STRUCTURE-FUNCTION ANALYSIS OF MURINE INTERFERON-ALPHA SUBSPECIES

STRUKTUUR-FUNKTIE ANALYSE VAN MUIZE INTERFERON-ALPHA SUBSPECIES

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ABBREVIATIONS

ATP	adenosine triphosphate
Bo	bovine
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CHO	chinese hamster ovary
CMV	cytomegaly virus
COS	CV-1, origin of replication, SV40
C-terminus	carboxyl terminus
CV-1	monkey kidney cell line
DNA	desoxyribonucleic acid
dsRNA	double-stranded RNA
E. coli	Escherichia coli
HIV	human immunodeficiency virus
HLA	human MHC antigen
HPLC	high performance liquid chromatography
HSP	heat shock protein
H-2	murine MHC antigen
Hu	human
IFN	interferon
IF-2α	initiation factor 2α
IRE	IFN regulatory element
IRS	IFN responsive sequence
ISG	IFN stimulated gene
kDa	kilo Dalton
MHC	major histocompatibility complex
mRNA	messenger RNA
MT	metallothionein
Mu	murine
NK	natural killer
N-terminus	amino terminus
PDGF	platelet-derived growth factor
PK	protein kinase
RNA	ribonucleic acid
SV40	simian virus 40
2-5A	2',5'-oligoadenylate synthetase
U/mg	units per milligram

1. INTRODUCTION

More than 30 years ago, Isaacs and Lindenmann (1957) discovered that cells, which had been exposed to heat-inactivated influenza virus, could confer resistance upon other cells against infection by the same or unrelated viruses. This resistance was transferred by a diffusable substance: a viral infection interfering agent or interferon (IFN). The conversion of a cell, after exposure to IFN, from a virus-susceptible to a resistant phenotype was shown to depend on de novo synthesis of mRNA and protein (Taylor, 1964). Some time after his discovery of IFN, Isaacs postulated that IFN could constitute a defence reaction of cells against intrusion by foreign (viral) nucleic acids (Isaacs, 1961, Isaacs et al, 1963). A few years later it was found that double-stranded (ds)RNA, in contrast to DNA, single-stranded RNA or DNA-RNA hybrids, was able to induce IFN synthesis (Field et al, 1967ab, Lampson et al, 1967, Tytell et al, 1967). This finding suggested that viral dsRNA, produced in the course of infection, was the IFN-inducing moiety of viruses. However, this hypothesis could not explain IFN induction by DNA viruses nor by various nonviral substances, like bacteria, endotoxin or certain mitogens or antigens. Until now no single hypothesis has been formulated to explain the different ways of IFN induction.

Before investigators became aware of the existence of IFN, they already had speculated about a general, first line defence mechanism against viral infection. IFN appeared to fulfil this characteristic, because it was produced locally and early after viral infection, did not act in a virus specific way and was not secreted by specialized cells. Thus, as a consequence of the action of IFN, a delay of viral spread will be obtained which enables the host to evoke an immune response against the virus. Direct proof of the importance of IFN for inhibition of viral infection became available after application of antibodies neutralizing its antiviral activity: viral infections developed more rapidly and resulted in a higher mortality (Gresser et al, 1976).

The discovery of the nonviral IFN inducers raised the question whether IFN possessed other properties besides antiviral activity. During the seventies it became established that IFN

was able to modulate certain aspects of the immune system. For example, IFN could activate macrophages (Schultz et al 1977) and the cytotoxic activity of natural killer (NK) cells (Trinchieri and Santoli, 1978, Herberman et al, 1979). Moreover, IFN was shown to inhibit cellular growth (Paucker et al, 1962, Knight, 1976, Gresser et al, 1979, Evinger et al, 1981a). These aspects of IFN activity led to speculations about IFN as a natural defence mechanism against tumor development and made IFN an attractive candidate for anticancer therapy. Recently, an additional function of IFN has been described. The signal generated from the early embryo to trigger maternal recognition of pregnancy was found to be an IFN (Imakawa et al, 1987, Pontzer et al, 1988).

All earlier studies on IFN were hampered by the lack of sufficient amounts of purified IFN. Only after considerable efforts methods for purification and large-scale production were established. Using purified IFN, it could finally be proven that all biological activities ascribed to IFN were indeed intrinsic properties of the protein.

Characterization of IFN produced by different cell types upon different induction protocols led to the discovery that IFN was heterogeneous, in the sense that some cell types secreted an acid stable and some an acid labile form. This observation led to the designation of type I (stable at pH 2) and type II (labile at pH 2) IFN. Subsequently, type I IFN was found to comprise two antigenically distinct species (Havell et al, 1975), called leukocyte and fibroblast IFN because they were produced after induction of either leukocytes or fibroblasts. As type II IFN was induced by immune recognition reactions, it was called immune IFN (see Table 1). However, after it was recognized that cells, especially murine cells, could produce both forms of type I IFN,

Table 1. Classification of IFNs

Old nomenclature	New nomenclature	Inducer	Cell type
Type I leukocyte	α	virus	all
fibroblast	β	"	"
Type II immune	γ	mitogen antigen	T lymphocytes

this nomenclature was changed. Now, IFNs are classified into three antigenically distinct species, IFN-α, -β and -γ, corresponding to the former designation leukocyte, fibroblast and immune IFN.

Natural IFN-α is heterogeneous, as has been demonstrated with several electrophoretic and chromatographic techniques. Earlier, this heterogeneity had been ascribed to a different extent of glycosylation of IFN-α or to partial degradation of the protein. However, determination of the amino acid sequence of peptides derived from human lymphoblastoid IFN showed the existence of at least five different but homologous proteins (Allen and Fantes, 1980).

The onset of recombinant DNA technology ensured the cloning of cDNAs for IFN-β (Taniguchi et al, 1979), -α1 (Nagata et al, 1980a, Goeddel et al, 1980) and -γ (Gray et al, 1982, Devos et al, 1982). Soon after the cloning of the first α cDNA, Goeddel et al (1981) described the isolation of eight different cDNA clones. Seven of these were able to encode functional IFN-α proteins, which displayed a homology of 80% at the amino acid level. The existence of multiple IFN-α subspecies was in agreement with the data of Allen and Fantes mentioned above and with the identification of at least eight IFN-α related genes (Nagata et al, 1980b). Southern hybridization analysis of digests of genomic DNA revealed the existence of a multigene family encoding IFN-α proteins in all species analyzed, whereas IFN-β was shown to be encoded by a single-copy gene in the majority of species (Wilson et al, 1983). The IFN-γ gene appeared to be single-copy in all species analyzed (Weissmann and Weber, 1986). The later described IFN-β2 (Weissenbach et al, 1980) turned out to be a B-cell stimulatory factor (BSF-2, Haegeman et al, 1986, Hirano et al, 1986) and is now renamed "interleukin 6" (Poupart et al, 1987).

Although IFN was originally believed to cause a major break through in cancer therapy, successful clinical application of IFN has up till now been limited to a selected number of malignancies. IFN has been successfully applied in two different kinds of tumors for which the human papilloma viruses are supposed to be the etiological agents. These tumors are juvenile laryngeal papillomatosis (Haglund et al, 1981, Goepfert et al, 1982, Lundquist et al, 1984), the most common benign tumor of the

larynx in children, and condylomata acuminata (Schonfeld et al, 1984, Gall et al, 1985), a wart-like affection of mucosa and skin of genitals and rectum. The latter has been associated with neoplastic change. In addition, a significant negative effect of a combination therapy of IFN and a virustaticum on development of herpes-keratitis has been reported (Sundmacher et al, 1978, Colin et al, 1983). In non viral tumors, hairy cell leukemia responds very well to IFN-treatment (Quesada et al, 1984, Janssen et al, 1984, Colomb et al, 1985, Ratain et al, 1985).

2. MOLECULAR BIOLOGY OF THE IFN SYSTEM

2.1. IFN genes and proteins

IFN-α and -β genes belong to a super family. They are clustered on chromosome 9 in man (Owerbach et al, 1981, Shows et al, 1982) and 4 in mouse (van der Korput et al, 1985, Dandoy et al, 1985) and diverged 200-300 million years ago from a single ancestral gene (Weissmann and Weber, 1986). The IFN-γ gene shows no clear structural relationship to the genes for α and β and is located on a different chromosome: 12 in man (Trent et al, 1982, Naylor et al, 1983) and 10 in mouse (Naylor et al, 1984). γ diverges from α and β not only in chromosomal localization and nucleotide sequence, but also in genomic organization. The IFN-α and -β genes belong to the few eukaryotic genes which are devoid of introns. In contrast, the IFN-γ gene contains three introns.

In Fig. 1 the amino acid sequence of the human (Hu) and murine (Mu) IFN-α consensus, -β and -γ proteins is depicted. Amino acids conserved in either all α, all β or all γIFNs are marked. The intraspecies homology between the various IFN-α proteins is 75 to 80% and between IFN-α and -β around 33%. The homology between α proteins from different species is about 70% and considerably larger than the interspecies homology between either β (50%) or γ (40-60%) IFNs. No obvious similarity exists between α and βIFNs on one hand and γIFN on the other. As α and βIFN were also shown to compete for the same receptor, whereas γ binds to a different one (Branca and Baglioni, 1981, Aguet et al, 1982), the classification into type I (α+β) and II (γ) IFN still retains its significance.

The molecular weights of the IFNs range from 16 to 27 kDa for IFN-α, from 19 to 35 kDa for IFN-β and from 16 to 25 kDa for IFN-γ. In most cases this heterogeneity can be ascribed to different degrees of glycosylation of IFN proteins. With the exception of most of the Hu and all bovine (Bo) IFN-α species, IFNs contain one or more N-glycosylation sites.

2.2. Regulation of IFN expression

IFN induction is mainly regulated at the level of

```
      * *     *                              ** **  *   *
Hu α  CDLPQTHSL- G-NRRTLM-L LAQM-GRISP FSCLKDRHDF GFPQEEFDGN
Mu α  .......N.- R-.K.A.T-. .V..-R.L.. L......K.. .....KV.AQ

        $  $      $      $$ $ $ $$          $     $   $ $
Hu β  MSYNLLGFLQ RSSNFQCQKL LWQLNGRLEY --CLKDRMNF DIPEEIKQLQ
Mu β  IN.KQ.QLQE .TNIRK..E. .E....KIN- ---.TY.AD. K..M.MTE--

                          # #   ###    #          #    #  ##
Hu γ            Q DPYVKEAENL KKYFNAGHSD --VADNGTLF LGILKNWKEE
Mu γ         CYCH GTVIESL.S. NN...SSGI. --.-EEKS.. .D.WR..QKD


                             *      *             **         *
Hu α  QFQK---AQA ISVLHEMIQQ TFNLFSTKDS SAAWDETLLD KFYTELYQQL
Mu α  .I..---... .P..S.LT.. IL.I.TS... ....NA.... S.CND.H...

         $             $ $$   $       $ $$$$ $$$    $$     $
Hu β  QFQKEDAALT I---YEMLQN IFAIFRQDSS STGWNETIVE NLLANVYHQI
Mu β  KM..SYT.FA .---Q..... V.LV..NNF. .........V R..DELHQ.T

         # #  ## #    ###   ##   ## #  #       #         #
Hu γ  SDRK-IMQSQ I---VSFYFK LFKNFKDDQS IQKSVETIKE D-MNVKFFNS
Mu γ  G.M.-.L... .---I...LR ..EVL..N.A .SNNISV.ES H-LITT..SN


                                      *     **       *     * *
Hu α  NDLEACVIQE VGVEETPLMN -E-DSI--LA VRKYFQRITL YLTEKKYSPC
Mu α  ...Q..LM.Q ...Q.P..TQ -.-..L--.. .....H...V ..R...H...

        $      $          $           $ $  $$      $$    $
Hu β  NHLKTVL-EE -KLEKEDFTR GKLMSS--LH LKRYYGRILH YLKAKEYSHC
Mu β  VF.....-.. -.-Q..RL.W -E-...TA.. ..S..W.VQR ...LMK.NSY

         #    #         #       #  #    ### #   #  #     ###
Hu γ  NKKKRDD-FE -KLTNYSVTD -LNVQRKAIH -ELIQVMAEL SPAAKTGKRK
Mu γ  S.A.K.A-.M -SIAKFE.NN -PQ...Q.FN -...R.VHQ. L.ESSLR...


      ***             *
Hu α  AWEVVRAEIM RSFSLSTNLQ ERLRRKE
Mu α  ........VW .AL.S.A..L A..SEEK

      $$  $   $   $      $$ $
Hu β  AWTIVRVEIL RNFYFI-NRL TGYLRN
Mu β  ..MV..A..F ...LI.-R.. .RNFQ.

      ##
Hu γ  RSQMLFRGRR ASQ
Mu γ  ..RC
```

Fig. 1. Amino acid sequence of Hu and MuIFN-α consensus, -β and -γ. HuIFN-γ is aligned with HuIFN-β according to DeGrado et al (1982). Residues conserved in: * all αIFNs, $ all βIFNs, # all γIFNs.

transcription (Weidle and Weissmann, 1983, Ohno and Taniguchi, 1983). In addition, evidence for posttranscriptional control of HuIFN-β mRNA turnover has been presented (Raj and Pitha, 1983, Nir et al, 1984).

Several groups have investigated the 5'-noncoding region of the HuIFN-β gene for sequences responsible for inducible expression. In the first reports which appeared on this subject large variations were observed in the position of the upstream boundary of regions found to be necessary for induction of IFN-β expression (Maroteaux et al, 1983, Tavernier et al, 1983, Zinn et al, 1983). A more detailed analysis of deletion mutants of the IFN-β promoter region revealed the presence of distinct domains required for maximal induction of IFN expression. These regions were located, with respect to the cap site, either between position -117 and -40 (Fujita et al, 1985), -79 and -39 (Goodbourn et al, 1985) or -111 and -75 (Dinter and Hauser, 1987). In the HuIFN-α1 promoter a similar domain was located between position -109 and -64 (Ryals et al, 1985). A common feature of the sequence of these so-called IFN regulatory elements (IREs) is the abundance of purine nucleotides. In addition, the IREs can function independently of their orientation (Fujita et al, 1985, Goodbourn et al, 1985, Ryals et al, 1985) and at various positions from the cap site (Goodbourn et al, 1985, Kuhl et al, 1987), reminding the properties of enhancer elements (Khoury and Gruss, 1983). Duplication of a -90/-51 fragment (Dinter and Hauser, 1987) or a 3- or 4-fold multimerization of a -109/-64 fragment (Kuhl et al, 1987) was shown to enhance several times the inducibility of respectively the β or α1 promoter. Between position -109 and -65 of the IFN-β promoter the presence of repetitious hexanucleotides was noted (Fujita et al, 1985). A synthetic fragment containing a four or six times tandemly repeated hexamer (AAGTGA) was shown to contribute substantially to virus-induced activation of transcription (Fujita et al, 1987, Kuhl et al, 1987).

As compared to the IFN-α and -β promoter region, the 5'-flanking sequence of the IFN-γ gene is far less purine-rich. No deletion mapping of putative regulatory domains in the γ promoter region has been performed.

Investigations concerning the mode of regulation of the

HuIFN-β promoter revealed the existence of two separable positive regulatory domains and an overlapping negative control sequence (Goodbourn et al, 1986, Goodbourn and Maniatis, 1988). Factors have been identified in uninduced and in induced cells which bind to these regulatory domains (Zinn and Maniatis, 1986, Keller and Maniatis, 1988, Miyamoto et al, 1988). Prior to induction a repressor molecule is bound to the IFN-β promoter region from position -63 to -37. After induction this repressor is inactivated and displaced from the DNA, enabling binding of positive regulators to two domains located between -77 and -55. Dirks et al (1986) detected derepression of the IFN-β promoter after competition with an excess of various promoter fragments. A repressor binding site was mapped between position -65 and -37, in agreement with the results of Goodbourn et al (1986). A positive regulatory factor, present after viral induction, could only be titrated out by a duplicated -91/-50 fragment. Competition experiments performed by Fujita et al (1987) and Xanthoudakis and Hiscott (1988) also proved the existence of a positive regulator binding to the DNA after induction, but the presence of a repressor molecule in uninduced cells could not be demonstrated. In one case, a relatively high level of constitutive expression was measured prior to induction (Fujita et al, 1987), suggesting that a repressor probably was not active in the system used (COS cells).

The data summarized above reveal regulatory domains in the HuIFN-β promoter region which differ in location respective to the cap site. These apparent discrepancies may be due to the action of different regulatory factors present in the various cell types used for the deletion mapping experiments, which bind to one or the other motif sequentially occurring in the IFN-β 5'-flanking region. Moreover, differences in factor requirement between the α1 and β promoter can, at least partly, be explained by the relatively independent regulation of α and β expression in human cells. In some cases, IFN-α is not or hardly expressed in IFN-β producing cells and vice versa. This phenomenon is dependent on the mode of induction (Havell et al, 1978) and on cell type (Hiscott et al, 1984) and does not occur in murine cells. In human 293 cells efficient virus-induced expression of the bacterial chloramphenicol acetyl transferase (CAT) gene was

monitored if placed downstream of the HuIFN-β promoter, whereas no CAT expression from the HuIFN-α1 promoter could be measured (Xanthoudakis et al, 1987). Fragments of the HuIFN-α1 (Xanthoudakis and Hiscott, 1987) or the the MuIFN-α4 (Hauser, pers. comm.) promoter could not compete for a protein associated with the upstream regulatory region of the HuIFN-β promoter. These experiments suggest that regulation of IFN-α and -β expression requires the action of different factors.

In addition to 5'-noncoding regulatory sequences, sequences in the 3'-noncoding region of IFN genes were found to affect IFN expression. Raj and Pitha (1981) have shown that IFN mRNA is very unstable, with a half life of 30 minutes. Expression studies of an IFN-α1 and a β-globin transcription unit under direction of the β-globin promoter, revealed a 10-100 times higher level of β-globin mRNA, as compared to the IFN-α1 level (Weidle and Weissmann, 1983). As the promoter region in the two constructs is similar, this observation suggests a difference in degradation rate for both mRNAs. A highly conserved 50-60 nucleotide A-T rich sequence found in the 3'-noncoding region of IFN and several other transiently expressed genes, like c-myc, c-fos and certain lymphokines (Caput et al, 1986), was shown to decrease mRNA stability (Shaw and Kamen, 1986). However, not only mRNA stability, but also mRNA translation seems to be affected by the nature of 3'-noncoding sequences. The translational efficiency of human IFN-β mRNA in certain in vitro translation systems (Xenopus oocytes, rabbit reticulocyte lysate) was shown to be increased when provided with Xenopus β-globin 3'-noncoding sequences. In addition, an IFN 3'-noncoding region placed downstream of a lysozyme cDNA severely inhibited the translational efficiency of the corresponding mRNA in both translation systems (Kruys et al, 1987). Our data show that exchange of an IFN 3'-noncoding region for that of the rabbit β-globin gene enhanced IFN expression at least four times (van Heuvel et al, 1986, appendix paper I). This result can be due to both phenomena described above.

In summary, IFN expression is tightly regulated by the action of negative and/or positive regulatory factors bound to the promoter region of IFN genes before and after induction. The fast turn over of IFN mRNA enables rapid intervention of regulatory mechanisms to shut off IFN synthesis.

3. MECHANISMS OF IFN ACTION

3.1. IFN-receptor interaction

IFN protein is synthesized from mRNA as a pre-IFN. Upon removal of a signal sequence of 19-23 amino acids a mature protein is secreted from the cell. Subsequent interaction of IFN with a specific cell surface receptor is necessary to trigger a biological response and, in addition, determines its relative species specificity. Cells showing undetectable binding of IFN are found to be unresponsive to its biological activity (Baglioni et al, 1982, Joshi et al, 1982, Yonehara et al, 1983b). IFN-α and -β bind to the same cellular receptor, whereas IFN-γ binds to a different one (Branca and Baglioni, 1981, Aguet et al, 1982). Depending on cell type, the amount of high affinity binding sites present on the cell surface varies from 200-6000 for type I IFN and from 1000-10,000 for type II IFN. Both receptors are glycoproteins. Using radioligand binding and cross-linking techniques, IFN-receptor complexes were shown to possess molecular weights varying from 90 to 300 kDa (see Pestka et al, 1987 for a review). After purification of the human type I and the human and murine type II receptors, their molecular weights turned out to be respectively 110 (Zhang et al, 1986) and 90-95 (Novick et al, 1987, Calderon et al, 1988, Basu et al, 1988) kDa. Various groups are attempting to clone the IFN receptor and reports on successful cloning can be expected soon, probably within a few months.

Binding of IFN to its receptor is followed by receptor mediated endocytosis (Branca et al, 1982, Zoon et al, 1983, Sarkar and Gupta, 1984). However, it is not clear as yet which events take place after internalization of the IFN-receptor complex. Most likely, IFN-receptor interaction triggers a second messenger system to transduce a signal from receptor to nucleus (see 3.2.). This implicates that internalization of IFN is not required for biological response. On the other hand, several groups have demonstrated intracellular action of IFN (see below).

A first indication that internalization is not necessary for a biological response was obtained by the demonstration that IFN-α/β, covalently linked to Sepharose, could induce an antiviral

state (Ankel et al, 1973). However, partial dissociation of IFN from Sepharose cannot be excluded. Studies on the influence of inhibition of IFN-receptor internalization on IFN-mediated cellular response (Yonehara et al, 1983a, Faltynek et al, 1988) neither gave a definite answer as to the necessity of internalization.

The ability of anti-receptor (Jacobs et al, 1978, Schechter et al, 1979, Couraud et al, 1981, Schreiber et al, 1981, 1983) or anti-idiotypic (Sege and Peterson, 1978, Obberghen et al, 1979, Schreiber et al, 1980, Farid et al, 1982) antibodies to mimic biological effects of certain ligands has been generally considered as a clear prove that interaction of a ligand with its receptor is sufficient to induce a biological response and that the receptor contains all biochemical attributes to perform this induction. In the IFN system one anti-IFN-α2 anti-idiotypic antibody has been described (Osheroff et al, 1985), which displayed antiviral activity and could compete with IFN-α2 for binding to bovine (MDBK) cells. The observation that a clear correlation exists between the biological activity of IFN and its affinity for a cellular receptor (Yonehara et al, 1983c, Aguet et al, 1984, Uzé et al, 1985, Hannigan and Williams, 1986, Meister et al, 1986, Shafferman et al, 1987) is also consistent with the idea that internalization of IFN is not required to initiate biological effects.

A few reports dealing with the ability of IFN to function inside the cell have been published. A direct role of IFN or IFN-receptor complexes in regulation of gene expression was suggested by one group of investigators (Kushnaryow et al, 1985, 1986, MacDonald et al, 1986). They observed high affinity binding of MuIFN-β or -γ to the nuclear membrane and its subsequent accumulation within the nucleus. A circumvention of the interaction with the receptor by artificial delivery of IFN inside the cell did not abolish a biological response, but abrogated species specificity. Hu and Mu γIFNs, encapsulated within liposomes, produced an activation of both human and murine macrophages (Fidler et al, 1985). In addition, murine cells transfected with a cDNA encoding a mature HuIFN-γ protein, which accumulated inside the cell, established a permanent antiviral state (Sancéau et al, 1987). In contrast, type I IFN could not

produce an antiviral state after microinjection in murine or human cells (Higashi and Sokawa, 1982, Huez et al, 1983).

3.2. IFN-mediated signal transduction

The hypothesis that binding of IFN to a cell surface receptor is sufficient to trigger a cellular response, presumes the action of one or more second messengers to transduce signals from the IFN-receptor complex to the nucleus.

Several groups have investigated if one or more of the currently known mechanisms of signal transduction are modulated after interaction of IFN with its receptor. After an IFN treatment of 15 to 30 minutes, no effect of IFN on phosphatidyl-inositide hydrolysis, cytoplasmic free Ca^{2+} levels or cytoplasmic alkalinization could be detected in several human cell lines, including Daudi, (Mills et al, 1985). On the other hand, PDGF induced stimulation of intracellular free Ca^{2+} was inhibited by pretreatment of cells with IFN, whereas no effect of IFN on PDGF induced increases in total inositolphosphate levels could be measured (Zagari et al, 1988). Conflicting data have been obtained on the involvement of protein kinase C in IFN mediated signal transduction. A rapid and transient increase in the level of diacylglycerol, a potent activator of protein kinase C, has been observed by Yap et al (1986 ab), within 30 seconds after IFN exposure. The magnitude of this increase could be correlated to the antiviral and the antiproliferative response and appeared to be proportional to the number of IFN receptors. In addition, a transient rise in inositol phosphates, but not in cytoplasmic free Ca^{2+} was measured. Protein kinase C itself was activated 5-fold three hours after IFN treatment of macrophages (Hamilton et al, 1985). Recently, it was shown that the transcriptional activation of an IFN-γ regulated gene in a macrophage-like cell line was also mediated by protein kinase C (Fan et al, 1988). On the contrary, the activity of protein kinase C was strongly decreased in two human tumor cell lines, Hep-2 and Khm-14, one hour after either IFN-β or -γ treatment (Ito et al, 1988). In Swiss 3T3 cells no changes in activity of protein kinase C were observed, but in this case activity was measured only up to ten minutes after the onset of IFN treatment (Mehmet et al, 1987). In

Table 2. IFN-induced genes and cDNAs.

Gene	IFN	Protein size (kDa)	Function/characteristics	Reference
HLA class I	$\gamma > \alpha,\beta$	44	heavy chain	Fellous et al (1982)
β2-microglobulin	$\gamma > \alpha,\beta$	14	small invariant cl I chain	Wallach et al (1982)
HLA class II	$\gamma \gg \alpha,\beta$	34	heavy and light chains	Rosa et al (1983)
2-5A	$\alpha,\beta > \gamma$	40,46,67,100	synthesis of oligoadenylates from ATP dsRNA dependent	Merlin et al (1983)
1-8	α,β,γ		not known	Friedman et al (1984)
9-27	α,β,γ		member of 1-8 family	"
6-16	α,β	13	secreted	"
MT-II	α,β,γ	7	metal detoxification, zinc homeostasis	"
ISG 15	α,β γ?	15	ubiquitin cross reactive protein	Korant et al (1984)
ISG 54	α,β	54	hydrophilic protein	Larner et al (1984)
ISG 56	$\alpha,\beta > \gamma$	56	cytoplasmic protein	Chebath et al (1983) Larner et al (1984) Wathelet et al (1986)
202	α,β	56	murine equivalent of ISG 56	Engel et al (1985)
Factor B	α,β,γ		complement protein	Strunk et al (1985)
C2	γ		complement protein	"
IP-10	$\gamma > \alpha,\beta$	10	homologous to platelet factor 4 and β-thromboglobulin	Luster et al (1985)
Mx	α,β	72	specific inhibition of influenza virus	Staeheli et al (1986)
p54	$\gamma > \alpha,\beta$	54	cytoskeleton-associated	Ulker et al (1987)
Indoleamine 2,3-dioxygenase	γ	40	tryptophan degradation	Takikawa et al (1988 Ozaki et al (1988)
Cytochrome B	γ	91	heavy chain	Newburger et al (1988
C5-4	$\gamma > \alpha,\beta$		not known	Caplan and Gupta (198
γ-1	$\gamma \gg \alpha,\beta$		"	Fan et al (1988)
IP-30	$\gamma > \alpha,\beta$	30,25	secreted, lysosomal?	Luster et al (1988)
Protein kinase	α,β,γ	69	phosphorylation of IF-2α dsRNA dependent, gene not cloned	Samuel (1979)

one case evidence has been presented that the antiproliferative activity of IFN on a macrophage cell line was mediated by cAMP (Nagata et al, 1984).

The different data presented above do not unambiguously show how a signal transduction pathway is modulated by IFN action. However, many variables can be indicated, such as cell type, growth state of cells (density inhibited /or proliferating), differences in culture conditions, length of incubation of cells with IFN, which may influence the outcome of an experiment.

3.3. IFN-regulated gene expression

As early as five minutes after exposure of a cell to IFN, the transcription of a series of genes is increased significantly (Friedman et al, 1984, Larner et al, 1984), indicating a rapid transfer of an IFN-generated signal from the cell membrane to the nucleus. During the last few years the isolation of a large number of IFN-induced cDNAs has been described. However, the functions of the protein products encoded by the corresponding genes are largely unknown. In Table 2 an overview is given of genes which are found to be induced by IFN. Indicated are the size of the protein product encoded by a particular gene and, if known, its function or other characteristics. Some genes are differentially regulated by type I and type II IFN, sometimes depending on the cell line in which the expression is assayed.

The accumulation of IFN-induced mRNAs is subject to transcriptional and post-transcriptional regulation (Friedman et al, 1984, Yoshie et al, 1984, Korber et al, 1987, Pascucci et al, 1988). After comparing 5'-flanking sequences of two HLA class I, one HLA class II and a MT-II gene, Friedman and Stark (1985) indicated the presence of a homologous sequence in the promoter region of these IFN-inducible genes. Deletion mapping of the promoter region of the murine MHC class I genes H-2 D, K and L (Israel et al, 1986, Korber et al, 1988, Sugita et al, 1987), 2-5A synthetase (Benech et al, 1987, Rutherford et al, 1988, Cohen et al, 1988), 6-16 (Porter et al, 1988) and ISG 54 (Levy et el, 1988) revealed the existence of an IFN responsive sequence (IRS) with the properties of an inducible enhancer element. The Friedman-Stark consensus sequence was present in all of these IR

Table 3. Homologies in flanking regions of IFN-inducible genes.

Gene		Strand	Position*	Reference
Hu Mx	CAGAAAC-GAAACT	-	-119/-131	Hug et al, 1988
Hu MT-II	AGAGAGGAGAAACT	-	-600/-613	Friedman
Hu HLA-A3	AGAGAAAAGAAACT	-	-147/-160	and Stark, 1985
Mu H-2 K	GCAGAAGTGAAACT	-	-138/-151	Kimura et al, 1986
Hu 6-16	GGGAAAATGAAACT	+	-112/-99	Porter et al, 1988
	GGGAAAATGAAACT	+	-153/-140	
Hu 2-5A	AGGAAAC-GAAACC	+	-100/-88	Cohen et al, 1988, Rutherford et al, 1988
Mu 2-5A	GGGAAATGGAAACT	+	-72/-59	Cohen et al, 1988
Hu ISG 15	GGGAAACCGAAACT	+	-108/-95	Reich et al, 1987
Hu ISG 54	GGGAAAGTGAAACT	-	-87/-100	Reich et al, 1987
Hu ISG 56	GGGAAAGTGAAACT	-	-106/-119	Wathelet et al, 1987
Mu 202	GGGAAATTGAAAGC	+	-144/-131	Samanta et al, 1986
Hu IP-10	TGGAAAGTGAAACC	+	-222/-209	Luster et al, 1987
Hu Fact B	AGGAAACAGAAACT	-	-127/-140	Wu et al, 1987

*Position is indicated with respect to the cap site, except for Hu and Mu 2-5A in which it is indicated with respect to the translation start site.

sequences and, in addition, could be detected in the promoter regions of all IFN-inducible genes sequenced sofar (see Table 3). With the exception of the MT-II gene, this sequence is located 100 to 200 nucleotides upstream from the transcription start site.

Two conserved hexanucleotides can be observed in the sequences depicted in Table 3. The NGGAAA motif is present in most of the human and murine IFN-inducible genes. In addition, it is found in the 5'-flanking region of several genes, which are not regulated by IFN (human c-fos, HSP, IFN-α and -β), and in the enhancers of SV40, CMV and HIV (Porter et al, 1988). On the contrary, the GAAACPy motif only occurs in IFN-inducible genes and TGAAAG in the IFN-inducible 202. Interestingly, a combination of GGAAA and TGAAAG is also present in the promoter region of the HuIFN-β gene. Recently, a number of papers have been published showing that some IFN-inducible genes can also directly be induced by dsRNA or virus (Tiwari et al, 1987, 1988, Wathelet et al, 1987, 1988, Hug et al, 1988). The physiological significance of this phenomenon is not clear as yet.

Analysis of IFN regulated expression of H-2 genes revealed the involvement of an additional region upstream of the IRS, called class I regulatory element or enhancer A. Absolute

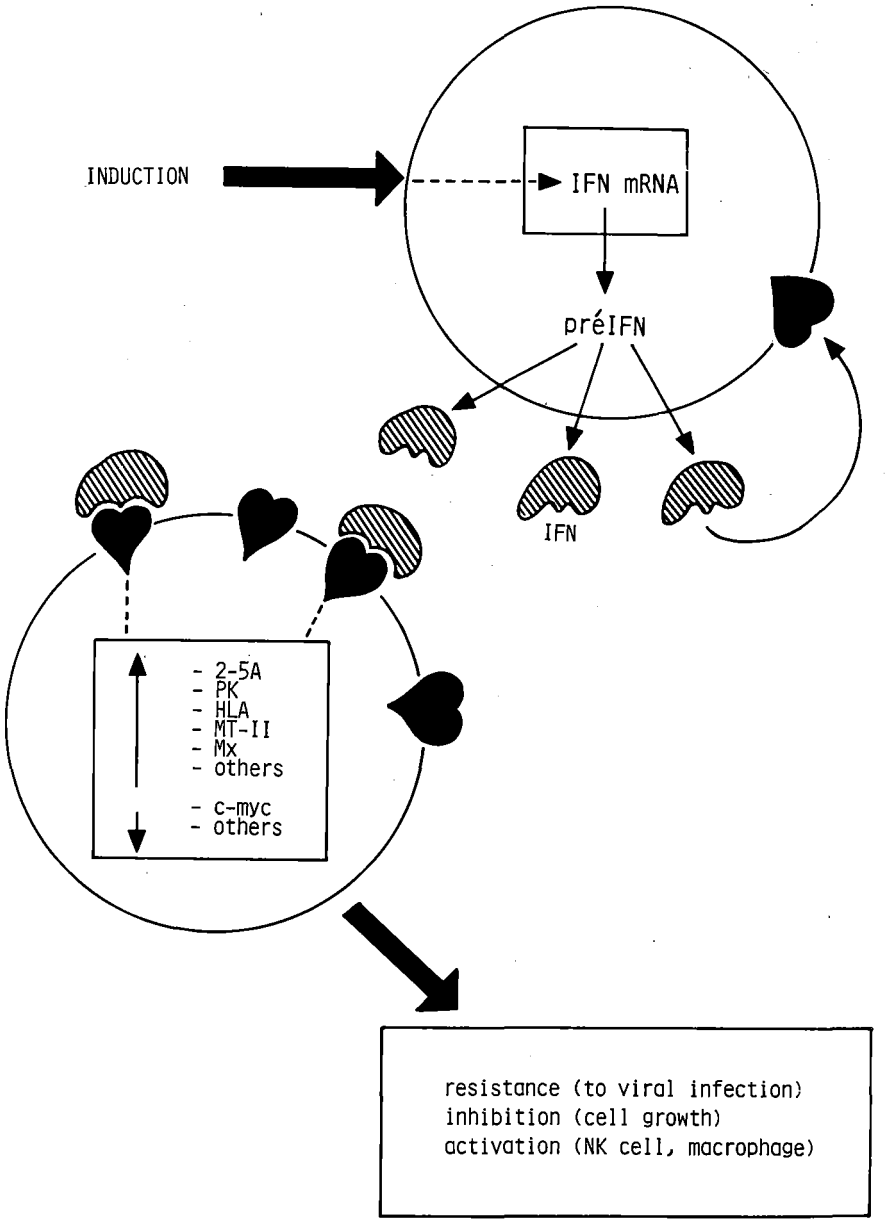


Fig. 2. Effects of IFN on cellular metabolism.

requirement of this region was shown to depend on the cell line and the IFN species used (Israel et al, 1986, Sugita et al, 1987, Pascucci et al, 1988, Korber et al, 1988).

With the use of band shift assays, several groups have detected specific binding of factors, present in nuclear extracts from uninduced and induced cells, to various IRS-containing promoter fragments (Porter et al, 1988, Rutherford et al, 1988, Cohen et al, 1988, Levy et al, 1988, Shirayoshi et al, 1988). Similar results were obtained with DNase I footprinting of H-2D (Korber et al, 1988) and ISG 54 (Levy et al, 1988) flanking sequences. In contrast, Israel et al (1987) could not detect proteins binding to the IRS of the H-2K gene. Point mutations in the IRS of ISG 54 (Levy et al, 1988) and the H-2L gene (Shirayoshi et al, 1988) prevented proper induction of these genes. In the 2-5A gene a repressor was shown to bind to a region between position +1 and +82 with respect to the translation start site. Derepression of the 2-5A promoter occurred with excess of a -122/+82 fragment (Benech et al, 1987), but not with a -155/+1 fragment (Rutherford et al, 1988). Until now, no data are available about the nature of the putative IRS-binding factors.

In addition to the ability of IFN to induce the expression of a large series of genes, IFN was shown to reduce c-myc mRNA levels in the human lymphoblastoid cell line Daudi (Jonak and Knight, 1984), a cell line which is extremely sensitive to the growth inhibitory effect of IFN. This down-regulation appeared to occur at the post-transcriptional level (Knight et al, 1985). Three IFN-repressed cDNAs were isolated by Kulesh et al (1987). The corresponding mRNA levels were also found to decrease when cell growth became naturally arrested by contact inhibition.

In Fig. 2 the effects of IFN on cellular metabolism are summarized. IFN expression has to be induced by for instance a virus. Secreted IFN binds to specific cell surface receptors on neighboring cells or on the producing cell itself. The latter results in an autocrine effect. Upon IFN binding the expression of several genes is increased or decreased. Finally, this will result in phenotypic changes, which depend on the nature of the target cell.

4. STRUCTURE-FUNCTION ANALYSIS OF IFN-α PROTEINS

4.1. Introduction

A first indication that IFN-α subspecies displayed divergent biological activities was obtained after separation of natural Hu leukocyte IFN with HPLC. At least 8 subspecies could be detected which showed different ratios of antiproliferative to antiviral activity and of antiviral activity on human to activity on bovine cells (Rubinstein et al, 1981, Evinger et al, 1981b). Natural MuIFN could also be separated into different components which were found to differ in their ability to protect murine or hamster cells against viral infection (Lemson et al, 1984).

Molecular cloning of IFN genes and cDNAs provided the possibility to analyze IFN gene structure and to investigate properties of individual proteins. As mentioned before, in all species analyzed sofar IFN-α is encoded by a multigene family. Most of our current knowledge of IFN-α comes from analysis of human and murine species. The human gene family can be divided into two subfamilies: α_I, containing 15 loci of which one is a pseudogene and α_{II}, containing 6 loci of which five are pseudogenes (see Weissmann and Weber, 1986 for a recent review). In the murine gene family 11 members have been described of which one is a pseudogene (Weissmann and Weber, 1986, Dion et al, 1986, Trapman et al, 1988, appendix paper V).

Mature IFN-α proteins are 165-167 amino acids long. Two exceptions have been observed: HuIFN-α_{II}1, which contains a C-terminal extension of six amino acids and MuIFN-α4, which contains a five amino acid deletion from position 103-107. All Hu and MuIFN-α subspecies contain four cysteines which are involved in disulfide bridges: one between C1 and C99 and one between C29 and C139 (Wetzel, 1981). An additional cysteine residue at position 86 is present in all Mu and in one Hu (α1) subspecies. Most HuIFN-α proteins are devoid of putative N-glycosylation sites; exceptions are GX-1 (at position 2), α14 (at positions 2 and 72) and α_{II}1 (at position 78). On the contrary, most Mu α subspecies, except for α6 and αA, contain a N-glycosylation site at position 78, and were shown to be glycosylated (van Heuvel et al, 1986, Trapman et al, 1988, appendix papers II and V).

Table 4. Specific antiviral activity of Hu and MuIFN-α subspecies on different cell types.

IFN	Specific antiviral activity*				Reference
	human	bovine	murine	hamster	
Hu α1 (D)	65	1300	3.2		Rehberg et al, 1982 Weber et al, 1987
α2 (A)	2100	1600	0.05		see α1
α4	1000	nd	nd		Lydon et al, 1984
α7 (J)	140	1200	nd		Langer et al, 1986
α8 (B)	2100	1600	0.17		Meister et al, 1986
Mu α1			200	200	van Heuvel et al,
α2			100	20	1986, 1988
α4			1000	2	
α6			200	400	
α10			15	15	Trapman et al, 1988

*Specific antiviral activity is depicted in units x 10^{-5}/mg. One unit IFN is the amount of protein required to protect 50 % of the target cells against virus-induced cell lysis in a cytopathic effect reduction assay.

Expression of cloned HuIFN-α subspecies in E. coli and subsequent purification of biologically active protein allowed a comparison of the specific antiviral activities of these proteins on various cell types (see Table 4 for those subspecies for which a specific activity has been determined). All HuIFN-α subspecies were found to display a similar high activity on bovine cells, whereas on human cells a considerable variation in antiviral activity occurs. HuIFNs possess very low activity on murine cells. Moreover, from Table 4 it is evident that a subspecies with a high activity on human cells does not necessarily possess the highest activity on murine cells.

In our laboratory five MuIFN-α subspecies have been isolated (Zwarthoff et al, 1985a, Trapman et al, 1988, appendix paper V) and expressed in CHO and monkey COS cells (Zwarthoff et al, 1985b, van Heuvel et al, 1986, Trapman et al, 1988, appendix papers II and V). Large variations in specific antiviral activity were found, both on mouse and on hamster cells (see Table 4). Of the five subspecies analyzed, MuIFN-α4 displayed the highest antiviral activity on mouse cells and the lowest activity on hamster cells.

The high homology in the amino acid and nucleotide sequences of members of the IFN-α gene family coupled to a considerable variation in biological activity of IFN-α proteins makes this

gene family an excellent tool for performing a detailed structure-function analysis. Various common restriction enzyme sites enable the construction of hybrid IFN molecules. In addition, with the method of in vivo homologous recombination hybrids can be generated with recombination sites scattered along the whole molecule. A comparison of the properties of hybrid versus parental molecules results in localization of domains responsible for a certain phenotype. The impact of a particular domain can be assessed in more detail by site-directed mutagenesis of relevant amino acid residues.

A first indication which domains of the IFN protein are involved in antiviral activity has been obtained with hybrids generated in vitro between several Hu and between several MuIFN-α subspecies, which were selected for their divergent antiviral activities on respectively human and murine or murine and hamster cells. Hybrids have been constructed between Hu α1 and α2 (Streuli et al, 1981, Weck et al, 1981, Rehberg et al, 1982), between Hu α1 and α8 (Meister et al, 1986), between Mu α1, α2 and α4 (van Heuvel et al, 1986, 1988, appendix papers II and III) and between Mu α1 and α10 (Trapman et al, 1988, appendix paper V). The amino acid sequence of these Hu and MuIFN-α proteins is depicted in Fig. 3. Residues conserved in either αIFNs or α plus βIFNs are marked. The regions which have been localized using these hybrids are too large to substantiate the role of individual amino acid residues. For this reason a more elegant approach, in vivo recombination, has been applied for the construction of hybrids between respectively Hu α1 and α2 and Mu α1 and α4. All hybrids obtained by this method were found to be biologically active IFN molecules (Weber et al, 1987, Zwarthoff et al, 1987, appendix paper IV). Site-directed mutants have been prepared of several Hu and MuIFN-α subspecies: Hu α1 (Nisbet et al, 1985, Beilharz et al, 1986), Hu α2 (Valenzuela et al, 1985, Camble et al, 1986, Edge et al, 1986, Weber et al, 1987), Mu α1 (Kerry et al, 1988,), Mu α2 (van Heuvel et al, 1988, appendix paper III), Mu α4 (Zwarthoff et al, 1987, appendix paper IV) and Mu α1, α2 and α6 (van Heuvel, unpublished results). Furthermore, interspecies hybrids have been generated between Hu α7 and Bo αC (Shafferman et al, 1987) and between several Hu and MuIFN-α subspecies (Raj et al, 1988).

```
          10          20          30          40          50
      * *   * $   *       $ *  **   $$  $$  $  $        $ $$
Hu α1 CDLPETHSLD NRRTLMLLAQ MSRISPSSCL MDRHDFGFPQ EEFDGNQFQK
   α2 ....Q....G S......... .R...LF... K......... ...-......
   α8 ....Q    G ...A.I.... .R....F... K.....E... ...-DK....

      **** *  *  $*  *  $   ****  ***$ **$ $$ *$   **    *
Mu α1 CDLPQTHNLR NKRALTLLVQ MRRLSPLSCL KDRKDFGFPQ EKVDAQQIKK
   α2 ....H.Y... .....KV.A. ....PF.... ...Q.....L ....N...Q.
   α4 ....H.Y..G ......V.EE ....P..... .........L ....N...Q.
   α10 .......... .........K .......... .......... A.......QE

            60          70          80          90          100
           *$   $* $ *$ *   $$        **      *  *$    * *
Hu α1 APAISVLHEL IQQIFNLFTT KDSSAAWDED LLDKFCTELY QQLNDLEACV
   α2 .ET.P....M .........S. .........T .....Y.... ..........
   α8 .Q.......M ...T....S. ......L..T ...E.YI..D .......S..

     $$ **    *$* *  $** *  $* $ *  *** *     $ ***$  *
Mu α1 AQAIPVLSEL TQQILNIFTS KDSSAAWNAT LLDSFCNDLH QQLNDLQGCL
   α2 .......RD. ...T..L... .A........ .......... .......T..
   α4 ....L..RD. ......L... ..L..T.... .......... ......KA.V
   α10 .......... .......... .......... ....V..... ..........

          110         120         130         140         150
      *               $     $*  $  $ $   *$$ $$ $**$$ $$*
Hu α1 MQEERVGETP LMNADSILAV KKYFRRITLY LTEKKYSPCA WEVVRAEIMR
   α2 I.GVG.T... ..KE...... R...Q..... .K........ ..........
   α8 ...VG.I.S. ..YE...... R...Q..... .......S.. ..........

      *         $$*$   $*    $**$***  $* *$** *$ $*$ $$$ *$
Mu α1 MQQVGVQEFP LTQEDALLAV RKYFHRITVY LREKKHSPCA WEVVRAEVWR
   α2 ........P. .......... .......... .......... ..........
   α4 ..------.P. .....S.... .T........ ..K....L.. ...I......
   α10 ..E.....LS .....S.... .........F .......... .......I..

          160
      *   *     *
Hu α1 SLSLSTNLQE RLRRKE
   α2 .F........ S..S..
   α8 .F...I...K ..KS..

      * ***   *  $*
Mu α1 ALSSSANVLG RLREEK
   α2 .....V.L.P ..S...E
   α4 .....T.L.A ..S...E
   α10 .......L.A ..R.K.
```

Fig. 3. Amino acid sequence of the HuIFNs α1, α2 and α8 and the MuIFNs α1, α2, α4 and α10. * Residues conserved in either Hu or Mu αIFNs. $ Residues conserved in either Hu or Mu α and β IFNs.

4.2. Localization of amino acid domains which influence antiviral activity

4.2.1. Characterization of HuIFN-α proteins

4.2.1.1. In vitro recombinants

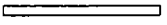
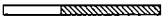
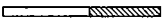
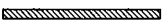
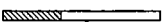
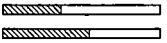
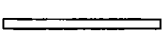
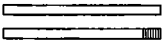
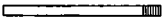
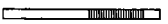
The antiviral activities of hybrids between the HuIFNs α1 and α2 (Streuli et al, 1981, Weck et al, 1981, Rehberg et al, 1982) and α1 and α8 (Meister et al, 1986) on human cells clearly proved the importance of a domain located in the N-terminal region of the IFN molecule. Rehberg and Meister calculated specific antiviral activities and a compilation of these data is depicted in Table 5. Given between brackets is the amino acid position behind which cross-over has occurred. The relative antiviral activities determined by Streuli and Weck were in good concordance with the data of Rehberg. The high antiviral activity of α2 and α8 on human cells ($1-3x10^8$ U/mg protein) is retained in all hybrids containing the N-terminal 60 amino acids of either α2 or α8. The activity of hybrids possessing this region from α1 was identical to or even lower than that of α1 ($4-7x10^6$ U/mg).

HuIFN-α subspecies have a low specific antiviral activity on murine cells, varying from $5x10^3$ U/mg for α2, $2x10^4$ U/mg for α8 to $3x10^5$ U/mg for α1. A remarkable phenomenon occurs with some of the hybrids derived from these subspecies. Hybrids, composed of the N-terminal part (position 1-60) of either α2 or α8 and the remaining part of α1, displayed an equally high activity on murine as on human cells ($1x10^8$ U/mg) (Rehberg et al, 1982, Meister et al, 1986). Hybrids in which, additionally, the 61-92 or the 93-150 region of α1 was replaced by α2 or α8 sequences had a 10- to 50-fold lower activity than the former hybrids. This was, however, still higher than the activity of the parental molecules on murine cells. These data suggested that at least three distinct regions are mediating the high activity of α2α1 (63) and α8α1 (60) on murine cells.

4.2.1.2. In vivo recombinants

In vivo recombination between two IFN-α genes results in a

Table 5. Specific antiviral activity of hybrids between Hu α1 and α2 (Rehberg et al, 1982) and α1 and α8 (Meister et al, 1986) on human and murine cells.

IFN		Specific antiviral activity*	
		human	murine
α1		44	0.78
α1α2 (63)		10	≤0.05
α1α2 (92)		27	0.48
α2		3200	0.05
α2α1 (63)		2800	1000
α2α1 (92)		3800	68
α2α1α2 (63,92)		430	3
α1		67	2
α1α8 (150)		80	2
α1α8α1 (60,92)		220	2
α1α8 (60)		22	0.2
α1α8 (92)		3.3	0.1
α1α8α1 (92,150)		7	0.3
α8		2100	0.2
α8α1 (150)		800	0.2
α8α1 (60)		1300	1000
α8α1 (92)		2300	22
α8α1α8 (92,150)		2000	98
α8α1α8 (60,92)		1300	51

*see Table 4.

set of hybrids with cross-over points from one gene to the other which are scattered throughout the molecule. As a consequence, differences in activity between these hybrids can be assigned to the area in between their respective cross-over points. Table 6 shows the areas or domains in which considerable transition of activity occurs. For instance, an α2α1 hybrid in which the 21 N-terminal amino acids are from α2 has an antiviral activity of 120×10^5 U/mg. The activity of the next hybrid, with a cross-over point at position 61, is about 8-fold higher (1000×10^5 U/mg). Thus, amino acids in between these cross-over points (e.g. domain 22-60) are responsible for this change.

With the α1α2 hybrids, domain 22-27 was responsible for a 50-fold drop in antiviral activity on human cells, whereas no effect on murine cells was detected (Table 6, α1α2 hybrids). In this region, α1 differs in three positions (22, 26 and 27) from α2 (see Fig. 3). At position 26 α2 possesses a leucine, whereas α1 and α8 possess a proline. Since α8, in contrast to α1, displays high activity on human cells, the nature of the residue

Table 6. Specific antiviral activity of human in vivo generated hybrids on human and murine cells (Weber et al, 1987). The domains or single amino acids indicated are responsible for the depicted transition in activity.

IFN	Domain	Specific antiviral activity*	
		human	murine
α1		62	5
α2α1	22-60	120-1000	11-150
	80		150-800
	86		800-50
	121		50-3.5
	125		3.5-0.02
	132		0.02-0.33
	152		0.33-0.13
α2		1100	0.05
α1α2	5-11		0.05-0.01
	22-27	1100-25	
	31	25-6	0.01-0.003
	69-86		0.003-0.04
	113-132	6-36	0.04-14
	152		14-2.8

*see Table 4

at position 26 seems to be of minor importance for antiviral activity. More likely, residues at position 22 and 27 are involved, since in both α2 and α8 arginine and phenylalanine residues are situated at these positions, whereas α1 contains two serines. At position 31 the presence of methionine (α1) instead of lysine (α2) decreased the antiviral activity on both human and murine cells a factor three to four.

In the C-terminal part of the HuIFN protein five amino acid residues were found to be important for activity on murine cells. At position 80, 86, 121, 125 and 132 the presence of respectively threonine (from α2), cysteine (from α1), lysine (from α1), arginine (from α1) and lysine (from α2) favoured the activity on murine cells (Table 6, α2α1 hybrids and Fig. 3). Remarkably, all Mu αIFNs contain threonine and cysteine at respectively position 80 and 86, except for Mu αA which has serine at position 80.

4.2.1.3. Site-directed mutants

A considerable number of HuIFN-α mutants have been produced with amino acid substitutions occurring throughout the whole protein. These mutants will be described starting from the N-

terminus.

Residues from position 1-4 were not involved in antiviral activity. Deletion of the N-terminal three (Edge et al, 1986) or four (Lydon et al, 1985) amino acids of respectively HuIFN-α2 or -α1 did not affect antiviral activity on human cells, nor did substitution of Hu α2 residues 2-7 for corresponding HuIFN-γ residues (Y2,C3,Q4,D5,P6,Y7, Edge et al, 1986).

A small N-terminal region was involved in two rather radical mutations. A Hu α2 mutant with only alanine residues from position 10-15 displayed a 100-fold lower antiviral activity than wild type α2. Deletion of the N-terminal 13 amino acids of α2 resulted in a complete loss of activity (Edge et al, 1986).

The importance of residues at position 27 and 31, demonstrated with the α1α2 in vivo recombinants (Table 6), was confirmed by the antiviral activity of a HuIFN-α2 mutant described by Edge et al (1986). This mutant possessed α1 residues at position 5, 27, 31 and 60 and displayed an antiviral activity on human cells comparable to that of α1 (Table 7). The nature of the residues at position 5 and 60 did not influence antiviral activity on human cells (Table 6, α1α2 hybrids).

A set of mutations was introduced into HuIFN-α2 (Camble et al, 1986) in a region close to the 22-27 domain discovered with the in vivo recombinants. This region, from position 30-33, is highly conserved in HuIFN-α,β and MuIFN-α, but to a lesser extent in MuIFN-β (Fig. 3). Substitution of the apolar leucine at position 30 for a polar residue led to a more than 100-fold drop in antiviral activity (Table 7). Especially, the integrity of the arginine at position 33 is important; even a replacement by the also positively charged lysine caused a 1000-fold drop in activity. Both residues are conserved in all α and βIFNs, in contrast to the aspartate at position 32, which is conserved in all αIFNs and in Hu βIFN, but not in Bo and Mu βIFNs. Replacement of the latter residue by alanine, glutamate or asparagine hardly altered the antiviral activity of the mutant protein. A total loss of biological activity occurred if all three residues discussed above were replaced by alanine.

Valenzuela et al (1985) suggested that a putative receptor binding site might be located in a region in HuIFN-α,β displaying a set of conserved amino acids which overlapped but

Table 7. Specific antiviral activity of HuIFN-α2 proteins with mutations in the N-terminal part of the molecule on human, murine and bovine cells.

IFN	Specific antiviral activity*			Reference
	human	murine	bovine	
α2	2000	nd	nd	Camble et al, 1986
α2, I30	600	nd	nd	
α2, N30	5	nd	nd	
α2, A32	4000	nd	nd	
α2, E32	2000	nd	nd	
α2, N32	700	nd	nd	
α2, A33	0.8	nd	nd	
α2, K33	3	nd	nd	
α2, M33	≤0.02	nd	nd	
α2, A30,32,33	inactive	nd	nd	
α2, E5,S27,M31,L60	80	nd	nd	Edge et al, 1986
α2, S48	1900	0.03	560	Valenzuela et al, 1985
α2, Y48	1400	0.02	2100	
α2, C48	1100	0.02	5500	
α2, H49	1200	0.02	1100	
α2, K62	1300	nd	nd	

*see Table 4

did not coincide with a set of conserved murine residues. Three amino acid residues were selected for site-directed mutagenesis: phenylalanine at position 48, occurring in HuIFN-β and in all Hu αs, except for $\alpha_{II}1$, glutamine at position 49, conserved in Hu β and in all Hu αs and glutamine at position 62, conserved in all α and βIFNs analyzed sofar (Fig. 3). None of the substitutions (serine, tyrosine or cysteine for F48, histidine for Q49 and lysine for Q62) resulted in an IFN protein with altered antiviral activity on human, bovine or murine cells (Table 7).

Site-directed mutagenesis of Hu α2 (Weber et al, 1987, Table 8) confirmed the impact of the residues at position 121, 125 and 132 on antiviral activity of HuIFNs on murine cells. A considerable increase (150-fold) of the antiviral activity of α2 on murine cells was obtained by substitution of glutamine at position 125 for the α1 residue arginine. Exchange of one basic residue (arginine) for another (lysine) at position 121 improved the antiviral activity of α2 15-fold. The same substitution in the mutant α2, R125 only resulted in a 3-fold enhancement. Analysis of the amino acid sequence of Mu αIFNs in this region revealed the presence of arginine or lysine at position 121 and a conserved histidine at position 125 (Fig. 3). However, a Hu α2

mutant with histidine at position 125 displayed a 15-fold lower activity than the mutant with arginine (Table 8). This observation suggests the possibility that the activity of MuIFN-α subspecies on murine cells can be improved by exchanging histidine at position 125 for arginine. At position 132 another conserved basic residue, arginine, can be observed in Mu αIFNs, whereas Hu αIFNs contain a polar threonine or methionine. Only Hu α2 and $\alpha_{II}1$ have a basic residue, lysine, at position 132, which was shown to enhance activity on murine cells a factor 10 (Table 8). The mutant displaying the highest activity on murine cells, α2, K121,R125, still has a 10- to 40-fold lower activity on murine cells than α2α1 hybrids recombined in the N-terminal part of the molecule. Possibly, exchange of tyrosine for cysteine at position 86 in α2, K121,R125 will enhance its antiviral activity on murine cells.

In the same region of the IFN molecule conserved aromatic residues occur at position 123, 124 and 130. The importance of tyrosine 123, which is conserved in all α and βIFNs analyzed sofar, was proven by its substitution for glycine. This mutation produced a protein without detectable antiviral activity on human cells and a 10-fold lower activity on bovine cells (see Table 8, Nisbet et al, 1985).

The extreme C-terminal part of the IFN molecule is not essential for antiviral activity, as Hu IFN-α proteins with deletions of the last 10-13 amino acids were shown to possess virtually the same antiviral activity as the parental molecules

Table 8. Specific antiviral activity of HuIFN-α1 and -α2 proteins with mutations in the C-terminal part of the molecule on human, murine and bovine cells.

IFN	Specific antiviral activity*			Reference
	human	murine	bovine	
α2	1100	0.05	1000	Weber et al, 1987
α2, K121	1300	0.86	910	
α2, R125	1200	7.4	1800	
α2, K121,R125	1400	21	1500	
α2, H125	1200	0.48	2300	
α2, K125	1300	0.26	1400	
α2, T132	1000	0.005	1100	
α1	200	5	3600	Nisbet et al, 1985
α1, G123	≤2.3	nd	320	

*see Table 4

Table 9. Specific antiviral activity of HuIFN-α1 proteins with one or more mutated cysteine residues (Nisbet et al, 1985, Beilharz et al, 1986).

IFN	Specific antiviral activity*		
	human	murine	bovine
α1	200	5	3600
α1, S1	9.2	0.26	450
α1, S29	15	0.22	190
α1, S99	22	0.55	990
α1, S86	78	14	3800
α1, S1,86	44	3.4	3800
α1, S86,99	38	3.8	6700
α1, S1,86,99	72	4.1	2900
α1, S1,29,86,99	≤0.2	nd	320

*see Table 4

(Levy et al, 1981, Franke et al, 1982).

4.2.1.4. Mutagenesis of cysteine residues

The four cysteine residues at position 1, 29, 99 and 139, conserved in all Hu and MuIFN-α subspecies, formed an evident target for mutagenesis (Nisbet et al, 1985, Beilharz et al. 1986). Chemical modification studies had already indicated the importance of the C29-C139 disulfide bridge for antiviral activity (Morehead et al, 1984). Proteolytic digestion of HuIFN-α2 was shown to generate a N-terminal fragment of 110 amino acids, which retained some biological activity (Ackerman et al, 1984). The fragment lacks disulfide bridge 29-139 suggesting that some degree of active conformation can exist without this disulfide bridge. The C1-C99 disulfide bridge was not essential for antiviral activity, since IFN proteins lacking the N-terminal three or four amino acids were shown to be as active as their wild type counterparts (Lydon et al, 1985, Edge et al, 1986).

The exchange of a cysteine residue for serine at position 1, 29 or 99 (Table 9) resulted in a 10- to 20-fold drop in antiviral activity on human, murine and bovine cells. Mutation of the unique cysteine residue of HuIFN-α1 at position 86 (conserved in all MuIFN-α subspecies) did not significantly alter its antiviral activity. The latter was also true for two double (S1,86 and S86,99) and one triple (S1,86,99) mutant. Formation of an aberrant disulfide bridge between C86 and a cysteine residue

which lacks its natural partner could explain the low activity of the single mutants. If the formation of one disulfide bridge is prevented, as occurs in the triple mutant, no severe loss of antiviral activity was observed. However, subsequent destruction of the second disulfide bridge, as occurs in the mutant S1,29,86,99, completely abolished antiviral activity on human cells. In contrast, antiviral activity on bovine cells decreased only 10-fold, reaching a value similar to that of the single mutants.

HuIFN-β contains three cysteine residues at position 17, 31 and 141, of which the last two can be aligned with cysteine 29 and 139 of IFN-α. Substitution of cysteine for tyrosine at position 141 of HuIFN-β completely abolished its antiviral activity (Shepard et al, 1981). In contrast, cysteine 17 could be mutated into a serine without change in antiviral activity (Mark et al, 1984). Remarkably, no disulfide bridges are required for MuIFN-β activity, because in this IFN only one cysteine residue is present at position 17. As in the HuIFN-β mutant described by Shepard, at position 141 of MuIFN-β a tyrosine is present. A possible explanation for the observation that tyrosine 141 is deleterious for HuIFN-β activity is that a nonnative disulfide bridge, formed between cysteine 17 and 31, prevents a correct folding of the protein.

4.2.2. Characterization of MuIFN-α proteins

4.2.2.1. In vitro recombinants

MuIFN-α subspecies possess either a high (α1, $2x10^7$ U/mg), intermediate (α2 and α10, $2x10^6$ U/mg) or low (α4, $2x10^5$ U/mg) antiviral activity on hamster cells. Comparison of the antiviral activities of these MuIFN-α subspecies and several series of hybrids on hamster cells revealed involvement of the same N-terminal region (from position 1-58 or 1-67, van Heuvel et al, 1986, 1988, appendix papers II and III) as found to mediate high activity of HuIFN-α2 and -α8 on human cells. The hybrids with this region from either α1, α2 or α4 displayed a similar high, intermediate or low activity on hamster cells as the parental proteins. Only in hybrids between Mu α1 and α10, presence of the

N-terminal part of α1 was not always accompanied by high activity on hamster cells. The middle part of the α10 protein, from position 68-123, was shown to be responsible for low activity on hamster cells, even if α1 residues were present from position 1-67 (Trapman et al, 1988, appendix paper V). The hybrids between α1 and α10 are the only ones which all displayed a similar activity on hamster and murine cells. Most likely, the absence of a proline residue at position 110 of α10, which can have structural consequences, determines its low activity on both hamster and murine cells.

MuIFN-α4 displays a 10-fold higher activity on murine cells ($1x10^8$ IU/mg) than both Mu α1 and α2. Three out of four hybrids possessing the C-terminal part of α4 (position 60-166) retained this α4 activity. It has been shown that the deletion of five amino acids, located in this region of α4 from position 103-107, when introduced into α2 and a α4α2 hybrid by site-directed mutagenesis did not produce the α4 phenotype (van Heuvel et al, 1988, appendix paper III). Preliminary results obtained with a series of hybrids between α2 and α4, recombined at position 58/59 and position 129/130, clearly indicated a role of the C-terminal part of α4, from position 130-166, in determining its high activity. In this region three amino acid residues of α4, at position 133 (lysine instead of glutamate), 137 (leucine instead of proline) and 143 (isoleucine instead of valine) (see Fig. 3), differ from residues conserved in all other MuIFN-α subspecies. The substitution of the acidic glutamate for the basic lysine is of special interest for antiviral activity, considering the results of Weber et al (1987). They proved the importance of basic residues in a region from position 121 to 132 for activity on murine cells (see 4.2.1.3.).

4.2.2.2. In vivo recombinants

The antiviral activities of hybrids generated in vivo between Mu α1 and α4 (Zwarthoff et al, 1987, appendix paper IV) are depicted in Table 10, in the same way as has been done for the human in vivo recombinants. Here, a 100-fold increase in activity on hamster cells occurred when residues from position 17-20 were derived from α1 instead of α4. The activity on murine

Table 10. Specific antiviral activity of murine in vivo generated hybrids on hamster and murine cells (Zwarthoff et al, 1987, appendix paper IV). The domains indicated are responsible for the depicted transition in activity.

IFN	Domain	Specific antiviral activity*	
		hamster	murine
α4		1.5	1000
α1α4	5-10		1000-3700
	17-20	1.5-150	3700-20,000
	55-67		20,000-600
	130-166		600-150
α1		150	150

*see Table 4

cells also increased in this area. It is close to the 22-27 domain found to be important for the antiviral activity of Hu αIFNs on human cells. In addition, a transition in MuIFN activity on murine cells occurred in domain 55-67. The α1α4 hybrids with cross-over points between residues 20 and 55 all showed a 10- to 100-fold higher activity on murine cells than both parental molecules. A combination of one or more α1 amino acid residues at position 10-20 and one or more α4 residues at position 55-67 is responsible for this phenomenon (Fig. 3). Two striking features of the α4 primary sequence in the 10-20 region can be observed: the presence of glycine at position 10 (all other Mu αIFNs contain arginine) and two negatively charged glutamate residues at position 19 and 20. The nature of the relatively similar leucine (α1) or valine (α4) at position 17 most likely has no or minor influence on antiviral activity.

An α4 mutant with arginine at position 10 was found to display the same properties as the α1α4 hybrid recombined between position 14 and 16 (α4, Q5.H7,R10). These data indicate that the small (4-fold) increase in antiviral activity of this hybrid on murine cells can be attributed to the presence of arginine instead of glycine at position 10.

In the 55-67 region α1 differs from α4 in four positions: 55, 58, 59 and 67. The residues at position 55 (leucine in stead of proline) and 58 (arginine instead of serine) are the most likely candidates for mediating the high activity of α1α4 hybrids on murine cells.

A small decrease in antiviral activity on murine cells

occurred if Mu α4 residues were replaced by α1 residues in a region past position 120 (Table 10). This is in agreement with the data obtained by α2α4 and α4α2 in vitro recombinants, which showed involvement of a region past position 130 in antiviral activity (4.2.2.1.).

The observation that a combination of two domains originating from different IFN subspecies considerably enhanced antiviral activity immediately raised the question as what might be the antiviral activity of the reverse hybrids. Analysis of antiviral activities of α4α1 in vivo recombinants revealed that hybrids recombined between position 10 and 67 have an extremely low antiviral activity on murine cells (Zwarthoff, unpublished results).

4.2.2.3. Site-directed mutants

Site-directed mutagenesis was used to remove the N-glycosylation site (at position 78) from Mu α1 and α2 and for introduction of this site into Mu α6 (van Heuvel, unpublished results). The latter protein is devoid of a N-glycosylation site. The presence or absence of a N-linked carbohydrate chain did not have any effect on either production (in COS cells), specific antiviral activity or stability of MuIFN-α proteins. Remarkably, α6 (glycosylated or not) was shown to be more stable at 37° C than all other MuIFN-α proteins.

Consistent with the importance of arginine at position 33 for HuIFN-α activity (4.2.1.3.), the substitution of this residue for glutamate in MuIFN-α1 resulted in a protein with undetectable antiviral activity (Kerry et al, 1988). Likewise, tyrosine 123 was shown to be important for both Hu and MuIFN-α activity. The conservative substitution of tyrosine for phenylalanine in Mu IFN-α1 decreased antiviral activity 10-fold; a replacement by serine, however, produced a 300-fold decrease. Only a slight decrease in antiviral activity was observed when cysteine 86, conserved in all MuIFN-α subspecies, was replaced by serine (Kerry et al, 1988).

```
                10          20          30          40
Bo αC CHLPHTHSLA NRRVLMLLGQ LRRVSPSSCL QDRNDFAFPQ EALGG
Hu α7 .D..Q....R ...A.I..A. MG.I..F... K..HE.R..E .EFD.

                10              2    5   20    0.3    15
```

Fig. 4. Amino acid sequence of the N-terminal region of BoIFN-αC and HuIFN-α7. In the lower line the enhancement of antiviral activity on human cells is indicated, occurring upon substitution of bovine amino acids for underlined human residues.

4.2.3. Antiviral activity of interspecies hybrids

Shafferman et al (1987) constructed hybrids between HuIFN-α7 and BoIFN-αC. The latter has an extremely low activity on human cells. Substitution of a region from position 10-44 in αC for corresponding α7 sequences enhanced antiviral activity on human cells 10^4-fold. As the specific activity of α7 on human cells is more than 10^5-fold higher than that of αC, amino acids past position 45 also affect antiviral activity, but to a far lesser extent. An evaluation of the data of Shafferman is depicted in Fig. 4. Amino acids at position 10, 31 and 42-44 are shown to have the largest impact on antiviral activity.

The antiviral activities of hybrids between Hu and MuIFN-α subspecies reported by Raj et al (1988) were consistent with the data on human and murine subspecies presented in the previous paragraphs. The origin of the N-terminal 122 amino acids was important for activity on human cells. High activity on murine cells was largely determined by a region located past position 122 and to a lesser extent by the middle part (position 63-122) of the molecule. The latter region, however, was mainly responsible for the high activity of Hu αIFNs on bovine cells, while a smaller contribution was provided by the N-terminal 62 amino acids.

4.3. Structural characteristics of IFN: implications for interaction with the receptor

4.3.1. Structural models of IFN

In the previous chapter several domains have been localized which affect the antiviral activity of Hu and MuIFN-α proteins.

These domains can either directly be involved in receptor binding, influence secondary or tertiary structure of the protein or display both characteristics. Knowledge of the tertiary structure of IFN proteins may provide data to discriminate between these possibilities. Due to the extensive structural homology between IFN-α proteins from different species, these proteins most likely display a similar tertiary structure. X-ray diffraction data are not yet available for IFN proteins and existing structural models are based upon secondary structure prediction methods. Several structural models for IFN indicate that its secondary structure is mainly α-helical and that α and βIFNs adopt a similar conformation (see below). Analysis of circular dichroism spectra of IFN-α proteins (Bewley et al, 1982, Manavalan et al, 1984, Davis et al, 1987) and data obtained by Raman spectroscopy (Williams, 1985) were consistent with this finding. In addition, the conformation of fibroblast and E. coli derived IFN-β is also highly α-helical, as shown by circular dichroism and nuclear magnetic resonance (Utsumi et al, 1986).

In Fig. 5 the location of α-helices according to four secondary structure models is indicated. Three models are based upon the amino acid sequences of HuIFN-β and one or two HuIFN-α subspecies (Sternberg and Cohen, 1982, Zav'yalov and Denesyuk, 1982, Ptitsyn et al, 1985). Raj et al (1988) used no β but several human and murine α subspecies. Sternberg and Cohen (1982) and Raj et al (1988) have used the empirical rules of Chou and Fasman (1978) and Garnier et al (1978) for their secondary structure prediction. In addition, Sternberg and Cohen included the method of Lim (1974) and Raj the method of Cohen and Parry (1986). These methods take into account the hydrophobic interactions occurring between α-helical segments. Zav'yalov and Denesyuk (1982) used their own method (Zav'yalov, 1973), combined with those of Chou and Fasman (1974) and Lim (1974). Ptitsyn et al (1985) also used their own method (Ptitsyn and Finkelstein, 1982), which takes into account local interactions within a chain and long-range interactions between different regions of the protein.

As can be seen in Fig. 5, the four models do not diverge significantly in their location of the helices A, B and C. Helix D was not found with the calculations of Sternberg and Cohen, but

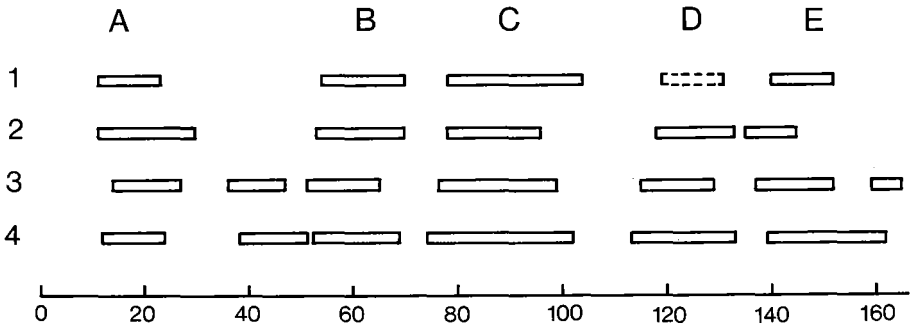


Fig. 5. Location of 5 α-helices in IFN-α,β according to four secondary structure models. 1. Sternberg and Cohen (1982), 2. Zav'yalov and Denesyuk (1982), 3. Ptitsyn et al (1985), 4. Raj et al (1988). In two models an additional helix between A and B is present. Helix D was not predicted by Sternberg and Cohen, but was suggested by these authors after examination of the primary structure in this region.

these authors did suggest the possibility of helix formation in a region from position 120 to 131. Regarding helix E, some variation in length and location can be observed. In two models, those of Ptitsyn and Raj, an additional helix around position 30 to 40 was predicted. In contrast to all other models, an alternative model of Ptitsyn et al (1985), which is not shown here, postulated the presence of β-sheets from position 125 to 155. Two isolated HuIFN-α fragments. a 16 residue (53-68) and a 34 residue fragment (117-150) were shown to be α-helical in nature (Clementi, 1985). In agreement with the location of helix D and E, the larger fragment contained two helices which were folded back upon each other.

4.3.2. Characteristics of regions involved in antiviral activity

Now that the approximate size and location of at least five

helical segments in the IFN protein are predicted, attempts can be made to assess regions found to be important for antiviral activity to either a helix or a segment in between two helices. In Table 10 an overview is given of those regions in which amino acid substitutions were shown to display an effect on antiviral activity. As can be seen, only the 5-11 and the 30-33 region are located outside an α-helix.

After examination of helix A no extensive clustering of apolar and polar residues at one side of the helix can be observed. Two negatively charged glutamate residues at position 19 and 20 of MuIFN-α4 have a disadvantageous effect on its activity on hamster and to a lesser extent on murine cells (4.2.2.2.). In helix A in none of the other IFN-α proteins negatively charged residues are found. In Hu αIFNs the presence of a positively charged arginine at position 22 and/or an apolar phenylalanine at position 27 enhance activity on human cells (4.2.1.2.).

Past helix A, from position 31-35, a cluster of five charged residues can be observed, conserved in Hu and Mu αIFNs. Only HuIFN-α1 contains an uncharged methionine at position 31. Upon replacement by lysine activity on human and murine cells is enhanced a factor four (4.2.1.2.). The mutants described by Camble et al (1986, 4.2.1.3.) and Kerry et al (1988, 4.2.2.3.) prove the importance of an apolar residue present at position 30 and arginine at position 33. The charge and chain length of the

Table 10. Location of regions or single amino acids involved in antiviral activity of Hu and Mu αIFNs on human, murine and hamster cells in a helix or an intrahelical segment.

Region	IFN Species	Cell type	Location
5-11	human	murine	just outside helix A
10	human	human	"
10	murine	murine	"
17-20	murine	murine,hamster	helix A
22-27	human	human	(end of) helix A
30-33	human	human,murine	intrahelical segment
33	murine	murine	"
55-67	murine	murine	helix B
80, 86	human	murine	helix C
68-123	murine	murine,hamster	
121-132	human	murine	(end of) helix D
123	murine	murine	"
130-166	murine	murine	

residue at position 32 does not seem to be very important. In contrast, arginine 33 cannot be replaced by another amino acid, not even by lysine, without deleterious effects on antiviral activity.

Helix B is strongly amphipathic, both in Hu and MuIFNs. Four residues in this helix were found to be involved in activity of Mu α1α4 hybrids on murine cells (4.2.2.2.). The changes at position 67 (at the apolar side of the helix) and at position 59 (exchange of one negatively charged residue for another) probably do not influence antiviral activity very much. More likely, the presence of a positively charged arginine at the polar side of helix B (at position 58) favours activity of α1α4 IFNs on murine cells, however, only in combination with the absence of one or two negatively charged residues in helix A. In addition, α4 contains leucine at position 55, which is proline in all other MuIFNs. This might extend helix B in N-terminal direction, which on its turn can alter the structure of the protein.

In the C-terminal part of the HuIFN protein, the presence of murine residues at position 80 (threonine) and 86 (cysteine) in helix C (also amphipathic in character) and of positively charged residues at position 121, 125 and 132 in helix D (weakly amphipathic, but residues 121, 125 and 132 are located at one side of the helix, Weber et al, 1987) enhance its activity on murine cells (4.2.1.2., 4.2.1.3.). Therefore it is not unlikely that in MuIFN-α4 the presence of another positively charged residue at position 133 is involved in its high activity on murine cells. In all other Hu and Mu αIFNs, except for Hu α16, a negatively charged glutamate occurs at this position. Remarkably, most of the substitutions which favour activity on murine cells, at position 10, 19 and/or 20, 31, 80, 125, 132 and perhaps 133, remove a negatively charged and/or add a positively charged residue. The conserved tyrosine at position 123 was shown to be important for both Hu and MuIFN-α activity (4.2.1.3., 4.2.2.3.).

From position 136 to 148 in Hu and Mu α and βIFNs, an extremely well conserved cluster of amino acids can be observed, which is largely located in helix E.

4.3.3. Hypothetical receptor binding sites

Since α and βIFNs bind to the same cellular receptor and, in addition, are structurally very similar, it is reasonable to postulate that their putative receptor binding sites are located in the same region of the molecule and contain several residues which are conserved within IFN-α and -β proteins. Domains located in the N-terminal (position 1-60) and in the C-terminal (position 121-132) region were shown to have a large impact on antiviral activity. Some experimental evidence has been obtained for the presence of at least one receptor binding site in the N-terminal part of the IFN protein. A HuIFN-α2 fragment comprising the N-terminal 110 amino acids displayed low antiviral activity (Ackerman et al, 1984). We have expressed truncated IFN-α genes in monkey COS cells and found that proteins which contained only the first 58 or 67 amino acid residues possessed a low, but distinct antiviral activity (van Heuvel, unpublished results).

Usually, sites for molecular recognition in proteins are found in loops or turns, whereas α-helices are more involved in maintaining the structural integrity of the protein. Loops or turns can serve as loci for receptor binding, antibody recognition or post-translational modification (Rose et al, 1985). From position 29-39 a region is located, which is conserved in α and βIFNs, is not α-helical in structure, and contains a cluster of charged residues (Fig. 3). For protein-protein interactions as occurring in antigen-antibody complexes, the presence of hydrophobic and aromatic residues was shown to be of special importance (Berzofsky, 1985, Huber, 1986). Aromatic and/or hydrophobic residues conserved in all α and βIFNs can also be observed in this region, at position 30 (leucine), 36 (phenylalanine), 38 (phenylalanine or hydrophobic) and 39 (proline). In addition, a conserved charged residue occurs at position 33 (arginine). The apolar character of the residue at position 30 and the integrity of arginine at 33 were shown to be of special importance for antiviral activity (Camble et al, 1986, Kerry et al, 1988). The observation that even minor alterations at position 33 are not allowed suggest the possibility that arginine forms a contact point with the receptor.

Interleukin-2 (Il-2) was shown to possess a similar

secondary structure as the structure predicted by Sternberg and Cohen (1982) for α and β IFNs (Cohen et al, 1986). A putative Il-2 receptor-binding site has been proposed in a region with a high proportion of hydrophobic and aromatic residues conserved between human and murine Il-2. This region was suggested to be structurally analogous to a region from position 26-38 in IFN-α (Cohen et al, 1986), which is postulated here to be involved in IFN receptor-binding. Mutation of phenylalanine at position 42 of the putative Il-2 receptor binding domain resulted in reduction of binding affinity without conformational peturbation of the Il-2 protein (Weir et al, 1988). In contrast, others suggested that residues in the above mentioned Il-2 region rather play a structural role than contribute to receptor-binding (Zurawski and Zurawski, 1988).

Besides conservation of important amino acids, a certain degree of variation must occur to account for species specificity and divergent antiviral activity of IFN-α subspecies. As has been shown, amino acid changes which affect antiviral activity are not limited to a distinct area but are distributed over the entire protein. For instance, the data on antiviral activity of HuIFN-α proteins on murine cells show that a change in species specificity can be accomplished by a series of amino acid substitutions occurring throughout the whole molecule, namely at position 10, 31, 80, 86, 121, 125 and 132. Considering the remarks made in the beginning of this paragraph, it is unlikely that these changes all interfere directly with receptor binding. Amino acid substitutions in the helices A and B, surrounding the receptor binding site postulated above, were shown to affect antiviral activity (Zwarthoff et al, 1987, appendix paper IV, Weber et al, 1987). This can occur through a structural change in one or both helices which on its turn modifies the conformation of the region in between these helices.

The existence of the C29-C139 disulfide bridge makes it plausible that the changes in helix D, described by Weber et al (1987), are also able to influence conformation of the domain around position 33. However, in helix D, from position 123-131, also conserved hydrophobic and aromatic residues can be observed. At position 123, 124 and 130 aromatic residues (tyrosine or phenylalanine) occur and at position 127 and 131 hydrophobic

residues. The two tyrosine residues at position 123 and 130 are located at one side of the helix, but at the opposite side of the residues at position 121, 125 and 132, identified by Weber et al (1987). The tyrosine at position 123 was shown to be important for antiviral activity (Nisbet et al, 1985, Kerry et al, 1988). Therefore, in addition to a structural role of helix D, it is not unlikely that one or more residues in this helix interact with the receptor.

In the extreme C-terminal part of the IFN protein no receptor binding site is present, because binding of IFN to its receptor did not prevent subsequent binding of a monoclonal antibody directed to the C-terminal 16 amino acids of the protein (Arnheiter et al, 1983).

4.4. Comparison of different biological activities of IFN

In addition to localization of a receptor binding site, a comparison of receptor binding and different biological activities of series of natural and hybrid IFN molecules can contribute to the elucidation of the molecular mechanism of IFN action. If the affinity of IFN for its receptor is the only determinant for the magnitude of the biological response, the ratio of various biological activities of different IFN subspecies will always be the same. The alternative possibility, the IFN receptor contains multiple binding sites which differentially respond to various domains on IFN molecules, will implicate that this ratio can vary between different subspecies.

A first comparison of antiviral and antiproliferative activities of various IFN-α subspecies has been performed by the group of Pestka using HuIFN-α subspecies purified from natural leukocyte IFN (Evinger et al, 1981b). The ratio between antiviral and antiproliferative activity was found to differ for each subspecies. Similar observations were made with recombinant IFN-α1 and -α2 and their hybrids (Rehberg et al, 1982). However, in both cases too much emphasis has been put on relatively small differences between the antiviral or the antiproliferative activities of various IFN proteins, which can easily lead to overinterpretation of the variation observed in ratios of antiviral and antiproliferative activity. The same argumentation

holds for the data of Stewart et al (1986) on antiviral and antiproliferative activities of various HuIFN-β mutants.

Those investigators who have compared absolute values of antiviral and antiproliferative activity of IFN-α subspecies (Goren et al, 1983) and their hybrids (Meister et al, 1987, Fidler et al, 1987) observed a clear similarity between these activities. We have compared the antiproliferative activity of two MuIFN-α subspecies with greatly divergent antiviral activities on hamster cells (van Heuvel et al, 1988, appendix paper VI) and our data indicated a strict correlation between these two activities. In addition, the antiproliferative response of hamster cells was nicely reflected by the IFN-induced expression of ISG 54 (van Heuvel et al, 1988, appendix paper VI and unpublished results).

In addition to biological activity, many investigators have determined the affinity of natural and hybrid IFNs for their cellular receptor (Yonehara et al, 1983c, Aguet et al, 1984, Uzé et al, 1985, Hannigan and Williams, 1986, Meister et al, 1986, Shafferman et al, 1987). In all cases a clear correlation between biological response and receptor binding could be detected.

In summary, all data on biological activity versus receptor binding favour the hypothesis that the affinity of IFN for its receptor is the only variable which determines the magnitude of the biological response.

4.5. Further investigation

A putative receptor binding site has been postulated in a conserved region located between helix A and B, whereas helix D was also suggested to possess characteristics of domains involved in protein-protein interaction. No experimental evidence has been delivered for this hypothesis and it cannot be excluded that one or more of the domains, which were suggested to influence conformation of the IFN-α protein, are directly involved in receptor binding.

Several options can be employed to investigate in more detail the relation between structure and activity of an IFN protein. The impact of certain domains or individual amino acids on biological activity or receptor binding can be investigated by

substitution of important amino acids for a series of more or less related residues using site-directed mutagenesis. However, it will be difficult to ascertain whether a particular domain exerts its influence directly, via binding to the receptor or indirectly, by affecting protein structure. Additionally, as it has been shown that several MuIFN-α1α4 hybrids have a higher activity than the parental proteins, knowledge of domains important for activity can be used to improve IFN proteins.

Synthetic peptides are used more and more frequently to investigate the involvement of particular protein domains in receptor binding. Several peptide fragments have been reported which compete with native ligand for binding to the receptor (Komoriya et al, 1984, Nestor et al, 1985, Heath et al, 1986, Appella et al, 1987, Keutmann et al, 1987) or even possess biological activity (Komoriya et al, 1984, Heath et al, 1986, Keutmann et al, 1987, Cardin et al, 1988). However, as compared to the native ligand, a large molar excess of the synthetic peptide, varying from 10^3 to 10^6 times, is required to establish these effects.

A completely different method to localize amino acids involved in receptor binding is chemical modification of amino acid residues. Differences in labelling of amino acids in free versus bound ligand identifies those residues which are protected against modification by bound receptor (Maly and Lüthi, 1988).

Molecular cloning of the IFN receptor is required to be able to investigate ligand-receptor interaction from the other end. With the progress in this area, as has been reported at international congresses, this will be more a matter of months than years.

Last but not least, only a model of the tertiary structure of IFN accomplishes the study on structure-activity relationships of IFN proteins. X-ray diffraction analysis of IFN and IFN-receptor complexes ultimately will define those domains which interact with the receptor.

5. SUMMARY

IFN has been defined as an antiviral agent, but, in addition, displays antiproliferative and immunomodulatory activities. It can be divided in three distinct subtypes: α, β and γ. IFN-α is encoded by a multigene family and comprises a group of structurally very related proteins. Despite this strong homology there exists a considerable variation in biological activity between the various IFN-α proteins. As a consequence, these proteins are an ideal subject for a detailed structure-function analysis. In this thesis data are presented on structure-activity relationships of several Mu IFN-α subspecies.

Normally cells do not express IFN. They start to secrete IFN molecules after exposure to an inducing agent, for instance a virus. In Chapter 2 molecular details with regard to the regulation of IFN expression are discussed. After IFN has left the cell it binds to a high affinity receptor situated on a neighboring cell or on the producing cell itself. This IFN-receptor interaction initiates a cascade of events, which ultimately leads to modulation of the expression of at least 25 genes (Chapter 3). It is not known as yet which part of the IFN protein is involved in interaction with the receptor and mediates biological activity. To elucidate this question several groups have constructed hybrid and mutant IFN molecules. Subsequently, the biological activities (mainly antiviral) of the modified IFNs have been compared to those of the parental proteins. Our current knowledge on structure-activity relationships of IFN proteins is, for a great deal, based on studies performed with Hu and Mu IFN-α proteins. An overview of the data obtained in this field is presented in Chapter 4. In the same chapter structural characteristics of the regions found to be responsible for changes in antiviral activity of modified IFN proteins are discussed and the possible location of receptor binding sites is suggested.

A technically simple and efficient expression system is required to obtain sufficient amounts of large series of natural and modified IFNs for initial comparison of their antiviral activities. In Paper I the transient expression of Mu IFN-α in

monkey COS and mouse TOP cells is described. In addition, several promoters have been analyzed for efficient IFN production. No large variations in IFN expression were observed between the promoters analyzed and between mouse and monkey cells. The substitution of the 3'-noncoding region of IFN for 3'-flanking sequences of the β-globin gene resulted in an at least 4-fold enhancement of IFN expression. As all Mu IFN genes isolated sofar have been cloned into the eukaryotic expression vector pSV328A (Paper II), this vector has been chosen for all further expression experiments (in COS cells).

Several Mu IFN-α genes have been isolated at our laboratory. In Paper II the specific antiviral activities of the protein products encoded by the α1, α2, α4 and α6 genes are presented. Considerable variation in antiviral activity on both murine and hamster cells was observed. The α4 protein, which contains a deletion of five amino acids (from position 103-107), displayed the highest activity on murine and the lowest on hamster cells. Except for α6, all Mu αIFNs contain a N-glycosylation site and were indeed found to be glycosylated. The unglycosylated α6 had a similar antiviral activity on both murine and hamster cells as the glycosylated α1. Improvement of the transfection method and digestion of IFN expression plasmids with restriction enzymes which practically removed all plasmid sequences resulted in an obvious increase in IFN production.

A first set of four hybrids between α1, α2 and α4 (Paper II), constructed by the use of common restriction enzyme sites within the genes, has been extended with additional series of hybrids (Paper III). Antiviral activity on hamster cells was influenced by a domain located in the N-terminal part of the protein (position 1-58). In contrast, a determinant for activity on murine cells was located in the C-terminal part (position 59-166). The five amino acid deletion present in this region of α4 did not affect antiviral activity when introduced into α2 or an α4α2 hybrid by site-directed mutagenesis. Preliminary evidence indicates involvement of residues past position 130 in mediating the high activity of α4 on murine cells.

To reduce the size of the regions which were shown to affect antiviral activity, an alternative method has been developed for the construction of hybrids between two IFN-α genes. Due to the

extensive homology in nucleotide sequence of the various IFN-α genes the homologous recombination system of E. coli can be used to generate hybrids with recombination sites throughout the whole molecule. This method was successfully applied to Mu IFN-α1 and α4 (Paper IV). The resulting hybrid genes were all shown to encode biologically active IFNs. A 100-fold increase in activity on hamster cells occurred when α4 residues were replaced by α1 residues from position 17-20. Remarkably, several α1α4 hybrids were obtained which displayed a 10- to 100-fold higher activity on murine cells than the parental proteins. A combination of α1 residues from position 10-20 and α4 residues from position 55-67 was responsible for this phenomenon.

The isolation of an additional Mu IFN gene, IFN-α10, is described in Paper V. Although at the amino acid level strong homology was observed with Mu α1, the antiviral activity of α10 on both murine and hamster cells was 10 times lower than the activity of α1 on these cells. A region from position 68-123 was found to be involved in the low activity of α10 on murine and hamster cells.

The growth inhibitory effect on hamster cells of two Mu IFN-α subspecies with divergent antiviral activity on these cells has been determined. A strong correlation between antiviral and antiproliferative activity could be observed (Paper VI). In addition, the magnitude of the antiproliferative response was found to be related to the level of induction of the ISG 54 mRNA. These observations are consistent with the hypothesis that the affinity of IFN for a cell surface receptor is the only determinant of the magnitude of the biological response (see Chapter 4.4. for a more detailed discussion on this subject). To obtain sufficient amounts of Mu IFN-α subspecies for further characterization, several CHO cell lines have been constructed which constitutively produce IFN. These cell lines were found to be resistant to the antiproliferative activity of IFN, but were still protected against viral infection by the Mu IFN they produced. This resistance was correlated to a strongly reduced level of ISG 54 mRNA. In combination with the data on the dose-response effect of IFN on antiproliferative activity and ISG 54 mRNA induction, this suggests that the ISG 54 gene product might be involved in the antiproliferative response of CHO cells to

IFN.

Several domains have been localized which can influence the antiviral activity of an IFN protein. Two possibilities exist: the domains act directly, via interaction with the receptor, or indirectly, by modulating the conformation of the receptor binding site(s) via structural changes in the protein. Obvious approaches for further investigation are: site-directed mutagenesis of important amino acids, analysis of activity/competition of synthetic peptides corresponding to different domains within the IFN protein, chemical modification studies and X-ray diffraction of IFN crystals. The latter will eventually result in a model of the tertiary structure of IFN.

SAMENVATTING

IFN is per definitie een eiwit met antivirale aktiviteit, maar heeft daarnaast ook celgroeiremmende eigenschappen en kan bepaalde funkties van het immuunsysteem moduleren. IFN kan onderverdeeld worden in drie verschillende subtypen: α, β en γ. IFN-α wordt gekodeerd door een genfamilie en bestaat uit een groep van struktureel zeer op elkaar lijkende eiwitten. Ondanks deze sterke homologie bestaat er een aanzienlijke variatie in biologische aktiviteit tussen de verschillende IFN-α eiwitten. Om die reden zijn deze eiwitten uitermate geschikt voor een gedetailleerde struktuur-funktie analyse. In dit proefschrift worden gegevens gepresenteerd betreffende struktuur-aktiviteit relaties van verschillende muize IFN-α subspecies.

Normaal gesproken komt IFN niet tot expressie in de cel. Cellen gaan pas IFN uitscheiden na in kontakt te zijn geweest met een induktor, bijvoorbeeld een virus. In Hoofdstuk 2 worden molekulaire details betreffende de regulering van IFN expressie besproken. Nadat IFN de cel verlaten heeft bindt het aan een receptor met een hoge affiniteit voor IFN, die zich op een naburige cel bevindt of op de producerende cel zelf. Deze IFN-receptor interaktie zet een reeks van gebeurtenissen in werking, hetgeen uiteindelijk leidt tot een verandering van de expressie van tenminste 25 genen (Hoofdstuk 3). Het is nog niet bekend welk deel van het IFN eiwit betrokken is bij interaktie met de receptor en biologische aktiviteit bewerkstelligt. Om dit vraagstuk op te helderen hebben verschillende groepen hybride en mutante IFN molekulen gemaakt, waarna de biologische aktiviteiten (voornamelijk antiviraal) van de gemodificeerde IFNen vergeleken zijn met die van de oorspronkelijke eiwitten. Onze huidige kennis van de struktuur-aktiviteit relaties van IFN eiwitten is voor een groot deel gebaseerd op studies die uitgevoerd zijn met humane en muize IFN-α eiwitten. Een overzicht van de gegevens die op dit terrein verkregen zijn wordt gepresenteerd in Hoofdstuk 4. In ditzelfde hoofdstuk worden strukturele kenmerken besproken van de gebieden die verantwoordelijk zijn voor veranderingen in antivirale aktiviteit van gemodificeerde IFN eiwitten en worden mogelijke posities voor receptorbindingsplaatsen gesuggereerd.

Een technisch eenvoudig en efficient expressiesysteem is nodig om van een grote reeks natuurlijke en gemodificeerde IFNen voldoende materiaal in handen te krijgen voor een eerste vergelijking van hun antivirale aktiviteiten. In Publikatie I wordt de kortdurende expressie van muize IFN-α in ape COS en muize TOP cellen beschreven. Bovendien zijn verschillende promotoren getest op hun vermogen IFN te produceren. Er werden geen grote verschillen in IFN expressie gevonden tussen de verschillende promotoren en tussen muize en ape cellen. De vervanging van het 3'-niet koderende gebied van IFN door 3'-flankerende sekwenties van het β-globine gen resulteerde in een minstens viervoudige verhoging van IFN expressie. Aangezien alle muize IFN genen die tot dan toe geïsoleerd waren in de eukaryote expressievektor pSV328A gekloneerd zijn, is deze vektor gekozen voor alle verdere expressie experimenten (in COS cellen).

In ons laboratorium zijn verschillende muize IFN-α genen geïsoleerd. In Publikatie II worden de specifieke antivirale aktiviteiten van de eiwitprodukten gekodeerd door de α1, α2, α4 en α6 genen gepresenteerd. Er werd een aanzienlijke variatie in antivirale aktiviteit op muize en hamster cellen gevonden. Het α4 eiwit, dat een deletie van vijf aminozuren bevat (positie 103-107), vertoonde de hoogste aktiviteit op muize en de laagste op hamster cellen. Alle muize α IFNen, behalve α6, bevatten een N-glycosyleringsplaats en bleken inderdaad geglycosyleerd te worden. Ongeglycosyleerd α6 had dezelfde aktiviteit op muize en hamster cellen als geglycosyleerd α1. Verbetering van de transfektiemethode en digestie van IFN expressieplasmiden met restriktie enzymen die bijna alle plasmide sekwenties verwijderden resulteerde in een duidelijke toename van expressie.

Een eerste groep van vier hybriden tussen α1, α2 en α4, gemaakt met behulp van gemeenschappelijke restriktie enzym knipplaatsen in de genen (Publikatie II), is uitgebreid met nieuwe series hybriden (Publikatie III). Antivirale aktiviteit op hamster cellen werd beïnvloed door een domein gelegen in het N-terminale gedeelte van het eiwit (positie 1-58). Een determinant voor aktiviteit op muize cellen was daarentegen gelokaliseerd in het C-terminale gedeelte (positie 59-166). De deletie van vijf aminozuren aanwezig in dit gedeelte van α4 had geen invloed op antivirale aktiviteit wanneer deze door middel van plaatsgerichte

mutagenese in α2 of een α2α4 hybride geïntroduceerd werd. Er zijn aanwijzingen dat residuen na positie 130 betrokken zijn bij de hoge aktiviteit van α4 op muizecellen.

Om de grootte van de gebieden die de antivirale aktiviteit bleken te beïnvloeden te verkleinen is een andere methode ontwikkeld om hybriden te maken tussen twee IFN-α genen. Ten gevolge van de uitgebreide homologie in nukleotide volgorde van de verschillende IFN-α genen, kan het homologe rekombinatie systeem van E. coli gebruikt worden om hybriden te vervaardigen met rekombinatieplaatsen door het gehele molekuul heen. Deze methode is met succes toegepast op muize IFN-α1 en -α4 (Publikatie IV). Alle hybride genen die op deze manier geproduceerd zijn kodeerden voor biologisch aktieve IFNen. Een 100-voudige toename in aktiviteit op hamster cellen trad op toen α4 residuen van positie 17-20 werden vervangen door α1 residuen. Opmerkelijk is dat verschillende α1α4 hybriden werden verkregen met een 10 tot 100 maal hogere aktiviteit op muize cellen dan de oorspronkelijke eiwitten. Een kombinatie van α1 residuen van positie 10-20 en α4 residuen van positie 55-67 was verantwoordelijk voor dit verschijnsel.

In Publikatie V wordt de isolatie van nog een muize IFN gen, IFN-α10, beschreven. Alhoewel een sterke homologie op aminozuur niveau werd waargenomen met muize α1, was de aktiviteit van α10 op muize en hamster cellen 10 maal lager dan de aktiviteit van α1 op deze cellen. Een gebied van positie 68-123 bleek betrokken te zijn bij de lage aktiviteit van α10 op muize en hamster cellen.

Het groeiremmend effekt op hamster cellen van twee muize IFN-α subspecies met uiteenlopende antivirale aktiviteit op deze cellen is bepaald. Er werd een sterke correlatie tussen antivirale en antiproliferatieve aktiviteit waargenomen (Publikatie VI). Bovendien bleek de hoogte van de anti-proliferatieve respons in verhouding te staan tot het niveau van induktie van ISG 54 mRNA. Deze waarnemingen zijn in overeenstemming met de hypothese dat alleen de affiniteit van IFN voor een membraanreceptor bepalend is voor de hoogte van de biologische respons (zie Hoofdstuk 4.4. voor een meer uitgebreide diskussie over dit onderwerp). Om voor verdere karakterisering een voldoende hoeveelheid muize IFN-α subspecies te verkrijgen zijn verschillende CHO cellijnen gekonstrueerd die konstitutief

IFN produceren. Deze cellijnen bleken resistent te zijn tegen de antiproliferatieve aktiviteit van IFN, maar werden nog wel beschermd tegen virale infektie door het muize IFN dat ze produceerden. Deze resistentie ging gepaard met een sterk verlaagd niveau van ISG 54 mRNA. Dit suggereert, in kombinatie met de gegevens over het dosis-respons effekt van IFN op antiproliferatieve aktiviteit en ISG 54 mRNA induktie, dat het ISG 54 gen produkt betrokken zou kunnen zijn bij de anti-proliferatieve respons van CHO cellen op IFN.

Er zijn verschillende domeinen gelokaliseerd, die de antivirale aktiviteit van een IFN eiwit kunnen beïnvloeden. Twee mogelijkheden bestaan: de domeinen werken direkt, via interaktie met de receptor, of indirekt, door wijziging van de konformatie van de receptor bindingsplaats(en) via strukturele veranderingen in het eiwit. Een duidelijke aanpak voor verder onderzoek is: plaatsgerichte mutagenese van belangrijke aminozuren, analyse van aktiviteit/kompetitie van synthetische peptiden overeenkomend met verschillende domeinen binnen het IFN eiwit, chemische modifikatie studies en Röntgen diffraktie van IFN kristallen. De laatste mogelijkheid zal uiteindelijk resulteren in een model voor de tertiaire struktuur van IFN.

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NAWOORD

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CURRICULUM VITAE

De auteur van dit proefschrift is geboren op 10 maart 1954 te Den Helder en heeft aldaar van 1966 tot 1971 de HBS-B opleiding gevolgd aan de openbare scholengemeenschap "Nieuwediep". Vervolgens heeft zij in 1974 aan het "Leids Opleidingsinstituut voor Analisten" het diploma HBO-B analytisch-chemische richting behaald en is zij van 1973 tot 1980 als analiste werkzaam geweest op een tweetal universiteitslaboratoria. In die tijd (in 1977) is een begin gemaakt met de biologiestudie (Bijzondere M.O.) aan de Rijksuniversiteit Utrecht. Het doktoraalexamen biologie is gedaan in 1984 aan de Rijksuniversiteit Leiden met als hoofdvak Molekulaire Genetika (Prof. Dr. P. van de Putte) en als bijvakken Medische Biochemie (Prof. Dr. W. Möller) en Molekulaire Plantkunde (Prof. Dr. K. Libbenga). Van 1984 tot 1988 is de auteur werkzaam geweest als wetenschappelijk assistent op de afdeling Pathologische Anatomie van de Erasmus Universiteit Rotterdam, alwaar het in dit proefschrift beschreven onderzoek uitgevoerd is. De laatste 1½ jaar zijn besteed aan een KWF projekt betreffende de rol van interferon in tumorgenese. Sinds 1 november 1988 is de auteur werkzaam op de afdeling Heterologe Genexpressie binnen de hoofdafdeling Research and Development van Gist-brocades N.V. te Delft.

PAPER I

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Transient expression of murine interferon-alpha genes in mouse and monkey cells

(Recombinant DNA; SV40 virus; enhancer; metallothionein promoter; long terminal repeat; plasmid vectors; Cos cells)

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SUMMARY

The coding regions of murine interferon-α (IFN-α) genes were combined with promoter and 3′-noncoding sequences from other eukaryotic genes. Transient expression of these fusion genes was achieved in monkey COS cells and in a mouse cell line (TOP cells) expressing polyoma virus (Py) large T antigen constitutively. The efficiency of the different expression plasmids was determined by measuring the amount of IFN secreted into the medium. Replacement of the 3′-noncoding region of an IFN-α gene by that of the rabbit β-globin gene resulted in a fourfold higher IFN-α production. The SV40 early promoter and the Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) produced similar amounts of IFN-α in COS cells. However, a tandem combination of the SV40 enhancer/early promoter and the mouse metallothionein-I promoter appeared fivefold more active than the SV40 early promoter. In TOP cells the MoMLV LTR was found to be threefold more active than the Py early promoter.

INTRODUCTION

IFNs are proteins with a complex range of activities which are found in a variety of higher organisms from fish to man. Among the activities of IFNs are their antiviral and antiproliferative properties and their ability to influence the immune system (see for review Finter, 1984). In mammals, there exist three antigenically distinct groups called IFN-α, -β and -γ. IFN-α and -β are transiently secreted by most tissues after viral induction. IFN-γ is, also transiently, secreted by lymphoid cells after mitogenic stimulation.

IFN-β and -γ are each encoded by a single gene. IFN-α consists of a group of highly related proteins, encoded by the IFN-α gene family (Weissmann and Weber, 1986). Despite the structural similarities between the IFN-α proteins there are profound differences in their biological properties, for instance

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Abbreviations: bp, base pair(s); DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; *gpt*, gene coding for *E. coli* xanthine-guanine phosphoribosyl transferase; IFN, interferon; IU, international units; kb, 1000 bp; LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; MT, mouse metallothionein-I; Nm, neomycin; nt, nucleotide(s); PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; Py, polyoma virus; ^{R}, resistance; RSV, Rous sarcoma virus; SV40, simian virus 40.

in their target-cell specificity and in the relative extent of their antiviral, antiproliferative and other responses (Lemson et al., 1984; Ortaldo et al., 1984; Rehberg et al., 1982; Van Heuvel et al., 1986).

We recently isolated several different murine IFN-α genes (Zwarthoff et al., 1985). To facilitate a characterization of the corresponding proteins we wished to express these genes efficiently and we decided to use a mammalian host system to obtain the products in a properly modified and secreted form. The ultimate goal of the experiments described here is to select the optimal construct for the establisment of cell lines producing large amounts of IFN constitutively. In stable transfectants the site of integration of the introduced DNA is thought to influence the level of expression. To avoid this problem we compared our constructs in a transient expression system. According to Mellon et al. (1981), insertion of fragments up to 8 kb into plasmids containing the SV40 origin of replication does not substantially influence the amount of replicating DNA in COS cells. This suggests that the differences found in expression levels between various plasmids in such a system can be related directly to the different regulatory regions. This report describes the construction of IFN expression plasmids, containing regulatory regions of several eukaryotic genes, and a comparison of their efficiencies in the production of IFN after transfer into mouse or monkey cells.

MATERIALS AND METHODS

(a) DNA manipulations

Plasmids were carried in *Escherichia coli* K-12 strain DH1 and were purified from chloramphenicol-amplified cultures by the alkaline-lysis method (Maniatis et al., 1982). Separation of plasmid DNA and RNA was achieved on a Biogel A-1.5m (BioRad) gel-filtration column. Restriction enzymes, alkaline phosphatase and T4 DNA ligase were from Boehringer Mannheim. DNA fragments were isolated from agarose gels by electroelution. All plasmid constructs were verified by restriction enzyme analysis.

(b) Cell culture and transfection

All cells were maintained in DMEM supplemented with 5% fetal calf serum, penicillin and streptomycin. For the construction of the TOP cell line cells were transfected by the calcium phosphate precipitation technique (Graham and Van der Eb, 1973). A selection marker was introduced by coprecipitation of a plasmid containing the Nm^R gene. Transfection of monkey COS (Gluzman, 1981) and mouse TOP cells (see RESULTS AND DISCUSSION, sections **b** and **c**) for transient expression was done either by calcium phosphate precipitation or by the DEAE-dextran method (McCutchan and Pagano, 1968). For these experiments the cells were seeded one or two days prior to transfection into 35-mm dishes and grown to about 30% confluency. For COS cells 100 μg/ml and for TOP cells 10 μg/ml of DEAE-dextran in serum free medium was used. In all transfections 0.3 pmol plasmid DNA per dish was added. After 0.5 to 4 h the medium containing DEAE-dextran was removed, and the cells were shocked with DMEM containing 10% DMSO for 2 min, washed twice and incubated in serum-containing medium. The next day the medium was changed and 72 h after transfection IFN activity was assayed in the medium. Induction of the MT promoter was achieved by addition of 5–30 μM cadmium sulfate 8 h prior to the IFN assay.

(c) Interferon assay

The IFN titre was determined in a cytopathic-effect reduction assay as described previously (Lemson et al., 1984) and calculated relative to the NIH reference standard G002-904-511 for mouse cells.

RESULTS AND DISCUSSION

(a) Effect of the 3′-noncoding region on expression

Plasmids used for the comparison of 3′-noncoding regions are shown in Fig. 1. They are based on the expression vector pSV328A. The construction of pSV328A, pSVα2 and pSVα4 will be described elsewhere (Van Heuvel et al., 1986). In

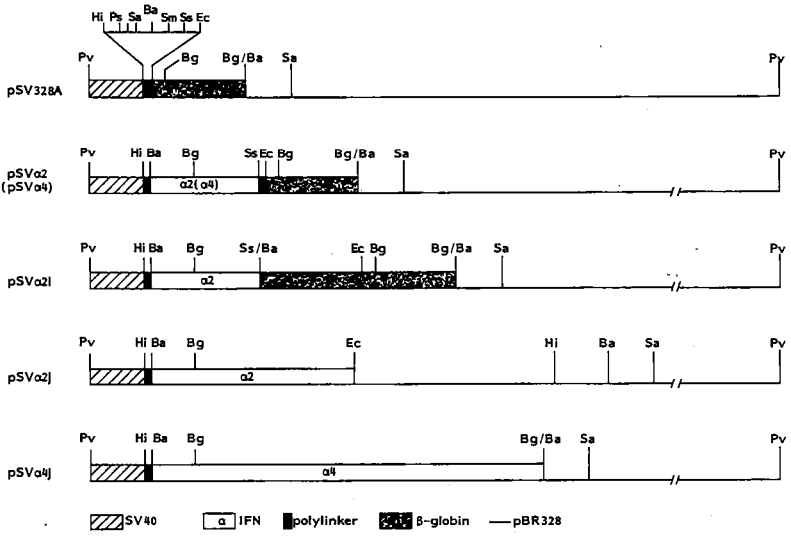

Fig. 1. Construction of plasmids used to compare the influence of the 3′-noncoding region on IFN expression. Only relevant restriction sites are shown: Ba, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Hi, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; Xb, *Xba*I. For the construction of pSVα2j the *Pvu*II-*Bgl*II fragment from pSVα2 (containing the SV40 promoter and the 5′-half of the gene) and a 1.3-kb *Bgl*II-*Eco*RI fragment (containing the 3′-half of the gene plus its 3′-noncoding sequences (Zwarthoff et al., 1985) were inserted into pBR328, between *Pvu*II and *Eco*RI. pSVα4j was obtained in a similar way by inserting the *Pvu*II-*Bgl*II fragment from pSVα4 together with a 2.1-kb *Bgl*II fragment (containing the 3′-half of the gene and its 3′-noncoding sequences) into pBR328A, between the *Pvu*II and *Bam*HI sites. The plasmid pSVα2i was constructed from pSVα2 by inserting a 0.64-kb *Bam*HI-*Eco*RI fragment (containing the last intron of the rabbit β-globin gene (Van Ooyen et al., 1979) between the *Sst*I and *Eco*RI sites, after both *Bam*HI and *Sst*I sites had been treated with Pollk to generate blunt ends.

pSVα2 and pSVα4 the coding regions of the IFN genes are inserted into the polylinker of pSV328A, thus placing the genes under control of the SV40 early promoter. In both plasmids the polyadenylation signal is provided by rabbit β-globin sequences. In pSVα2j and pSVα4j, both IFN genes are followed by their own 3′-noncoding sequences.

Monkey COS cells were transfected with equimolar amounts of the plasmids depicted in Fig. 1. The amount of IFN activity that was produced by each construct was assayed 72 h after transfection. The results of a representative experiment are presented in column four of Table I. To compare the efficiencies of the different constructs, the amount of IFN produced by the plasmids pSVα2j and pSVα4j was taken as 1 in every experiment and the activities produced by equimolar amounts of the other plasmids were related to these values. From a number (N) of experiments a mean ratio was calculated. This ratio ± the standard error is also given in Table I. In these experiments and in those described below the ratios found were independent of the transfection method used. Also different batches of plasmid DNA had no significant effect on the relative amount of IFN produced. It appeared that when the 3′-noncoding region of the IFN genes is replaced by that of the β-globin gene the IFN expression is increased at least 4-fold (e.g., pSVα2 vs. pSVα2j and pSVα4 vs. pSVα4j). A possible explanation for this effect is that the addition of a piece of β-globin mRNA stabilizes the resulting fusion mRNA. This assumption is based on the finding that β-globin mRNA has a half-life of 90 h (Vollock and Housman, 1981) whereas IFN mRNAs are thought to be very unstable (Raj and Pitha, 1981). It is surprising that the introduction of an intron, although a

TABLE I

Influence of the 3′-noncoding region on expression in COS cells

Plasmid (1)	3′-Noncoding (2)	Intron (3)	IFN[a] (4)	Mean ± S.E.[b] (5)	N[c] (6)
pSVα2j	α2	–	48	1	3
pSVα2	β-globin	–	240	5.3 ± 1.3	3
pSVα2i	β-globin	+	320	6.7 ± 1.3	3
pSVα4j	α4	–	640	1	3
pSVα4	β-globin	–	2560	4.0 ± 0.0	3

[a] IFN production is given in IU/ml.

[b] Mean activity calculated from a number of experiments [N, column (6)].

[c] Number of experiments.

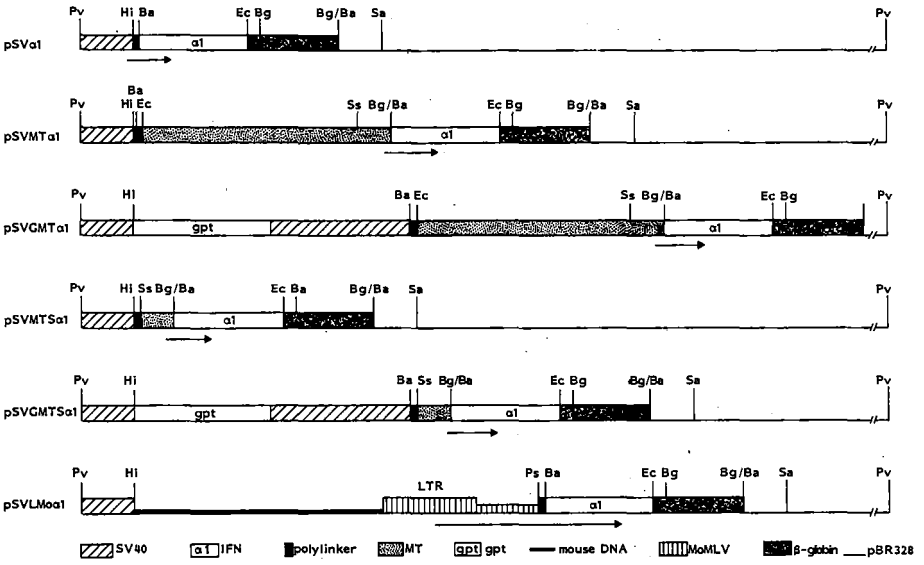

Fig. 2. Construction of plasmids used to compare the influence of different promoters on IFN expression in COS cells. For restriction sites see Fig. 1. For the construction of pSVMTα1, a 1.6-kb *Eco*RI-*Bgl*II fragment containing the MT promoter (Mayo et al., 1982) was first inserted into pSV328A, between the *Eco*RI and *Bgl*II sites. The MT promoter fragment was isolated from the resulting plasmid by digestion with *Bam*HI + *Bgl*II and subsequently inserted into the *Bam*HI site of pSVα1, thus creating pSVMTα1. pSVMTsα1 was constructed from pSVMTα1 by digestion with *Sst*I and recircularization of the plasmid. This resulted in a deletion of mouse MT sequences upstream of nt −150 from the transcription start point. The plasmids pSVGMTα1 and pSVGMTsα1 were constructed by insertion of a 1.8-kb *Hin*dIII-*Bam*HI fragment of pSV2gpt (Mulligan and Berg, 1981) between the *Hin*dIII and *Bam*HI sites of pSVMTα1 and pSVMTsα1, respectively. This fragment contains the *gpt* sequences followed by an SV40 polyadenylation signal. The *Hin*dIII-*Pst*I fragment of pMV5 (kindly provided by Dr. P. Luciw), containing the left LTR of MoMLV and flanking mouse DNA, was inserted into pSVα1, between the *Hin*dIII and *Pst*I sites, resulting in pSVLMOα1. Arrows represent the direction of transcription of the IFN-α1 gene.

prerequisite for the expression of some genes (Gruss and Khoury, 1980), did not significantly influence IFN expression (e.g., pSVα2i vs. pSVα2). It should be noted, however, that IFN-α and -β genes belong to the class of intronless genes.

(b) Influence of different promoters on expression in COS cells

Plasmids used for the comparison of different promoters are shown in Fig. 2. They are also based on the vector pSV328A (Fig. 1). All plasmids contain the murine IFN-α1 coding region and the 3'-noncoding region of the rabbit β-globin gene. The construction of pSVα1 will be described elsewhere (Van Heuvel et al., 1986). The promoters analyzed are the SV40 early promoter (pSVα1), the MoMLV LTR (pSVLMOα1) and the MT promoter. The latter has been used for several constructs which differ in the length of the MT promoter fragment (e.g., pSVMTα1 vs. pSVMTsα1) and in the absence or presence of the *gpt* gene (e.g., pSVMTα1 vs. pSVGMTα1).

Several transfection experiments were carried out with the plasmids depicted in Fig. 2. The data obtained are summarized in Table II. Column 2 shows the amount of IFN produced in a representative experiment. The relative activities of all constructs were calculated from a number (N) of experiments, taking the constitutive activity of the MT promoter in pSVMTα1 as 1 in every experiment. The values obtained are specified in column 3 of Table II. The data show that the activities of the MT promoter in pSVMTα1, the SV40 early promoter (pSVα1) and the MoMLV LTR (pSVLMOα1) do not differ significantly. From Table II it also appears that the constitutive activity of the MT promoter is twelve-fold higher when it is situated close to the SV40 enhancer/early promoter than when it is far removed from the SV40 sequences (compare pSVMTsα1 with pSVGMTα1 and pSVGMTsα1). This construct is also approx. five-fold more active than the SV40 promoter. Gorman et al. (1982) compared the RSV LTR with the SV40 early promoter in a transient expression experiment in monkey CV-1 cells (COS cells are CV-1 cells that produce SV40 large T antigen; Gluzman, 1981). They found that the avian virus LTR was about twice as active as the SV40 promoter, which, when combined with the results presented here, places its activity between that of the MoMLV LTR and the SV40-MT combination. To account for our results we propose that the increased activity of the MT promoter in pSVMTsα1 is at least partly due to a greater effect of the SV40 72-bp repeats when the MT promoter is brought closer to the SV40 sequences. In this construct the SV40 early promoter is situated between the enhancers and the MT promoter and it is known that enhancers primarily affect the nearest promoter (Wasylyk et al., 1983). Thus, perhaps an even stronger effect can be obtained if the enhancer is placed next to the MT promoter. Part of the activity of this tandem construct can also be assigned to transcription starting on the SV40 promoter. However, an addition of the activities of the two promoters alone is not sufficient to explain the increase in expression that we observed. The MT promoter can be induced by heavy metal ions, such as cadmium (Mayo et al., 1982). With our constructs containing the MT promoter, addition of cadmium sulphate resulted in induction levels that varied considerably between different transfection experiments. Maximum induction found was approx. eight-fold with pSVMTα1 and pSVMTsα1 (results not shown).

TABLE II

Comparison of different promoters regarding their ability to direct IFN synthesis in COS cells[a]

Plasmid (1)	IFN (2)	Mean ± S.E. (3)	N (4)
pSVMTα1	320	1	11
pSVGMTα1	160	0.5 ± 0.0	3
pSVMTsα1	1920	6.2 ± 0.9	6
pSVGMTsα1	160	0.5 ± 0.1	3
pSVα1	480	1.3 ± 0.2	10
pSVLMOα1	640	1.8 ± 0.4	3

[a] See Table I.

(c) Influence of different promoters on expression in TOP cells

The MT promoter and the MoMLV LTR are promoters that normally function in mouse cells. To compare them in a homologous environment we have constructed a transient expression system based on mouse cells. To this end a mouse cell line

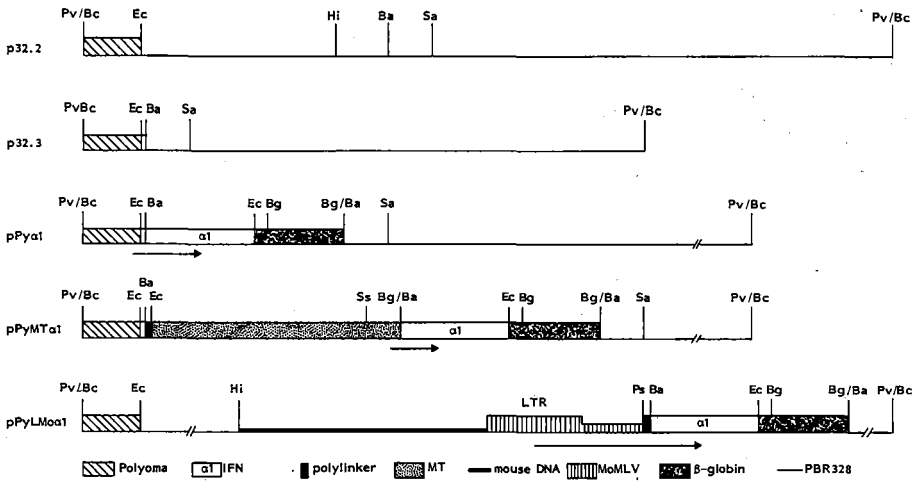

Fig. 3. Plasmids used for IFN expression in mouse TOP cells. For restriction sites see Fig. 1. For the construction of the expression plasmids p32.2 was first converted by the addition of a *Bam*HI linker to the Pollk-treated *Eco*RI site (this restores the *Eco*RI site) and subsequent removal of the thus generated *Bam*HI fragment, which resulted in the vector p32.3. Plasmids pPyα1 and pPyMTα1 were created by inserting the *Bam*HI-*Sal*I fragments from pSVα1 and pSVMTα1 (Fig. 2) into p32.3, between *Bam*HI and *Sal*I sites. pPyLMOα1 was constructed by ligation of the *Hin*dIII-*Bam*HI and *Bam*HI-*Sal*I fragments of pSVLMOα1 and the largest *Hin*dIII-*Sal*I fragment of p32.2. For arrows see Fig. 2.

was constructed (TOP cells) that expresses Py large T antigen constitutively. This was achieved by cotransfection of 3T6 cells with p48.19, a plasmid which contains a linearized Py genome with a defective origin of replication (kindly provided by Dr. G.M. Veldman), and a plasmid containing the Nm^R gene. Isolated Nm^R clones were analyzed for their ability to produce IFN after transfection of plasmids containing an IFN gene and the Py origin of replication. A clone, which produced the highest amounts of IFN, was selected for further experiments.

Plasmids used for expression experiments in TOP cells are shown in Fig. 3. They are based on plasmid p32.2 (provided by Dr. G.M. Veldman), which contains the 366-bp *BclI-BglI* Py fragment spanning the origin of replication and early promoter. All plasmids contain the murine IFN-α1 coding region and the rabbit β-globin 3′-noncoding region. The promoters analyzed are the Py early promoter (pPyα1), the MoMLV LTR (pPyLMOα1) and the MT promoter (pPyMTα1). We did not include the SV40 promoter in these experiments because other workers have shown that this promoter has only scant activity in mouse cells (Gorman et al., 1982; McIvor et al., 1985).

Several separate transfection experiments were carried out with the plasmids depicted in Fig. 3. Again the IFN production was measured 72 h after transfection. The IFN production and the calculated relative activities (related to pPyMTα1) are given in Table III. It should be stressed that a direct comparison of the results found with COS and TOP cells is not possible because it cannot be assumed that these cell types have similar transfection and/or replica-

TABLE III

Comparison of different promoters regarding their ability to direct IFN synthesis in mouse TOP cells[a]

Plasmid (1)	IFN (2)	Mean ± S.E. (3)	N (4)
pPyMTα1	128	1	8
pPyα1	64	0.6 ± 0.1	6
pPyLMOα1	256	1.9 ± 0.6	8

[a] See Table I.

tion efficiencies. Table III shows that the MoMLV LTR is approx. three times as active as the Py early promoter and twice as active as the MT promoter in pPyMTα1. Thus, also in mouse cells the MT promoter has a considerable constitutive activity, which is in concordance with the results of other workers (McIvor et al., 1985; Valerio et al., 1984).

In our hands induction of the MT promoter gave variable results in both COS and TOP cells. We presume that this is due to differences in the sensitivity of the cells to cadmium ions from one experiment to another. When the cells are kept in culture after cadmium treatment a large percentage of the cells die. This and the relatively low induction levels suggest that constitutive expression from the MT promoter is a better option for the construction of stable producer lines, especially when this activity can be increased by the introduction of an enhancer next to the MT promoter. At present, we are constructing expression vectors for both mouse and monkey cells which combine enhancer regions directly with the MT promoter.

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PAPER II

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Properties of Natural and Hybrid Murine Alpha Interferons

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SUMMARY

Four natural murine interferon-α genes (MuIFN-α1, -α2, -α4 and -α6) and four hybrid genes (α1α4, α2α4, α4α1 and α4α2) were transiently expressed in monkey COS cells under the transcriptional control of the simian virus 40 early promoter. The proteins were labelled with [^{35}S]methionine during a 16 h incubation and proteins secreted by the cells during this period were separated by polyacrylamide gel electrophoresis and subsequently visualized by fluorography. Under the conditions used, the IFNs represented 5 to 10% of the total amount of secreted proteins. All genes were found to encode biologically active IFN subspecies, including α4 which has a deletion of five amino acids. When the specific activities of the proteins were compared, it appeared that the specific antiviral activity of α4 on mouse cells was three- to sixfold higher than the activities of the other natural IFN subspecies. The specific activities of the hybrid proteins were similar to those of the natural proteins, except for the α2α4 hybrid which had a higher specific activity than the original proteins. The ability of the natural and hybrid subspecies to protect hamster cells against viral infection was determined using MuIFN-α1 as a standard. Large differences in activity were found, with α6 as the most and α4 as the least active subspecies.

INTRODUCTION

Type I interferons (IFNs) are proteins produced by mammalian cells upon induction with a virus or a double-stranded polyribonucleotide. Once secreted by the producing cells they are able to protect surrounding cells against viral infection. In addition to their antiviral activities, type I IFNs are able to inhibit cell growth and they can influence the immune system, e.g. by stimulating macrophages and natural killer cells (for reviews, see Finter, 1984). To elicit their biological properties it is necessary that the IFNs bind to specific surface receptors on the target cells. Subsequently, the expression of several genes within the target cells is affected (Fellous *et al.*, 1982; Friedman *et al.*, 1984; Jonak & Knight, 1984; Larner *et al.*, 1984; Shulman & Revel, 1980). So far, however, little is known about the precise relationship between the different biological and molecular changes induced by IFNs.

In man and mouse, type I IFNs comprise a group of closely related proteins called IFN-α and a single antigenically distinct protein called IFN-β (for review, see Weissmann & Weber, 1986). The IFN-α proteins are encoded by the IFN-α gene family, consisting of over 10 members. These genes are tightly clustered in the genome. The single IFN-β gene is closely associated with the IFN-α gene family (Ohlson *et al.*, 1985; Trent *et al.*, 1982; Van der Korput *et al.*, 1985). The IFN-α and -β genes do not contain introns and encode proteins of 186 to 190 amino acids, including a signal peptide of 23 amino acids. It is assumed that the IFN-α and -β genes share a common ancestor and that the IFN-α gene family arose by repeated duplications of the ancestral α gene. The IFN-α genes are relatively well conserved. Within a species the mean homology between two proteins is about 70%. Between human (Hu) and murine (Mu) IFN-α proteins the homology is 50 to 60%.

Despite these structural similarities, there are differences in the biological activities of the different IFN-α subspecies. They are for instance relatively species-specific: most HuIFNs have only a low activity on mouse cells (Weck *et al.*, 1981*a*). Moreover, the relative efficacy of antiviral compared to antiproliferative or other responses may vary from one subspecies to another (Ortaldo *et al.*, 1984; Rehberg *et al.*, 1982). To investigate the properties of different IFN subspecies *in vivo*, we have chosen the mouse as a model organism. We have recently reported the isolation of four MuIFN-α genes (Zwarthoff *et al.*, 1985*b*). In this paper, we show that these genes encode biologically active IFN subspecies when introduced into a transient expression system. We found that the specific antiviral activity on mouse cells varied between the different proteins and that there are great differences in the abilities of the proteins to protect heterologous Chinese hamster ovary (CHO) cells against viral infection. In addition, we have constructed hybrid genes and compared the properties of their protein products to those of each parent.

METHODS

DNA manipulations. Plasmids were carried in *Escherichia coli* K12 strain DH1 and were purified from chloramphenicol-amplified cultures by the alkaline lysis method (Maniatis *et al.*, 1982). Separation of plasmid DNA and cellular RNA was achieved on a Bio-Gel A-1·5m (Bio-Rad) gel filtration column. DNA fragments were isolated from agarose gels by electroelution. All cloning procedures were as described in Maniatis *et al.* (1982). Plasmid constructs were verified by restriction enzyme analysis.

Cell culture and transfection. Cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% foetal calf serum, penicillin and streptomycin. COS-1 cells (Gluzman, 1981) were seeded 1 to 2 days prior to transfection in 35 mm Petri dishes and grown to 30% confluence. Transfection was performed using the DEAE-dextran technique (McCutchan & Pagano, 1968). To this end, the medium was removed and replaced by DMEM (1 ml) containing 1 μg *Pvu*II- and *Hpa*II-digested plasmid DNA and 100 μg DEAE-dextran. Digestion of the plasmid with these enzymes removes practically all plasmid sequences leaving the eukaryotic DNA intact. This treatment results in a higher expression of the IFN genes (I. Seif & J. De Maeyer-Guignard, personal communication). After 2 h, the mixture was removed and the cells were treated with 0·1 mM-chloroquine in DMEM for 4 h and they were subsequently fed with DMEM plus serum. The medium was changed once 24 h after transfection. After 72 h the medium was removed and the cells were washed extensively with Hanks' balanced salt solution and incubated for 16 h with DMEM with one-tenth the normal methionine concentration plus 45 μCi [^{35}S]methionine but without serum. The resulting specific radioactivity of methionine was 63·5 × 10^6 d.p.m./μg. This medium was used for PAGE and IFN assays.

IFN assay. IFN titres were determined in a cytopathic effect reduction assay, using vesicular stomatitis virus as a challenge. IFN titres on mouse cells were calculated relative to the NIH reference standard G002-904-511. Because no standard for hamster cells is available, titres on these cells were calculated relative to the activity of MuIFN-α1 on these cells, which was set at 100% of its activity on L cells.

PAGE. The ^{35}S-labelled proteins (40 μl culture medium) were separated on a 12·5% gel according to the procedure described by Laemmli (1970). The proteins were visualized by fluorography (Bonner & Laskey, 1974). Total radioactivity incorporated into secreted proteins was determined by gel filtration of the culture medium. These values and densitometric scans of the fluorogram were used to calculate the amount of label incorporated in each IFN band.

RESULTS

Construction of expression plasmids

All plasmids used for expression in COS cells were based on the expression plasmid pSV328A (Fig. 1). This plasmid was constructed from pSV10EF (Zwarthoff *et al.*, 1985*a*) by removing the *Hin*dIII–*Eco*RI fragment containing the coding region of MuIFN-10EF (= MuIFN-α1) and by replacing it by the *Hin*dIII–*Eco*RI polylinker fragment of bacteriophage M13mp11 (Messing, 1983). The coding regions of the MuIFN-α genes (Fig. 1) were subsequently inserted into the polylinker region of pSV328A, thus bringing them under the control of the simian virus 40 (SV40) early promoter. The polyadenylation signal is provided by the rabbit β-globin sequences.

For the construction of pSVα1, the *Hin*dIII site 20 nucleotides upstream of the ATG codon was converted into a *Bam*HI site by the addition of a *Bam*HI linker. Subsequently, the *Bam*HI–*Eco*RI fragment containing the entire coding region was inserted into pSV328A, between the

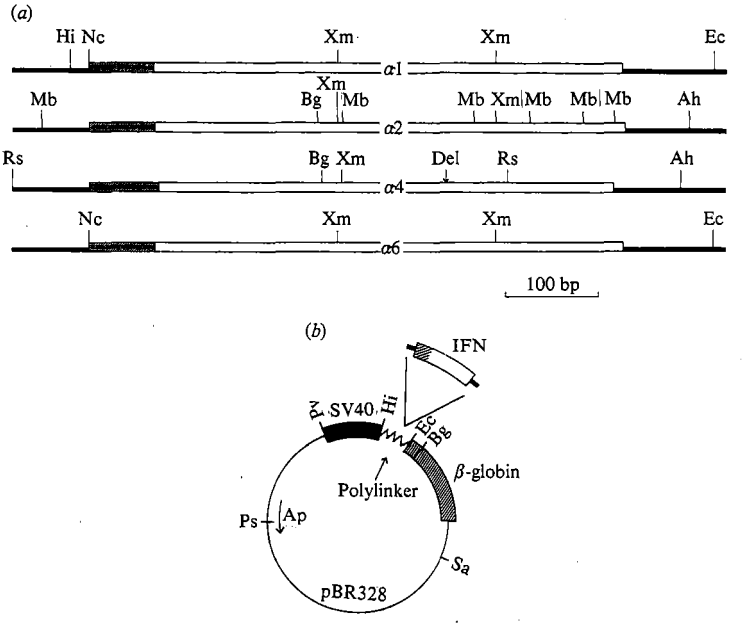

Fig. 1. Construction of expression plasmids containing IFN genes. (*a*) Physical maps of the IFN-α genes used. The portion coding for the signal peptide within the IFN coding region is hatched. Del, Position of the 15 nucleotide deletion in the α4 gene. (*b*) Outline of the expression vector pSV328A. The coding regions of the IFN genes are inserted in the polylinker of this plasmid. See text for details concerning the constructions. In all maps only the relevant restriction enzyme sites are shown: Ah, *Aha*III; Bg, *Bgl*II; Ec, *Eco*RI; Hi, *Hin*dIII; Mb, *Mbo*II; Nc, *Nco*I; Pv, *Pvu*II; Ps, *Pst*I; Rs, *Rsa*I; Sa, *Sal*I; Xm, *Xmn*I. Ap, Ampicillin resistance gene.

*Bam*HI and *Eco*RI sites. For the construction of pSVα2 the *Mbo*II fragment spanning the left half of the coding region was isolated and treated with nuclease S1 to create flush ends. The fragment was then cut with *Bgl*II and the *Mbo*II–*Bgl*II fragment containing the 5′ end of the gene was inserted in pSV328A between *Sma*I and *Bgl*II. This fragment was again isolated from the resulting plasmid by digestion with *Bam*HI and *Bgl*II. It was subsequently ligated together with the *Bgl*II–*Aha*III fragment containing the 3′ two-thirds of the α2 gene to pSV328A cut with *Bam*HI and *Sma*I, thus creating pSVα2. For the construction of pSVα4, first the *Rsa*I fragment spanning the left two-thirds of the gene was isolated and treated with nuclease Bal31 so as to remove approximately 60 nucleotides from the ends. After digestion with *Bgl*II the left part of the fragment was inserted in pSV328A between *Sma*I and *Bgl*II. The Bal31 digestion was analysed by nucleotide sequencing and a clone with 20 nucleotides left upstream of the ATG codon was selected for the subsequent construction of the expression plasmid pSVα4, which from this point on went analogously to the construction of pSVα2. The plasmid pSVα6 was created from pSVα1 by replacing the *Nco*I–*Eco*RI fragment of the latter for the analogous fragment from gene α6.

Hybrids between IFNs α1 and α4 (α1α4 and α4α1) were constructed by ligating the left *Xmn*I site in α1 to the only *Xmn*I site in α4 (see Fig. 1). The crossover point in these hybrid genes is between amino acids 67 and 68. Hybrids between α2 and α4 (α2α4 and α4α2) were constructed using their single *Bgl*II site (see Fig. 1). In these the crossover point was at position 60. All hybrid genes were inserted into the polylinker of pSV328A (Fig. 1) in an analogous way to the constructions used for the parent genes.

Table 1. *Antiviral activity produced by expression plasmids as measured on mouse (L929) and hamster (CHO) cells*

IFN	L929*	CHO†
α1	3200	3200
α2	2400	640
α4	25600	16
α6	3200	5120
α1α4	3200	256
α2α4	51200	640
α4α1	1600	32
α4α2	3200	64

* IFN titres on L cells are given in IU/ml.
† Titres on CHO cells were determined as described in Methods and are given in U/ml.

Expression in COS cells

The expression plasmids described in the preceding section were digested to remove all vector sequences and they were subsequently transfected into monkey COS cells. Seventy-two h after transfection the medium was removed and replaced with medium containing [^{35}S]methionine but without serum, and 16 h later the medium was collected. The amount of antiviral activity secreted during this period was assayed on mouse and hamster cells in a cytopathic effect reduction assay. Titres on CHO cells were related to the activity of MuIFN-α1 on these cells, which is equal to its activity on L cells (Zwarthoff *et al.*, 1985*a*). Table 1 shows the titres found in a representative experiment. It appeared that all the natural and hybrid IFN genes coded for IFN subspecies with biological activity on mouse as well as on hamster cells. Surprisingly, the α4 gene, which contains an in-phase deletion of 15 nucleotides (Zwarthoff *et al.*, 1985*b*), also encoded a biologically active IFN. Moreover, α4 and the α2α4 hybrid produced considerably more antiviral activity on mouse cells than the other genes. The results obtained with hamster cells showed that α4 had only a low activity on these cells. In contrast, α6 was even more active on hamster cells than on mouse cells.

To determine whether the differences in activity found were indeed due to differences in specific activity of the various proteins we decided to compare the amount of IFN produced by the transfected COS cells. To this end the proteins secreted by the cells during the 16 h labelling period described above were separated on a polyacrylamide gel. Fig. 2 shows the fluorograph of such a gel. The genes used for transfection are indicated on top of each lane. Most IFNs can be seen as bands of approximately 22000 mol. wt. (22K), moving slightly slower than the 18 K mol. wt. marker. IFN-α6 ran at 18K. In the control lane transfected with pSV328A no bands were visible in this area. The molecular weights observed were consistent with the nucleotide sequence data, which suggests that α6 lacks the *N*-glycosylation site at positions 78 to 80 in the protein that is present in α1, α2 and α4 and as a consequence also in their hybrids (Zwarthoff *et al.*, 1985*b*). Densitometric analysis of the fluorogram indicated that 5 to 10% of the label incorporated into secreted proteins was present in the IFNs (results not shown). The amount of label in the IFN bands (except α6) varied slightly between the different lanes; this was partly due to differences in total incorporation and partly to actual differences in the amounts of IFN present. That the α6 band contained significantly less label than the other IFN bands was caused by the fact that α6 has only one methionine residue, whereas all the other subspecies contain two methionines.

Specific activities of natural and hybrid IFNs

Specific activities of the different subspecies were calculated from the antiviral titre of the medium and the amount of label present in IFN in the same sample as described in Methods. The specific activities are presented as units × 10^3/d.p.m. in the second and fourth columns of Table 2. It should be noted that the specific activity of α6 when expressed in this way was overestimated because the protein contains only one methionine residue. Using the known specific activity of the added [^{35}S]methionine, the specific activities were also calculated as

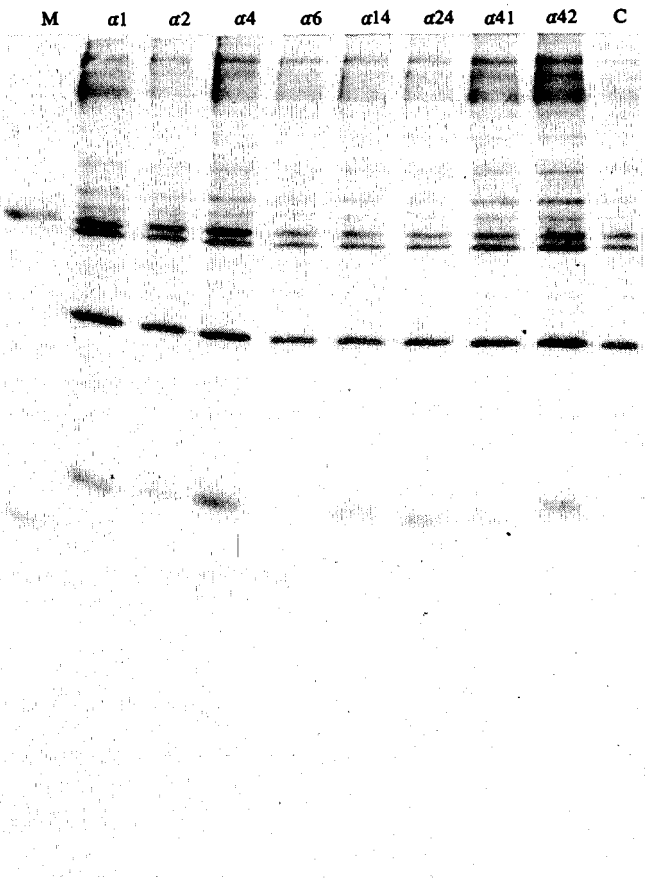

Fig. 2. Fluorograph of SDS–polyacrylamide gel containing ^{35}S-labelled proteins secreted by COS cells transfected with IFN expression plasmids. The IFN genes used are indicated on top of each lane (α14 stands for α1α4, etc.). M, Molecular weight markers of 97K, 69K, 46K, 30K and 18K respectively. The 46K and 18K markers are the stronger bands. C, Control cells transfected with pSV328.

units/mg protein present in the IFN band. This also corrected the specific activity of α6. The values found are displayed in columns three and five of Table 2. The results show that the differences in antiviral activity produced by the different genes as displayed in Table 1 were largely due to differences in the specific activity of the corresponding proteins. Thus, the specific activity of α4 on mouse cells was considerably higher than that of the other natural subspecies. It

Table 2. *Specific activities of natural and hybrid IFNs on mouse (L929) and hamster (CHO) cells*

	L929		CHO	
IFN	IU × 10^3/d.p.m.	IU/mg	U × 10^3/d.p.m.	U/mg
α1	48	5 × 10^7	48	5 × 10^7
α2	72	8 × 10^7	19	2 × 10^7
α4	284	3 × 10^8	0·2	2 × 10^5
α6	192	1 × 10^8	307	1·6 × 10^8
α1α4	84	9 × 10^7	6·7	7 × 10^6
α2α4	731	8 × 10^8	9	1 × 10^7
α4α1	50	5 × 10^7	1	1 × 10^6
α4α2	33	3 × 10^7	0·7	7 × 10^5

is interesting to see that the α2α4 hybrid which also contained the 15 nucleotide deletion had an even higher specific activity. However, α1α4 in which the deletion was also present, had a specific activity that was only about one-third that of α4. The α6 subspecies displayed a considerable activity on mouse and an even higher activity on hamster cells. Thus, the absence of *N*-glycosylation had no obviously deleterious effect upon the biological activity of this protein. In contrast to the considerable activities of α1, α2 and α6 on hamster cells, the activity of α4 on these cells was at least 100-fold lower. The activities of the hybrid proteins on hamster cells were found to differ from those of their parents; instead of the high (α1, α2) or low activities (α4), intermediate values were found.

DISCUSSION

In this study, we have investigated the expression of several MuIFN genes in a transient expression system. Under the conditions used, the IFNs represent 5 to 10% of the proteins secreted by the COS cells. The data obtained demonstrate that all the genes analysed encode biologically active IFN subspecies. Moreover, we found that MuIFN-α4 has three- to sixfold higher activity on mouse cells than the other α proteins. This is a surprising result since the α4 gene contains a unique deletion of five amino acids. Expression of MuIFN-α4 has also been achieved in *E. coli* (Kelley & Pitha, 1985). However, the results obtained led the authors to conclude that the specific activity of this subspecies is 100-fold lower than that of MuIFN-α1 or an α4α2 hybrid IFN. We do not know at present what the reason for this discrepancy might be.

When mouse L cells are induced with Sendai virus and the individual IFN mRNAs are visualized in a S1 nuclease experiment, the mRNAs transcribed from the α4 and β genes are at least 10 times more abundant than the mRNAs from the α1, α2 and α6 genes (Zwarthoff *et al.*, 1985*b*). When these results are combined with the relatively high specific activity of α4, it is highly probable that IFN-α4 represents the major part of the IFN-α activity produced by mouse fibroblasts. This hypothesis is supported by the results obtained in the experiments on CHO cells. An IFN-α preparation from L cells has only a low activity on CHO cells (Lemson *et al.*, 1984). We now find that, not only IFN-α1, but also IFN-α2 and IFN-α6 are active on CHO cells, whereas IFN-α4 has only a very low activity (see Table 2). The finding that the MuIFN-α6 subspecies is highly active on hamster cells suggests that it could be useful in experiments *in vivo* with hamsters.

The hybrid genes were constructed with the objective of localizing the different phenotypic properties displayed by the different α proteins to certain regions within the genes. Studies using hybrids between HuIFN-α1 (D) and -α2 (A) show that this is, at least to some extent, possible (Rehberg *et al.*, 1982; Streuli *et al.*, 1981; Weck *et al.*, 1981*b*). The behaviour of the hybrid IFNs on heterologous hamster cells shows that their activities are intermediate between those of either parent, but that hybrids with the N-terminal ends of α1 or α2 have significantly higher activities than constructs with the identical part of α4. This suggests that the origin of the N-terminal end has some influence on the behaviour of the hybrid with respect to hamster cells. When the

activities of the hybrids on mouse cells are compared it is interesting to see that the α2α4 hybrid has an even higher activity on mouse cells than α4. The most obvious difference between α4 and the other subspecies in the C-terminal part of the protein is the 15 nucleotide deletion. However, since the α1α4 hybrid has a lower activity than α4, the absence of the five amino acids is apparently not sufficient by itself to explain the relatively high activity of α4 and α2α4. At present, we are studying the effect of the deletion in more detail using site-directed mutagenesis. The α2α4 hybrid shows that it is possible to create IFN subspecies with a specific activity that is higher than that of the natural proteins. Preliminary results obtained in our laboratory with other hybrids suggest that this is not an isolated case and that the construction of hybrids might be a way to 'improve' the IFNs.

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PAPER III

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Structure-Function Analysis of Murine Interferon-α: Antiviral Properties of Novel Hybrid Interferons

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ABSTRACT

As described earlier the protein products of the murine interferon (IFN) genes MuIFN-α_1, -α_2, and -α_4 differ in their antiviral activity on hamster (CHO) and mouse (L929) cells. For structure-function analysis, hybrids were prepared between the three genes using common restriction enzyme sites. Natural and hybrid genes were transiently expressed in monkey COS cells. Under the conditions used IFN constituted 20-30% of the total amount of secreted proteins. Using a panel of hybrids either between α_1 and α_2 or between α_1, α_2, and α_4, the amino-terminal region of the protein, from amino acids 10 to 58, was found to determine its antiviral activity on hamster cells. On mouse cells, the antiviral activities of hybrids between α_4 and either α_1 or α_2 were compared. The high activity of α_4 (five to ten times that of α_1 or α_2) was not transmitted to hybrids having the amino-terminal part of α_4, but coincided with the presence of the α_4 carboxy-terminal region in all but one hybrid construct. The deletion of five amino acids (positions 103-107) located in this region of α_4 did not affect antiviral activity when introduced into MuIFN-α_2 and a MuIFN-α_{42} hybrid by site-directed mutagenesis.

INTRODUCTION

LIKE HUMAN INTERFERON-α (HuIFN-α), murine (Mu) IFN-α comprises a group of closely related proteins.[1] The MuIFN-α gene family contains 15 or more different genes; nine of these have been isolated and shown to encode a biologically active protein as deduced from expression in eukaryotic cells or *Escherichia coli*[2-9] (Trapman, unpublished results). In addition, one pseudogene has been described.[10]

Mature MuIFN-α proteins are 166 or 167 amino acids long, with the exception of MuIFN-α_4, which contains a deletion of five amino acids corresponding to positions 103-107 in other subspecies.[4,5] The mutual structural homology of the various proteins varies from 74 to 91%, whereas

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the homology between MuIFN-α species and their HuIFN-α counterparts is 50–60%. Although highly homologous, MuIFN-α proteins display distinct antiviral properties. Their specific antiviral activity as measured on mouse cells can vary a hundredfold[7] (Trapman, unpublished results). Moreover, large differences exist between species in relative antiviral activity on hamster cells.[7]

The availability of very homologous MuIFN-α genes of which the protein products display different biological activities allows a detailed structure-function analysis. To this purpose we prepared a large series of hybrid genes using common restriction enzyme sites. In this study we describe the antiviral properties of hybrids derived from MuIFN-α_1 and -α_2 and extend our earlier observations[7] on hybrids between MuIFN-α_4 and either MuIFN-α_1 or -α_2. In addition we investigate the possible effect of the deletion of five amino acids present in natural MuIFN-α_4 on its antiviral properties.

MATERIALS AND METHODS

DNA manipulations

All cloning procedures were as described in Maniatis *et al.*[11] After RNase treatment, plasmid DNA was separated from RNA by gel filtration over a Bio-Gel A 1.5 m (Bio-Rad) column.

Isolation of MuIFN-α_2 ∇ (103–107)

The 0.6-kb IFN-α_2 *Bam*HI-*Eco*RI fragment was cloned into the RF of M13mp8 and single-stranded phage DNA prepared using standard procedures. M13mp8 DNA containing the IFN gene was mixed with the *Eco*RI-*Bgl*I fragment of M13mp18 (0.2 pmole of each in 10 μl 10 m*M* Tris (pH 7.5), 10 m*M* $MgCl_2$, 1 m*M* DTT, 50 m*M* NaCl) boiled for 5 min and incubated for 3 h at 65°C. Next, 10 pmole of a phosphorylated oligonucleotide (GTCTGATGCAGGAACCTCCTCTG, a 23-mer spanning the deletion of α_4) in 2 μl of H_2O was added, the mixture incubated for another 15 min at 65°C and for 1 h at 15°C. Subsequently, 10 μl of 20 m*M* Tris-HCl (pH 7.5) containing 10 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* ATP, dNTPs (1 m*M* each), 2 U Klenow DNA polymerase, and 3 U T4 DNA ligase was added. Incubation at 15°C was continued for another 4 h. The reaction was stopped by addition of 100 μl 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA.

E. coli HB2154 was transformed with an appropriate amount of this mixture and plated in soft agar after addition of *E. coli* HB2151. Plaques were transferred to duplicate nitrocellulose filters which were hybridized overnight at 60°C in 6× SSC/10× Denhardt/0.1% SDS with the α_4 probe or an α_2 probe (GTGTGATGCAGCAGGTGGGGGTGCAGGAACCTCCTC). Subsequently filters were washed in 6× SSC at room temperature. Plaques which hybridized more intensely with the α_4-specific probe were picked, and phages were grown and characterized by restriction enzyme mapping and sequencing. Using this method the α_2 ∇ (103–107) deletion mutant was isolated. A similar approach for isolation of α_4 insertion mutants was unsuccessful.

Cell culture and transfection

Cells were propagated in Dulbecco's MEM (DMEM) supplemented with 5% fetal calf serum and antibiotics. COS-1 cells[12] were seeded 1–2 days prior to transfection in 35-mm petri dishes and grown to 30% confluency. At that time culture medium was replaced by DMEM (1 ml) containing 1 μg of the appropriate expression plasmid and 100 μg of DEAE-dextran. Prior to this, plasmid DNA was digested with *Pvu*II and *Hpa*II which removes almost all pBr328 sequences and leaves the eukaryotic DNA intact. After a 2-h incubation, the mixture was removed and the cells were treated for 2–3 h with 0.1 m*M* chloroquine in DMEM and subsequently fed with DMEM plus serum. Medium was changed 24 h and 72 h after transfection. Supernatants harvested at 88 h were used for IFN assays.

In vivo *labeling of secreted proteins*

Culture medium was removed 72 h after transfection and the cells were washed twice with Hanks' balanced salt solution and incubated for 16 h with MEM (Eagle) containing one-tenth the normal methionine concentration supplemented with 40 μCi [^{35}S]methionine (1,265 Ci/mmole, Amersham). Total radioactivity incorporated into secreted proteins was determined by quantitative TCA precipitation. To this end, 10 μl of culture medium was spotted on a Whatman 3MM filter and proteins were precipitated during a 10-min incubation in 10% TCA at 0°C. The filters were washed twice with 5% TCA for 10 min at 80°C, rinsed in cold 100% ethanol, air-dried, and counted in a liquid scintillation counter using a toluene-based counting liquid.

Polyacrylamide gel electrophoresis and determination of the specific activity of IFN

^{35}S-Labeled proteins (40 μl of culture medium plus 10 μl of sample buffer, boiled for 5 min) were separated on a 12.5% polyacrylamide-SDS gel.[13] The proteins were visualized by fluorography[14] using enlightening (NEN).

Densitometric scans of the fluorograph were used to determine the relative amount of IFN present in the labeled proteins. Those exposures were used in which the density of the various bands was proportional to the amount of label present. The relative amount of IFN together with the total radioactivity incorporated into secreted proteins and the specific activity of the [^{35}S]methionine were used to calculate the specific activity of an IFN subspecies.

IFN assay

IFN titers were determined in a cytopathic effect reduction assay, using vesicular stomatitis virus (VSV) as a challenge. IFN titers on mouse (L929) cells were calculated relative to the NIH reference standard G002-904-511. Titers on hamster (CHO) cells were calculated relative to the activity of MuIFN-α_1 on these cells, which was set at 100% of its activity on mouse cells.[7]

RESULTS

Construction of MuIFN-α expression plasmids and their expression in COS cells

One of the main differences between human and mouse IFN-αs is that most mouse subspecies are *N*-glycosylated, whereas most human IFN-αs are not.[1] Because of this glycosylation natural and hybrid MuIFN-α genes were cloned into an eukaryotic expression vector behind the SV40 early promoter. Construction of the plasmids pSVα1, pSVα2, and pSVα4, containing the coding region of MuIFN-α_1, -α_2, and -α_4, respectively, was described previously.[7] Hybrids between MuIFN-α_1 and -α_2 were prepared using the common restriction enzyme sites (outlined in Fig. 1) for *Mst*II (between corresponding amino acid positions 9 and 10), *Xmn*Ia (between positions 67 and 68), and *Xmn*Ib (between positions 123 and 124). The series of hybrids between MuIFN-α_4 and either α_1 (common *Xmn*Ia) or α_2 (common *Bgl*II, between positions 58 and 59) described earlier[7] was extended with hybrids between the three genes. These were constructed by exchanging the *Hin*dIII-*Mst*II fragments of pSVα14 and pSVα24. The five amino acid deletion of α_4 was introduced into MuIFN-α_2 using site-directed mutagenesis, resulting in pSVα2∇(103–107). The hybrid pSVα42∇(103–107) was prepared in essentially the same way as pSVα42 by use of the common *Bgl*II site.

For analysis of antiviral activities of MuIFN-α species, we collected supernatants of COS cells transiently expressing the various natural and hybrid genes. IFN proteins produced in this way turned out to be stable for over 1 week at 4°C and for at least 24 h at 37°C (results not shown).

Therefore, and because of the high degree of purity, COS cell supernatants seemed to be a good source of IFN for planned biological assays and we did not purify the protein further. Using this approach we avoided IFN inactivation that frequently was observed during handling of small samples.

For quantitation of IFN production, *in vivo* ^{35}S-labeled proteins secreted by COS cells were separated on a SDS polyacrylamide gel. The fluorograph shown in Fig. 2 is an example of the expression of a series of different natural and hybrid IFN-α species. IFN shows a molecular weight of

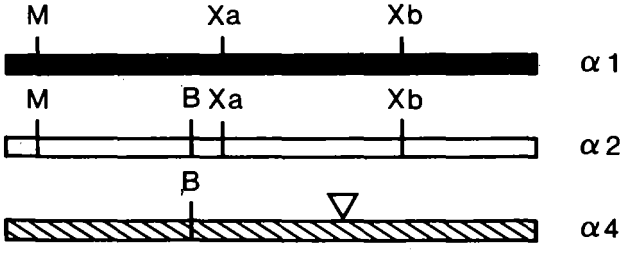


FIG. 1. Outline of the regions encoding the mature MuIFN-α_1, -α_2, and -α_4 proteins. Restriction enzyme sites used for construction of hybrid genes are indicated. M (*Msl*II) cleaves between corresponding amino acids 9/10, B (*Bgl*II) between 58/59, Xa (*Xmn*I) between 67/68, and Xb (*Xmn*I) between 123/124. (▽) Deletion spanning amino acids 103–107.

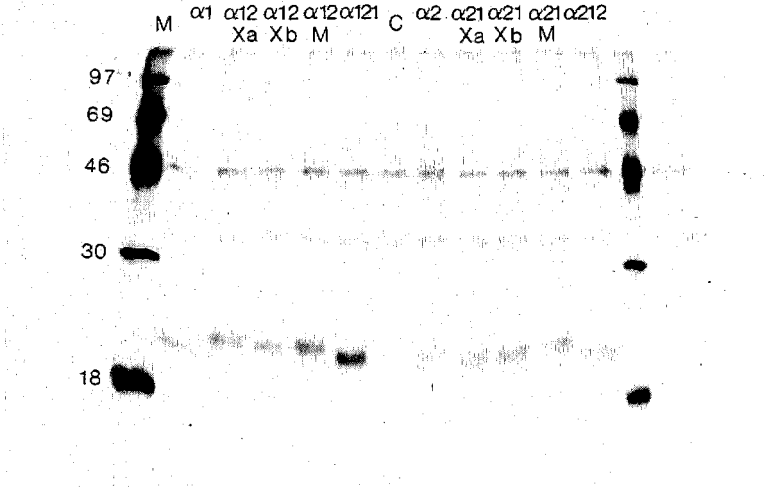


FIG. 2. Fluorograph of a SDS-polyacrylamide gel containing ^{35}S-labeled proteins secreted by COS cells transfected with IFN expression plasmids. IFN genes used are indicated on top of each lane. C, Control cells transfected with pSV328A; M, molecular weight markers.

about 21 kD; the IFN band is absent in the control lane. As can be seen, similar amounts of ^{35}S-labeled IFNs are present in the various lanes. Densitometric scanning of the fluorograph of a SDS polyacrylamide gel reveals that IFN represents 20–30% of the total amount of secreted proteins. The specific antiviral activities of natural MuIFN-α species as calculated from the antiviral titers and [^{35}S]methionine incorporation (see Materials and Methods and Tables 1 and 2) are in general somewhat lower than earlier described values.[7] This correction was made as a result of densitometric scanning of a number (five or more) of different fluorographs.

Antiviral activities of natural and hybrid IFNs on hamster (CHO) cells

MuIFN-α_1 displays similar antiviral activity on hamster as on mouse cells. The activity of α_2 on hamster cells is 10 times lower than that of α_1; α_4 is even less active on these cells[7] (Table 1). Therefore, hybrids between α_1 and α_2 and between α_4 and either α_1 or α_2 were suitable for analysis of protein domains which contribute to antiviral activity on hamster cells. Table 1 summarizes the

TABLE 1. ANTIVIRAL ACTIVITY OF NATURAL AND HYBRID MuIFN-α's ON HAMSTER (CHO) CELLS

IFN		Antiviral act.* (U/ml)	Spec. antiviral act. (U/mg)
α 1		3200	2×10^7
α 21	(M)	6400	3×10^7
α 12	(Xa)	3200	2×10^7
α 12	(Xb)	3200	1×10^7
α 212	(M, Xa)	4800	2×10^7
α 2		320	2×10^6
α 12	(M)	240	7×10^5
α 21	(Xa)	240	1×10^6
α 21	(Xb)	240	1×10^6
α 121	(M, Xa)	240	7×10^5
α 4		48	2×10^5
α 41	(Xa)	48	1×10^5
α 42	(B)	64	1×10^5
α 14	(Xa)	160	5×10^5
α 214	(M, Xa)	2400	8×10^6
α 24	(B)	640	1×10^6
α 124	(M, B)	640	1×10^6

*Antiviral activity is expressed in arbitrary units (see Materials and Methods).

TABLE 2. ANTIVIRAL ACTIVITY OF NATURAL AND HYBRID MuIFN-α'S ON MOUSE (L929) CELLS

IFN	Antiviral act. (IU/ml)	Spec. antiviral act. (IU/mg)
α1	3200	2 x 10^7
α2	2400	1 x 10^7
α4	32000	1 x 10^8
α2 ∇ (103-107)	3200	2 x 10^7
α14 (Xa)	3200	2 x 10^7
α214 (M,Xa)	24000	8 x 10^7
α24 (B)	64000	2 x 10^8
α124 (M, B)	64000	2 x 10^8
α41 (Xa)	3200	1 x 10^7
α42 (B)	3200	7 x 10^6
α42 ∇(103-107)(B)	4800	2 x 10^7

relative and specific antiviral activities of the various hybrids and the parental proteins on hamster cells. The data obtained with hybrids between α_1 and α_2 unambiguously show that the high activity on hamster cells of IFN-α_1 is retained by hybrids containing the amino-terminal part of α_1 (positions 1-67), even if the first 9 amino acids of α_1 in α_{12} (Xa) are replaced by the corresponding part of α_2 [compare α_{12} (Xa) with α_{212} (M, Xa)]. All hybrids with the amino-terminal part of α_2 between positions 10 and 67 possess the properties of α_2. Moreover, the data obtained with hybrids between α_4 and either α_1 or α_2 confirm that the low activity of α_4 on hamster cells is transmitted to the two hybrids possessing the amino-terminal part of α_4.

Similarly, from the four hybrids containing the carboxy-terminal part of α_4, α_{24}, and α_{124} display the phenotype of α_2, whereas α_{214} displays the α_1 phenotype. Only the α_{14} hybrid shows an aberrant behavior, its activity is 20–40 times lower than expected. We have no clear-cut explanation for this finding. It is not the consequence of cloning or expression artifacts, because identical results were obtained with two independently constructed α_{14} hybrids.

Antiviral activities of natural and hybrid IFNs on mouse (L929) cells

The antiviral activities of MuIFN-α_1, -α_2, and -α_4 do not diverge as much on mouse cells as on hamster cells. The activity of α_4 is 5–10 times higher than the activities of both α_1 and α_2 (the latter two show comparable activities on mouse cells). So, only the hybrids between α_4 and either α_1 or α_2 are suitable for structure-function analysis on mouse cells.

In contrast to the results obtained on hamster cells as described above, the antiviral activities measured on mouse cells[7] (Table 2) prove that the high activity of α_4 is not transmitted to the two hybrids possessing its amino-terminal part. These hybrids (α_{41} and α_{42}) display an activity compa-

rable to that of α_1 and α_2. From the hybrids containing the carboxy-terminal part of α_4 only the α_{124} hybrid and not α_{14} retained the high activity on mouse cells which is characteristic of α_4. The α_{124} hybrid, in which the first nine amino acids of α_2 are replaced by corresponding sequences of α_1, behaves identically to α_{24}: It has the same high specific activity on mouse cells. The α_{214} hybrid shows an activity which is about four times higher than that of α_{14} and almost as high as that of α_4. So with one exception (α_{14}) the carboxy-terminal part of α_4 is largely responsible for high activity on mouse cells. Note that α_{14} also behaves aberrantly on hamster cells.

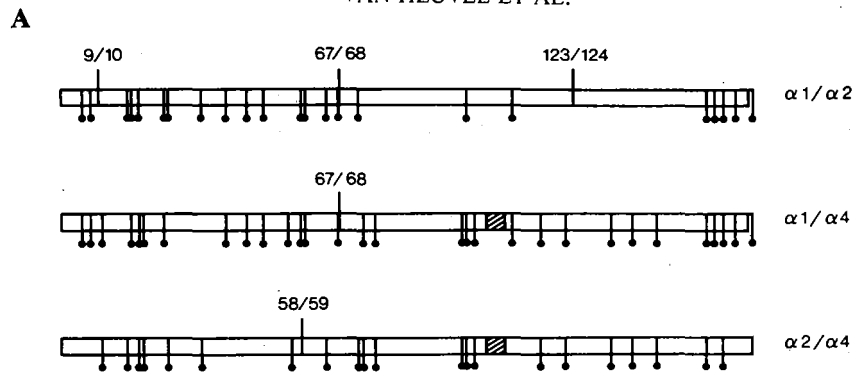
The most prominent difference between MuIFN-α_4 and other α-species in the carboxy-terminal part is the deletion of five amino acids at positions 103–107, present in the α_4 protein. To investigate in more detail whether this deletion is responsible for the high activity of proteins with carboxy-terminal sequences derived from α_4, the same deletion was introduced into α_2 and the α_{42} hybrid. The data presented in Table 2 show that the antiviral activity of the mutated α_2 [$\alpha_2 \nabla$ (103–107)] as well as of the mutated α_{42} [$\alpha_{42} \nabla$ (103–107)] does not reach the α_4 value. From this it must be concluded that the presence or absence of the deletion has no significant influence on the antiviral properties of a MuIFN-α molecule.

DISCUSSION

As described previously[7] and in this study, MuIFN-α subspecies display distinct antiviral activities on both mouse and hamster cells. To define molecular regions that are responsible for a certain phenotype, hybrids have been constructed between three MuIFN-α subspecies (α_1, α_2, and α_4). Earlier results obtained with a limited series of α_1/α_4 and α_2/α_4 hybrids[7] did not provide satisfactory information in this respect. By preparing a large number of α_1/α_2 hybrids and hybrids between the three genes much better interpretable data were obtained.

Our results clearly show the location of an important domain for activity on hamster cells in a amino-terminal fragment ranging from amino acids 10 to 58. As can be seen in Fig. 3A MuIFN-α_1 and MuIFN-α_2 differ in 10 positions in this region and are more conserved in other parts of the molecule. Large differences between α_4 and α_1 or α_2 are also found in the 10–58 region. In addition, α_4 diverges considerably from α_1 and α_2 in the carboxy-terminal half of the protein (see Fig. 3A). Direct comparison of amino acid sequences (Fig. 3B) reveals that in the 10–58 region only one position can be found in which all three subspecies are different (amino acid 19 is a valine in α_1, alanine in α_2, and glutamic acid in α_4). Furthermore, a series of three varying amino acids are situated in the close vicinity at positions 16, 17, and 20. At 19 and 20, α_4 is the only subspecies that contains two negatively charged residues. All other MuIFN-αs isolated so far lack negatively charged amino acids in a wide surrounding area (from 3 to 31). It is tempting to speculate that the amino acid sequence from position 16 to 20 plays a direct or indirect (by conformational changes) role in the interaction of an IFN molecule with its receptor on hamster cells. Results obtained with hybrid MuIFN-α species constructed by *in vivo* recombination also point in this direction (E. Zwarthoff, in preparation). However, other explanations are possible and site-directed mutagenesis experiments and direct receptor binding studies have to provide a definite answer.

The carboxy-terminal portion of α_4 (from amino acids 59 to 167) is important for its high antiviral activity on mouse L cells. An even higher activity can be reached if α_4 sequences at the amino-terminal part are replaced by α_2 or α_{12} hybrid sequences. A role of the deletion at positions 103–107 in the activity of α_4 could be ruled out. Examination of the amino acid composition of MuIFN-α subspecies in the carboxy-terminal part reveals that, besides the deletion, in this region MuIFN-α_4 differs from both MuIFN-α_1 and -α_2 subspecies at 12 positions (see Fig. 3). Preliminary results obtained with hybrids between α_2 and α_4 using the common *Rsa*I site at corresponding amino acid position 129/130 reveal that the 130–167 region seems to be largely involved in the high activity of MuIFN-α_4 (data not shown). Possibly amino acid substitutions in the further highly conserved 130–155 region[1] at position 133 (lysine instead of glutamic acid) and 138 (leucine instead of proline) are of interest. As pointed out above for studies on hamster cells, here also the introduction of spe-



B

```
                1                  10                  20                  30                  40                  50
                .                  .                   .                   .                   .                   .
α1  C D L P Q T H N L R N K R A L T L L V Q M R R L S P L S C L K D R K D F G F P Q E K V D A Q Q I K K
α2  . . . . H . Y . . R . . . . . K V . A Q . . . . P F . . . . . . Q . . . . . L . . . . N . . . Q .
α4  . . . . H . Y . . G . . . . . T V . E E . . . . P P . . . . . . K . . . . . L . . . . N . . . Q .

                               60                  70                  80                  90                  100
                               .                   .                   .                   .                   .
α1  A Q A I P V L S E L T Q Q I L N I F T S K D S S A A W N A T L L D S F C N D L H Q Q L N D L Q G C L
α2  . . . . P . . R D . . . . T . . L . . . . . A S . . A . . . . . . . . . . . . . . . . . . Q T . L
α4  . . . . L . . R D . . . . I . . L . . . . . D L . . T . . . . . . . . . . . . . . . . . . K A . V

                                   110                 120                 130                 140                 150
                                   .                   .                   .                   .                   .
α1  M Q Q V G V Q E F P L T Q E D A L L A V R K Y F H R I T V Y L R E K K H S P C A W E V V R A E V W R
α2  . . . . . . . . P . . . . . . A . . . . . K . . . . . . . . . . E . . . . P . . . . . V . . . . . .
α4  . . . . . - - - - - P . . . . . S . . . . T . . . . . . . . . . K . . . . L . . . . . I . . . . . .

                                   160
                                   .
α1  A L S S S A N V L G R L R E E K -
α2  . . . . . V . L . P . . S . . . E
α4  . . . . . T . L . A . . S . . . E
```

FIG. 3. A. Amino acid differences between MuIFN-α_1, -α_2, and -α_4. Numbers indicate amino acid positions used for construction of hybrid molecules. B. Amino acid sequence of MuIFN-α_1, -α_2, and -α_4.

cific point mutations coupled to receptor binding studies on mouse L cells are needed to substantiate our findings.

Similar experiments as described here for mouse IFN-αs have been carried out with human IFN-αs. In these studies two series of hybrids between different subspecies (α_1 and α_2[15–17] or α_1 and α_8[18]) have been used. When measured on mouse cells, the activity pattern obtained is very complex. Hybrids can be constructed that are far more potent in their activity on mouse cells than either of the parental molecules and several parts of the protein seem to play a role in this high

activity. Results obtained with both sets of hybrids on human cells indicate that the amino-terminal 1–60 segment is important for antiviral activity and also for receptor binding.[18] Interestingly, at position 16–20, as postulated here to be important for activity of MuIFN-αs (on hamster cells), HuIFN-α_1, -α_2, and -α_8 are completely identical. Therefore, the series of hybrid proteins as described here provides a valuable extension of our knowledge of the properties of IFN-α and is important starting material for more detailed studies.

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PAPER IV

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Two domains in alpha interferons influence the efficacy of the antiviral response

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Summary: Murine interferon-α1 and murine interferon-α4 share 80% of their amino acids, yet the proteins differ considerably in their ability to protect mouse or hamster cells against viral infection. With the aim of localizing areas within these proteins which influence the biological response we have constructed hybrid α1α4 genes by means of homologous recombination of the parent genes. When the antiviral activities of these proteins were compared, it appeared that there are at least two domains that affect the biological response to these proteins: area A (amino acids 10 - 20) and area B (amino acids 55 - 67). These areas are presumably involved in the interaction between ligand and receptor. Most interestingly, hybrids in which area A from IFN-α1 is combined with area B from α4, have antiviral activities on homologous cells that are one to two orders of magnitude higher than those of the parent proteins.

Interferon-α (IFN-α) is a group of closely related proteins which are able to protect cells against viral infection, can inhibit cell growth and posses immunomodulatory activities (1). These effects are mediated through binding to a specific cell surface receptor (2), followed by a change in the expression of several genes (3). The IFN-α proteins are encoded by a large gene family, which presumably arose by repeated duplication of an ancestral IFN-α gene (4).

Despite the homology between the IFN-α proteins, they can differ considerably in their biological activities (5, 6). For instance, murine IFN-α1 is 100 times as active in the protection of hamster cells against viral infection as is murine IFN-α4 (6), yet the two proteins differ in only 20% of their amino acids (7). Several groups have studied this phenomenon by the investigation of hybrid proteins, constructed by the use of common restriction enzyme sites within the genes. From these studies the rough picture emerged that several parts of an IFN-α molecule influence the magnitude of the biological response (6, 8-11). Due to the nucleotide sequence homology between the IFN-α genes it is also

possible to obtain hybrid genes by means of homologous recombination between two parent genes in Escherichia coli (12, 13). We have used this approach for the construction of hybrids between the murine IFN-α1 and -α4 genes and we have analysed the behaviour of the corresponding hybrid proteins.

MATERIALS AND METHODS

Construction of hybrid genes. These experiments were performed largely according to methods given in Maniatis et al., (14). The exact cross-over point was established by nucleotide sequencing of fragments inserted into M13 vectors (15), using the enzymatic method described by Sanger et al., (16).

Cell culture and transfection. COS cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% foetal calf serum, penicillin and streptomycin. The cells were seeded in 35 mm Petri dishes and grown to 30% confluence. Transfection was performed using the DEAE-dextran technique (17). To this end the medium was removed and replaced by DMEM (1 ml), containing 1 μg PvuII- and HpaII-digested plasmid DNA and 100 μg DEAE-dextran (6, 18). After 2 h the mixture was removed and the cells were treated with 0.1 mM chloroquine in DMEM for 2 to 4 h. They were subsequently fed with DMEM plus serum. This was changed once 24 h after transfection. After 72 h the medium was removed and the cells were washed extensively with Hanks' balanced salt solution and incubated for 16 h with DMEM with one-tenth the normal methionine concentration plus 50 μCi [^{35}S]-methionine but without serum. This medium was used for polyacrylamide gel analysis and IFN assay.

IFN analysis. IFN titres were determined in a cytopathic effect reduction assay, using vesicular stomatitis virus as a challenge. IFN titres on mouse cells were calculated according to the NIH reference standard G002-904-511. Titres on hamster cells were calculated relative to the activity of murine IFN-α1 (6). Proteins secreted by transfected COS cells were separated on a 12.5% polyacrylamide gel according to the procedure described by Laemmli (19). The proteins were visualized by fluorography (20). Total radioactivity incorporated into secreted proteins was determined by precipitation with trichloroacetic acid. These values and densitometric scans of the fluorograph were used to calculate the amount of IFN in each sample.

RESULTS

Construction and expression of hybrid IFN genes. The approach followed is shown in Figure 1. First a PvuII-EcoRI fragment from pSVα1 containing the murine IFN-α1 coding region (6) was inserted into pBR328, resulting in pAGA. This plasmid was subsequently used for the construction of pAGB, by the insertion of a BamHI-SalI fragment from pSVα4 (6), which contains the α4 coding sequences. These cloning steps were carried out in a recA$^-$ host strain. Plasmid pAGB was subsequently linearized with ClaI and the linear construct was introduced into a recA$^+$ Escherichia coli strain. Resulting colonies which carried hybrid α1α4 genes were identified by their failure to hybridize to a BglI-NcoI fragment derived from pBR328 which is located next to the ClaI site in pAGB. In total 48 hybrid α1α4 genes were isolated, they were called α14-1, α14-2 etc. The cross-over points in these hybrids were established by analysis with restriction enzymes. From a preliminary analysis of the hybrid proteins 10 hybrid genes (α14-4, -6, -8, -10, -11, -12, -15, -16, -17 and -60) were selected for further experiments. With the exception of hybrids α14-4 and -8 the

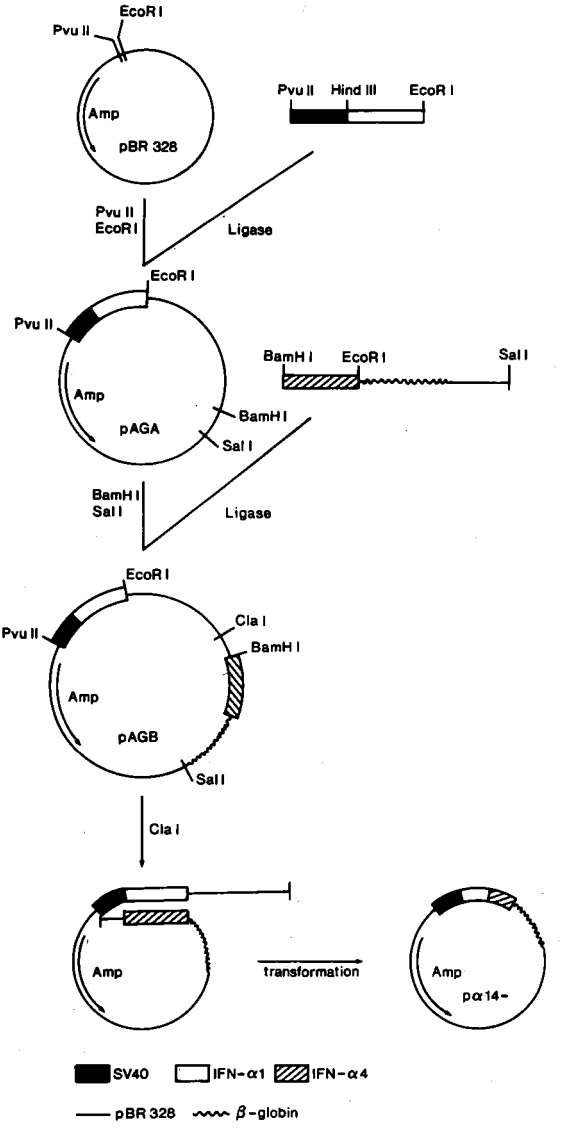

Figure 1. Construction of murine IFN-α1α4 hybrid genes. Only the relevant restriction enzyme sites are shown. Amp: ampicillin resistance gene.

cross-over points were further defined by nucleotide sequence analysis. An outline of the hybrids used throughout this study is shown in Figure 2, where the area in which cross-over has taken place is shown in black. Hybrid α1α4 was constructed using a common XmnI site and has been described before (6). As a source of the corresponding hybrid proteins we used the culture medium of monkey

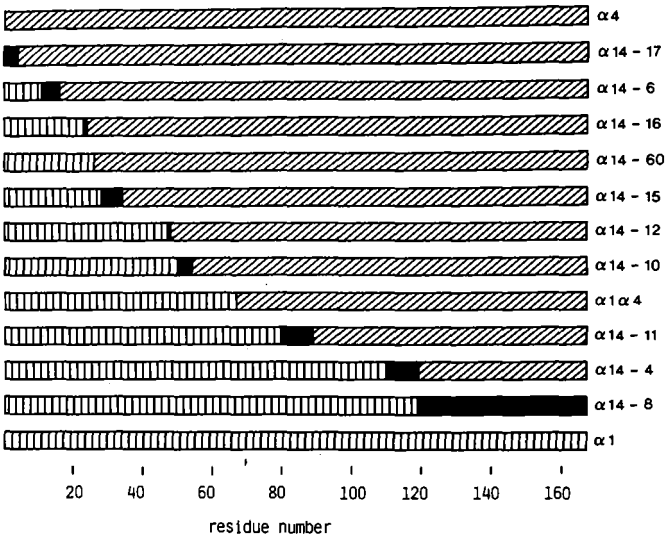

Figure 2. Outline of the murine IFN-hybrid genes used in this study. In each hybrid the area in which cross-over has taken place is shown in black. The cross-over areas of hybrids α14-4 and -8 were only roughly defined by restriction enzyme analysis. Hybrid α1α4 was constructed using a common XmnI site, as a consequence its cross-over point is exactly known. In all other hybrids the cross-over area was defined by nucleotide sequencing. The 5'-border of a cross-over area is defined by the last α1-specific nucleotide and its 3'-border by the first α4-specific nucleotide. Within the cross-over area both sequences are identical. The exact position of the cross-over with respect to the amino acid sequence is indicated by the amino acid residue number on the lower line.

COS cells (21) transfected with the hybrid IFN genes. Three days after transfection the culture medium was replaced with serum-free medium containing [^{35}S]-methionine. After a 16 h incubation this medium was collected and the proteins secreted during this period were separated by gel electrophoresis and visualized by fluorography (Figure 3). A band of approximately 21 kD, which represents the various (glycosylated) IFNs, is clearly visible in the lanes containing medium from COS cells transfected with IFN genes.

Activity of hybrid IFNs on chinese hamster ovary (CHO) cells. The specific antiviral activity of the different IFN species was determined from the antiviral titre and the amount of protein present as IFN (see Materials and Methods). The activities on CHO cells are given in Figure 4. We have previously shown that the specific activity of murine IFN-α1 on hamster cells is about two orders of magnitude higher than that of murine IFN-α4 (6). The activities calculated for the hybrid IFNs show that when the length of the N-terminal α1-fragment is increased from 16 (α14-6) to 22 amino acids (α14-16) the activity of the hybrid IFN increases 100-fold from the low α4-level (α14-6) to the high α1-level (α14-16). Due to the high degree of homology between the

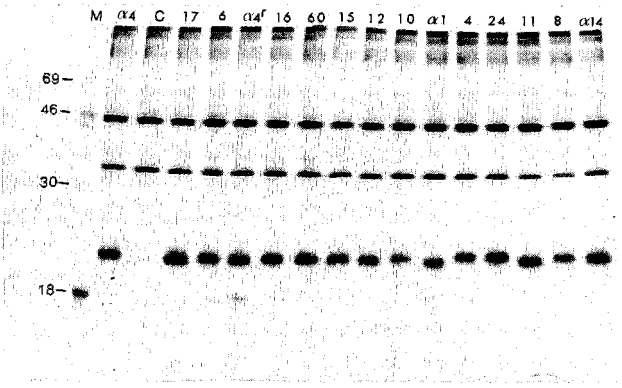

Figure 3. Fluorograph of a polyacrylamide gel containing ^{35}S-labelled proteins secreted by COS cells transfected with IFN expression plasmids. Lane M, molecular weight markers; lane C, medium from COS cells transfected with a plasmid not containing IFN sequences; other lanes, medium from COS cells transfected with (hybrid) IFN genes.

parent α1 and α4 proteins the actual difference between the two hybrids is only 3 amino acids (position 17, 19 and 20 (7); see also Figure 6). Thus, it is conceivable that these amino acids represent, or are part of, a domain that has a profound influence on the specific activity of the proteins. All other hybrids in which the length of the N-terminal α1-fragment exceeds 22 amino acids

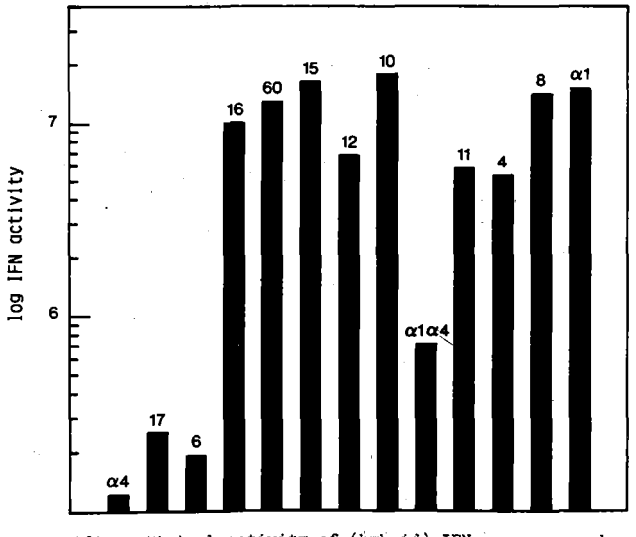

Figure 4. Specific antiviral activity of (hybrid) IFNs as measured on hamster CHO cells. The activities are represented as the logarithm of the specific antiviral activity (units per mg IFN).

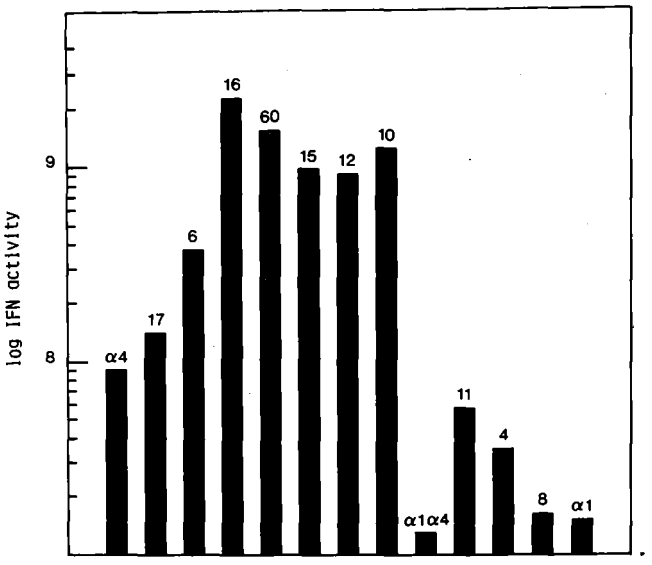

Figure 5. Specific antiviral activity of (hybrid) IFNs as measured on mouse L929 cells. The activities are represented as the logarithm of the specific antiviral activities (international units per mg IFN).

were found to have activities similar to that of α1, with the exception of the previously described hybrid α1α4. To exclude any possible errors made while constructing this hybrid, we assembled it once more and analysed two independent

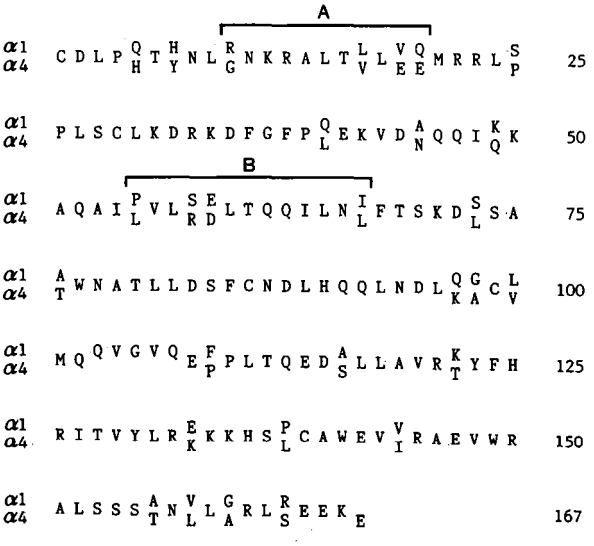

Figure 6. Amino acid sequence of murine IFN-α1 and -α4 as described previously (7). Differences between α1 and α4 are presented as follows: upper line, α1 residues; lower line, α4 residues. All other amino acids are identical for the two proteins. Areas A and B which influence the antiviral response are marked.

clones. Both show exactly the same behaviour as the original hybrid, thus the specific activity must be accurate. This could point to two other domains which influence the antiviral response. The first is confined by the cross-over points of hybrids α14-10 and α1α4 and the second by the cross-over points of hybrids α1α4 and α14-11 (Figure 2). Also in these areas the actual difference between the active and less active hybrids is only a few amino acids (amino acids 55, 58, 59 and 67 in the first area and amino acids 73 and 76 in the second area; see Figure 6).

Activity of hybrid IFNs on mouse cells. In Figure 5 the specific activities of the (hybrid) proteins as measured on mouse (L929) cells are given. The natural α4 protein is about 6 times more active on mouse cells than α1 as we have shown previously (6). It now appears that with a combination of α1 and α4 sequences far more active proteins can be obtained. Thus, hybrid α14-16 has a 15-fold higher activity than α4 and hybrid α14-17. The amino acids responsible for this effect are thus confined by the cross-over points of hybrids α14-17 and α14-16 (amino acids 4 through 22). In this area positions 5, 7, 10, 17, 19 and 20 differ between the two hybrids. That amino acids 5 and 7 probably do not contribute to the observed difference is suggested by the the behaviour of an α4 mutant protein ($\alpha 4^r$) in which glycine at position 10 is replaced by arginine (Van Heuvel et al., to be published). The specific activity of this protein on mouse and hamster cells was found to be identical to the activity of α14-6 (results not shown). Thus, amino acids 10, 17, 19 and 20, or a subset of these, must be responsible for the observed difference in activity between α14-17 and α14-16. When the α1-fragment is further increased in length from 22 to 54 amino acids, the specific activities of the hybrid proteins stay high. However, a sudden 100-fold drop in activity occurs in the area between amino acids 55 and 67 (compare the activity of hybrids α14-10 and α1α4 in Fig. 5). This again points to an important role for amino acids 55, 58, 59 and 67, the amino acids in which these two hybrids differ. Past amino acid 67 no significant differences in specific activity were found.

DISCUSSION

So far it has been difficult to investigate the impact of certain amino acids on the biological activity of the relatively large IFN proteins. For instance, mutagenesis of conserved residues in human IFN-α2 did not lead to proteins with an altered behaviour (22). A comparison of sets of overlapping hybrid proteins as presented here, makes it possible to define two domains within the proteins (area A: amino acids 10 through 20 and area B amino acids 55 through 67) which affect the specific antiviral activity of these murine IFNs on mouse as well as on hamster cells. A combination of area A from α1 with area B from α4 even leads to hybrid IFNs which are far more active on homologous cells than the

natural IFNs. In both areas the actual difference between active and less active hybrids is only 4 amino acids (Figure 6). Thus identified it is possible to explore their impact separately by site-directed mutagenesis. It is reasonable to assume that more amino acids in area A and B than the ones that differ between active and less active hybrids influence the efficacy of these proteins. Now that these areas have been identified the importance of these amino acids can also be investigated. In view of the considerable homology between alpha IFNs from different species (4), it is conceivable that our findings are not restricted to the murine species but are also valid for alpha IFNs of other species.

The appearance of an antiviral state is a consequence of the interaction between IFN molecules and a cellular receptor (8). Indeed in many cases the biological effects of IFNs appeared to be directly proportional to receptor binding (2, 11, 23, 24). In view of these observations the most obvious explanation for the results presented here is that both area A and area B are involved in binding of the ligand to its receptor, either because they represent actual receptor binding sites or because they influence the tertiary structure of the molecules in such a fashion that a binding site is more (un)favorably positioned. A murine α1 fragment comprising the N-terminal 67 amino acids and similar fragments of other murine IFNs have a low but distinct antiviral activity (M. van Heuvel, unpublished results); this suggests that at least one receptor binding site is present in the N-terminal 67 amino acids. Experiments with hybrid IFNs, constructed by means of common restriction enzyme sites, by us (6; M. van Heuvel and J. Trapman, unpublished results) and others (8-11) show that more parts of the IFN molecule may contribute to the magnitude of the biological response. Thus, a comparison of other sets of overlapping hybrids could lead to the identification of additional domains that affect the efficacy of these proteins.

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PAPER V

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Key words: *interferon (MuIFN-α)/hybrid gene/gene structure*

Structure–Function Analysis of Mouse Interferon Alpha Species: MuIFN-α10, a Subspecies with Low Antiviral Activity

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(*Accepted 24 September 1987*)

SUMMARY

A mouse interferon alpha gene (MuIFN-α10) was isolated from a BALB/c cosmid genomic library. The gene was located on a 1·8 kb *Hin*dIII fragment and a 5·1 kb *Eco*RI fragment. The coding region and parts of the 5′ and 3′ non-coding regions were sequenced. The results showed that the MuIFN-α10 gene encoded a protein of 167 amino acids. Like most other MuIFN-α species it contained a putative *N*-glycosylation site at amino acid positions 78 to 80. It also possessed cysteine residues at positions 1, 29, 86, 99 and 129. In the signal peptide, in addition to cysteine 21, which is present in all MuIFN-α species sequenced so far, a cysteine was found at position 22. At the amino acid level MuIFN-α10 showed strong homology to MuIFN-α1 (only 15 out of 167 amino acids were different). The MuIFN-α10 gene was transiently expressed in monkey COS cells under the direction of the simian virus 40 early promoter. The protein product secreted by COS cells was equally active on mouse (L929) and hamster (CHO) cells. However, as compared to MuIFN-α1 and MuIFN-α4 the specific activity on mouse cells of the protein was 10- to 100-fold lower. To find out which region of its structure was responsible for this low activity, hybrids of the genes encoding MuIFN-α10 and MuIFN-α1 were constructed using the two common *Xmn*I sites which correspond to positions between amino acids 67 and 68 and 123 and 124, respectively. The data showed that hybrid constructs which were MuIFN-α1-like from amino acid 68 or MuIFN-α10-like from position 124 to the C terminus possessed high antiviral activity. Other hybrid constructs were hardly active at all. This implied that the amino acid 68 to 123 region was mainly responsible for the low antiviral activity of MuIFN-α10. In this part of the molecule MuIFN-α1 and MuIFN-α10 differed in only five amino acids. A serine at position 110 and a valine at 85 were unique to MuIFN-α10 as compared to all known MuIFN-α and human IFN-α subspecies.

INTRODUCTION

Three antigenically distinct types of interferons (IFNs) can be recognized. These are commonly known as IFN-α, IFN-β and IFN-γ (for a recent review, see Weissmann & Weber, 1986). IFN-αs are encoded by a multigene family. In the mouse system, nine different complete genes have so far been isolated and characterized. Eight of these [α1, α2, α4, α5, α6T, α6P, αA (α7) and α9] encode biologically active proteins (Shaw *et al.*, 1983; Daugherty *et al.*, 1984; Zwarthoff *et al.*, 1985; Kelley & Pitha, 1985*a*; Seif & DeMaeyer-Guignard, 1986; Kelley *et al.*, 1986) and the other is a pseudo-gene (LeRoscouet *et al.*, 1985). Results so far show that all mouse (Mu) IFN-α genes are clustered on chromosome 4 (Kelley *et al.*, 1983; Lovett *et al.*, 1984; Dandoy *et al.*, 1984, 1985; Van der Korput *et al.*, 1985).

The mature MuIFN-α species are 166 or 167 amino acids long. An exception is MuIFN-α4, which possesses a deletion of five amino acids corresponding to amino acids 103 to 107 in the

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other proteins (Zwarthoff *et al.*, 1985; Kelley & Pitha, 1985*b*). The mutual structural homology of the eight different subspecies varies from 74% to 88%. Most MuIFN-α species [excluding MuIFN-α6T and MuIFN-αA (α7)] contain an *N*-glycosylation site (Asn-Ala-Thr) at positions 78 to 80 of the mature protein. Although highly homologous, MuIFN-αs show different antiviral properties. Their specific activity as measured on mouse cells varies about tenfold (Van Heuvel *et al.*, 1986, 1987). Moreover, there are large differences in antiviral activity on hamster cells between the various species.

The availability of a family of very homologous genes of which the protein products show different biological activities provides the opportunity to perform a detailed structure–function analysis of these proteins. During the course of our study on this subject we isolated a MuIFN gene (MuIFN-α10) which was closely related to MuIFN-α1, but showed a tenfold lower specific activity on mouse (and hamster) cells. Here we describe the characterization of this IFN species and of hybrid proteins derived from MuIFN-α1 and MuIFN-α10 by recombination of the natural genes using common restriction enzyme sites.

Preliminary data have been published elsewhere (Trapman *et al.*, 1986). In that abstract the gene described as MuIFN-α10 here was tentatively named MuIFN-α9. Because of the recent isolation of another gene which was also called MuIFN-α9 (Seif & DeMaeyer-Guignard, 1986) the nomenclature has been changed.

METHODS

A cosmid mouse genomic library prepared from WEHI cells was the kind gift of Dr A. de Klein, Department of Genetics, Erasmus University. The MuIFN-α10 gene was detected under standard stringent hybridization conditions using MuIFN-α1 as a probe. Preparation of genomic and plasmid DNA, restriction enzyme digestions, ligations, nick translation and Southern blotting were essentially as described by Maniatis *et al.* (1982).

Sequencing was done by the dideoxy chain termination method (Sanger *et al.*, 1977). The appropriate fragments were cloned in M13mp18/19 (Messing *et al.*, 1981).

Transfection of COS cells, PAGE of ^{35}S-radiolabelled supernatant proteins and calculation of the specific activity of MuIFN-αs were as described previously (Van Heuvel *et al.*, 1986, 1987).

Interferon titrations were by the cytopathic effect reduction assay on mouse L929 cells or Chinese hamster ovary (CHO) cells using vesicular stomatitis virus as a challenge. G-002-904-511 was used as an International Standard on L929 cells. MuIFN-α1 was used as a standard on CHO cells (Van Heuvel *et al.*, 1986).

RESULTS

Isolation of the MuIFN-α10 gene

A BALB/c mouse genomic library was screened with a MuIFN-α1 gene fragment as a probe. One of the positive clones was further characterized by restriction enzyme mapping. The MuIFN-α gene could be localized on a 5·1 kb *Eco*RI fragment. This fragment was subcloned and analysed in more detail, its restriction map is depicted in Fig. 1 (*a*). Fine mapping showed that the gene was situated on a 1·8 kb *Hin*dIII fragment and a 1·2 kb *Hin*dIII–*Pst*I fragment.

To ensure that we had isolated a naturally occurring MuIFN-α gene, rather than a recombinant gene arising from two different genes by homologous recombination during propagation of the cosmid library, we hybridized mouse genomic DNA with the 1·2 kb and 0·6 kb *Hin*dIII–*Pst*I fragments (Fig. 1 *b* and *c*). Hybridization with the 1·2 kb probe showed a large series of bands both in the *Eco*RI and *Hin*dIII digests, representing the MuIFN-α multigene family. Among the most prominent bands were a 5·1 kb *Eco*RI and a 1·8 kb *Hin*dIII fragment, corresponding in size to the respective restriction enzyme fragments of the isolated clone. When the 0·6 kb flanking fragment was used as a probe, a single band was observed (also 5·1 and 1·8 kb in size in both digests). From these results we concluded that we had isolated a natural MuIFN-α gene (MuIFN-α10).

Structure of the MuIFN-α10 gene and protein

The coding region and parts of the 5′ and 3′ flanking regions of MuIFN-α10 were sequenced (Fig. 2). The results indicated that it was a functional MuIFN-α gene. The presence of an open reading frame of 570 bp was obvious. Furthermore, a TATTTAA box, which is a characteristic

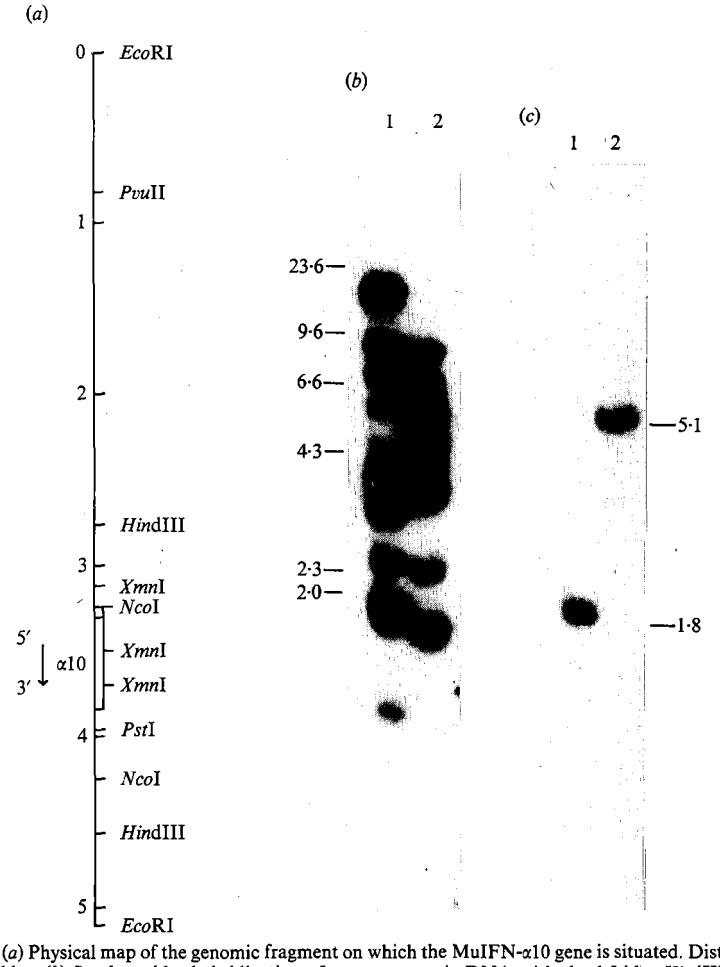

Fig. 1. (*a*) Physical map of the genomic fragment on which the MuIFN-α10 gene is situated. Distances are in kbp. (*b*) Southern blot hybridization of mouse genomic DNA with the 1·2 kbp *Hin*dIII–*Pst*I MuIFN-α10 probe. Lane 1, *Hin*dIII digest; lane 2, *Eco*RI digest. (*c*) Southern blot hybridization of mouse genomic DNA with the 0·6 kbp *Pst*I–*Hin*dIII MuIFN-α10 flanking probe. Lane 1, *Hin*dIII digest; lane 2, *Eco*RI digest. Markers in kbp are *Hin*dIII fragments of phage λ.

of IFN-α genes, was found about 100 bp upstream from the ATG start codon, while 32 bp downstream from the (modified) TATA box an AG sequence was found, which is thought to represent the transcriptional start of IFN-α mRNAs. In the small stretch of 3′ non-coding nucleotides sequenced, no polyadenylation signal could be detected.

Fig. 3(*a*) shows the amino acid sequence of the signal peptide of the MuIFN-α10 protein as deduced from the nucleotide sequence; Fig. 3(*b*) compares the primary structure of the mature MuIFN-α10 protein with the sequence of other MuIFN-α proteins. In the signal peptide MuIFN-α10 diverged from all other known sequences at one position. In addition to the Cys residue on S21, MuIFN-α10 contained a Cys at S22, where normally Ser is found. The mature protein was composed of 167 amino acids. It possessed the five cysteine residues observed in all MuIFN-α species at positions 1, 29, 86, 99 and 139. It also contained the putative *N*-glycosylation site (Asn-Ala-Thr) at 78 to 80 found in MuIFN-α species with the exception of

```
125                    -100                                    -50
AAAAATAGAAACTAGTGTTTTTCTCCTATTTAAGACACATTCACCCAGGATGGTCTTCAGAGAACCTAGAGGGAAGGATCAGGACCAAACAGCCCAGAAGACCAG
                       1                                                                   50
CAGCATTGGCAACATTCACC ATG GCT AGG CTC TGT GCT TTC CTG ATG GTT CTG GCG GTG ATG AGC TAC TGG CCA ACC TGT TGT
                                                    100
CTA GGA TGT GAC CTT CCT CAG ACT CAT AAC CTC AGG AAC AAG AGA GCC TTA ACC CTC CTG GTA AAA ATG AGG AGA CTC
         150                                                                 200
TCC CCT CTC TCC TGC CTG AAG GAC CGG AAG GAC TTT GGA TTT CCC CAG GCA AAG GTG GAT GCC CAG CAG ATC CAG GAG
                                              250
GCT CAA GCC ATC CCT GTC CTG AGT GAG CTG ACC CAG CAG ATC CTG AAC ATC TTC ACA TCA AAG GAC TCA TCT GCT GCT
 300                                                                 350
TGG AAT GCA ACC CTC CTA GAC TCA GTC TGC AAT GAC CTC CAC CAG CAG CTC AAT GAC CTG CAA GGC TGT CTG ATG CAG
                                    400                                                                 450
GAG GTG GGG GTG CAG GAA CTT TCC CTG ACC CAA GAA GAC TCC CTG CTG GCT GTG AGG AAA TAC TTC CAC AGG ATC ACT
                                                          500
GTG TTC CTG AGA GAG AAG AAA CAC AGC CCC TGT GCC TGG GAG GTG GTC AGA GCA GAA ATC TGG AGA GCC CTG TCT TCC
                         550                                                                 600
TCA GCC AAC TTG CTG GCA AGA CTG AGT GAG AAG AAG GAG TGA GTCCTGAGACAAAGTAGAGAGGATCTCCAGGATTAGGACACTGCACC
                                    650                                                 699
TCTCTGTCCAGATTCTACCATCTCAAAAATATCATACAATTTTGCATTAATTGAAGCAAGTTGCCTAGATCTTCTGCAG
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Fig. 2. Nucleotide sequence of the MuIFN-α10 gene. TATA box, ATG start codon, TGT codon encoding the first amino acid in the mature protein and TGA stop codon are underlined.

(*a*)

```
      S1       S10       S20
α10   MARLCAFLMVLAVMSY-WPTCCLG
α1    .....................S..
α2    ........VM.I......SI.S..
α4    .........I.VM...Y.SA.S..
α5    ...........P.L........S..
α6T   ...................S..S..
α6P   .............L........S..
α9    ...PF......V.I....S..S..
αA    .........T.L......S..S..
```

(*b*)

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      1        10        20        30        40        50        60        70        80
α10   CDLPQTHNLRNKRALTLLVKMRRLSPLSCLKDRKDFGFPQAKVDAQQIQEAQAIPVLSELTQQILNIFTSKDSSAAWNATLLDS
α1    .....................Q.....................E.......KK.....................................
α2    ....H.Y.........KV.AQ....PF.......Q.....LE...N....K.......RD....T..L....A.............
α4    ....H.Y..G......V.EE....P..............LE...N....K....L..RD.......L.....L..T.........
α5    ..........................................E..G.....................V.....................
α6T   .......K,..........IQ.....................E...TLK..KEK.......V.................D.....T
α6P   ..........................................E..G..............T.......TL....................
α9    ............KI....AQ.....................E.........................TL...................
αA    .....................Q................R...E........N.......Q.....V.............D.S....

      90        100       110       120       130       140       150       160
α10   VCNDLHQQLNDLQGCLMQEVGVQELSLTQEDSLLAVRKYFHRITVFLREKKHSPCAWEVVRAEIWRALSSSANLLARLSEKKE
α1    F.................Q.....FP.....A.............Y.................V.........V.G..R.E.-
α2    F.............T....Q.....PP.....A.............Y.................V.......V...P....E..
α4    F............KA.V..------.PP............T.......Y..K....L.....I...V.......T.........E..
α5    F..EV.......KA.V..Q.....SP.....................Y.................V.......V.......KEE-
α6T   F....Y.......A..V.Q.RL..PP....V..............Y.................V.........V.G..R.E.-
α6P   F......L..........Q.EI.A.P...........T.....................V.........K.....N.DE-
α9    F.TG...L..........L..MK..P......Q..MK.........Y.................V.......V.........E..
αA    F............KA.V.........PP.....Y.....T.......Y.................V...M....K.......E..
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MuIFN-α6T and MuIFN-αA (α7). MuIFN-α10 showed the highest homology to MuIFN-α1 (only 15 out of 167 amino acids are different) and the lowest to MuIFN-α4 (36 differences). MuIFN-α10 contained unique amino acids at four positions, conserved in other MuIFN-α proteins: positions 41 (Ala instead of Glu), 85 (Val instead of Phe), 110 (Ser instead of Pro) and 148 (Ile instead of Val).

Antiviral properties of the MuIFN-α10 protein

To obtain information on the antiviral properties of the MuIFN-α10 protein, the gene was inserted into the eukaryotic expression vector pSV328A (Van Heuvel *et al.*, 1986) which contains the origin of replication and early promoter of simian virus 40 (SV40) and the polyadenylation signal of the rabbit β-globin gene. pSV328A was digested with *Pst*I (partially) and *Hin*dIII. The 1·2 kb *Hin*dIII–*Pst*I fragment of MuIFN-α10 was cloned into the partially digested pSV328A, resulting in pSVα10HA. Next, the *Nco*I fragment of pSVα10HA was replaced by the *Nco*I fragment of pSVα1, thus removing the MuIFN-α10 promoter. Alternatively, the *Mst*II fragment of pSVα10HA was isolated and exchanged with the *Mst*II fragment of pSVα1. The latter approach was possible because in the coding region of the mature proteins the first amino acids of MuIFN-α1 and -α10 were identical. In this part MuIFN-α1 and -α10 differed only in the signal peptide sequence (see Fig. 3).

Both constructs were transiently expressed in monkey COS-1 cells and the supernatant was monitored for antiviral activity and protein production. Fig. 4 shows the results of SDS–PAGE of MuIFN-α10 (*Nco*I) secreted by COS cells as compared to control IFNs. Table 1 summarizes the corresponding antiviral activity. The protein pattern illustrates that comparable amounts of different proteins (MuIFN-α1, -α4 and -α10) were present; however, the amount of antiviral activity measured in the different samples varied considerably. The Cys residue in the signal peptide on S22 had no effect on the production of the protein [compare MuIFN-α10 (*Nco*I) and MuIFN-α10 (*Mst*II) in Table 1]. As shown earlier (Van Heuvel *et al.*, 1986) MuIFN-α4 possesses the highest specific activity, while the activity of MuIFN-α10 is more than 100-fold less and at least 10-fold lower than that of MuIFN-α1. Using an independent isolate of the MuIFN-α10 gene from a different library, identical values were obtained (data not shown). From these results, MuIFN-α10 was the mouse IFN subspecies with the lowest specific antiviral activity on mouse cells. The antiviral activity on hamster (CHO) cells was relatively high and identical to that on mouse cells (see Table 1).

Antiviral properties of MuIFN-α10α1 hybrid proteins

To obtain a more detailed insight into the region of MuIFN-α10 that is responsible for its low biological activity we constructed a series of hybrids between the highly homologous MuIFN-α10 and MuIFN-α1 genes using the two common *Xmn*I sites which correspond to amino acid positions 67 to 68 and 123 to 124, respectively. The hybrid genes were transiently expressed in COS cells and supernatants were analysed for protein composition and antiviral activity (Fig. 5, Table 2). Varying amounts of the different hybrid proteins were present in the cell culture medium. MuIFN-α1α10 (Xb) and MuIFN-α10α1 (Xa) (Fig. 5, lanes 5 and 7) were present in amounts comparable to the parental proteins, no MuIFN-α1α10 (Xa) and a decreased amount of MuIFN-α10α1 (Xb) were seen (Fig. 5, lanes 4 and 8). From these findings we concluded that there is a relationship between high activity and the presence of MuIFN-α1 sequences in the middle part (from amino acid 68 to 123) of the protein molecule (Table 2). If MuIFN-α10 sequences are present in this region no IFN or an IFN with a much lower specific activity is detected. This strongly suggests that amino acids which differ between MuIFN-α1 and -α10 in this part of the protein are responsible for the low antiviral activity of MuIFN-α10.

Fig. 3. (*a*) Amino acid sequence of the signal peptide of the MuIFN-α10 protein as deduced from the nucleotide sequence compared to the signal peptides of other MuIFN-αs. (*b*) Amino acid sequence of the mature MuIFN-α10 protein as compared to other MuIFN-α subspecies.

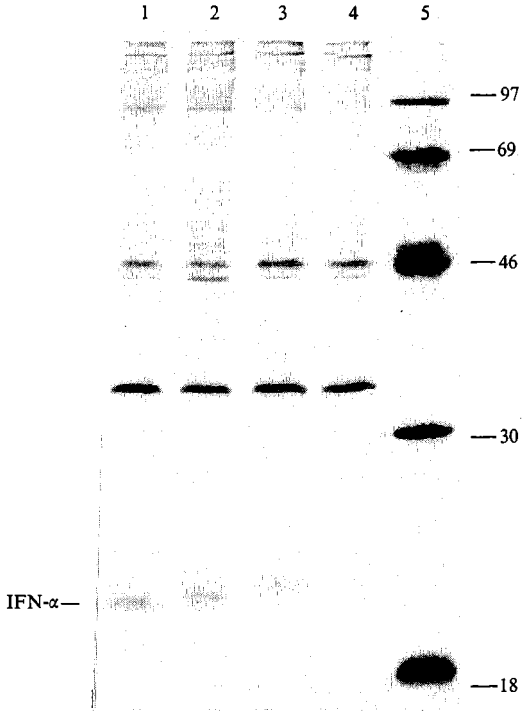

Fig. 4. Fluorograph of an SDS–polyacrylamide gel containing ^{35}S-labelled proteins secreted by COS cells which had been transiently transfected with IFN expression plasmids. Lane 1, MuIFN-α1; lane 2, MuIFN-α4; lane 3, MuIFN-α10 (*Nco*I); lane 4, control COS supernatant; lane 5, molecular weight markers ($M_r \times 10^{-3}$).

Table 1. *Antiviral activity of MuIFN-α subspecies on mouse (L929) and hamster (CHO) cells*

MuIFN	Antiviral activity on L929 cells (IU/ml)	Antiviral activity on CHO cells (U/ml)
α1	3200	3200
α4	32000	48
α10 (*Nco*I)	240	320
α10 (*Mst*II)	240	320

Table 2. *Antiviral activity of MuIFN-α1α10 hybrid proteins on mouse (L929) cells*

MuIFN	Antiviral activity (IU/ml)	Specific activity (IU/mg protein)
α1	3200	$1{\cdot}5 \times 10^7$
α1α10 (Xa)	<1	ND*
α1α10 (Xb)	1600	$0{\cdot}8 \times 10^7$
α10	240	$1{\cdot}5 \times 10^6$
α10α1 (Xa)	3200	$1{\cdot}5 \times 10^7$
α10α1 (Xb)	2	$3{\cdot}1 \times 10^4$

* ND, Not done.

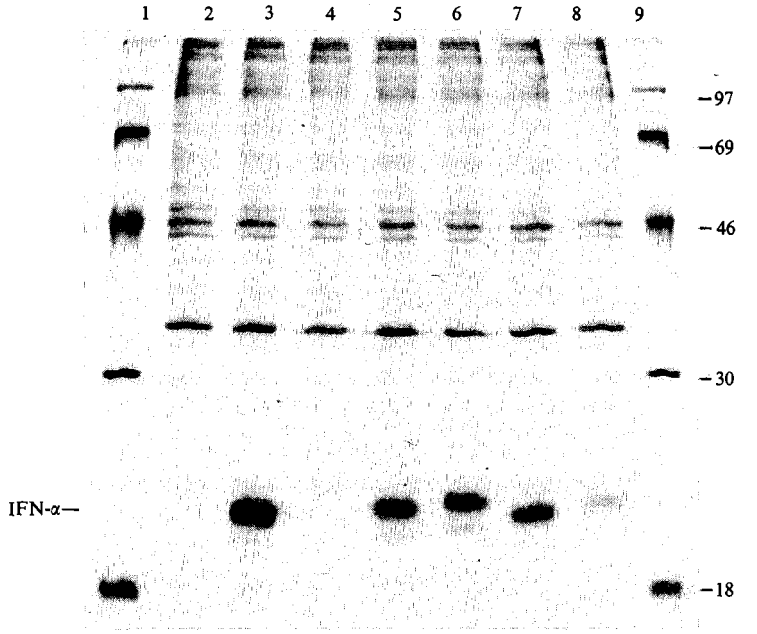

Fig. 5. Fluorograph of an SDS–polyacrylamide gel containing ^{35}S-labelled proteins secreted by COS cells transfected with MuIFN-α1α10 hybrid expression plasmids. Lanes 1 and 9, molecular weight markers ($M_r \times 10^{-3}$); lane 2, control COS supernatant; lane 3, MuIFN-α1; lane 4, MuIFN-α1α10 (Xa); lane 5, MuIFN-α1α10 (Xb); lane 6, MuIFN-α10; lane 7, MuIFN-α10α1 (Xa), lane 8, MuIFN-α10α1 (Xb). Xa and Xb indicate *XmnI* sites in the corresponding MuIFN-α genes used for the construction of hybrid genes (see also Table 2).

DISCUSSION

In this study we describe the characterization of the protein product of the MuIFN-α10 gene. Direct comparison of the primary structure of MuIFN-α10 with that of other MuIFN-α species (Fig. 3) revealed a considerable degree of homology (91% to 78%). MuIFN-α10 differs from all other MuIFN-α species evaluated so far by its much lower antiviral activity on mouse cells. The at least 10-fold difference in antiviral activity between MuIFN-α10 and MuIFN-α1 combined with the high degree of mutual sequence homology provided us with a tool for a more detailed structure–function analysis. Hybrids were constructed between the two genes using common restriction enzyme sites and the protein products were analysed. Previously we have used a similar approach to characterize the regions that are involved in the high antiviral activity of MuIFN-α1 on hamster cells and that of MuIFN-α4 on mouse cells (Van Heuvel *et al.*, 1987). These data indicated a role of N-terminal sequences in high activity of MuIFN-α species on hamster cells and the importance of the C-terminal part of the MuIFN-α4 for its high activity on mouse cells. Our data here show that a third region (amino acids 68 to 123) is important for the low antiviral activity of MuIFN-α10 on mouse cells. In this part of the molecule MuIFN-α1 and MuIFN-α10 differ in only five amino acids, at positions 85, 103, 109, 110 and 116. Of these, the Val residue at 85 and the Ser residue at 110 are unique to MuIFN-α10 as compared to all other MuIFN-α species. All MuIFN-αs possess a Phe and a Pro residue at positions 85 and 110, respectively. It is conceivable that the aberrant composition of MuIFN-α10 at one of these highly conserved positions is of importance to its diminished antiviral properties. The absence of a Pro residue at 110, which can lead to structural changes of the protein, is especially striking.

Because of the large differences in antiviral activity between hybrids containing the middle part of α10 and the parental α10 protein, these hybrids are not well suited for a more detailed structure–function analysis of MuIFN-α10 than that done so far. The low activity of these hybrids is most probably explained by additional structural changes of the protein and not by a direct reduction of the affinity of the binding domain(s) for the IFN receptor. Site-directed mutations of the various positions discussed above are needed to extend our observations and to confirm our hypothesis of the importance of Pro 110 (and Phe 85) to the antiviral activity of MuIFN-α species.

The human (Hu) IFN-α multigene family is composed of at least 14 different members (Weissman & Weber, 1986). Comparison of the consensus HuIFN-α amino acid sequence (Weissman & Weber, 1986) with that of MuIFN-αs shows a mutual homology of around 70%. The most striking structural differences between HuIFN-αs and MuIFN-αs are *N*-glycosylation of MuIFN-αs (*N*-glycosylation site at positions 78 to 80) and the presence of a Cys residue at amino acid position 86 in MuIFN-αs, which is absent in HuIFN-αs with the exception of HuIFN-α1 (D). Cys residues at positions 1, 29, 99 and 139 are found in all MuIFN-α and HuIFN-α species examined. Similarly, Pro residues are seen at positions 4, 26, 39, 110 and 138 in almost all IFN-αs from both species [MuIFN-αs contain an additional Pro at 55, which is also present in HuIFN-α2 (A)]. Like MuIFN-α10, HuIFN-α16 lacks Pro 110. Unfortunately, data about the antiviral properties of HuIFN-α16 or hybrids derived from it, which would be interesting for comparison to MuIFN-α10, are not available.

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PAPER VI

J. CELL. BIOCHEM., in the press

IFN PRODUCING CHO CELL LINES ARE RESISTANT TO THE ANTIPROLIFERATIVE ACTIVITY OF IFN: A CORRELATION WITH GENE EXPRESSION.

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CHO cell lines were constructed which constitutively produce the murine IFN-α subspecies α4 and α6. The producer cell lines were protected against viral (vesicular stomatitis virus) infection by the IFN species secreted, but were resistant to the growth inhibitory activity of the IFN species. As compared with α4, the α6 protein displayed a high antiproliferative activity when added to normal CHO cells. This correlates completely with the high antiviral activity of α6 on these cells. Three mRNA species, which are normally induced in CHO cells by IFN treatment (1-8, 2-5A synthetase and ISG 15) were constitutively present in CHO producer cell lines. The level of another mRNA (ISG 54), however, was very low in the producer cells as compared with its expression in short-term IFN-treated cells. These data indicate that 1-8, 2-5A synthetase and ISG 15 are not involved in the antigrowth activity of IFN in this system, but rather suggest a function of ISG 54 in this respect.

INTRODUCTION

Interferon-α (IFN-α) comprises a group of highly homologous proteins encoded by a multigene family of at least 15 members [1]. To exert its various biological activities (antiviral, antiproliferative and immunomodulatory), IFN binds to a specific cell surface receptor, whereupon the expression of a series of different genes is induced [2]. The function of the protein products of most of these genes is unknown. For some of the proteins, e.g. 2-5A synthetase (2-5A) [3] or metallothionein-IIA [4], which have a known activity, it is difficult to correlate this activity to one of the IFN-specific biological functions. In contrast, the protein product of the IFN-induced Mx gene has been proved specifically to inhibit influenza virus replication in mice [5]. However, in this case the properties of the Mx protein are not understood.

We have isolated several murine (Mu) IFN-α genes and characterized their protein products [6-8]. The specific antiviral activities of five different subspecies, as measured on mouse cells, were found to diverge considerably. Even larger differences were found when antiviral titers were measured on hamster cells. To obtain sufficient amounts of the various proteins for further characterization, CHO cell lines were constructed which constitutively produce MuIFN-α proteins. In addition to their function as IFN producers, we used these cell lines to investigate the effects of long-term IFN exposure on cellular functions. In this study we describe some properties of CHO IFN producer cell lines and correlate the biological response of cells to continuous and short-term IFN exposure with the expression of IFN-induced genes.

METHODS

Cell culture and transfection

Cells were propagated in a 1:1 mixture of Ham's F10 and Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (FCS), penicillin and streptomycin. Transfections were performed according to the calcium phosphate precipitation technique [9]. Selection of transfectants was done in the presence of G-418 sulphate (Gibco).

Cell growth assay

Normal and IFN-producing CHO-9 and CHO-12 cells were grown to confluency and left for two days on medium containing 0.5% FCS, whereupon the cells were seeded at a density of 10^3 cells/cm^2 in 12 well clusters in medium containing 5% FCS. One day after seeding IFN was added to the culture medium. The supernatant of IFN-producing CHO cells was used as a source of the various IFN species. The medium was changed every other day (without or with added IFN). At indicated time points, the cell density was determined by the dye uptake method of Kohase et al. [10].

Antiviral assay

IFN antiviral titers were determined in a cytopathic effect reduction assay using vesicular stomatitis virus (VSV) as a challenge by essentially the method as described by Armstrong [11]. IFN titers on mouse (L929) cells were calculated relative to the NIH reference standard G002-904-511. Titers on hamster (CHO) cells were calculated relative to the activity of MuIFN-α1 on these cells, which was set at 100% of its activity on mouse cells [6].

Northern hybridization analysis

Total cellular RNA was isolated by the guanidinium thiocyanate method [12]. 20 μg RNA was denatured by glyoxal treatment, separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (GeneScreen), using the method described by the manufacturer (NEN, Boston). Filters were hybridized with cDNA probes labelled as described [13]. Overnight hybridization at 42° C in 50% formamide and washing of the filters were as described by NEN. Filters were exposed to a Kodak X-Omat AR film at -70° C using intensifying screens.

RESULTS

Establishment of CHO IFN-α producer cell lines

MuIFN-α subspecies display different specific antiviral activities on mouse cells. The protein showing the highest activity is α4 ($1x10^8$ IU/mg protein) [8] and the one with the lowest activity α10 ($1.5x10^6$ IU/mg) [7]. When specific activities of IFN-α's are measured on hamster cells, α6 shows the highest activity ($4x10^7$ HU/mg) [6,8 and unpublished results], whereas α4 is 200-fold lower in activity ($2x10^5$ HU/mg) [8].

CHO cells are frequently used for the introduction of foreign genes to generate cell lines synthesizing large amounts of the protein product of the introduced gene. We used these cells for construction of cell lines with integrated MuIFN-α genes. To this end, two different CHO cell lines, CHO-9 [14] and

Table I. Constitutive IFN production by CHO cell lines transfected with the MuIFN-α4 or -α6 gene.

Cell type	clone	IFN	IFN production*) IU/10^6 cells/day	IU/ml/day	HU/ml/day
CHO-9	61	α4	$1.6x10^4$	$1.6x10^4$	32
	106	α6	$4x10^3$	$4x10^3$	$8x10^3$
CHO-12	15	α4	$3.2x10^4$	$6.4x10^4$	128
	28	α6	$8x10^3$	$1.6x10^4$	$3.2x10^4$

*) IFN production as measured on mouse cells in international units (IU) or as arbitrary antiviral units on hamster cells (HU).

CHO-12 [15], were transfected with a coprecipitate of the selection plasmid pKOneo and the IFN expression plasmids pSVα4 or pSVα6 [6]. In these expression plasmids an IFN-α coding region is placed downstream of the SV40 early promoter and followed by rabbit β-globin 3'-noncoding sequences. After 2 weeks of G-418 selection single colonies could be isolated. During this time the α6 producer clones grew at a slower rate than did the α4 clones. This growth inhibition turned out to be transient, because when the clones could be expanded in culture this difference in growth rate was much less pronounced.

IFN production by the isolated clones was variable. Approximately 5% of the CHO-9 and 15% of the CHO-12 clones secreted $\geq 10^3$ IU/ml IFN-α per day. Regardless of the IFN-α subtype, CHO-12 clones produced about four times more IFN than CHO-9 clones (as measured in IU/ml/day). This is at least partly due to the higher cell density of CHO-12 cells, as compared with CHO-9, in confluent cultures. However, CHO-9 cells have the advantage that they can be maintained in a confluent state for a longer time than CHO-12 cells, enabling a longer period of serial overnight IFN collections. The maximal amount of IFN synthesized by selected high-producer CHO-9 and CHO-12 clones, expressed in antiviral units per 10^6 cells or per ml, is shown in Table I. α4 producers secrete four times more IFN (in antiviral units as measured on mouse cells) than α6 producers. These data, combined with the specific antiviral activity of both subspecies on mouse cells ($1x10^8$ IU/mg for α4 [8] and $2x10^7$ IU/mg for α6 [6,8 and unpublished results]), indicate that comparable amounts of IFN-α4 and -α6 protein are secreted by CHO cell lines. However, as measured in antiviral units on hamster cells, α6

producers synthesize 250-fold more IFN than α4 producers.

Proliferation and virus resistance of established CHO IFN producers and short-term IFN-treated CHO cells

The transient cell growth delay of newly generated IFN-producing CHO clones (see previous section) urged us to investigate in more detail the growth characteristics of CHO cells in the presence of IFN. The growth rate of the various CHO IFN producers was determined and compared with that of control CHO cells and CHO cells incubated with exogenous IFN-α4 or -α6. The resulting growth curves are depicted in Fig.1. and clearly show that control CHO cells are sensitive to the antiproliferative activity of IFN, whereas the established IFN-producing CHO cells grow as fast as nonproducing CHO cells. The latter is true even for the α6-producing clones (CHO-9, 106 and CHO-12,

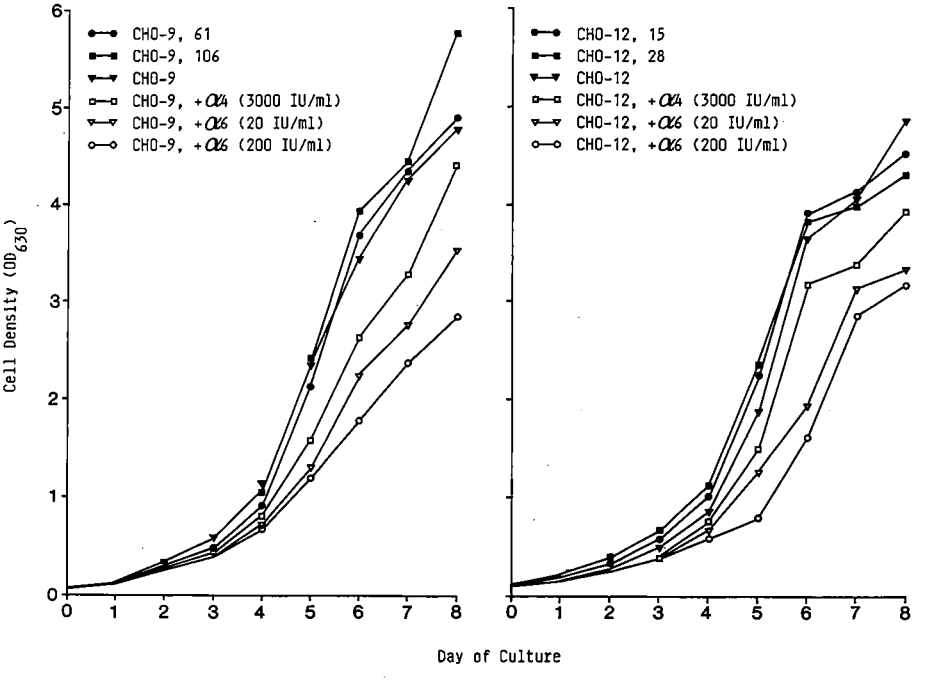

Fig. 1. Proliferation of CHO cells, CHO cells grown in the presence of MuIFN-α4 (3000 IU/ml, corresponding with 6 HU/ml) or -α6 (20 or 200 IU/ml, corresponding with 40 or 400 HU/ml) and IFN-producing CHO cell lines (see Table I for IFN production).

28), which synthesize up to 3.2×10^4 HU/ml/day (see Table I). The experiments with exogenously added α4 and α6 show a clear IFN dosage effect: a dose of 20 IU/ml α6, corresponding to 40 HU/ml, causes a stronger growth inhibition than a dose of 3000 IU/ml α4, corresponding to 6 HU/ml. Because HU are defined as antiviral units on hamster cells, this indicates that a strict correlation exists between the antiviral and the antiproliferative activity on hamster cells of both subspecies. We have also investigated the influence of exogenously added IFN on the growth of the producer cell lines, because, as a consequence of low cell density, at the onset of the proliferation experiments only small amounts of IFN were present in the culture medium. A slight inhibitory effect of exogenously added IFN-α6 on the growth of the producer cell lines was observed (Fig. 2). Inhibition measured at an IFN dose of 100 IU/ml (200 HU/ml) was identical to

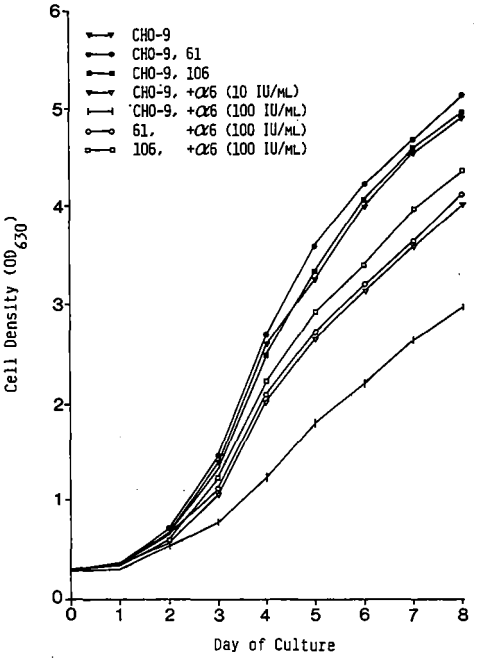

Fig. 2. Proliferation of control and IFN-producing CHO-9 cells in the presence or absence of MuIFN-α6. Control CHO cells were incubated with 10 or 100 IU/ml α6, corresponding with 20 or 200 HU/ml, and IFN producers with 100 IU/ml α6.

or even smaller than that found with control CHO cells at an IFN concentration of 10 IU/ml. Similar data were obtained, when these

experiments were done with the CHO-12 cell lines (results not shown).

All IFN producer cell lines were analyzed for their ability to support replication of VSV. One day after VSV infection the control CHO cells were already lysed by the virus, whereas the IFN producer cell lines were completely protected. This clearly shows that IFN producer cell lines, including the α4 producers, which secrete only a limited amount of IFN as measured in antiviral units on hamster cells (32-128 HU/ml/day), still respond to the antiviral activity of IFN. In addition, several clones producing smaller amounts of α4 (10-20 HU/ml) were also protected against VSV-induced cell lysis.

Combined, the growth characteristics and the viral protection properties of established CHO IFN producer cell lines suggest an uncoupling of the IFN-induced growth inhibition and virus resistance pathway in this system.

IFN-induced gene expression in CHO cells

The complete protection against VSV infection shown by IFN-producing CHO cells indicates that the first common step in all biological responses to IFN, which is binding to the specific IFN receptor, is still intact in these cells. However, our experiments did not exclude modification (decreased binding capacity) or down regulation of the receptor. Most likely, an uncoupling of antiviral and antiproliferative activity of IFN can be explained by a selective change in IFN-regulated gene expression following long term exposure to IFN.

A large number of IFN-induced mRNAs have been described in the human [16-19] and in the mouse [20] system. We used probes obtained from several laboratories to investigate IFN-induced gene expression in hamster cells, grown under various conditions. Five of these probes cross-hybridized sufficiently with hamster RNA. The mRNAs detected with the probes representing 1-8, 6-26 [16], ISG 15 (15k) [19] and ISG 54 (54k) [17], proved to be of similar size as mRNAs found in IFN-treated human cells (0.8, 0.8, 0.7 and 2.9 kb respectively). The 2-5A probe [18] hybridized to a mRNA of about 3 kb in CHO cells.

The 6-26 gene showed a basically high and uninducible

expression (data not shown). A similar observation was made by others in Hela cells [21]. Expression of the other four genes (1-8, ISG 15, 2-5A and ISG 54) was induced in normal CHO cells by IFN treatment for 8, 16 and 24 hours (Fig. 3, lanes 1-7 and 10-12). In general, the level of induced mRNA was higher in α6-treated ($4x10^3$ IU or $8x10^3$ HU/ml) than in α4-treated cells

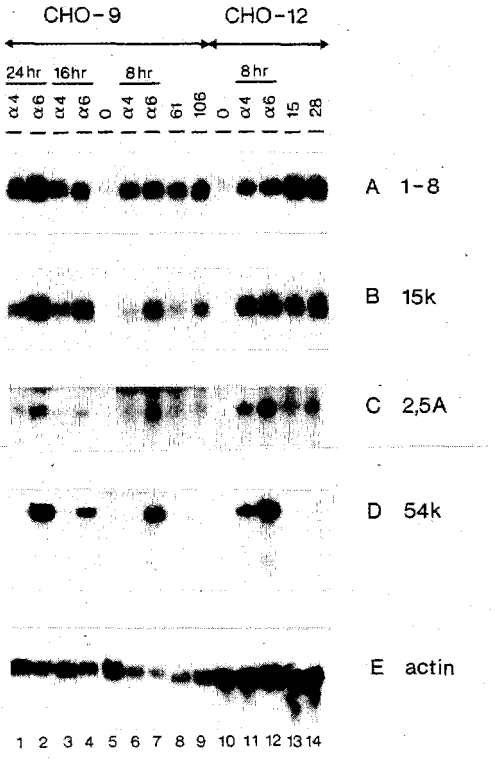

Fig. 3. Northern hybridization analysis of IFN-induced gene expression. The expression of 1-8 (panel A), ISG 15 (15k, B) 2-5A (C), ISG 54 (54k, D) and actin (E) was monitored in CHO-9 and CHO-12 cells (lanes 5 and 10), in CHO-9 cells treated with $1.6x10^4$ IU/ml (32 HU/ml) α4 or $4x10^3$ IU/ml ($8x10^3$ HU/ml) α6 for 8 (lanes 6 and 7), 16 (lanes 3 and 4) and 24 hours (lanes 1 and 2), in CHO-12 cells treated with the same amount of α4 and α6 for 8 hours (lanes 11 and 12) and in the CHO producer cell lines 61, 106, 15 and 28 (lanes 8,9,13 and 14; see Table I for IFN production).

($16x10^3$ IU or 32 HU/ml). This effect was by far the most pronounced for ISG 54 (Fig. 3, panel D, lanes 1-7 and 11,12) and hardly detectable for 1-8 expression (Fig. 3A, lanes 1-7 and 11,12). 2-5A and ISG 15 expression represent intermediate situations in this respect.

Three of the IFN-induced mRNA species (1-8, ISG 15 and 2-5A) were clearly found to be present in the CHO IFN producer cell lines (Fig. 3A,B,C, lanes 8,9,13,14). This is the case both for α4 (15,61) and for α6 producers (28,106). In contrast, the ISG 54 transcript was undetectable or present at a very low level in producer cells (Fig. 3D, lanes 8,9,13,14). Exogenously added IFN-α6 did not enhance this level (results not shown). These data indicate that 1-8, ISG 15 and 2-5A most probably are not involved in the antiproliferative activity of IFN. On the other hand, the ISG 54k protein seems to be an intriguing candidate for such a role and not to be essential for the antiviral activity of IFN.

DISCUSSION

In this study we report the construction and properties of CHO cell lines constitutively producing large amounts of the MuIFN-α subspecies α4 and α6. The IFN produced by these cell lines is very well suited for further characterization. Earlier, we and others described the application of CHO cells for production of γIFNs from different species [22-24], of human (Hu) IFN-α [25] and -β [26,27] and of MuIFN-α1 [28]. In most of these studies, except for the amount of IFN produced, little attention has been given to other properties of the producer cells. Because γIFNs show no or only minute biological activity on cells of different species [29], the γIFN producers will not be affected in a specific way by the IFN-γ protein. However, this probably is not true for Hu α and βIFNs [26,29,30]. A HuIFN-β producing CHO cell line constructed by McCormick et al [27] was found to be insensitive to the growth inhibitory activity of IFN, however, in contrast to our results, also to its antiviral activity, even after adding exogenous IFN-β. This phenomenon was not investigated in more detail. During construction of a MuIFN-α1 CHO producer cell line, we have observed a similar transient growth inhibition as described here for the α6 producer [28].

Interestingly, the α1 protein also displays high antiviral activity on hamster cells [6].

A method different from the one described here to generate IFN resistant cell lines is by long-term culture of cells in the presence of increasing amounts of IFN. This method has been applied for establishment of sublines of Daudi [31], L1210 [32], Friend [33] and RSa [34] cells. Most of the resulting cell lines appear to be resistant to both the antiviral and the antiproliferative activity of IFN. In one resistant cell line, L1210, IFN insensitivity is accompanied by a complete loss of functional IFN receptors from the cell surface [35]. On other resistant cell lines receptors are present [36,37], although in some cases a reduction in binding of IFN to its receptor has been observed [38,39]. Uncoupling of growth inhibitory and antiviral activity has been described for an IFN resistant subline of human RSa cells [34] and for a human yolk-sac tumor cell line [40]. Few data are available on specific gene expression in both cell lines. Two enzymatic activities, 2-5A synthetase and 73k protein kinase, are equally well induced in resistant and in sensitive RSa cells [41], whereas in the yolk-sac tumor cells 2-5A synthetase, the only enzymatic activity which has been measured, displays a proper induction [40].

Analysis of IFN-induced mRNA expression in a Daudi cell line which is completely resistant to the antiproliferative and partially resistant to the antiviral activity of IFN [42] has revealed three genes (1-8, 6-16 and 9-27) which fail to respond to IFN in the resistant cells, whereas 2-5A synthetase mRNA is induced equally well in resistant and sensitive cells. We have not included 6-16 and 9-27 in our analysis, because they do not cross-hybridize with hamster RNA. Regarding the 1-8 gene, our results do not point to involvement of this gene in growth inhibition in CHO cells (see below).

The resistance of the IFN-producing CHO cells to the antigrowth activity of IFN is correlated with a very low level of ISG 54 (54k) transcripts. In contrast, three other IFN-inducible mRNAs (1-8, ISG 15 (15k) and 2-5A synthetase) are clearly present in the producer cells and not or hardly detectable in uninduced cells. This observation suggests that 1-8, ISG 15 and 2-5A synthetase do not play a role in growth inhibition in this

system. Moreover, because ISG 54 expression correlates properly with growth inhibition (see Figs. 3D and 1), its gene product is a likely candidate for mediating the growth inhibition induced by IFN. As can be seen from Fig. 3A such a correlation cannot be established for 1-8 expression, as maximal induction of 1-8 already occurs with 32 HU/ml α4, a dose unable to elicit a maximal inhibition of cell growth. A function of ISG 54 in cell growth regulation might be elucidated by the introduction of the ISG 54 coding region, cloned downstream of a strong constitutive or inducible promoter in respectively antisense or sense direction, into the appropriate cells and determination of the growth characteristics of the resulting cell lines. Although the cDNA has been completely sequenced, so far the properties of the 54k protein are completely unknown. No similarities with other proteins have been observed, except for a small region of 14 amino acids which shows homology to a 13 amino acid stretch present in the mouse Mx protein [43].

To obtain a more detailed insight into the mechanism of resistance to IFN in the CHO producer cell lines, we are presently extending the analysis of IFN modulated genes. Special attention is being given to those genes known to be involved in regulation of cell proliferation and also shown to be modulated by IFN, for example c-myc [42,44,45] or the receptors for certain growth factors, like EGF [46], transferrin [47] and insulin [48].

Another approach for detecting specific genes involved in IFN mediated growth inhibition can be provided by a comparison of the effects of IFN-α,β with those of IFN-γ on cellular functions. For example, IFN-γ has been shown to restore the antiviral state in IFN-α,β resistant Friend cells, a phenomenon accompanied by an IFN-γ-induced 67k protein kinase activity but not by induction of 2-5A synthetase activity [49]. Unfortunately, the hamster IFN-γ is not available, thus in this system these experiments cannot be done as yet.

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