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Cloning and expression of the chromosomal immune interferon gene of the rat

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The chromosomal immune interferon gene of the rat (IFN-γ) was identified by screening a recombinant rat λ phage library with a human IFN-γ cDNA probe. In contrast to the genes of other rat IFNs, this rat IFN-γ chromosomal gene contains introns and its structural organization closely resembles that of the human and murine IFN-γ genes. The rat IFN-γ gene encodes a signal sequence of 19 amino acids followed by the mature IFN-γ protein of 137 amino acids. The gene was expressed under control of the simian virus 40 (SV40) early promoter in Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase (DHFR) after co-transformation with a plasmid containing the mouse DHFR gene. Initial transformants with a DHFR$^+$ phenotype produced IFN-γ titres ranging from 20 to 1600 units/ml. After stepwise increases in the concentration of methotrexate (MTX) in the growth medium of transformed CHO cells, MTX-resistant clones producing 80 000 – 100 000 units per ml were isolated. Protein analysis of supernatants of these MTX-resistant cells by polyacrylamide gel electrophoresis revealed a product with an apparent mol. wt. of 18 000 daltons which was not detectable in the growth medium of DHFR$^+$ transformants that did not produce IFN. The product was identified as rat IFN-γ and constituted ~5% of the proteins excreted from these cells.

Key words: rat immune interferon/gene library/DNA sequence/transient expression/co-transformation

Introduction

Based on antigenic properties, three types of interferons (IFNs) can be distinguished: IFN-α, IFN-β and IFN-γ (Stewart *et al.*, 1980; Havell *et al.*, 1975). These also differ in a number of biological and physicochemical characteristics. IFN-γ can be induced in cultured lymphocytes or mononuclear leucocytes isolated from peripheral blood by stimulation with antigens or mitogens (Wheelock, 1965; Perussia *et al.*, 1980; Dianzani *et al.*, 1979; Green *et al.*, 1969). In contrast to both IFN-α and IFN-β, it is not induced by viruses. IFN-γ has been shown to have a more potent anticellular activity (Blalock *et al.*, 1980; Crane *et al.*, 1978) and can activate natural killer cells to a greater extent than do other IFNs (Claeys *et al.*, 1982). Furthermore, the antiviral and antitumor effects of virally induced (α- and β-) IFNs can be potentiated by IFN-γ (Fleischmann *et al.*, 1979, 1980). There is also evidence suggesting different mechanisms of action for IFN-γ as compared with IFN-α and -β, such as difference in affinities for gangliosides (Ankel *et al.*, 1980), membrane receptors (Branca and Baglioni, 1981), induction profiles of intracellular polypeptides (Weil *et al.*, 1983) and effects on HLA antigens (Wallach *et al.*, 1982).

Initial efforts to define the range of actions of IFN-γ were hampered by the lack of sufficient purified material. Therefore, it was necessary to use crude preparations which contained many other lymphokines and factors affecting cell functions. Only recently has extensive purification of IFN-γ been achieved, resulting in a more precise determination of the molecular size and revealing the glycosylated nature of the protein (Yip *et al.*, 1982).

An alternative approach to elucidate the functions of IFN-γ is the molecular cloning of the gene and its expression in suitable host-vector systems leading to IFN-γ preparations free of other lymphokines (Gray *et al.*, 1982; Gray and Goeddel, 1982; Devos *et al.*, 1982; Taya *et al.*, 1982; Haynes and Weissmann, 1983; Scahill *et al.*, 1983). Whereas IFN-α and IFN-β exhibit some antiviral activity against cell lines derived from different species (Stewart, 1979), IFN-γ has a more restricted species specificity. So, human IFN-γ cannot be tested in rats or mice, but only in higher primates. Recombinant DNA-derived rat IFN-γ enables the evaluation of existing antiviral and antitumor models of the rat which may be extrapolated towards the clinical application of human IFN-γ.

Here, we describe the identification and characterization of the chromosomal rat IFN-γ gene and the synthesis of rat IFN-γ by cultured mammalian cells after transfection or co-transformation with the genetically engineered rat IFN-γ gene under the control of viral promoters.

Results

The human IFN-γ cDNA was used as a hybridization probe to screen a recombinant λ Charon 4A/rat genomic library. Under low stringency conditions for hybridization and washing, two individual hybridizing signals were observed. We designated these as phages λRIF-γ_1 and λRIF-γ_2. The phages were plaque-purified and characterized by Southern blotting, using 5′ and 3′ parts of the human IFN-γ cDNA as a probe. Treatment of the DNA of phage λRIF-γ_1 with *Eco*RI resulted in a 5.5-kb fragment hybridizing with the 5′ probe and a 2.1-kb fragment hybridizing with the 3′ probe. The DNA of phage λRIF-γ_2 only contained the 5.5-kb *Eco*RI fragment that hybridized with the 5′ human IFN-γ cDNA probe. Otherwise, the rat genomic DNA of both phages proved to be identical. The IFN gene present in the λRIF-$\gamma_1$was subcloned as a 5.5-kb *Pst*-1 fragment in the unique *Pst*I site of pBR322 and characterized by DNA sequence analysis. Figure 1 gives the schematic representation of the rat IFN-γ gene structure and the strategy followed for DNA sequencing of the four exon regions. Figure 2 shows the total nucleotide sequence of the coding regions of the rat IFN-γ gene and its flanking regions. Because of the high DNA sequence homology with the published murine IFN-γ cDNA clone (Gray and Goeddel, 1983), we were able to identify with some certainty the promoter-related sequences, the putative start site(s) of mRNA initiation, the ATG translation initiation codon and the signal sequence cleavage sites. This extrapolation results in a rat IFN-γ protein sequence containing a 19 amino acid long signal sequence and a 137 amino

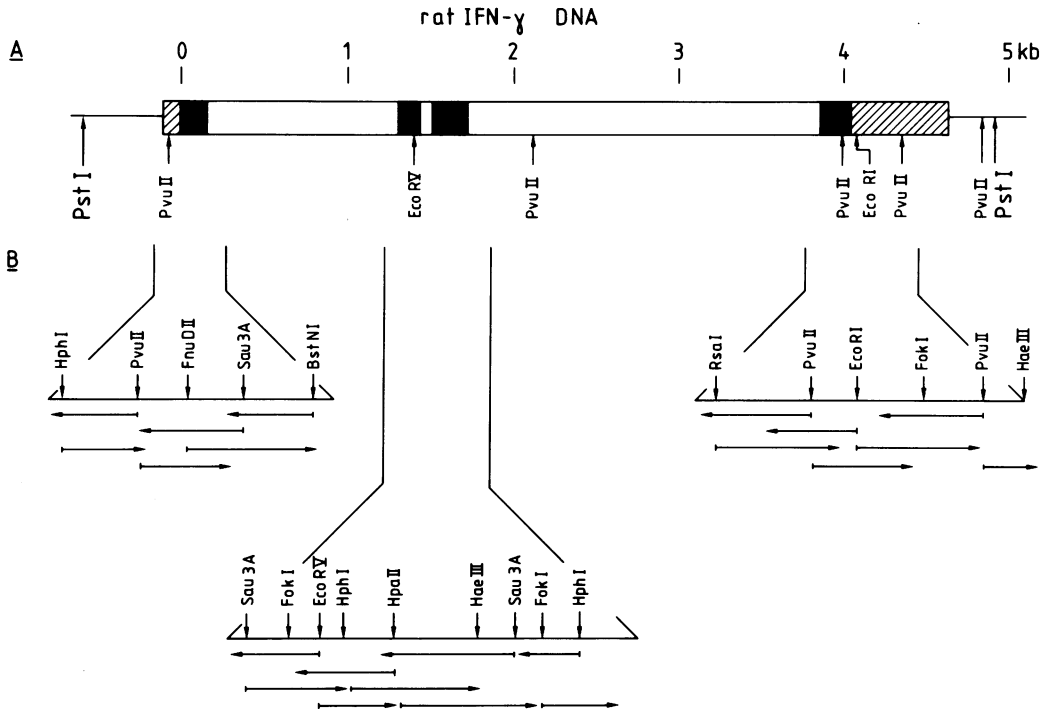

Fig. 1. Structural organization and restriction endonuclease map of the chromosomal IFN-γ gene of the rat as determined by DNA sequence comparison with the murine IFN-γ cDNA (**A**). The 5′- and 3′-untranslated regions of the rat IFN-γ gene are shown as hatched boxes, coding regions (exons) as solid boxes and intervening non-coding regions (introns) as open boxes. The DNA sequence strategy was performed as outlined (**B**). Each sequence was determined from the marked position and sequenced in the direction of the arrow; the length of the sequence determined corresponds to the length of the arrow.

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GTCACAAACCATAGCTATAATGCAAAGTAACTAGCTCCCGCCACCTATCTTTCACCATCTTAACTTAAAAAAAAAACCTGTGAAAATACGTAATCCCAAG

AAGCCTTCGGTCATGTATAAAACTGGAAGCAAGAGAGGTGCAGCGTATAGCTGCCATCGGCTGATCTAGAGAAGACACATCAGCTGATTCTTCGGACTC

                                                    Met Ser Ala Thr Arg Arg Val Leu Val Leu Gln
TCTGACTTAATACAGGAGTTCTGGGCTTTCCCTGCCTTGGCCTAGCTCTGAGACA ATG AGT GCT ACA CGC CGC GTC TTG GTT TTG CAG

Leu Cys Leu Met Ala Leu Ser Gly Cys Tyr Cys Gln Gly Thr Leu Ile Glu Ser Leu Glu Ser Leu Lys Asn Tyr
CTC TGC CTC ATG GCC CTC TCT GGC TGT TAC TGC CAA GGC ACA CTC ATT GAA AGC CTA GAA AGT CTG AAG AAC TAT

Phe
TTT GTAAGTATGATCTTTTCATAGTGCCTGTGGTTGACGGTGGCTGGTGTTGACTCCCTGTAGTGAACGCTAGACTGCCATCTCTGGCCCACAGTCAT

                                                                                          Asn Ser
TTTG-----(INTRON1-1150bp)-----CAGATATTTTCAGGGCAGTTTGGTGAAATAATTACAAATCGATCTTTTCTCTTCTCCTCAG AAC TCA

Ser Ser Met Asp Ala Met Glu Gly Lys Ser Leu Leu Leu Asp Ile Trp Arg Asn Trp Gln Lys
AGT AGC ATG GAT GCT ATG GAA GGA AAG AGC CTC CTC TTG GAT ATC TGG AGG AAC TGG CAA AAG GTGAGCTGAATATCC

                                  -----(INTRON2-103bp)-----                               Asp Gly
CCCCCAACACACTCCCCTGCCTCCCCTGCTTTCCTGTTGTTTCTAATGAACCGGTTCTCACAATACTCTCTTTGTGTTGTTTCCCAAG    GAC GGT

Asn Thr Lys Ile Leu Glu Ser Gln Ile Ile Ser Phe Tyr Leu Arg Leu Phe Glu Val Leu Lys Asp Asn Gln Ala
AAC ACG AAA ATA CTT GAG AGC CAG ATT ATC TCT TTC TAC CTC AGA CTC TTT GAA GTC TTG AAA GAC AAC CAG GCC

Ile Ser Asn Asn Ile Ser Val Ile Glu Ser His Leu Ile Thr Asn Phe Phe Ser Asn Ser Lys Ala Lys Lys Asp
ATC AGC AAC AAC ATA AGT GTC ATC GAA TCG CAC CTG ATC ACT AAC TTC TTC AGC AAC AGT AAA GCA AAA AAG GAT

Ala Phe Met Ser Ile Ala Lys Phe Glu
GCA TTC ATG AGC ATC GCC AAG TTC GAG GTGAGACAGCTTTGCAAACTACCGTATTATTGTTTGTTTCACATTGTCTTTGAATTATCAGAC

AGTAGAAATTAGCTACTCATCAGTTGATAAAGCTGAGAGATGTTTCCCCACCGCAGGCAGATTGGGAGGAATCTGCCTTTTTTTTTTG----------

-----(intron3-2150bp)-----ATTGCTGTACTACTTTGTTAAGAGGAATATTTTCATTTTCACTGACCATGATGTCAAGAAGAATAGTCCAATG

                                                    Val Asn Asn Pro Gln Ile Gln His Lys Ala Val Asn Glu
ACTTATATGCTTGGAATTAATTTCATTTCCCTCCCCACTCCATTAG  GTG AAC AAC CCA CAG ATC CAG CAC AAA GCT GTC AAT GAA

Leu Ile Arg Val Ile His Gln Leu Ser Pro Glu Ser Ser Leu Arg Lys Arg Lys Arg Ser Arg Cys ***
CTC ATC AGA GTG ATT CAC CAG CTG TCA CCA GAA TCT AGC CTA AGG AAG CGG AAA AGG AGT CGG TGC TGA TTCTGGG

GTAGAGAGTGTGCCAATAAGAAGAATTCTGCCAGCACTATTTGAATTTTAAAACTAAACCTATTTATTAATATTTAAATTTATTTATATGGAGAATATA

TTTTAGACTCATCAACCAAAGAAGTATTTATAGTAACAACTTATATGTGATAAGGATGAATTTCTATTAATATATGTGTTATTTATAATCTCTGTGTCC

TTAATTATTCCTCTTTGACCAATCATTCTTTCTGACTAATTAACCCAGACTGTGATTATGAAGTTGTATCTGGGGTGGGGGGACAGCCAACCAGCTGAC

TGAACTCACACTGTGGCTTGTGCACTTACTTCACTTGCCAACGGGGAACATTCAGAACTGCAATGACCCCGTGAGGTGCTGCTGACCAGAGGAATGTCT

ATACATCGGCC
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Fig. 2. Partial nucleotide sequence of the chromosomal IFN-γ gene of the rat. Indicated are the putative mRNA start site (indicated by an asterisk) and the signal peptide cleavage site (indicated by a vertical arrow).

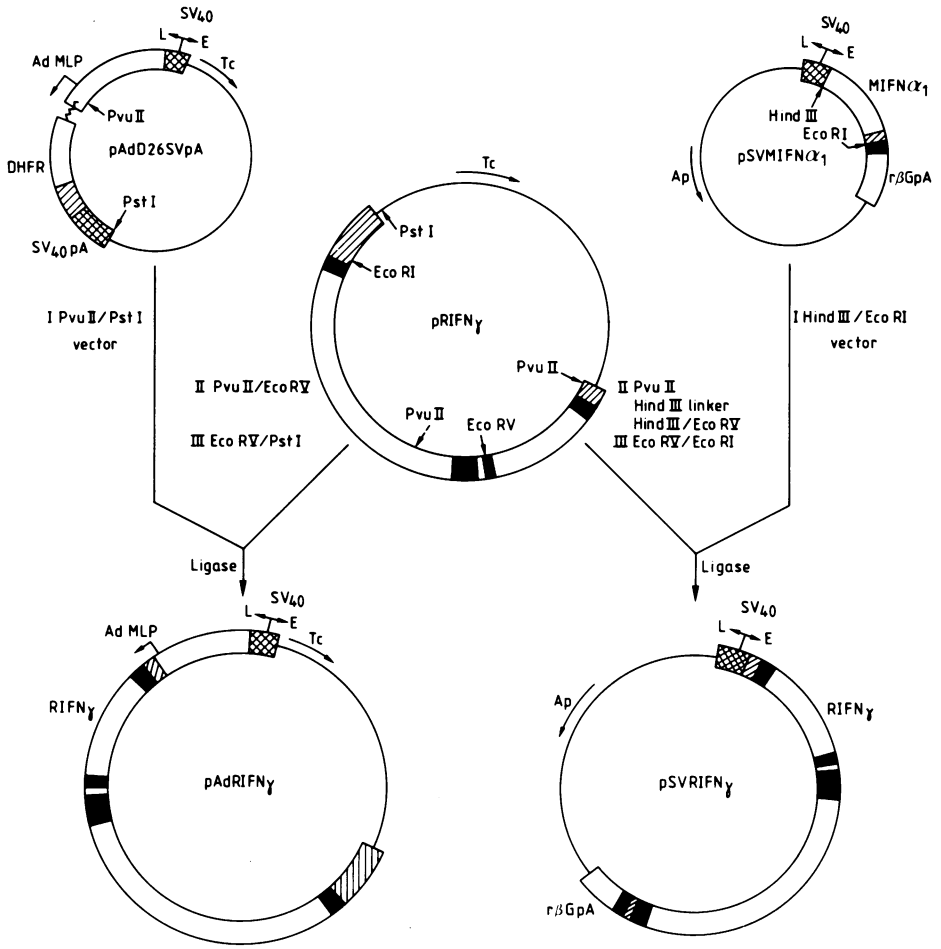

Fig. 3. Construction of viral-based rat IFN-γ expression vectors. The constructions are described in Materials and methods. Only the relevant *Pvu*II sites of pRIFN-γ are indicated. Ap and Tc stands for ampicillin and tetracycline resistance, respectively; AdMLP, adenovirus 2 major late promoter; SV40, simian virus 40 origin/promoter region.

acid long mature protein (Figure 2). To prove the identity of this DNA sequence as the rat IFN-γ, we expressed the genetic information in mammalian cells to see whether the resulting protein had biological activities characteristic for rat IFN-γ. As outlined in Figure 3 and in Materials and methods, the rat IFN-γ gene was inserted into an adenovirus major late promoter- and a SV40 early promoter-based expression vector. The resulting expression vectors (pAdRIFN-γ and pSVRIFN-γ, respectively) were transfected into the monkey cell lines COS-1 and COS-7, which both endogenously express the SV40 T antigen necessary for replication of such SV40 origin-based hybrid vectors (Gluzman, 1981). Transfected cells were grown for 10 days and the culture medium was assayed for antiviral activity on a continuous rat cell line (Ratec). Interferon activity could be detected in the supernatants. Titers reached a maximum of 400 units per ml for pAdRIFN-γ and 1600 units per ml for pSVRIFN-γ at 48 h after transfection. Co-transformation of CHO cells with pAdRIFN-γ and the *Escherichia coli gpt* gene-containing vector pEV_1gptH^+ (provided by Dr. J.Hoeijmakers) as well as co-transformation of $DHFR^-$ cells with pSVRIFN-γ and the modular murine DHFR gene-containing vector pAdD26SVpA-3 (Scahill *et al.*, 1983) resulted in cells secreting interferon activity into their culture medium. Co-transformants with the *gpt* gene were found to produce 100 – 200 units/ml, whereas co-transformants with the murine DHFR gene yielded titers ranging from 200 to 1600 units/ml. One such cell line transformed with pSVRIFN-γ and pAdD26SVpA-3 produced a stable interferon level of 1600 U/ml up to the 15th passage. This initial transformed cell line was grown in monolayer culture and passaged in increasing concentrations of methotrexate (MTX). Cells were initially suspended in 10^{-8} M MTX and clones growing at this concentration were picked and maintained independently in culture. MTX selection was continued with these low resistant sublines by increasing the MTX concentration to 400 nM over a 3 month period. Two cell lines resistant to 400 nM MTX were isolated and grown to confluency. They were found to produce ~100 000 units IFN per ml per day. The activity was constantly excreted into the culture medium for at least 6 days, provided that the medium was changed every 24 h.

Southern blot analysis of total cellular DNA from these cells showed an ~200-fold increase in the number of copies of the IFN-γ gene in comparison with the initial transformed cell line producing 1600 units per ml per day.

To characterize the IFN produced, crude preparations were concentrated and partially purified by adsorption/desorption on controlled pore glass (CPG) beads (DeLey *et al.*, 1980). The beads retained 96% of the activity and it was possible to elute >90% of the total applied activity with 0.5 M tetramethyl ammonium chloride or 50% ethyleneglycol in the presence of 1.4 M sodium chloride. The degree of purification achieved by this method was determined by analyzing the proteins by SDS-polyacrylamide gel electrophoresis (see Figure 5). Such an

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          1                                  10                                  20
HUMAN     CYS TYR CYS GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN
           *   *   *   *                       *       *   *       *   *   *                   *
RAT       CYS TYR CYS GLN GLY THR LEU ILE GLU SER LEU GLU SER LEU LYS ASN TYR PHE ASN SER SER SER MET ASP ALA MET GLU GLY
           *   *   *       *   *       *   *   *   *   *   *   *       *   *   *   *   *   *           *           *
MURINE    CYS TYR CYS HIS GLY THR VAL ILE GLU SER LEU GLU SER LEU ASN ASN TYR PHE ASN SER SER GLY ILE ASP VAL     GLU GLU

               30                                  40                                  50
HUMAN     GLY THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER GLN ILE VAL SER PHE TYR
               *       *       *           *   *                       *   *           *   *   *       *   *   *
RAT       LYS SER LEU LEU LEU ASP ILE TRP ARG ASN TRP GLN LYS ASP GLY ASN THR LYS ILE LEU GLU SER GLN ILE ILE SER PHE TYR
           *   *   *       *   *   *   *   *   *   *   *   *   *           *   *   *       *   *   *   *   *   *   *
MURINE    LYS SER LEU PHE LEU ASP ILE TRP ARG ASN TRP GLN LYS ASP GLY ASP MET LYS ILE LEU GLN SER GLN ILE ILE SER PHE TYR

                   60                                  70                                  80
HUMAN     PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE
               *   *               *   *       *       *                   *                                       *
RAT       LEU ARG LEU PHE GLU VAL LEU LYS ASP ASN GLN ALA ILE SER ASN ASN ILE SER VAL ILE GLU SER HIS LEU ILE THR ASN PHE
           *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *       *
MURINE    LEU ARG LEU PHE GLU VAL LEU LYS ASP ASN GLN ALA ILE SER ASN ASN ILE SER VAL ILE GLU SER HIS LEU ILE THR THR PHE

                       90                                  100                                 110
HUMAN     PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA
           *               *       *       *       *                   *                               *       *   *
RAT       PHE SER ASN SER LYS ALA LYS LYS ASP ALA PHE MET SER ILE ALA LYS PHE GLU VAL ASN ASN PRO GLN ILE GLN HIS LYS ALA
           *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
MURINE    PHE SER ASN SER LYS ALA LYS LYS ASP ALA PHE MET SER ILE ALA LYS PHE GLU VAL ASN ASN PRO GLN VAL GLN ARG GLN ALA

                               120                                 130                                 140
HUMAN     ILE HIS GLU LEU ILE GLN VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER GLN MET LEU PHE ARG
               *   *   *       *                   *   *   *                   *   *   *   *   *
RAT       VAL ASN GLU LEU ILE ARG VAL ILE HIS GLN LEU SER PRO GLU SER SER LEU ARG LYS ARG LYS ARG SER ARG CYS
               *   *   *   *   *       *   *   *       *   *   *   *   *   *   *   *   *   *   *   *   *
MURINE    PHE ASN GLU LEU ILE ARG VAL VAL HIS GLN LEU LEU PRO GLU SER SER LEU ARG LYS ARG LYS ARG SER ARG CYS

HUMAN     GLY ARG ARG ALA SER GLN
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Fig. 4. Comparison of human (**upper**), rat (**middle**) and murine (**lower**) IFN-γ mature protein sequences. Identical residues are indicated with an asterisk. Potential N-glycosylation sequences (asn-X-thr and asn-X-ser) are underlined.

analysis revealed that the eluate from the CPG beads gave a major band expected for IFN-γ at 18 K. This band was absent when the medium of a transformed non-producing CHO cell clone was used. In the absence of 2-mercaptoethanol in the sample buffer some additional minor bands with higher mol. wts. (37, 55 and 74 K) became visible in the gel pattern of the high-producing cell line (not shown). Obviously, IFN-γ can exist in multimeric forms which are converted to the monomeric form in the presence of a reducing agent.

To study the species specificity of this IFN, the spectrum of antiviral activity in different mammalian cell lines was compared. It was found that the factor was unable to induce an antiviral state in human and hamster cells (HEP-2, Amnion, CHO, BHK-B1 and BHK-21). On the other hand it was highly active on rat and mouse cells. In L929 cells challenged with vesicular stomatitis virus (VSV), the antiviral activity was 1.3 times lower on average than in Ratec cells infected with VSV. Additional evidence for the identification of the antiviral activity as being the result of rat IFN-γ was obtained by experiments that showed a synergistic enhancement of the antiviral activity of natural and recombinant DNA derived rat IFN α/β. The potentiation factors (determined by dividing the observed with the expected titers) were 11 and 4, respectively. As reported for the murine system (Fleischmann *et al.*, 1980), such a demonstration of synergism is only found between combinations of IFN α/β with IFN-γ and not in mixtures of IFN-α and IFN-β.

A major characteristic of immune interferon is its susceptibility ty to low pH. Figure 6 shows the sensitivity of CHO cell-derived interferon to different hydrogen ion concentrations during a 20 h period at 4°C. The IFN preparation lost >90% of its activity at pH values as low as 3. Also, the heat stability of the interferon was tested. After an incubation of 2 min at temperatures varying from 37 to 85°C, a T_i^{50} (temperature at which 50% of the biological activity is lost) of 66°C was found, indicating that this type of IFN is rather heat stable. All of these data strongly suggested that we were dealing with rat gamma interferon.

Discussion

We have identified the chromosomal rat IFN-γ gene by screening a recombinant rat λ phage library with a human IFN-γ cDNA probe. Under similar conditions of hybridization and washing, no signals in chromosomal rat liver DNA were observed in Southern blots with this probe, presumably because of different copy numbers of the gene present in a phage plaque in comparison with a chromosomal DNA blot. Extensive screening of the rat DNA library cloned in the λ Charon 4A phage revealed only two positive phages, which were shown to be derived from the same chromosomal DNA segment by DNA restriction enzyme analysis. This number of positive phages is what one would ex-

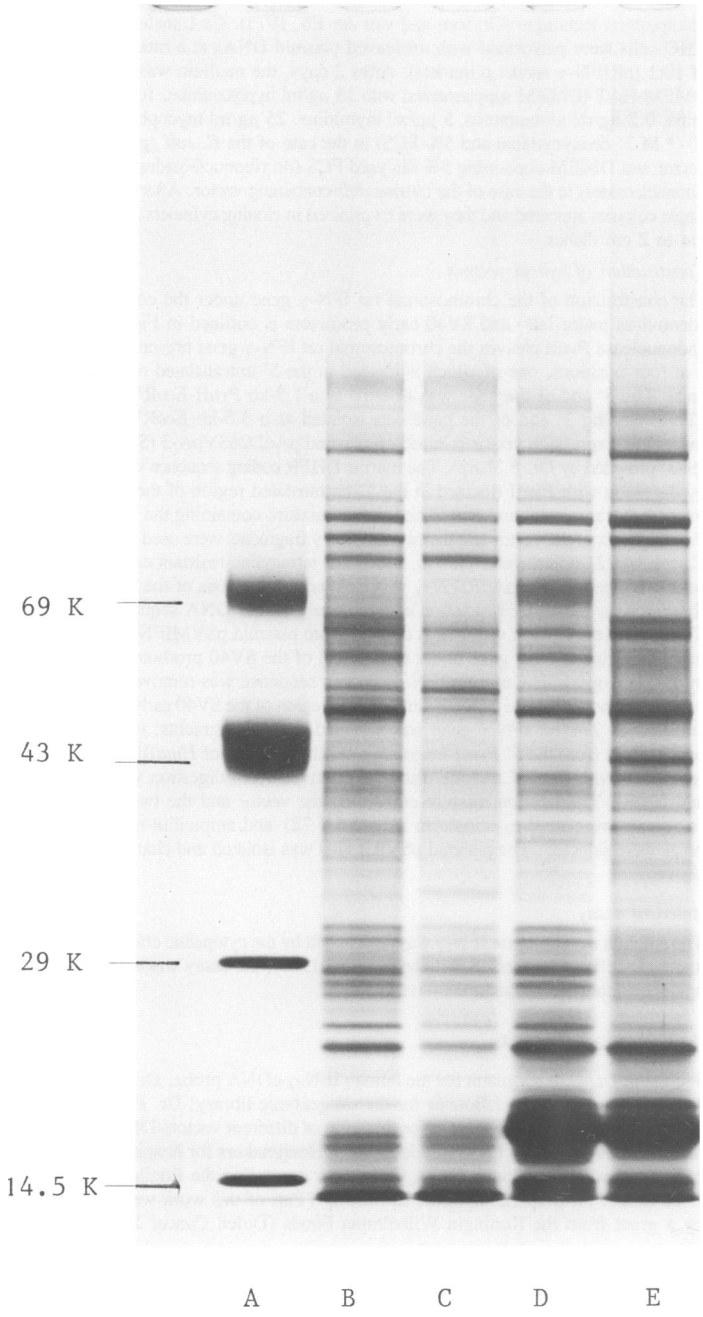

Fig. 5. SDS-polyacrylamide gel electrophoresis of partially purified rat IFN-γ in a 5–30% gradient gel. Approximately 250 μg of protein was placed in each slot and subjected to electrophoresis. As mol. wt. standards, we used (from top to bottom) bovine serum albumin (69 K), ovalbumin (43 K), carboxyanhydrase (29 K) and lysozyme (14.5 K). The gel was run as described by Laemmli (1970) and stained with Coomassie brilliant blue. **Lane A**, marker proteins; **lane B**, 40–85% (saturation) ammonium sulfate pellet fraction of a non-producing (<20 U/ml) CHO cell line; **lane C**, 40–85% (saturation) ammonium sulfate pellet fraction of a low-producing (~1200 U/ml) CHO cell line; **lane D**, 40–85% (saturation) ammonium sulfate pellet fraction of a high-producing (~100 000 U/ml) CHO cell line; **lane E**, CPG bound fraction of the same high-producing CHO cell line. In contrast to the first two cell lines, the high-producing clone was cultured in the presence of 100 nM methotrexate.

pect for a single copy gene (Dijkema *et al.*, 1984). The structural organization of the chromosomal rat IFN-γ gene is very similar to that of the murine (Gray and Goeddel, 1983) and human IFN-γ genes (Gray and Goeddel, 1982; Taya *et al.*, 1982). There are three intervening sequences (introns I and III are estimated

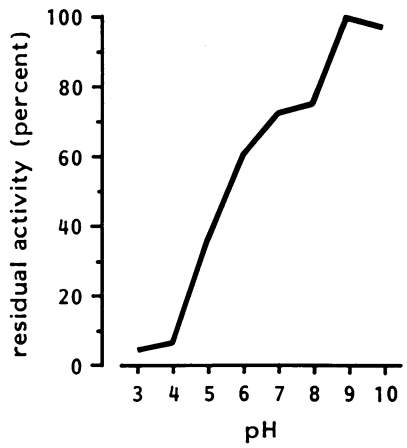

Fig. 6. The effect of the hydrogen ion concentration on the stability of rat IFN-γ. Crude IFN samples of 50 ml (culture fluid diluted 30 times in a 50 mM sodium bicarbonate buffer supplemented with 1% FCS and 6 mM β-mercaptoethanol) containing ~3000 antiviral units in tightly capped tubes were exposed at different pH values at 4°C for 20 h. The pH of the samples were brought to the indicated values by the addition of 1 M HCl or 1 M NaOH. After 30 and 60 min these values were checked and if necessary adjusted to the original value. After 20 h at 4°C the samples were directly assayed for interferon activity.

to be 1150 and 2150 bp, respectively; intron II is 103 bp), all having the consensus 5′ (GT.....AG) 3′ sequence (Breathnach *et al.*, 1978). Each intron interrupts the reading frame exactly between codons. The coding regions (i.e., the four exons) have a homology of 60% with the human IFN-γ gene and a 90% homology with the murine IFN-γ gene. Like the murine IFN-γ gene, the rat IFN-γ gene has three nucleotides less in the signal coding sequence (codon 19) than has human IFN-γ. However, rat IFN-γ has three nucleotides (codon 26) more than the murine IFN-γ gene. Consequently, the overall length of the mature rat IFN-γ is 137 amino acids *versus* 136 for murine IFN-γ. A comparison of the mature protein sequences of human, murine and rat IFN-γ is presented in Figure 4. The protein homology of rat IFN-γ is 39% as compared with human IFN-γ and 87% in comparison with murine IFN-γ. As was to be expected from the observed amino acid homology between rat and murine IFN-γ, we indeed found a strong cross species specificity of rat IFN-γ on mouse cells. Rat and murine IFN-γ also have three cysteine residues and two potential N-glycosylation sequences at identical positions.

Since IFN-γ produced by stimulated lymphocytes is glycosylated and IFN-γ contains introns and post-translational signal peptide cleavage, the rat IFN-γ gene was cloned in eucaryotic host-vector systems. Two hybrid vectors containing either the adenovirus major late (pAdRIFN-γ) or the SV40 early promoter (pSVRIFN-γ) were used for this purpose. There is a 4-fold difference in expression between the pSVRIFN-γ and pAdRIFN-γ (1600 units/ml and 400 units/ml, respectively) in transfected COS cells. We do not know whether this difference simply reflects a different promotor strength or whether the polyadenylation signal derived from the rabbit β-globin gene present in pSVRIFN-γ increases the expression of the rat IFN-γ gene over the authentic IFN-γ 3′ region present in pAdRIFN-γ.

To obtain constant expression, CHO cells were transformed with these hybrid vectors together with vectors bearing dominant selection markers. Transformants obtained with both combinations, pAdRIFN-γ-pEV1gptH$^+$ (*E. coli* XGPRT) and pSVRIFN-γ-pAdD26SVpA-3 (murine DHFR), produced IFN-

γ. Again, there was a difference in the SV40-directed IFN-γ synthesis *versus* adeno-directed IFN synthesis; perhaps, apart from the above mentioned explanations, there may be a selection marker-dependent integration. In addition to more efficient production, the advantage of using genetic engineered eucaryotic cells is the absence of other lymphokines which are abundantly present in natural preparations.

The number of rat IFN-γ genes integrated into the chromosomal DNA of the host cell can be amplified by drugs like methotrexate, which may lead to increasing yields of IFN-γ.

Initial transformants contained about three copies of the IFN-γ gene, but, after MTX selection up to 400 nM concentration, the number was increased to ~200 copies and the production of IFN-γ increased to 100 000 units/ml day. Clones of these cells synthesized a protein with a mol. wt. of 18 K which was absent in a non-producing CHO cell line. This polypeptide represented ~5% of the total excreted protein. The production of this protein in MTX-resistant cells represented an ~60-fold increase over the level in the initial MTX-sensitive transformants. To characterize the substance produced as interferon, the antiviral and anticellular activities of the preparation were determined in human, hamster, mouse and rat cells. The preparation exhibited antiviral and antiproliferative activity only against rat and mouse cells and was inactive in human and hamster cells. Also, the demonstration of a synergistic interaction between CHO cell-derived IFN and naturally derived rat IFN α/β and recombinant DNA derived rat IFN-α_1 is highly suggestive that the substance is indeed rat IFN-γ. Treatment at low pH resulted in a >90% loss of activity. In addition, the factor shows a remarkable stability at temperatures above 56°C. The latter phenomenon may indicate that rat IFN-γ is glycosylated. A major characteristic of a glycosyated IFN species is its remarkable stability at high temperatures (Grossberg, 1972). All of these biological and physicochemical characteristics are in good agreement with the properties reported for IFN-γ in general.

Materials and methods

Phage library screening

A λ Charon 4A phage recombinant library constructed by Sargent *et al.* (1979) containing a partial *Eco*RI digest of rat genomic DNA was used. About 750 000 phages were screened by the *in situ* phage hybridization procedure (Benton and Davis, 1977; Maniatis *et al.*, 1978) using the *Pst*I-excised insertion of a human IFN-γ cDNA clone as a probe (Haynes and Weissmann, 1983). A density of 75 000 phages/15 cm diameter plate was used after adsorption onto two sequentially placed nitrocellulose filters. Hybridizations were performed under conditions of low stringency in 25% formamide, 5 x SSC at 28°C and the filters were washed twice with 2 x SSC, 0.1% SDS at room temperature (Gray and Goeddel, 1983). Only duplicate spots were selected for further study. Two distinct plaques were picked out and plaque-purified. Recombinant phage DNA was prepared, digested with various restriction enzymes, subjected to electrophoresis on 0.6% horizontal agarose gels and analysed with Southern blotting by hybridization with (parts of) the human IFN-γ cDNA probe.

Subcloning

The *Pst*I fragment of hybrid phage λRIFN-γ_1 was subcloned into the single *Pst*I site of pBR322, resulting in pRIFN-γ.

Sequence analysis

DNA sequencing was performed according to the procedure described by Maxam and Gilbert (1977).

Cell culture

COS-1, COS-7 (Gluzman, 1981), and CHO cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 5 – 10% fetal calf serum (FCS); *dhfr*$^-$ CHO cells (Chasin and Urlaub, 1980) were grown in DMEM supplemented with 5% FCS and 10 mg/l of adenosine, deoxyadenosine and thymidine.

Transfection and co-transformation

Fresh monolayers of COS-1 and COS-7 cells in 9 cm diameter plates were transfected with 10 μg of uncleaved plasmid DNA by the calcium phosphate precipitation technique (Graham and van der Eb, 1973). Co-transformations of CHO cells were performed with uncleaved plasmid DNAs at a ratio (mol/mol) of 10:1 (pRIFN-γ *versus* p marker). After 2 days, the medium was changed to DMEM-HAT (DMEM supplemented with 15 μg/ml hypoxanthine, 10 μg/ml xanthine, 0.2 μg/ml aminopterin, 5 μg/ml thymidine, 25 μg/ml mycophenolic acid, 10^{-5} M 2′ deoxycytidine and 5% FCS) in the case of the *E. coli gpt*-containing vector and DMEM containing 5% dialysed FCS (no ribonucleosides and deoxyribonucleosides) in the case of the murine *dhfr*-containing vector. After ~2 weeks, single colonies appeared and they were trypsinized in cloning cylinders and transferred to 2 cm dishes.

Construction of hybrid vectors

The construction of the chromosomal rat IFN-γ gene under the control of the adenovirus major late- and SV40 early promoters is outlined in Figure 3. The endonuclease *Pvu*II cleaves the chromosomal rat IFN-γ gene present in pRIFN-γ at four positions, one of which is located in the 5′-untranslated region of the gene. The 5′ end of the gene was isolated as a 1.5-kb *Pvu*II-*Eco*RV fragment. The remaining 3′ end of the gene was isolated as a 3.5-kb *Eco*RV-*Pst*I fragment. The expression vector is based on plasmid pAdD26SVpA-3 (Scahill *et al.*, 1983; provided by Dr. P.Sharp). The murine DHFR coding sequence was removed by digestion with *Pvu*II (located in the 5′-untranslated region of the adenovirus major late transcripts) and *Pst*I. The ligation mixture containing the large *Pvu*II-*Pst*I fragment of the vector and the two rat IFN-γ fragments were used to transform *E. coli* JA 221 (Clarke and Carbon, 1978) and tetracyline-resistant colonies were selected. The plasmid pAdRIFN-γ was isolated on the basis of the proper configuration as shown by restriction enzyme analysis and DNA sequencing. The SV40-based expression vector was derived from plasmid pSVMIFN-α_1 containing the murine IFN-α_1 gene under the control of the SV40 promoter (provided by Dr. E.Koper). The murine IFN-α_1 coding sequence was removed by digestion with *Hind*III (located in the 5′-untranslated region of the SV40 early transcripts) and *Eco*RI. The rat IFN-γ gene was supplied as two fragments; the proximal part derived from the 5′ *Pvu*II fragment after the addition of *Hind*III linkers and restriction with *Eco*RV and the distal part derived from digestion with *Eco*RV and *Eco*RI. The ligation mixture containing the vector and the two rat IFN-γ fragments were used to transform *E. coli* JA 221 and ampicillin-resistant colonies were selected. The plasmid pSVRIFN-γ was isolated and characterized as above.

Antiviral assay

The antiviral activity of rat IFN-γ was determined by the cytopathic effect reduction assay as earlier described (Schellekens *et al.*, 1980); the assay was standardized against a laboratory standard of rat IFN-γ.

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