Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5842–5846, October 1983
Biochemistry

Cloning and expression of murine immune interferon cDNA

(interferon-γ/antiviral activity/protein homology/simian virus 40 promoter/*trp* promoter)

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Communicated by K. Frank Austen, June 3, 1983

ABSTRACT **The murine immune interferon (IFN-γ) gene was cloned and expressed under control of the simian virus 40 early promoter in the monkey COS-1 cell line. A protein is secreted from these cells having the biological, antigenic, and biochemical characteristics of natural murine IFN-γ. Cloned murine IFN-γ cDNAs were obtained by using RNA from both mitogen-induced murine spleens and the transfected COS cells, and both code for identical proteins. The mature murine IFN-γ encoded is 136 amino acids long, 10 amino acids shorter than human IFN-γ. The nucleotide homology between the murine and human IFN-γ genes is 60–65%, whereas the encoded proteins are only 40% homologous. Murine IFN-γ cDNA was expressed in *Escherichia coli* under *trp* promoter control.**

Murine immune interferon (MuIFN-γ) has been isolated from mitogen-induced mouse spleen cultures (1–4) and T-cell lines (5, 6). These preparations have not been purified to homogeneity but nevertheless contain potent *in vitro* antiviral and anticellular activities (1–6). MuIFN-γ may also have *in vivo* antitumor efficacy (7).

We previously reported the isolation and expression of the cDNA (8) and gene (9) for human IFN-γ (HuIFN-γ). This gene encodes a protein of 166 amino acids; the first 20 residues serve as a signal sequence for the secreted, mature protein. HuIFN-γ is distinctly different from HuIFN-α (leukocyte IFN) and HuIFN-β (fibroblast IFN): HuIFN-γ is encoded by a single gene on chromosome 12 (10), which contains three introns (9). The HuIFN-α gene family and single HuIFN-β gene are encoded by chromosome 9 (11) and contain no introns (12–17). Furthermore, the DNA sequence and encoded amino acid sequence of HuIFN-γ are unrelated to the HuIFN-α sequences (8, 12, 18) or the HuIFN-β sequence (15–17). Whereas HuIFN-α and HuIFN-β generally exhibit some antiviral activity on cell lines of other species (19), HuIFN-γ has a strict species specificity. Consequently, murine model systems, which have been useful in the study of potential antitumor agents, may not be suitable for examination of HuIFN-γ. Therefore, a source of MuIFN-γ could prove to be quite valuable in murine model systems and may aid the evaluation of the clinical potential of HuIFN-γ.

MuIFN-α (20) and MuIFN-β (T. Taniguchi, personal communication) genes have recently been cloned and characterized. However, no structural data on MuIFN-γ has been determined. The properties of MuIFN-γ are similar in some respects to HuIFN-γ; the antiviral activity of MuIFN-γ is sensitive to pH 2 and temperature (65°C for 1 hr) (1, 2, 5). Antibodies prepared against MuIFN-α or MuIFN-β do not neutralize MuIFN-γ antiviral activity (3–5). In an effort to determine the structure of MuIFN-γ, to further characterize its properties, and to provide material for animal testing, we isolated the MuIFN-γ gene from a recombinant murine-λ phage library and effected its expression in monkey cells and *Escherichia coli*.

MATERIALS AND METHODS

Isolation of the MuIFN-γ Gene. A ^{32}P-labeled DNA probe containing the entire coding region of human IFN-γ [873-base-pair (bp) *Sau*3A fragment; ref. 7] was prepared by the calf thymus priming method (21). The probe was hybridized to a recombinant murine-λ phage library (prepared from M600 murine genomic DNA in λCh4A, generously provided by C. Simonsen) essentially as described by Maniatis *et al.* (22). Hybridization was performed at a low stringency in 20% formamide, and filters were washed twice in 0.3 M sodium chloride/0.03 M sodium citrate/0.1% NaDodSO$_4$ at room temperature. Several phage that hybridized with the probe were plaque-purified (22). Phage DNA was prepared (22, 23), digested with various restriction endonucleases, subjected to electrophoresis through 1% agarose gels, and analyzed with Southern blots (24) by hybridization with the human cDNA probe. The 10.5-kbp *Bam*HI fragment from phage λMG9 that hybridized with the human cDNA was isolated by agarose gel electrophoresis, electroeluted, and subcloned into the *Bam*HI site of pBR322 to yield the plasmid pmg10.5. The sequence of >6,000 bp of the insert of pmg10.5 was determined by a combination of the Maxam and Gilbert chemical degradation technique (25) and the dideoxy chain-termination method of Sanger and colleagues (26).

Expression of the MuIFN-γ Gene in Monkey Cells. The MuIFN-γ gene was ligated into a simian virus 40 (SV40)-based expression vector as outlined in Fig. 1. The endonuclease *Pvu* II cleaves the plasmid pmg10.5 at six positions, one of which is in the 5′ untranslated region (71 bp upstream of the ATG initiation codon) of the gene. The 5′ end of the gene was isolated as a 348-bp fragment from this *Pvu* II site to a unique *Cla* I site in the first intron. The 3′ end of the MuIFN-γ gene was isolated as a 6,400-bp *Cla* I–*Bgl* II fragment. The expression vector is derived from the plasmid p342E (27), which was provided by A. Levinson. Hepatitis B surface antigen-encoding sequences were removed by digestion with *Eco*RI and *Bam*HI, and the *Eco*RI site was filled in by using deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I to create a blunt end. The ligation mixture containing the vector and the two MuIFN-γ gene fragments was transformed into *E. coli* 294, and ampicillin-resistant colonies were selected. The plasmid pSVEmuγ was isolated, which contained the three fragments in the proper configuration as shown by restriction endonuclease analysis.

Plasmid DNA was prepared and used to transfect COS-1 cells (originally provided by Y. Gluzman; ref. 28) by a dextran sulfate

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Abbreviations: IFN, interferon; MuIFN, murine IFN; HuIFN, human IFN; IFN-α, -β, and -γ, leukocyte, fibroblast, and immune IFN, respectively; bp, base pair(s); SV40, simian virus 40.

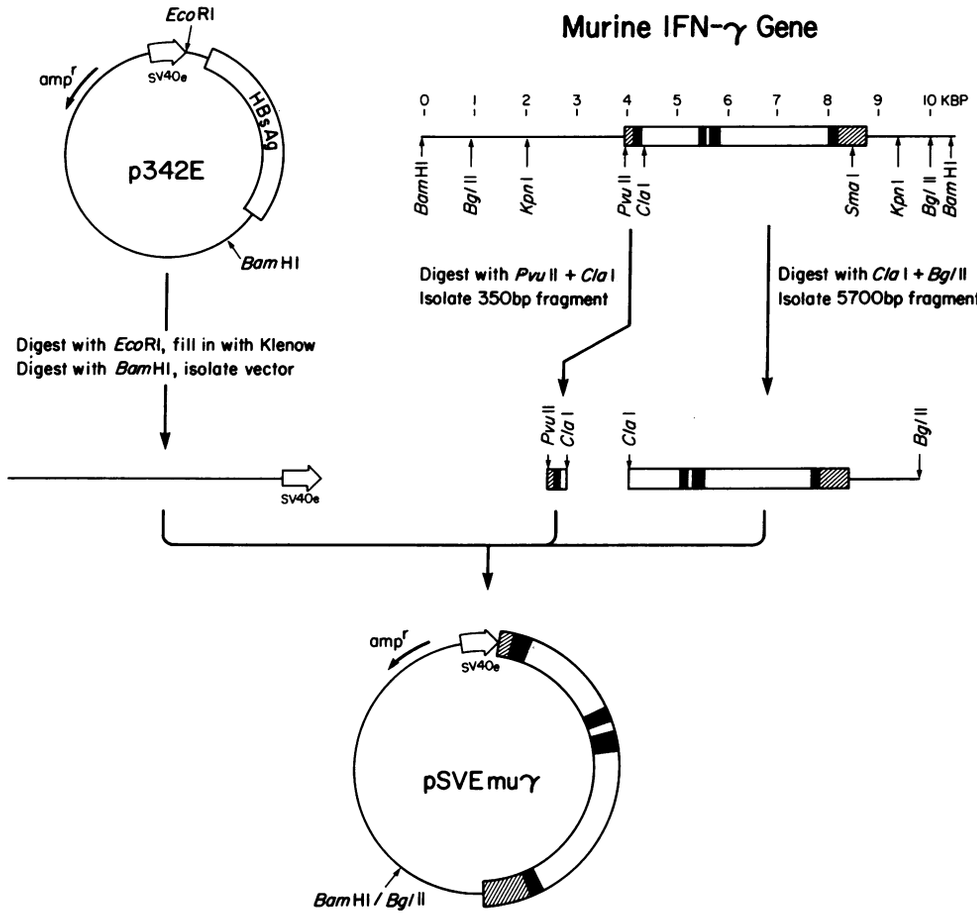

FIG. 1. Construction of the MuIFN-γ expression vector for COS-1 cells. The MuIFN-γ gene is presented as a boxed structure; the solid portions represent the four exons, open boxes show introns, and hatched regions represent the 5′ and 3′ untranslated segments of the mRNA. The insert from pmg10.5 is shown in the 5′-to-3′ direction from left to right.

precipitation method. Medium was removed from COS cells 48 hr after transfection and assayed for antiviral activity in a cytopathic effect inhibition assay (19) with encephalomyocarditis virus as a challenge virus and murine L929 cells as the target. The assay was standardized against MuIFN-α and MuIFN-β of known activity (Lee Biomolecular Laboratories, San Diego, CA).

Isolation of MuIFN-γ cDNA Clones from Transfected COS-1 Cells and Murine Spleens. RNA was isolated by the method of Berger and Birkenmeier (29) from COS-1 cells 48 hr after transfection with the MuIFN-γ expression plasmid pSVEmuγ or from concanavalin A-induced (30) spleen cells from CD1 mice after a 24-hr induction. The purified RNAs were passed over oligo(dT)-cellulose to enrich for mRNA. Double-stranded cDNA was prepared from 5 μg of each mRNA and cloned in *E. coli* by standard procedures (31). Transformants were screened by using the 3′ end of the MuIFN-γ gene as a probe (800-bp *Pst* I fragment containing the entire fourth exon).

Expression of MuIFN-γ cDNA in *E. coli*. A MuIFN-γ cDNA (pmc14) was tailored for expression in *E. coli* by the primer extension method, previously described for the expression of HuIFN-β (32). An *Eco*RI fragment (712 bp; 5 μg) of pmc14 was denatured by heat with 200 pmol of the 5′ ^{32}P-labeled synthetic octadecanucleotide d(A-T-G-T-G-T-T-A-C-T-G-C-C-A-C-G-G-C) (provided by M. Vasser). This primer was extended in a reaction containing 50 mM NaCl, 6 mM Tris·HCl (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 10 units of the Klenow fragment of *E. coli* DNA polymerase I, and deoxynucleoside triphosphates, each at 0.5 mM (32). The reaction proceeded at 37°C for 4 hr and then was extracted with phenol/chloroform, and the DNA was ethanol precipitated. The DNA was digested with *Pst* I (New England BioLabs) and subjected to electrophoresis through a 5% polyacrylamide gel. The primer extension product of 145 bp was identified by autoradiography and isolated by electroelution. This DNA was ligated to a vector used to express IFN-αD (33); the vector, pLeIFDtrp11, was digested with *Xba* I, filled in with deoxynucleotides and Klenow polymerase, digested with *Pst* I, and subsequently isolated by gel electrophoresis. The ligation reaction was transformed into *E. coli* 294, and a tetracycline-resistant colony, pmγtrp1, was identified as having the proper construction by restriction endonuclease mapping and DNA sequence determination. The 644-bp *Pst* I fragment from pmc14, containing sequences coding for the 3′ end of the cDNA, was ligated into the plasmid pmγtrp1. The resulting *E. coli* expression plasmid, pmγtrp6, was characterized by sequence analysis of the MuIFN-γ coding region. *E. coli* containing plasmid pmγtrp6 were grown in M9 media to an OD_{550} of 1.0, harvested by centrifugation, resuspended in phosphate-buffered saline, and sonicated. This extract was diluted and assayed for antiviral activity.

RESULTS

The HuIFN-γ cDNA (8) was used as a hybridization probe to screen a recombinant murine-λ phage library for the MuIFN-γ gene. By utilizing low-stringency conditions of hybridization and washing, several hybridizing signals were observed on duplicate filters. Corresponding phage were plaque-purified, and

phage DNA was prepared. The DNA of phage λMG9 contained a 10.5-kbp *Bam*HI fragment that hybridized to both 5′ and 3′ probes of the HuIFN-γ cDNA (Southern analysis not shown). The 10.5-kbp *Bam*HI fragment was subcloned in pBR322 and was characterized by DNA sequence analysis. The sequence revealed a 6,000-bp stretch of DNA having high homology (60%) to the HuIFN-γ gene. (This DNA sequence will be published elsewhere.)

A schematic representation of the MuIFN-γ gene structure is seen in Fig. 1. The MuIFN-γ gene has a structure similar to the HuIFN-γ gene (9), containing four exons and three introns. The potential coding regions of the MuIFN-γ gene were determined by homology comparisons with the human gene. The predicted protein sequence was confirmed by sequence analysis of the cDNA, which is presented below. Southern analysis of mouse genomic DNA suggests that there is a single MuIFN-γ gene per haploid genome (data not shown), as observed for the HuIFN-γ gene.

To confirm the identity of this DNA sequence as the MuIFN-γ gene, the DNA was engineered for expression, and the resulting protein was shown to have antiviral activity. As outlined in Fig. 1, a restriction fragment containing the putative 5′ coding portion (*Pvu* II–*Cla* I, 348 bp) and a fragment containing the remaining 3′ coding sequences (*Cla* I–*Bgl* II, 6,400 bp) were ligated to a SV40-based expression vector, p342E, which has been shown to efficiently synthesize hepatitis B surface antigen (27). The resulting MuIFN-γ expression plasmid was transfected into the monkey cell line COS-1 (28), which endogenously produces T antigen and allows replication of plasmids containing a SV40 origin of replication (34, 35). The transfected cells were grown for 2 days, and then the culture supernatant was assayed for antiviral activity by using a murine L929 cell assay challenged with encephalomyocarditis virus (19). Media from transfected COS-1 cells contained 6,000 units per ml of IFN activity, whereas media from nontransfected COS-1 cells contained no detectable activity.

The antiviral activity secreted from transfected COS-1 cells was identified as MuIFN-γ both antigenically and biochemically (Table 1). The activity was completely neutralized by a rabbit antibody preparation prepared against natural MuIFN-γ derived from mitogen-induced murine spleen cells (provided by H. M. Johnson and E. A. Havell). Furthermore, the IFN antiviral activity from transfected COS cells is heat labile (100% of activity destroyed at 65°C for 1 hr) and sensitive to pH 2; natural MuIFN-γ has these same properties (1–5). The recombinant MuIFN-γ has no measurable antiviral activity on human cells (HeLa) or bovine cells (MDBK).

The coding sequence of MuIFN-γ can be predicted from the total gene sequence by homology comparisons with the HuIFN-γ gene sequence (9). Intron–exon splice junctions appear to occur at positions homologous with the HuIFN-γ gene (9). To confirm the coding sequence of MuIFN-γ, we isolated and determined the sequence of MuIFN-γ cDNA clones derived from mRNA from pSVEmuγ transfected COS cells. In order to further show that this gene is expressed during mitogenic stimulation of lymphocytes, we also isolated a cDNA clone derived from mRNA isolated from concanavalin A-induced murine spleen cultures. The cDNA libraries (3,200 clones from transfected COS-1 cell mRNA and 6,000 clones from spleen mRNA) were independently screened with a ^{32}P-labeled probe prepared from the 3′ end of the gene. Seventeen of the COS-1-derived cDNA clones and a single spleen-derived clone hybridized with the probe. The chemical degradation method of Maxam and Gilbert (25) was used to determine the sequence of the cDNA inserts of one of the COS-1-derived clones, pmc14, and the spleen-derived clone, pms10. Both pmc14 and the shorter pms10 contained the entire coding region of the MuIFN-γ gene (Fig. 2).

The MuIFN-γ cDNA (pmc14) was tailored for expression in *E. coli* by the primer extension method (32) as described. *E. coli* 294 harboring the resulting MuIFN-γ expression plasmid pmγtrp6 produced ≈50,000 units per ml of bacterial culture in the murine antiviral assay. The characteristics of this activity are presented in Table 1. *E. coli*-derived MuIFN-γ has biological and biochemical properties that are similar to the activity derived from transfected COS-1 cells and induced murine spleen cultures.

Table 1. Characteristics of recombinant MuIFN-γ

Treatment*	MuIFN-γ activity remaining, %		
	Natural	COS	*E. coli*
Titer on murine L929 assay	100	100	100
Titer on human HeLa assay*	0	0	0
Titer on bovine MDBK assay*	0	0	0
55°C for 1 hr	67	50	60
65°C for 1 hr	0	0	5
pH 2	5	8	15
Rabbit anti-IFN-γ	0	0	0
Rabbit preimmune serum	100	100	100

*Assays were performed with encephalomyocarditis virus as a challenge virus on mouse L929 cells (31), except for the human and bovine assays. These assays utilized vesicular stomatitis virus on HeLa cells or MDBK cells, respectively (31).

DISCUSSION

The MuIFN-γ gene was identified from a murine–λ phage recombinant library by hybridization with a HuIFN-γ cDNA probe. The protein encoded by this gene has properties indistinguishable from natural MuIFN-γ. The recombinant MuIFN-γ is neutralized by antibodies specific for MuIFN-γ and is sensitive to heat and pH 2. Furthermore, the strict species specificity of antiviral activity is retained with the recombinant-DNA-derived material.

The structures of the MuIFN-γ gene and cDNA are very similar to those of HuIFN-γ. The overall nucleotide homology at the cDNA level is 64%. The area of highest homology is found in the 3′ untranslated regions, which are 68% homologous. The MuIFN-γ cDNA has a deletion of three nucleotides in the signal coding sequence (codon 19) and a deletion of three nucleotides in the mature coding sequence (codon 26) when compared with the HuIFN-γ cDNA (Fig. 3). The termination codon TGA follows residue 136. Consequently, the overall length of the mature MuIFN-γ is only 136 amino acids, 10 residues shorter than HuIFN-γ. The predicted smaller size of MuIFN-γ is consistent with a slightly greater mobility observed in $NaDodSO_4$/polyacrylamide gel electrophoresis of HuIFN-γ and MuIFN-γ synthesized in *E. coli* (results not shown). HuIFN-γ analogs have been prepared in *E. coli* by introducing termination codons in the carboxyl-terminal coding region (unpublished results). These shortened HuIFN-γs, with up to 11 residues removed, are very active in antiviral assays, suggesting that the carboxyl terminus is not required for this activity. In divergence from HuIFN-γ, the natural MuIFN-γ contains a deletion of the last nine amino acids. This also suggests that the carboxyl-terminal residues are unnecessary for antiviral activity.

A comparison of the protein sequences of HuIFN-γ and MuIFN-γ is presented in Fig. 3. The overall protein homology is only 40%, in contrast to the higher DNA homology. This low homology may explain the observed strict species specificity of IFN-γ (19). HuIFN-α has antiviral activity on a variety of other species (19, 33), including mouse, hamster, bovine, rabbit, and monkey cells. This is reflected in the higher protein homologies

FIG. 2. Nucleotide and encoded amino acid sequence of the cDNA for MuIFN-γ. The entire sequence of pmc14 is shown; pms10 begins at nucleotide 20 (marked with an asterisk). The putative signal peptide residues are shown in lower case (S1–S19). Numbers above each line refer to amino acid position and numbers below each line refer to nucleotide position.

of IFN-α. For example, HuIFN-αs are about 60% homologous with MuIFN-αs (20) at the amino acid level. MuIFN-α2 is quite active on human cells, whereas MuIFN-α1 is inactive (20). HuIFN-β, which is about 60% homologous with bovine IFN-β (unpublished results), has low activity on bovine cells (32).

MuIFN-γ has several structural features similar to HuIFN-γ. Both contain two potential *N*-glycosylation sequences (36), although at different positions (Fig. 3). Both proteins contain an excess of basic residues, although HuIFN-γ is more highly charged than MuIFN-γ: HuIFN-γ contains 31 basic residues and 19 acidic residues, whereas MuIFN-γ contains 22 basic residues and 15 acidic residues. The measured isoelectric point of natural MuIFN-γ is between pH 5.5 and 6.0 (4, 5); the discrepancy between this value and the predicted basic nature may be due to sialic acid residues added during glycosylation.

MuIFN-γ contains three cysteine residues at positions 1, 3, and 136. HuIFN-γ contains only two cysteines at positions 1 and 3 of the mature protein, which may be too close for the

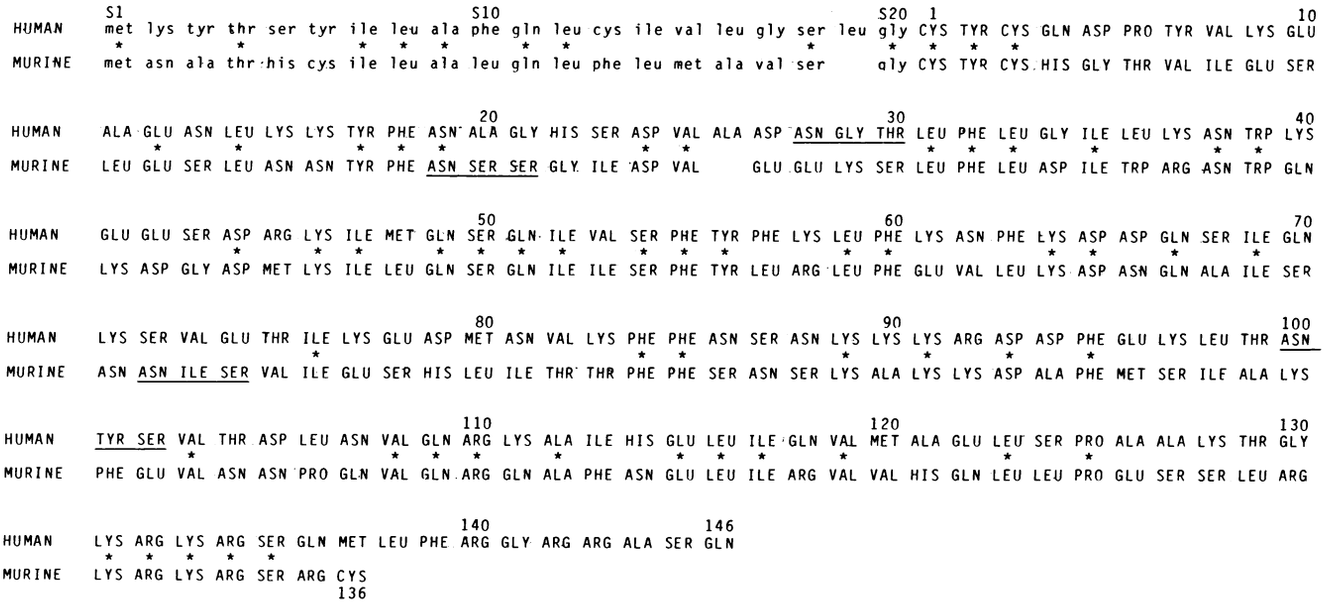

FIG. 3. Comparison of human (8) and murine IFN-γ protein sequences. Identical residues are shown with an asterisk. The signal sequences are numbered S1–S20 and are shown in lower case. Potential *N*-glycosylation sequences are underlined.

formation of a disulfide bond due to steric hindrance. The MuIFN-γ has the potential for an intramolecular disulfide bridge between either of the amino-terminal cysteine residues and position 136. Such a disulfide bond may confer added stability to the molecule, and, indeed, we have observed an increased temperature lability profile of antiviral activity when recombinant MuIFN-γ from COS-1 cells (melting temperature t_m = 55°C) and HuIFN-γ (t_m = 45°C) are compared (unpublished observations). Incubation of HuIFN-γ at 55°C for 1 hr totally destroys antiviral activity, but only 50% of MuIFN-γ is lost under these conditions. Other investigators have observed this increased thermostability of natural MuIFN-γ (5).

Estimated values of the size of natural MuIFN-γ range from M_r 38,000 to M_r 80,000 (2, 4, 5) and are based on molecular sieve chromatography. The encoded size of the mature protein based on the DNA sequence is only 15,894. This discrepancy between the observed and predicted sizes is probably due to glycosylation and aggregation. A similar situation was observed with HuIFN-γ. The cloned DNA sequence encoded a mature protein of 17,110 (8); however, gel filtration had suggested a M_r range of 40,000–70,000 (37). Two polypeptides containing antiviral activity have been eluted from $NaDodSO_4$/polyacrylamide gels of highly purified natural human IFN-γ at M_rs 20,000 and 25,000 (38). These two forms may differ in their degree of glycosylation and may aggregate to form the observed higher M_r species.

Availability of recombinant MuIFN-γ will greatly aid the biological studies of IFN-γ action. Besides having potent antiviral activity, this molecule may have anticellular activity against transformed cell lines. Furthermore, MuIFN-γ may stimulate the immune system; it appears to activate natural killer cell activity (39) and to induce Ia antigen synthesis (40). These studies were performed with material from mitogen-induced lymphocyte cultures that were not purified to homogeneity; other lymphokines may contaminate these preparations and affect the observed activities attributed to MuIFN-γ. The recombinant MuIFN-γ should clarify these results and also provide sufficient material for *in vivo* studies.

The authors thank Pamela Sherwood and Julie Jarrett for excellent technical assistance, Dr. Christian Simonsen for performing COS-1 cell transfections, Drs. H. M. Johnson and E. A. Havell for antibodies prepared against natural MuIFN-γ, and Dr. Sang He Lee for concanavalin A-induced murine spleen cultures and IFN assays.

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