic secretory protein signal peptides in general[53,54] and more specifically to the IFN-αs for which DNA sequences have been determined[11-16]. It has the same potential cleavage site (Gly-Cys) that is likely to be the signal peptide cleavage sequence common to all IFN–αs[14]. Furthermore, the three amino acids (Ser-Leu-Gly) preceding this potential cleavage site are identical to those encoded by 10 of the 11 IFN-α genes sequenced to date[11-16].

The only other homology between this potential IFN-γ sequence and IFN-α or IFN-β covering more than three consecutive amino acids occurs at amino acids 15–18 (Lys-Lys-Tyr-Phe). These same four amino acids are found at positions 121–124 of two IFN-αs (IFN-αD and -αF; ref. 14). The 146 amino acid polypeptide estimated to constitute the mature protein has a calculated molecular weight of 17,110, much smaller than the molecular weights reported previously for IFN-γ (refs 18–21). The reasons for this discrepancy are not clear, but it should be noted that the molecular weights reported

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Table 1 Characterization of IFN-γ produced by *E. coli* and monkey cells

Treatment	IFN-α standard	IFN-β standard	IFN-γ standard	*E. coli* W3110/pIFN-γtrp48 extract	COS-7 cell/pSVγ69 culture medium
	Antiviral activity (U ml^{-1})				
Untreated	375	125	250	250	62.5
*p*H 2	375	125	<6	<12	<4
0.1% SDS	375	—	<4	<8	<4
Rabbit anti-IFN-α	<8	125	250	250	62.5
Rabbit anti-IFN-β	375	<8	187	250	62.5
Rabbit anti-IFN-γ	375	125	<4	<8	<4

An overnight culture of *E. coli* W3110/pIFN-γtrp48 in Luria broth[68] containing 5 μg ml^{-1} tetracycline was diluted 1:100 in M9 medium[68] containing 0.2% glucose, 0.5% casamino acids and 5 μg ml^{-1} tetracycline at a 1:100 dilution. Indole acrylic acid was added to a final concentration of 20 μg ml^{-1} when A_{550} was 0.1–0.2. Samples (10 ml) were collected by centrifugation at A_{550} = 1.0 (~3.5 × 10^8 cells ml^{-1}) and resuspended immediately in 1 ml phosphate-buffered saline containing 1 mg bovine serum albumin (PBS–BSA). Cells were opened by sonication and cleared of debris by centrifugation. The supernatants were stored at 4 °C until assay. The plasmid pSVγ69 was introduced in COS-7 cells[62] using a modification of the DEAE-dextran technique[69]. Fresh monolayers of COS-7 cells in 6-cm diameter plates were transfected with 1 μg of pSVγ69 in 1.5 ml of Dulbecco's minimal essential medium (DMEM; Gibco), 200 μg ml^{-1} DEAE-dextran (500,000 molecular weight; Pharmacia), 0.05 M Tris-HCl (*p*H 7.5). After 16 h at 37 °C, the plates were washed twice with DMEM, and 1.5 ml of fresh DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μg ml^{-1} penicillin G and 50 mg ml^{-1} streptomycin was added to each plate. The medium was replaced the following day with serum-free DMEM. Fresh serum-free medium was then added every day. The collected media were stored at 4 °C until assayed. IFN-α and IFN-β control samples were NIH reference standards. The IFN-γ control was partially purified (~10^6 units per mg) from induced PBLs. The anti-IFN-α and -β were obtained from the National Institute of Allergy and Infectious Diseases. The anti-IFN-γ antisera was prepared using authentic IFN-γ (5–20% purity) purified from stimulated PBLs. Samples for antibody neutralizations were diluted, if necessary, then incubated for 2 h at 4 °C with 1:20 dilutions of rabbit anti-human leukocyte, fibroblast or immune interferon antisera. Samples were centrifuged for 3 min at 12,000*g* before assay. To test *p*H 2 stability, samples were adjusted to *p*H 2 by addition of 1 M HCl, incubated for 2 h at 4 °C and neutralized by addition of 1 M NaOH before assay. To test SDS sensitivity, samples were incubated with an equal volume of 0.2% SDS for 2 h at 4 °C before assay. The IFN assay was performed as described in Fig. 1 legend.

tionally, IFN-γ is glycosylated *in vivo*[20,21,55], which could affect molecular weight estimates. Consistent with this observation is the presence of two potential *N*-glycosylation sites[56] in the predicted protein sequence, at amino acids 28–30 (Asn-Gly-Thr) and 100–102 (Asn-Tyr-Ser). As the only two cysteines present in the deduced amino acid sequence occur at positions 1 and 3, the tertiary structure of the polypeptide is probably not dependent on intramolecular disulphide bonds. This is in agreement with the finding that, in contrast to IFN-α and IFN-β, disulphide linkages are not critical for the antiviral activity of IFN-γ (ref. 21). The mature protein sequence shown in Fig. 3*b* is quite basic in character, having 27 basic amino acids (Arg + Lys) and 19 acidic residues (Asp + Glu). This is consistent with the observation of Yip *et al.*[20] that IFN-γ has a high isoelectric point (*p*I = 8.6–8.7), whereas IFN-α and IFN-β have been shown to have distinctly lower *p*Is[20,57].

Synthesis of IFN-γ in *E. coli* and monkey cells

As IFN-γ has not been purified to homogeneity, nothing is known directly about its amino acid sequence. Therefore, the only direct means of proving that the cDNA sequence of p69 actually codes for IFN-γ is by demonstrating that the polypeptide obtained through expression of this cloned gene has the properties of natural IFN-γ. Two independent expression systems involving *E. coli* and monkey cells were used for this purpose. The *E. coli* system was selected because it has been used to produce biologically active IFN-αs and IFN-β (refs 10, 13). The mammalian cell system was chosen because, unlike *E. coli*, it should be able to perform specific processing (for example, glycosylation) which might be required for the expression of a biologically active product.

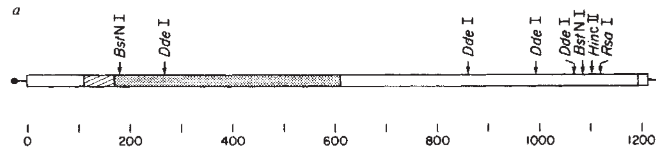

Fig. 3 *a*, Restriction endonuclease map of the cDNA insert of p69. The cDNA insert is bounded by *Pst*I sites (dots at both ends) and poly(dG-dC) tails (single lines). The number and size of fragments produced by restriction nuclease cleavage were estimated by electrophoresis through 6% polyacrylamide gels. Positions of sites were confirmed by nucleic acid sequencing (presented below). The shaded region indicates the coding sequence of the putative mature protein, the cross-hatched region represents the putative 20 residue signal peptide coding sequence, and the open regions show the 3′- and 5′-noncoding sequences. *b*, Nucleotide sequence and deduced amino acid sequence of plasmid p69 cDNA insert. The putative signal sequence is represented by the residues labelled S1 to S20. The entire sequence was determined by both the dideoxynucleotide chain termination method[47] after subcloning fragments into the M13 vector mp7[48] and the chemical method of Maxam and Gilbert[46]. Numbers above each line refer to amino acid position (S refers to presumed signal peptide) and numbers below each line to nucleotide position.

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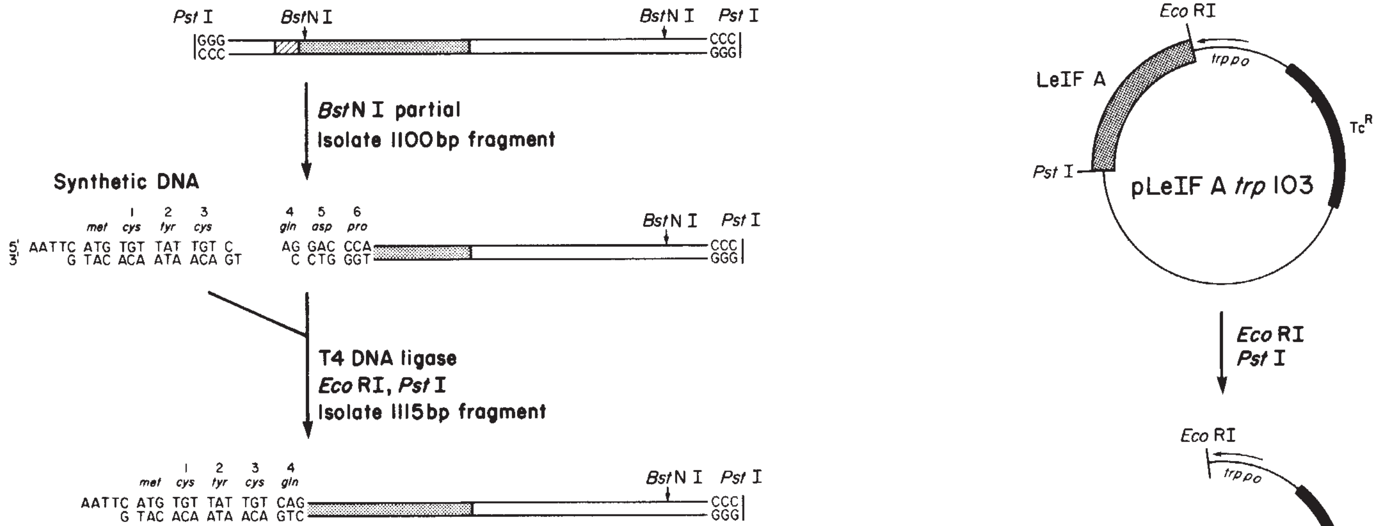

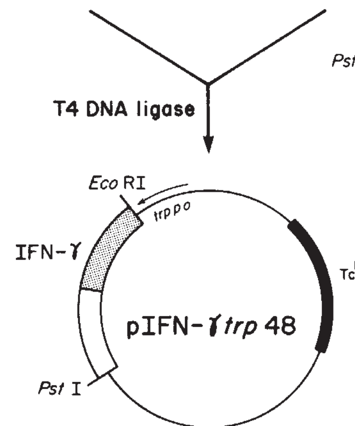

Fig. 4 Construction of a plasmid coding for the direct synthesis of mature IFN-γ in *E. coli.* 5 μg of the 1,250 bp *Pst*I insert of plasmid p69 were isolated and partially digested with 3 units of *Bst*NI (Bethesda Research Laboratories) for 15 min at 37 °C and the reaction products resolved on a 6% polyacrylamide gel. Approximately 0.5 μg of the desired 1,100 bp *Bst*NI*Pst*I fragment was recovered by electroelution. The two indicated deoxyoligonucleotides, 5′-dAATTCATGTGTTATTGTC and 5′-dTGACAATAACACATG, were synthesized by the phosphotriester method[49] and phosphorylated as follows. 100 pmol of each deoxyoligonucleotide were combined in 30 μl of 60 mM Tris-HCl (*p*H 8), 10 mM $MgCl_2$, 15 mM β-mercaptoethanol and 240 μCi [γ-^{32}P]ATP (Amersham, 5,000 Ci $mmol^{-1}$). 12 units of T4 polynucleotide kinase were added and the reaction allowed to proceed at 37 °C for 30 min. 1 μl of 10 mM ATP was added and the reaction allowed to proceed for an additional 20 min. After phenol/$CHCl_3$ extraction, the oligomers were combined with 0.25 μg of the *Bst*NI–*Pst*I 1,000 bp fragment and ethanol precipitated. These fragments were ligated at 20 °C for 2 h in 30 μl of 20 mM Tris-HCl (*p*H 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP and 10 units T4 DNA ligase. The mixture was digested for 1 h with 30 units of *Pst*I and 30 units of *Eco*RI (to eliminate polymerization through ligation of cohesive termini) and electrophoresed on a 6% polyacrylamide gel. The 1,115 bp product (110,000 c.p.m.) was recovered by electroelution. The plasmid pLeIFAtrp103 is a derivative of the plasmid pLeIFA25 (ref. 13) in which the *Eco*RI site distal to the LeIF A gene has been removed. 3 μg of pLeIFAtrp103 were digested with 20 units of *Eco*RI and 20 units of *Pst*I for 90 min at 37 °C and electrophoresed on a 6% polyacrylamide gel. The large (~3,900 bp) vector fragment was recovered by electroelution and the 1,115 bp *Eco*RI–*Pst*I IFN-γ DNA fragment ligated into 0.15 μg of this prepared vector. Transformation of *E. coli* K-12 strain 294[40] gave 120 tetracycline-resistant colonies. Plasmid DNA was prepared from 60 of these transformants and digested with *Eco*RI and *Pst*I. Three of these plasmids contained the desired 1,115 bp *Eco*RI-*Pst*I fragment. DNA sequence analysis verified that these plasmids had the desired nucleotide sequence at the junctions between the *trp* promoter, synthetic DNA and cDNA. One of these plasmids, pIFN-γtrp48, was chosen for additional study.

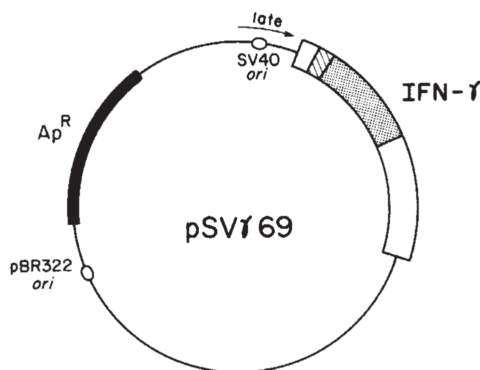

Fig. 5 Plasmid constructed for the expression of IFN-γ in monkey cells. The 342 bp *Hin*dIII–*Pvu*II fragment encompassing the SV40 replication origin[60] was converted to a fragment bounded by *Eco*RI restriction sites. The *Hin*dIII site was converted to an *Eco*RI site by addition of a synthetic oligomer (5′dAGCTGAATTC) and the *Pvu*II site was converted by ligation to an *Eco*RI site which had been filled-in using DNA polymerase I (Klenow fragment). The *Eco*RI fragment containing the SV40 origin was ligated into the *Eco*RI site of pML-1[59]. The resulting expression vector was chosen which contained the SV40 late promoter oriented to read away from the ampicillin-resistance gene of pML-1. The *Eco*RI site nearest the ampicillin-resistance gene was destroyed by partial *Eco*RI digestion, filling in with the Klenow fragment of DNA polymerase I, and subsequent ligation (as outlined in ref. 73). The 1,250 bp *Pst*I insert of p69 was converted to a fragment bounded by *Eco*RI sites by insertion in the *Pst*I site of pNCV[13], as

The procedure followed to express the cDNA insert of p69 directly in *E. coli* was similar to that used previously for human growth hormone[39], IFN-β (ref. 10) and several IFN-αs[13,16], and is outlined in Fig. 4. A *Bst*NI restriction site located at codon 4 of the presumed mature coding sequence was used to remove the signal peptide coding region. Two synthetic deoxyoligonucleotides were designed which restore the codons for amino acids 1–4, incorporate an ATG translational initiation codon and create an *Eco*RI cohesive terminus. These two oligomers were ligated to the remainder of the cDNA insert to construct a 1,115 bp synthetic–natural hybrid gene coding for a polypeptide of 147 amino acids and bounded by *Eco*RI and *Pst*I sites. This gene was inserted into the plasmid pLeIFAtrp103 (a derivative of pLeIFA25 (ref. 13) in which the *Eco*RI site distal to the LeIF *A* gene was destroyed) between the *Eco*RI and *Pst*I sites to give the expression plasmid pIFN-γ*trp48*. In this plasmid the cloned gene is transcribed under the control of a 300 bp fragment of the *E. coli trp* operon[13]. This fragment contains the *trp* promoter, operator and the Shine–Dalgarno sequence of the *trp* leader peptide[58], but lacks the ATG sequence for initiation of translation of the leader peptide. *E. coli* W3110 strains transformed with either pIFN-γtrp48 or p69 were grown and extracted for interferon assay (see Table 1). Extracts from cultures of W3110/pIFN-γtrp48 exhibited definite interferon activity (~250 units per ml of extract), whereas no activity (<3 units per ml) was detected in extracts of W3110/p69.

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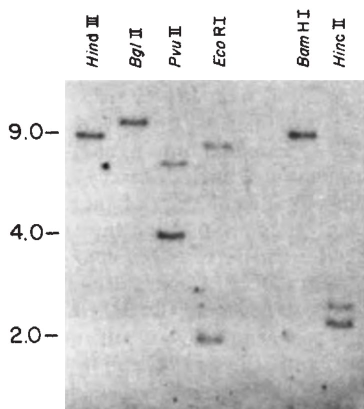

Fig. 6 Hybridization of human genomic DNA with a ^{32}P-labelled cDNA probe from plasmid p69. High molecular weight human lymphocyte DNA (5 μg) prepared by the procedure of Blin and Stafford[74] was digested to completion with various restriction endonucleases, electrophoresed on 1.0% agarose gels and blotted to a nitrocellulose filter[67]. The ^{32}P-labelled DNA probe was prepared from a 594 bp *Dde*I fragment (nucleotides 266–860 of Fig. 3*b*) of the cDNA insert of p69 as described previously[45]. 1×10^7 c.p.m. of the probe were hybridized with the filter for 16 h and then washed as described elsewhere[14]. Two hybridizing DNA fragments were observed with three endonuclease digests: *Pvu*II (6.7 and 4.0 kbp), *Eco*RI (8.8 and 2.0 kbp) and *Hinc*II (2.5 and 2.2 kbp). Three endonuclease digestion patterns provide only one hybridizing DNA fragment: *Hind*III (9.0 kbp), *Bgl*II (11.5 kbp) and *Bam*HI (9.5 kbp).

was constructed from the following three components: (1) the plasmid pML-1 (pML-1 is a derivative of pBR322 in which sequences known to inhibit the replication of SV40–pBR322 recombinants in simian cells have been deleted, but which still contains the ampicillin-resistance selectable marker and an *E. coli* replication origin[59]); (2) a 342 bp *Pvu*II–*Hind*III fragment of SV40 virus providing the replication origin of SV40[60] and which is thought to encompass the promoter for both the early and late SV40 transcriptional units[61]; and (3) the entire cDNA insert from plasmid p69.

The cDNA insert and the SV40 origin region were oriented in pSVγ69 such that the interferon structural gene should be transcribed from the promoter for the late transcriptional unit (Fig. 5). This vector was introduced by DNA transfection into the transformed monkey cell line COS-7 (ref. 62). COS-7 endogeneously expresses SV40 large T antigen and has been shown to permit the propagation of recombinant plasmids containing pML-1 and SV40 origin sequences[59,60]. Cell media from transfected cultures were assayed daily for the presence of interferon activity. Yields of 50–100 units per ml were obtained 3 or 4 days after transfection, indicating that the interferon was being secreted into the culture medium by the COS-7 cells. No interferon activity was observed in the media from cells transfected with plasmids in which the cDNA coding region was inserted in the opposite orientation to that shown in Fig. 5 (data not shown).

Characterization of interferon activity produced by *E. coli* and monkey cells

The properties of the interferon activity present in extracts of *E. coli* W3110/pIFN-γtrp48 and secreted from COS-7/pSVγ69 cells were compared with authentic human IFN-γ produced by stimulated PBLs and with authentic IFN-α and IFN-β. Distinguishing characteristics of IFN-γ include *p*H 2 lability and lack of neutralization by antisera prepared against human IFN-α or IFN-β (ref. 2). As indicated in Table 1, the interferon produced by *E. coli* and monkey cells, like the IFN-γ produced by SEB/desacetylthymosin α_1-stimulated PBLs, was not neutralized by antisera to either IFN-α or IFN-β, although these antisera were effective in neutralizing authentic IFN-α or IFN-β. Antisera prepared against partially purified PBL-produced IFN-γ (E. Rinderknecht and V. Anicetti, unpublished results) completely neutralized the interferon activity synthesized by *E. coli* and monkey cells while having no effect on the activity of IFN-α and IFN-β (Table 1). Furthermore, the complete loss of activity observed when these interferon preparations were treated with *p*H 2 or 0.1% SDS is characteristic of IFN-γ, and distinct from IFN-α and IFN-β. Therefore, the polypeptide encoded by the cloned cDNA sequence of p69 has

A single gene for IFN-γ

The human interferon gene family comprises 12 or more distinct IFN-α genes having 80–95% DNA sequence homology[11–16,63] and at least one IFN-β gene (IFN-β_1; refs 64–66) which is 40–50% homologous with the IFN-α genes. To estimate the number of related IFN-γ genes in the human genome, human DNA of one individual was digested with several restriction enzymes, electrophoresed through agarose gels, transferred to nitrocellulose paper[67] and hybridized to a ^{32}P-labelled IFN-γ cDNA probe (Fig. 6). *Hinc*II is the only one of the six restriction enzymes used that cleaves the IFN-γ cDNA sequence, but the *Hinc*II site is not located in the DNA fragment which was used as probe. A unique, intronless gene would thus have appeared as a single hybridizing DNA fragment in each lane of the gel. Instead, two hybridizing DNA fragments were observed for three of the endonuclease digestions (*Pvu*II, *Eco*RI, *Hinc*II) and single hybridizing fragments of about 9 kilobase pairs (kbp) each were found for the other digestions (*Hind*III, *Bgl*II, *Bam*HI). Similar genomic blots using DNA from other individuals gave the same hybridization patterns. There are two possible explanations for these results: first, there are two homologous and closely linked (<8 kbp apart) IFN-γ genes separated by *Pvu*II, *Eco*RI and *Hinc*II sites, and second, there is a single IFN-γ gene which contains one or more introns containing *Eco*RI, *Pvu*II and *Hinc*II sites. This second possibility was supported by repeating the hybridizations with a ^{32}P-labelled probe prepared from the 3′-untranslated region of IFN-γ cDNA. In this experiment only single *Pvu*II, *Eco*RI and *Hinc*II fragments hybridized to the probe (data not shown). The gene structure of IFN-γ is therefore distinctly different from that of IFN-α and IFN-β, which contain no introns[15,63–66].

Discussion

Poly(A) mRNA from human lymphocytes producing IFN-γ was fractionated on a denaturing agarose gel. The 18S size fraction was used to prepare a cDNA library in *E. coli*. Twenty-two bacterial clones containing potential IFN-γ sequences were identified by specific hybridization to ^{32}P-cDNA probes prepared using mRNA from induced lymphocytes and failure to hybridize to similar probes prepared from uninduced mRNA. Restriction mapping and DNA hybridization experiments revealed that all 22 induced cDNA clones were related and that the frequency of these clones in the cDNA library was about 1:120. Therefore, the frequency of IFN-γ mRNA in our most active poly(A) RNA preparation is ~1:2,400 because the mRNA used to prepare this cDNA library had been enriched about 20-fold in IFN-γ sequence by the agarose gel fractionation procedure.

The 1,194 bp cDNA insert (excluding poly(A) sequence and dG·dC tails) of p69 codes for a polypeptide of 166 amino acids, 20 of which are probably cleaved during the process of secretion of mature IFN-γ from lymphocytes. The encoded amino acid sequence, which bears little resemblance to those of IFN-α or IFN-β, has two potential glycosylation sites and is quite basic in character. Identification of this sequence as IFN-γ was obtained by expression of the cDNA sequence of p69 in bacterial and mammalian cells. In both systems the expression product was shown to have antiviral activity which was immunologically indistinguishable from that of the IFN-γ produced by stimulated human lymphocytes. Additionally, the SDS and *p*H 2 sensitivity observed for the IFN-γ produced by bacteria and monkey cells are distinguishing features of authentic IFN-γ (refs 2, 18–21).

The amounts of antiviral activity recovered from *E. coli* producing IFN-γ are significantly lower than those obtained for IFN-α or IFN-β using similar expression vectors[10,13,16]. If a specific activity range of 10^8–10^9 units per mg is assumed for IFN-γ (ref. 20), between 8 and 80 active molecules are present per cell. However, if the specific activity of the interferon depends to some degree on its state of glycosylation, consider-

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