
Human interferon γ is encoded by a single class of mRNA

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ABSTRACT

Polyadenylated RNA from human peripheral blood lymphocytes and spleen cells, treated with different inducers for IFN- γ production, was fractionated on denaturing sucrose gradients. Two IFN- γ mRNA peaks at 12S and 16S were consistently observed. Nucleotide sequence analysis of cDNA clones showed that the 12S IFN- γ mRNA from the different sources is identical to the gel fractionated 18S IFN- γ mRNA which gave rise to the IFN- γ cDNA clone p69 (1). Nucleotide sequence analysis of several IFN- γ cDNA clones showed the presence of a CGA (Arg) codon at position 140 of mature IFN- γ in contrast with the CAA (Gln) codon, which is found in p69 (1). Specifically primed cDNA extension on total induced polyadenylated RNA revealed the presence of a single mRNA species having a 5' untranslated region of 125-130 nucleotides. The nucleotide sequence of this region has been obtained. These data suggest that a single human IFN- γ gene, which has very little polymorphism, gives rise to a single size class of mRNA.

INTRODUCTION

The use of recombinant DNA technology has brought a major breakthrough in the study of human interferons (IFNs). Several groups have cloned and determined the structure of the complementary DNAs (cDNAs) for the human IFNs- α (leukocyte) (2-7) and IFN- β (fibroblast) (8-10). This has led to high level synthesis of human interferons in *E. coli* (3,8,11) and yeast (12). We have recently also described the cloning and the structure of a cloned cDNA encoding human IFN- γ (immune) and its expression in *E. coli* and monkey cells (1).

Analysis of the cloned cDNAs and the chromosomal genes for the human IFNs- α reveals the presence of a multigene family, consisting of at least 20 distinct but homologous genes (4-7, 13-17). These genes encode IFNs which differ from each other in about 20 to 30 of their 166 amino acid residues (4). In addition, minor differences have been observed in IFN- α cDNA and genomic sequences, which results in 1 or 2 amino acid substitutions, probably a reflection of allelism at these loci. In the

case of human IFN- β , sequence analysis of cloned cDNAs and the corresponding gene by several groups (8-10, 18-22) has revealed the structure of only one species. A possible allelism in the nucleotide sequence has been found, which does not result in any change in the amino acid sequence for IFN- β . Here the Tyr at position 30 in the mature protein is encoded by a UAC or UAU (8-10, 18-22). Also, Shepard *et al.* (23) isolated a variant IFN- β cDNA coding for a Tyr instead of the Cys at position 141. The protein encoded by this cDNA is biologically inactive.

In the case of human IFN- γ , a single gene corresponding to the cloned cDNA has been detected (1). In contrast to the genes for IFN- α (4, 13-16) and IFN- β (19-22) this gene contains three intervening sequences (1, P.W.G. and D.V.G., unpublished results). This paper reports the results of the sequence analysis of several IFN- γ cDNAs prepared using mRNA from both induced peripheral blood lymphocytes and induced spleen lymphocyte cultures. We also report the nucleotide sequence of the 5' untranslated region of the IFN- γ mRNA, as determined from specifically primed cDNA synthesis on total induced mRNA.

MATERIALS AND METHODS

Preparation and size fractionation of mRNA. The induction of peripheral blood lymphocytes (PBLs) for IFN- γ production with *Staphylococcus* enterotoxin B (SEB) and desacetylthymosin- α_1 , and the preparation of total mRNA has been described (1). Spleen lymphocytes were induced with *Staphylococcus* enterotoxin A (SEA, 10 μ g/ml) as the only inducing agent. The mRNA was fractionated by centrifugation in a 5 to 25 percent sucrose gradient in 70 percent formamide (24). Centrifugation took place in a Beckman SW40 rotor at 35,000 rpm for 19 hrs at 20°C.

RNA dot blot analysis. RNA samples were immobilized onto nitrocellulose (Schleicher and Schuell, BA85) by the procedure of Thomas (26), adapted for dot blot analysis (27) and hybridized with a 32 P-labelled IFN- γ cDNA probe. This probe was prepared by isolation of a 595 bp *Dde*I fragment of p69 (ref. 1) on a 5 percent polyacrylamide gel and 32 P-labelling *in vitro* by the random calf thymus priming method (28).

cDNA cloning and colony hybridization. Double stranded cDNA was prepared from the fractionated mRNA (1). After fractionation on a 5 percent polyacrylamide gel, this cDNA was extended with deoxy C-residues and annealed to deoxy G-tailed *Pst*I-cleaved pBR322 (29) and then used to transform *E. coli* 294 (30,31). Colonies were probed with the

³²P-labelled IFN- γ specific 595 bp DdeI fragment (1,32).

Plasmid DNA preparation and digestion. Plasmid DNA preparation from 5 ml bacterial cultures was done by the procedure of Birnboim and Doly (33). Larger DNA preparations from 500 ml cultures were performed by a standard cleared lysate procedure (34). The plasmid DNA was digested with 20 μ g RNase A for 5 minutes at room temperature and purified by column chromatography on Biogel A-50 M (Bio-Rad). Restriction endonucleases were purchased from New England Biolabs or Bethesda Research Laboratories and used as recommended by the suppliers.

DNA sequencing. Plasmid DNAs were sequenced by the dideoxynucleotide chain termination method (35), using synthetic DNA primers, complementary to the positive strand of the IFN- γ cDNA insert (see Results). The single-stranded deoxyoligonucleotides were synthesized by the modified solid phase phosphotriester method (36). The only change in the synthetic procedure was the use of 1-(2,4,6-tri-isopropylbenzenesulphonyl)-3-nitro-1,2,4-triazole (37) rather than 1-(2,4,6-tri-isopropylbenzenesulphonyl)-tetrazole as the condensing reagent. Plasmid DNA was denatured in 0.2 M NaOH, 0.2 mM EDTA, for 5 minutes at room temperature and neutralized by addition of 0.13 M Ammonium acetate (pH 4.5). The primer was then hybridized to the plasmid DNA for 10 minutes at 42°C in 6 mM Tris-HCl, pH 7.6, 6 mM MgCl₂, 50 mM NaCl. The sequencing procedure, using the Klenow fragment of DNA polymerase I, was performed essentially as described (35,38).

The chemical degradation method for DNA sequence analysis (39) was done using 5' terminally ³²P-labelled restriction fragments. The strategy for sequencing of the cDNA inserts of p52, p67, p72, p12S γ 8 and pS12-10 is shown in Fig. 2.

Specifically primed cDNA synthesis. The synthetic DNA primer 5'dTCGTTCCGAGAG 3', complementary to nucleotides 98-110 of the p69 insert (1), was synthesized by the modified phosphotriester method (36,37). About 200 pmoles of the primer were phosphorylated by incubation in a 60 μ l mixture, containing 60 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 15 mM β -mercaptoethanol, 500 pmoles of [γ -³²P]-ATP (2,500 Ci mmol⁻¹; New England Nuclear) and 4 units of T4 polynucleotide kinase (New England Nuclear), for 30 minutes at 37°C and then purified on a Sephadex-G50 column. The 200 pmoles of the ³²P-labelled synthetic primer and 20 μ g of total induced PBL mRNA were denatured together at 100°C for 3 minutes and quickly chilled. Specifically primed complementary DNA was synthesized

using reverse transcriptase in a 60 μ l reaction mixture containing 15 mM Tris-HCl (pH 8.3), 20 mM KCl, 8 mM MgCl₂, 30 mM β -mercaptoethanol, 0.3 mM of each dNTP and 40 units RNAsin (Biotec) for 30 min at 43°C. The cDNA product was sized on a denaturing 8 percent polyacrylamide 7 M urea gel (39) and autoradiographed. The discrete cDNA band was excised, recovered by electroelution and sequenced by the Maxam-Gilbert method (39).

RESULTS

Fractionation of induced mRNA. The induced polyadenylated mRNA from either peripheral blood lymphocytes or spleen cells was fractionated by sucrose gradient centrifugation (24). Fractions were assayed for the presence of IFN- γ mRNA by translation in Xenopus oocytes, followed by a cytopathic inhibition antiviral interferon assay (1). A typical profile is shown in Fig. 1. This profile was consistently obtained for both the SEA-induced spleen RNA and the RNA from PBLs induced with SEB and desacetylthymosin- α_1 . Two different peaks of IFN activity were present in the gradient, corresponding to 12S mRNA and 16S mRNA. In contrast, a single peak at 18S is observed when the mRNA is fractionated on a denaturing agarose gel (1). These activities are apparently due to IFN- γ , since no activity was detectable when the same translated mRNA fractions were assayed on bovine cells (MDBK), which are protected by IFN- α and - β (8), but not by human IFN- γ (1,40). The difference between the values of 16S and 18S can easily be due to inherent properties of the mRNA in these two experimental systems. The presence of two separate peaks in the

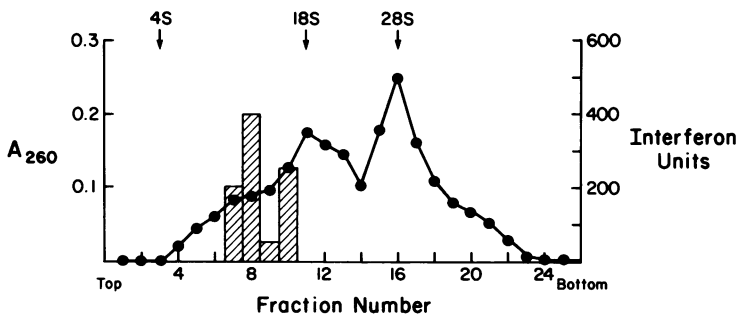


Fig. 1. Separation of total polyadenylated RNA from induced PBLs on 5-25 percent sucrose gradient in 70 percent formamide (24). The A₂₆₀ profile is shown with a solid line. The interferon activity determined in the medium of injected Xenopus oocytes is shown in a hatched histogram.

gradient and the significant difference between these S-values encouraged us to investigate the nature of the 12S IFN- γ mRNA.

Therefore, samples from the RNA gradient fractions, either derived from induced PBLs or spleen cells, were immobilized on nitrocellulose paper and probed using the RNA dot blot method (26,27) with a ^{32}P -labelled IFN- γ specific DdeI fragment from the plasmid p69 (Fig. 2). Both the 12S and 16S RNA fractions from the sucrose gradient hybridized with this probe and the intensity of hybridization coincided with the IFN- γ mRNA profile (data not shown). This result clearly indicates that the 12S and 16S mRNA fractions both contain IFN- γ nucleotide sequences.

cDNA cloning of 12S IFN- γ mRNA. To further investigate the nature of the 12S IFN- γ mRNA peaks, cDNA clones were prepared using the fractionated mRNA from both PBLs and spleen cells. A cloned cDNA library in E. coli 294 was constructed by standard techniques (1). About 1000 bacterial clones from induced PBL mRNA and 2500 clones from induced spleen mRNA were screened for the presence of IFN- γ specific sequences by colony hybridization (32) again using the ^{32}P -labelled DdeI fragment of p69. About 1 percent of the clones hybridized with the probe. Plasmid DNA was then prepared from these clones and the cDNA inserts were characterized using different restriction endonucleases. The plasmids from all hybridizing clones from both PBL and spleen mRNA had restriction maps corresponding to the cDNA insert of p69. In addition, the longest IFN- γ cDNA inserts, detected among the spleen or PBL cDNA clones, were only slightly shorter than the insert in p69 (Fig. 2); all cDNA clones examined contained the complete 3' noncoding sequence as detected in p69 (data not shown). These data suggest that IFN- γ mRNA in the 12S peak from both cellular sources is very similar or identical to the gel fractionated 18S IFN- γ mRNA which gave rise to p69. However, minor differences in nucleotide sequence cannot be excluded on this basis.

Nucleotide sequence of IFN- γ cloned cDNA. The IFN- γ cDNAs were sequenced to detect possible minor structural differences, especially in the coding region. Five different IFN- γ cDNA clones were investigated. The plasmids p52, p67 and p72 are IFN- γ cDNA clones which were prepared at the same time as p69 using the 18S gel fractionated mRNA. Plasmids p12S γ 8 and pS12-10 came from the 12S mRNA gradient peak from induced PBLs and spleen cells respectively. For the dideoxy sequencing of the cDNA coding sequence, three synthetic single-stranded DNA primers were used: DAGTCAGCTTTTCG (primer 1), dTTGGCTCTGCATT (primer 2) and dAAATATTGCAGGC

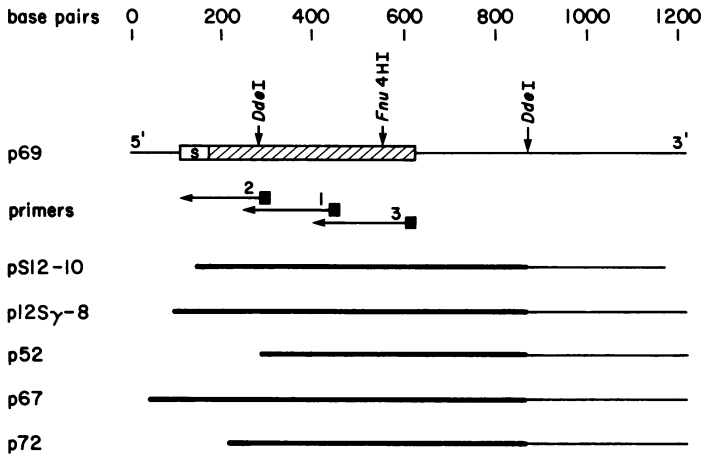


Fig. 2. Schematic diagram showing the length of the sequenced clones and the regions for which the nucleotide sequence was obtained. The upper diagram represents schematically the IFN- γ cDNA insert of p69, with the boxed hatched coding sequence and the putative signal sequence (S) and a scale representing the length of the cDNAs in basepairs. The restriction sites on the cDNA which have been used for the 5' end labelling are indicated. The localization of the synthetic primers, complementary to the cDNA, and the regions sequenced by the dideoxy chain termination method are indicated by arrows. The lengths of the five analyzed cDNAs are schematically drawn with the sequenced regions shown as a thick line.

(primer 3), complementary to nucleotides 453-465, 309-321 and 620-632 respectively in the cDNA sequence of p69 (1). Restriction fragments were 5'-labelled at the *Fnu4HI* or the *DdeI*-sites for the Maxam-Gilbert sequencing method. The sequencing strategy and the analyzed regions are shown in Fig. 2.

All cDNAs were shorter than the cDNA insert of p69 (Fig. 2). With the exception of one position their sequences were identical to each other and to the previously established sequence of p69 (1). In all five plasmids we found a CGA triplet, coding for arginine at amino acid position 140 of the mature protein, in contrast with a CAA coding for glutamine at the same position in p69 (1).

The 5' untranslated region of IFN- γ mRNA. The results described above clearly show structural identity for the different IFN- γ mRNA fractions. However, heterogeneity in the 5' untranslated region might have been undetectable. Indeed, a heterogeneity in the 5' untranslated region,

preceding identical coding sequences has been reported in the case of mouse liver and salivary gland α -amylase mRNA (41,42) and of mouse dihydrofolate reductase mRNA (43). To investigate this and to obtain additional nucleotide sequence information of the 5' untranslated region of the IFN- γ mRNA, a primer extension experiment was performed. The specific 13 mer dTCGTTTCCGAGAG, complementary to the region of mRNA immediately preceding the IFN- γ start codon, was synthesized and hybridized with total polyadenylated RNA from induced PBLs. The specific elongation with reverse transcriptase resulted in the synthesis of a single product of a length of 125-130 nucleotides, as determined by denaturing gel electrophoresis in a 8 percent polyacrylamide, 7 M urea gel (Fig. 3a). Comparison with the

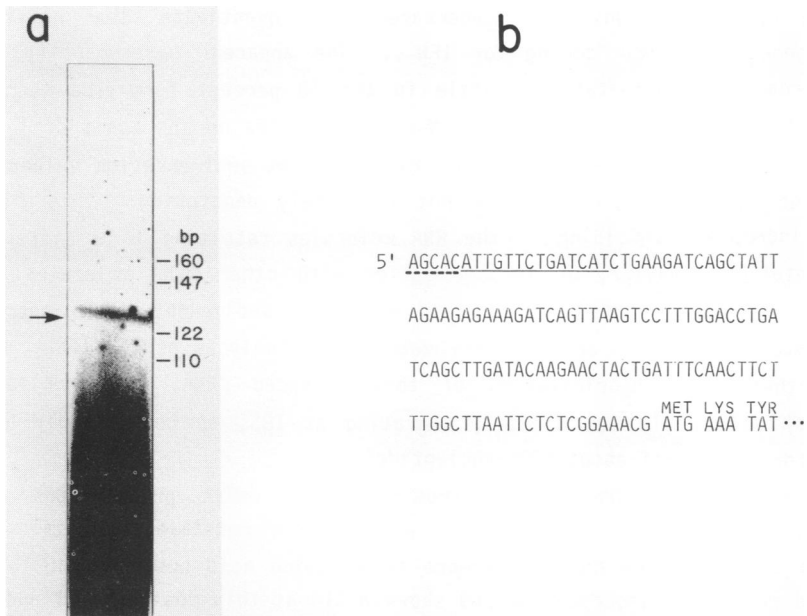


Fig. 3. Analysis of the specifically primed extension product from IFN- γ mRNA.

- The autoradiogram shows the specific product (arrow) obtained after specifically primed extension on total mRNA. The size in basepairs of ^{32}P -labelled restriction fragments used as size markers is shown.
- The 5' untranslated sequence of IFN- γ mRNA. The 5' terminal residues underlined with a dotted line are derived from analysis of the chromosomal gene. The rest of this sequence was determined by sequence analysis of the primed extension product and of the p69 cDNA. The underlined nucleotides are not present in the cDNA of p69.

cloned cDNA (1) indicates that p69 is missing only 15–20 nucleotides from the 5' end of the mRNA. It also indicates that only a single IFN- γ mRNA species is detectable, which is in agreement with the previous findings from Southern hybridizations with total genomic DNA and from Northern hybridization experiments with total mRNA (1). The labelled 5' specific extension product was sequenced by the Maxam-Gilbert method (39). The sequence is in complete agreement with the p69 cDNA sequence of the 5' flanking region and extends it an additional 14 nucleotides (Fig. 3b). The presence of five bases preceding these is apparent from the sequencing gel but they could not be identified with certainty.

DISCUSSION

The present study was undertaken to investigate the possible heterogeneity of mRNA coding for IFN- γ . The apparent heterogeneity, as seen from the sedimentation profile in the 70 percent formamide sucrose gradient, is not due to different IFN- γ mRNA species on the basis of these studies. A probable explanation for the different sedimentation values is that the conditions employed are not completely denaturing. This could cause incomplete unfolding of the RNA molecules resulting in a different sedimentation behavior, or in aggregation with other mRNA molecules. A similar phenomenon has been observed with the sedimentation in sucrose gradients of rabbit α - and β -globin mRNA (44). Analysis of the IFN- γ mRNA by Northern blot hybridization of total induced mRNA (1, unpublished results) shows only one species, migrating at 18S, an unexpectedly high value for an mRNA of about 1200 nucleotides.

Several IFN- γ cDNA clones from different mRNA preparations and purifications have been sequenced in a search for possible allelism. The results show that the only difference is at amino acid position 140. The deduced amino acid sequence for p69 shows a Gln at this position (1) and an Arg for p52, p67, p72, p12S γ 8 and pS12-10. These might represent two allelic forms of the IFN- γ gene. However, since the Gln has been found for only one cDNA, it is possible that a transcription error has occurred during the in vitro cDNA synthesis which gave rise to p69, especially since the Arg at position 140 is also found in the sequence of the chromosomal gene for IFN- γ (P.W.G. and D.V.G., unpublished results). The IFN- γ cDNA of p67 has been inserted in a SV40 expression vector, as was previously done for the p69 cDNA (1). The levels of IFN- γ synthesized after transfection of these plasmids into monkey cells were indistinguishable from each other

in biological IFN-activity (D.W.L. and C.C. Simonsen, unpublished results).

The analysis of the mRNA and the corresponding cloned cDNAs clearly shows that the same species of IFN- γ mRNA is induced in lymphocytes of different tissue origins by the two induction schemes used. Neutralization experiments using different antisera and IFN- γ preparations also indicate that only antigenically related species or a single species of IFN- γ are produced by human lymphocytes upon different inductions (Y.K. Yip and J. Vilcek, personal communication).

The experiments with specific primer extension of the mRNA revealed that the 5' untranslated region of the mRNA is homogeneous in length and 125 to 130 nucleotides long. An additional 14 residues have been determined past the 5' end of the p69 cDNA insert. From the analysis of the primed extension product it was clear that a few residues were preceding these 14 nucleotides. From the sequence determination of the chromosomal gene (P.W.G. and D.V.G., unpublished results) and the observation that eukaryotic mRNAs mostly start with a capped purine residue, we conclude that the 5' terminal sequence of the mRNA is as shown in Fig. 3b, starting with a capped A-residue. The total length of the IFN- γ mRNA is then 1214 nucleotides excluding the polyA-tail, with a 128 nucleotides long 5' untranslated region. The 5' untranslated region of IFN- γ is contrastingly longer than the 5' untranslated sequences of IFN- α (67 nucleotides, ref. 13) and IFN- β (73-75 nucleotides, ref. 21).

In conclusion, the data presented in this report clearly show that only a single mRNA for human IFN- γ has been found from different lymphocyte sources using different induction conditions. These results, together with those from the analysis of genomic DNA (ref. 1, P.W.G. and D.V.G., unpublished results) and from Northern blot analysis of the RNA (1), point to the existence of a single species of human IFN- γ .

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