REGULATION OF EXPRESSION AND PROPERTIES OF THE INTERFERON-INDUCED ISG54K/56K GENE FAMILY

(REGULATIE VAN EXPRESSIE EN EIGENSCHAPPEN VAN DE INTERFERON-GEINDUCEERDE ISG-54K/56K GENFAMILIE)

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"Als je alles zou begrijpen wat ik zeg, zou je mij zijn."

-Miles Davis-

CONTENTS

LIST OF ABBREVIATIONS

bp	: base pairs
CAT	: chloramphenicol acetyltransferase
cDNA	: complementary deoxyribonucleic acid
CSF	: colony-stimulating factor
EGF	: epidermal growth factor
EMSA	: electrophoretic moblity shift assay
FcγR	: Fc-γ receptor
FISH	: fluorescent in situ hybridization
GAS	: gamma activating factor
GBP	: guanylate binding protein
GM-CSF	: granulocyte-macrophage colony-stimulating factor
GRR	: gamma response region
Ha	: hamster
hr	: hour
Hu	: human
IBP	: ISRE binding protein
ICSBP	: ISRE consensus sequence binding protein
IE	: inducible element
IFN	: interferon
IL	: interleukin
IP-10	: inflammatory protein-10 kDa
IRF	: interferon reponse factor
ISG	: interferon-stimulated gene
ISGF	: interferon-stimulated gene factor
ISRE	: interferon-stimulated responsive element
IU	: international units
JAK	: "just another kinase"
kB	: kilo bases
kDa	: kilo dalton
LIF	: leukemia inhibitory factor
MAPK	: mitogen-activated protein kinase
MHC	: major histocompatibility complex

min	: minutes
Mo	: monkey
Mu	: murine
mRNA	: messenger ribonucleic acid
nt	: nucleotides
oligo	: oligodeoxyribonucleotide
ORF	: open reading frame
PCR	: polymerase chain reaction
PDGF	: platelet derived growth factor
poly(A)	: poly adenylation
PTK	: protein tyrosine kinase
RACE	: rapid amplification of cDNA ends
SIF	: sis-inducible factor
SH2	: src homology domain 2
STAT	: signal transducer and activator of transcription
SV40	: simian virus 40
2-5AS	: 2',5'-oligoadenylate synthetase
VRE	: virus responsive element
VSV	: vesicular stomatitus virus

CHAPTER 1

INTRODUCTION

INTERFERON-INDUCED GENE EXPRESSION

1.1.1 Introduction

Interferons (IFNs) were discovered over 30 years ago by Isaacs and Lindenmann (1957), who observed that supernatants from virus-infected cell cultures contained a protein that could react with cells to render them resistant to infection by many viruses. Since then much has been learned about the IFN system, including its defensive role in vivo and applicability to treatment of disease (Pestka, 1986a; Baron et al., 1987; Dianzani and Antonelli, 1989; Nelson and Borden, 1989; Taylor and Grossberg, 1990). Although IFNs were first recognized for their potent antiviral properties, it has now been established that they may profoundly affect other vital cellular and body functions, including antiproliferative, antitumor, immunomodulatory and hormonal actions (Pestka, 1986a; Baron et al., 1987; Dianzani and Antonelli, 1989; Nelson and Borden, 1989; Taylor and Grossberg, 1990). Clinically, IFN treatment proved to be benificial in the case of numerous human diseases including viral infections and cancer (Baron et al., 1991).

IFNs are a family of molecules which can be divided into three species: IFN-α, IFN-β, and IFN-γ. IFN-α and IFN-β are also known as Type I IFNs, IFN-γ as Type II IFN. Type I IFNs can be produced by many cell types and induced by many substances, but the amount produced and the subspecies induced can vary per cell type and per inducer. Viruses and synthetic double stranded RNAs are amongst the best inducers. Type II IFN is largely produced by T lymphocytes stimulated by foreign antigens or mitogens (Baron et al., 1991).

In all mammalian species studied to date, the α IFNs are encoded by a family of closely related genes (Weissman and Weber, 1986). In man there are at least 14 "classical" IFN-α genes (IFNA) (Diaz, 1993) and 4 pseudogenes (IFNP), clustered on the short arm of chromosome 9 (Trent et al., 1982; Henco et al., 1985; Olopade et al., 1992). The human IFN-α genes exhibit a high degree of sequence homology, ranging from 80 to almost 100% within their coding regions. An additional, IFNA-related member of the human IFN-α superfamily has been identified: IFN-ω1 (W1) (previously designated IFN-α class II-1), which exhibits approximately 70% sequence homology with the other human IFN-α genes (Capon et al., 1985; Hauptmann and Swetly, 1985). Seven closely related, albeit non functional, human IFN-W pseudogenes (IFNWP) have also been described (Feinstein et al., 1985; Weissman and Weber, 1986; De Maeyer and De Maeyer-Guignard, 1988; Diaz, 1993). Multiple functional IFN-W genes have been described in cattle, horses,

and sheep (Capon et al., 1985; Himmler et al., 1986; Adolf et al., 1991). In contrast, the IFN-W genes appear to be absent in dogs and mice (Himmler et al., 1987; De Maeyer and De Maeyer-Guignard, 1988).

Human IFN-β (or IFN-B) (Diaz, 1993) is encoded by a single copy gene, which exhibits approximately 50% homology at the nucleotide level to the IFN-α genes, and which is physically closely associated with the IFN-α gene family. The mouse, in common with man, possesses a single IFN-β gene, whereas cattle, sheep, and pigs possess multiple IFN-β genes (Weissman and Weber, 1986; De Maeyer and De Maeyer-Guignard, 1988).

In human, type I IFN genes (IFN-A, -W, and -B) are located within the band p22 on the short arm of chromosome 9 (Trent et al., 1982; Olopade et al., 1992). Recently, the first complete physical map of the type I IFN gene cluster has been established (Diaz et al., 1991; Olopade et al., 1992).

In all animal species studied, type I IFN genes are devoid of introns and encode proteins of 186 to 190 amino acids, including a signal peptide of 23 amino acids (Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988). It is assumed that type I IFN 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 homology between the proteins is 70% or more. Between human and murine IFN-α proteins the homology is 50 to 60%. Despite these structural similarities, IFNαs are relatively species-specific: most human IFNs have only a low activity on mouse cells (Weck et al., 1981). However, certain mouse IFNαs reveal high antiviral activity on hamster cells (Van Heuvel et al., 1986). Human IFN-γ is encoded by a single gene, which contains three introns, and which is located on the long arm of chromosome 12 (Kellye et al., 1983; Lovett et al., 1984). Type II IFNs contain approx. 146 amino acids and show no significant sequence homology with type I IFNs (Gray et al., 1982; Trent et al., 1982; Pestka et al., 1987). All other animal species studied to date, including the mouse, cattle, sheep, and pig, also possess a single IFN-γ gene.

Sheep, cattle, and other ruminants possess a distinct class of genes which code for the trophoblast IFNs. These are proteins which are secreted by trophoblasts during early pregnancy and which regulate maternal recognition of pregnancy (Cross and Roberts, 1991). In addition, the trophoblast IFNs exhibit antiviral activity in common with other IFNs, and are able to bind the same receptor as IFN-α, IFN-β and IFN-ω (Stewart et al., 1987).

To elicit their biological properties, it is necessary that the IFNs bind to specific receptors on the target cells. The interaction of IFNs with their cognate receptors activates

cytoplasmic signals that enter the nucleus to stimulate the induction of a set of primary genes which are central in mediating the biological response. This transcriptional activation is rapid and does not require protein synthesis (Levy and Darnell, 1990a; Williams, 1991; Sen and Lengyel, 1992; Pellegrini and Schindler, 1993). The delayed activation of other genes is also part of this response, however, pathway(s) that activate late genes are likely to be indirect, less specific and are not yet well understood. Similar genetic activation of cells by IFN appears to be required for most of its biological actions, e.g. antiviral, cell growth inhibitory and immunomodulatory activities.

1.1.2 The IFN-α/β and IFN-γ signalling pathways (Model for polypeptide-dependent gene activation)

Initiation of the signal transduction pathway occurs when IFNs interact with their cognate receptors. Receptor occupancy rapidly triggers signalling cascades through the activation of tyrosine kinases which culminate in the activation (by tyrosine phosphorylation) of cytoplasmic signalling proteins. Once activated, a ligand-specific set of cytosolic proteins transduce the signal to the appropriate DNA target sequences in the nucleus (Levy and Darnell, 1990a; Pellegrini and Schindler, 1993).

The IFN-α/β and IFN-γ signalling pathways serve as models for cell receptors whose occupation ultimately stimulates transcription through a DNA-binding protein that recognizes a specific consensus element in the DNA, via a specific cytoplasmic counterpart (receptor-recognition protein). The pathway that would conserve specificity includes at least four highly specific reactions: (I) the ligand-receptor interaction; (II) the recognition of the intracellular domain of the receptor by a cytoplasmic protein capable of assisting in the assembly of active transcription factors; (III) the formation and transport of the activated transcription factors and (IV) the recognition in the nucleus of the specific DNA site. Each of the steps, involved in IFN-α/β and IFN-γ activated transcription will be discussed below.

1.1.3 IFN receptors

All cellular responses to IFNs require the interaction of the ligand with a low number of high-affinity, species specific cell surface receptors. Although it was originally thought that a single receptor existed for all IFNs, it is now known that there are two types of receptors, one which interacts with type I IFNs, and a second receptor that binds type II IFN (Pestka, 1986b; Pestka et al., 1986c; Aguet et al., 1988; Langer and Pestka, 1988; Uze et al., 1990; Uze, 1992).

1.1.3a IFN-α/β or type I IFN receptor

The type I receptor permits the productive binding of IFN-β and the many related subtypes of IFN-α (Uze, 1992). IFN-α/β subspecies differ considerably in their affinity for the receptor, which correlates with the level and type of biological activity (Hu et al., 1990).

The human receptor cDNA contains an open reading frame encoding a protein of 64 kDa (Uze et al., 1990), the corresponding gene has been mapped to the long arm of human chromosome 21 (21q22.1; Langer et al., 1990; Lutfalla et al., 1990). Cross-linking studies, however, yielded apparent molecular weights (Mr) in the range of 110-140 kDa for the Hu IFN-α/β receptor. This discrepancy may be due to the presence of glycosyl groups at any of the 15 different potential glycosylation sequences (Uze et al., 1990). Complexes of higher Mr's have also been observed, an indication that the receptor may interact with one or more other polypeptides.

The extracellular region of the type I receptor contains the ligand binding domain and is composed of a duplicated region which suggests the existence of two functional ligand binding domains with varying affinity for one or another homolog of type I IFN (Bazan, 1990a; Bazan, 1990b). Binding of one ligand to a particular domain may affect binding of another to the second domain. This mechanism may account for the multiplicity of binding affinities and receptor occupancies for IFN-α subtypes (Uze et al., 1985; Mogensen et al., 1989). The intracellular domain is very short, but is probably involved in the signal transduction pathway, although no specific functions have been assigned (for example, it lacks a tyrosine kinase domain or multiple membrane spanning domain). Indirect evidence suggesting a multi-subunit receptor or species specific coupling system between the receptor and cellular machinery has been presented by several groups (Jung and Pestka, 1986; Uze et al., 1990). When overexpressed in mouse cells treated with Hu IFN-αB and -β, the human type I receptor confers the antiviral phenotype. In addition it will

mediate MHC induction when transfected into mouse NIH3T3 cells. However, the product of this receptor alone is not sufficient to elicit a response to some other Hu IFN-α species in the transfected cells, suggesting the requirement for an accessory component(s) to reconstitute the response to all type I IFNs. Some of these factors may be species specific (Jung and Pestka, 1986; Uze, 1992).

1.1.3b IFN-γ or type II IFN receptor

The native IFN-γ receptor has been purified and characterized from several cell lines and human placenta (Rashidbaigi et al., 1985; Aguet and Merlin, 1987; Calderon et al., 1988; Fountoulakis et al., 1989; Stefanos et al., 1989; Van Loon et al., 1991). Like the IFN-α/β receptor, the IFN-γ receptor is a single chain glycoprotein. It has an apparent Mr of about 90 kDa (Rashidbaigi et al., 1985; Aguet and Merlin, 1987; Calderon et al., 1988; Fountoulakis et al., 1989; Stefanos et al., 1989; Van Loon et al., 1991). It has an extracellular, a transmembrane and an intracellular domain. The apparent Mr of the receptor from the different cell lines showed variations from 90 to 110 kDa, which could result from differences in glycosylation of the extracellular domain (deglycosylation resulted in a Mr of 70-75 kDa). Glycosylation is not essential for the interaction with the ligand (Fountoulakis et al., 1989).

The human IFN-γ receptor cDNA (Aguet et al., 1988) encodes a protein of 489 amino acids, the gene has been mapped to human chromosome 6q (Rashidbaigi et al., 1986; Jung et al., 1987). The complete extracellular domain of the mature human IFN-γ receptor carrying the ligand binding site has been expressed in E. coli, insect and eukaryotic cells (Fountoulakis et al., 1990a; Fountoulakis et al., 1991). The soluble IFN-γ receptor expressed in E coli, binds IFN-γ in its dimeric form (Fountoulakis et al., 1990b). Comparison of the extracellular domain protein sequences for the type II and type I receptors suggests a similar ligand binding domain of approx. 210 residues (albeit repeated in the type I structure) with characteristic cysteine pairs at both amino- and carboxy-termini (Bazan, 1990a). Two distinct regions of the intracellular domain play an important role in mediating the functional activity of the IFN-γ receptor (Farrar et al., 1991): a short membrane-proximal region of 48 amino acids, which contains a consensus motif found in the intracellular domains of a variety of rapidly internalized receptors, such as the transferrin receptor (Collawn et al., 1990) and the mannose phosphate receptor (Canfield et al., 1991), and three carboxy-terminal amino acids (Tyr-Asp-His) (Farrar et al., 1992). The intracellular region of IFN-γ receptor, like the IFN-α/β receptor, does not contain any obvious catalytic domains.

Human IFN-γ receptors expressed in mouse cell lines with or without human chromosome 21, were fully capable of binding, internalizing and directing the degradation of ligand. However, only the man/mouse somatic hybrids carrying human IFN-γ receptor and human chromosome 21 were responsive to human IFN-γ. To reconstitute functional IFN-γ receptor the presence of a species-specific component(s) encoded by human chromosome 21, interacting with the extracellular domain of the IFN-γ receptor, is required (Hemmi and Auget, 1991; Hibino et al., 1991; Kalina et al., 1991; Soh et al., 1993).

Comparison of extracellular domains of different cytokine receptors supports the view that the receptors for growth factors, IFNs, and lymphokines may exhibit a convergence of structure, that is not evident in the amino acid sequence (Bazan, 1990a; Bazan, 1990b). An analysis of the sequences, which may be predictive for the secondary structure, further supports the idea that the extracellular domains of the IFN receptors share a common three-dimensional architecture formed by amphipathic β strands typically found on globular proteins. Therefore, the multisubunit structure of several cytokine receptors might serve as a paradigm for the organization of the IFN receptors. The finding that the intracellular regions of IFN-α/β receptor and IFN-γ receptor do not possess any obvious catalytic domains, suggests that they interact with crucial signalling components, as has been demonstrated for other cytokine receptors.

1.1.4 IFN-inducible genes

As previously mentioned, the type I and type II IFNs, through their interaction with their different receptors, induce the expression of partially overlapping sets of cellular genes. Some of these genes are induced in common by the type I and type II IFNs, whereas others seem to be preferentially induced by one of the IFN types. The similarities and differences in the biological properties of the type I and type II IFNs may be a reflection of partially overlapping and differential regulation of cellular genes by the two types of IFNs.

A search for IFN-induced proteins responsible for mediating the antiviral and other effects of IFNs led to the identification of novel enzymes [e.g., two double stranded RNA-activated enzymes: a protein kinase and a 2'-5'-oligoA synthetase (2-5AS), cell surface molecules (e.g., MHC class I and class II antigens, Fc receptors] and a number of new proteins (Sen and Lengyel, 1992). A direct role in the antiviral actions of IFN has been demonstrated for the Mx protein and the 2-5AS systems. It is, however, beyond the scope

of this thesis, to cover the aspects of the numerous properties of IFN-induced proteins. For a more detailed review concerning this topic see refs. Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1989; Sen and Lengyel, 1992.

1.1.4a IFN-α/β-stimulated genes

A group of approx. 16 directly IFN-α/β regulated genes (IFN-stimulated genes or ISGs) has been identified. The characterization of their transcriptional response to IFN-α by using in vitro nuclear transcription assays and in vivo promoter analyses has revealed distinctive features of ISG regulation (Friedman et al., 1984; Larner et al., 1984; Faltynek et al., 1985; Friedman and Stark, 1985; Larner et al., 1986; Levy et al., 1986; Williams, 1991; Sen and Lengyel, 1992). ISG mRNAs are detected in cells within about one hour of treatment with type I IFN. In some cases, the mRNAs accumulate to a steady-state level which is maintained for many hours. In other cases, the mRNAs are induced to a peak level which then declines, despite the continued presence of IFN (Friedman et al., 1984; Larner et al., 1984; Revel and Chebath, 1986; Sen and Lengyel, 1992). The induction of gene expression by IFNs is often of great magnitude, reaching high rates of transcription from nearly undetectable levels. Although the induction is primarily at the transcriptional level (Friedman et al., 1984; Larner et al., 1984), additional regulation at a post-transcriptional level has been proposed in certain cases (Friedman et al., 1984). Induction of transcription will occur even in the absence of cellular protein synthesis. The transcriptional response of ISGs correlates with receptor occupancy (Hannigan and Williams, 1986). Analysis of a number of ISGs has allowed the identification of regulatory sequences that determine their inducibility by IFNs. This led to a refinement of the IFN-responsive regulatory sequence. A consensus sequence NAGTTTCNNTTTC/TNN [where N is any nucleotide], designated as the interferon-stimulated response element (ISRE), has been determined. A list of genes containing the ISRE is presented in Table 1. The ISRE exists in ISGs in either orientation, sometimes in multiple copies, and (minor) variations from the consensus sequence have been found in individual ISRE sequences. Functional analysis of the ISG-15K gene (Reich et al., 1987; Reich and Darnell, 1989), and a variety of other inducible genes (Levy et al., 1986; Israel et al., 1986; Benech et al., 1987; Sugita et al., 1987; Wathelet et al., 1987; Cohen et al., 1988; Hug et al., 1987; Kessler et al., 1988a; Levy et al., 1988; Porter et al., 1988; Rutherford et al., 1988; Dale et al., 1989a; Reid et al., 1989) has demonstrated that ISRE is necessary for IFN induction. It appears, therefore, that the IFN-α/β-induced expression of cellular genes is mediated through a common response enhancer element, the ISRE.

TABLE 1 REPRESENTATIVE ISRE SEQUENCES

Gene	Species	ISRE	Ref.
ISG-15	human	CAGTTTCGGTTTCCC	Reich et al., 1987
ISG-54	human	TAGTTTCACTTTCCC CAATTTCACTTTCTA	Wathelet et al., 1987
ISG-56	human	TAGTTTCACTTTCCC CCCTTTCGGTTTCCC	Wathelet et al., 1987
6-16	human	GAGTTTCATTTTCCC CAGTTTCATTTTCCC	Porter et al., 1988
9-27	human	AAGTTTCTATTTCCT	Reid et al., 1989
GBP	human	TACTTTCAGTTTCAT	Lew et al., 1991
HLA class I	human	CAGTTTCTTTTCTCC	Pellegrini and Schindler, 1993
2-5AS	human	TGGTTTC-GTTTCCT	Cohen et al., 1988
IP-10	human	AGGTTTCACTTTCCA	Ohmori et al., 1993
Factor B	human	CAGTTTCTGTTTCCT	Wu et al., 1987
MxA	human	AGGTTTC-GTTTCTG GAGTTTC-ATTTCTT	Chang et al., 1991
H-2K	mouse	CAGTTTCACTTCTGC	Pellegrini and Schindler, 1993
H-2D	mouse	CAGTTTCACTTTTGC	Pellegrini and Schindler, 1993
H-2L	mouse	CAGTTTCCCTTTCAG	Pellegrini and Schindler, 1993
202	mouse	CAGTTTCTCATTTAC	Pellegrini and Schindler, 1993
Mx	mouse	GAGTTTCGTTTCTGA	Hug et al., 1988
2-5AS	mouse	CAGTTTCCATTTCCC	Cohen et al., 1988
β2m	mouse	CAGTTTCATGTTCTT	Pelligrini and Schindler, 1993
Consensus		NAGTTTCNNTTTCNN T	

1.1.4b Genes regulated by IFN-γ

There are also genes that are known to be activated immediately at the transcriptional level by IFN-γ. In this respect, the study of the guanylate-binding protein (GBP) gene, which is inducible by both IFN-γ and IFN-α/β, has led to the identification of the IFN-γ activation site or GAS, in addition to an ISRE (Decker et al., 1989; Lew et al., 1989; Lew et al., 1991). In HeLa S3 cells, GBP mRNA accumulated in response to IFN-α or IFN-γ. For boths IFNs, the induction was transcriptional and primarily direct. The kinetics of GBP mRNA accumulation in response to types I and II IFN were markedly different, with IFN-γ producing a slowly-developing and long-lasting transcriptional induction, whereas in IFN-α treated HeLa cells the GBP gene was transcribed with an abrupt onset and a rapid decay. A GBP promoter-reporter construct, containing an ISRE sequence, could be

activated by IFN-γ or IFN-α. Deletion of the ISRE core abolished the response to both IFNs, but interestingly, base substitutions, which crippled the ISRE homolog for response to IFN-α failed to affect induction by IFN-γ. However, a promoter-proximal element overlapping the ISRE and termed GAS (see Table 2) was absolutely required for IFN-γ to utilize the GBP fragment as an inducible enhancer.

TABLE 2 IFN-γ-RESPONSIVE SEQUENCES

Gene	Species	GAS	Ref.
GBP	human	ATTACTCTAAA	Lew et al., 1991
FcγRI	human	TTTCCCAGAAA	Pearse et al., 1991
ICSBP	human	TTTCTCGGAAA	Pellegrini and Schindler, 1993
IFP-53	human	ATTCTCAGAAA	Strehlow et al., 1993
IRF-1	human	TTTCCCCGAAA	Pellegrini and Schindler, 1993
IRF-1	mouse	TTTCCCCGAAA	Pellegrini and Schindler, 1993
Ly6E	mouse	ATTCCTATAAA	Khan et al., 1993
mig	mouse	CTTACTATAAA	Pellegrini and Schindler, 1993
Consensus		NTTCCCNTAAA ATT G	

Recently, it has become clear that primary activation of other genes by IFN-γ operates through a similar GAS-like element (see Table 2 for a partial list; consensus, NTT(C/A)(C/T)(C/T)N(T/G)AAA). Characterization of the IFP 53 promoter (Strehlow et al., 1993) led to the detection of an IFN-γ response region containing a GAS but no ISRE, while a 9 nt core region in the 3' domain of the GRR (IFN-γ response region) of the FcγRI gene promoter also resembles the GAS (Pearse et al., 1993). These results support the conclusion that the GAS site has a more general role in the induction of transcription by IFN-γ, comparable to the ISRE in ISG induction upon IFN-α treatment (see I.1.4a).

Evidence accumulates that IFN-α/β and IFN-γ induction of gene expression is not as straight forward as presented as above. As already mentioned there is considerable overlap between IFN-α/β and IFN-γ regulated genes. This overlap cannot be explaned by the presence of both an ISRE and a GAS, as found in the GBP gene. The study of the Ly-6A/E gene (Khan et al., 1993), which is transcriptionally induced in cells exposed to IFN-

α/β or IFN-*γ*, indicates that immediate IFN transcriptional response through the GAS element cannot only be used in genes inducible by IFN-*γ*, but also by genes induced by IFN-*α*. Alternatively, in some cases not the GAS element, but the ISRE sequence has shown to be involved in immediate IFN-*γ* transcriptional response. The ISRE present in the IP-10 promoter (Ohmori and Hamilton, 1993) for example, was able to confer IFN-*γ* sensitivity upon a heterologous promoter. Analysis of promoter constructs containing native and mutated ISREs suggested that this motif (see Table 1) is essential for the response of the 9-27 gene to IFN-*γ* as well as IFN-*α/β* (Reid et al., 1989). The human 6-16 gene, which contains ISRE sequence elements (highly homologous to the 9-27 ISRE; see Table 1) in the upstream region (Porter et al., 1988; Reid et al., 1989), is induced selectively by IFN-*α* and not by IFN-*γ*, but the ISRE sequence of the 6-16 gene can confer inducibility to a reporter gene by both types of IFN (Reid et al., 1989). It was surmised that the context of the ISRE may determine the specificity of the response. A further study of the 6-16 gene did not reveal the presence of any IFN-*γ*-specific negative regulatory element (Reid et al., 1989). So, in conclusion it seems possible that under certain conditions, the IFN-*γ* pathway can end up in activation of an ISRE, and IFN-*α/β* might also be able to activate a GAS element.

Further complexity of the system is derived from the indirectly activated genes. An example of this group are the MHC class II genes (Kappes and Strominger, 1988), their induction by IFN-*γ* requiring ongoing protein synthesis (Blanar et al., 1988; Amaldi et al., 1989). An ISRE sequence, present in the upstream regulatory region of the HLA-DR*α* gene (member of the MHC class II genes), does not appear to be important for this secondary response to IFN-*γ* (Basta et al., 1988). The upstream regulatory region of MHC class II genes contains other unique features, which include several highly conserved sequence motifs (W, X and Y box) (Benoist and Mathis, 1990) that are required for IFN-*γ* inducibility. Homologs of W, X and Y box sequences are also found in the invariant chain gene and are required for their constitutive, as well as IFN-*γ* inducible expression (Eades et al., 1990; Brown et al., 1991).

1.1.5 IFN-regulated DNA-binding factors

1.1.5a Factors regulated by IFN-*α/β*

Using electrophoretic mobility shift assay (EMSA), several protein factors have been identified which recognize the ISRE: ISGF1, -2 (IRF1) and -3, ICSBP, IBP-1 and IRF2 (Table 3). The question now is, do all of these factors have a role in IFN-regulated gene

expression, and if so, what is this role. Interferon response factors 1 and 2 (IRF-1 and IRF-2), IBP1 and ICSBP (Fujita et al., 1988; Miyamoto et al., 1988; Blanar et al., 1989; Harada et al., 1989; Imam et al., 1990; Pine et al., 1990) bind the central ~11 bp core element of the ISRE which is shared with the IFN-β gene enhancer (Kessler et al., 1988a).

TABLE 3 ISRE-BINDING FACTORS

Name(s)	Binding Specificity	Protein Composition	Potential Function	Ref.
ISGF1	ISRE core 9 bp	?	Suppressor of basal expression	Levy et al., 1988
IRF-1 (ISGF2)	ISRE core 9 bp	37.3 kDa nuclear	Activation of IFN-β gene	Miyamoto et al., 1988 Pine et al., 1990
IRF-2	ISRE core 9 bp	39.4 kDa nuclear	Repression of activated IFN-β gene	Harada et al., 1989
ICSBP	ISRE core 9 bp	48.2 kDa IRF-related	Repression of ISG transcription	Driggers et al., 1990
IBP-1	ISRE core 9 bp	59 kDa nuclear	IFN-γ induced form of IRF-1	Blanar et al., 1989
ISGF3	ISRE	43.7, 84, 91 and 113 kDa	Activation of ISG transcription	Kessler et al., 1990

However, the entire ~15 bp ISRE sequence is required for transcriptional activity in response to IFN-α/β (Kessler et al., 1988a; Dale et al., 1989a). In addition, these factors are induced in a protein-synthesis-dependent manner following exposure of cells to IFN-α/β, even though initial transcription of ISGs does not require new protein synthesis (Levy et al., 1988; Miyamoto et al., 1988; Harada et al., 1989; Driggers et al., 1990; Imam et al., 1990). Moreover, the timing of their induction is delayed, compared to the kinetics of transcription. These results suggest that, although these factors bind specifically to the ISRE, they are not involved in the (early) transcriptional activation of ISGs (see below). Interferon Stimulated Gene Factor 3 (ISGF3), a slowly migrating gel-shift complex, thusfar, is the only factor that fulfils the above criteria, therefore implicating it in transcriptional activation of ISGs (Levy et al., 1989).

Characteristics and regulation of ISGF3

The factor ISGF3 is induced very rapidly by IFN-α/β without novel protein synthesis. The kinetics of appearance and decline of ISGF3 and its lack of dependence on protein synthesis correlate with the transcriptional activation and deactivation of IFN-α/β-inducible genes (Levy et al., 1988; Rutherford et al., 1988; Dale et al., 1989a; Levy et al., 1989). The sequence requirements for ISGF3 binding at the ISRE correlate precisely with the requirements for transcriptional activation and its absence from IFN-α-resistant cell lines also correlates with ISG transcription (Kessler et al., 1988a; Kessler et al., 1988b; Levy et al., 1988; Cohen et al., 1989; Dale et al., 1989a; Reich et al., 1989). Furthermore, mutations in ISRE which affect the formation of the ISGF3 complex also affect its transcriptional response to IFN-α (Kessler et al., 1988a). Finally, ISGF3 stimulates in vitro transcription from a template containing several copies of the ISRE sequence (Fu et al., 1990). Thus, ISGF3 apparently serves as a positive regulator in IFN-α/β-mediated induction of cellular genes (Kessler et al., 1988a; Kessler et al., 1988b; Reich et al., 1989; Levy et al., 1989; Fu et al., 1990).

The activity of ISGF3 is apparently produced from pre-existing polypeptides (Levy et al., 1989; Dale et al., 1989b). ISGF3 could be induced in cytoplast preparations (free of nuclei) by treatment with IFN-α, but not in nucleoplast preparations (Dale et al., 1989b). Moreover, it was observed that the level of active ISGF3 in cytoplasmic extracts of IFN-α treated cells could be increased by addition of extracts from untreated cells or, more dramatically, from cells treated with IFN-γ (Levy et al., 1989). These results have led to the conclusion that ISGF3 is formed from two components which have been designated ISGF3α and ISGF3γ.

The ISGF3γ component is active and mostly cytoplasmic in untreated cells, but following IFN-α treatment it accumulates in the nucleus (Kessler et al., 1990). Its induction by IFNγ could contribute to the synergy between IFN-α and IFN-γ (Levy et al., 1990b). ISGF3γ was identified as a single polypeptide with an apparent Mr of 48 kDa, therefore also indicated as p48. It serves as the DNA recognition subunit and by itself already binds to the ISRE sequence with the same specificity as ISGF3, but with a much lower affinity (Kessler et al., 1990). Recently, a cDNA encoding this protein has been isolated (Veals et al., 1992). The deduced sequence of ISGF3γ revealed at the amino terminus significant similarity to the three members of the interferon response factor (IRF) family of DNA-binding proteins (IRF-1/ISGF2, IRF-2 and ICSBP; Veals et al., 1992). Moreover, the conserved amino termini of these proteins are related to the DNA-binding domain of the c-myb-encoded oncoprotein (Howe et al., 1990; Kanei-Ishii et al., 1990; Gabrielsen et al.,

1991) (Figure 1), suggesting that they use a similar structural motif (a tryptophan-cluster helix-turn-helix) for DNA recognition (Veals et al., 1992). The carboxyl-terminal regions of these proteins diverge significantly.

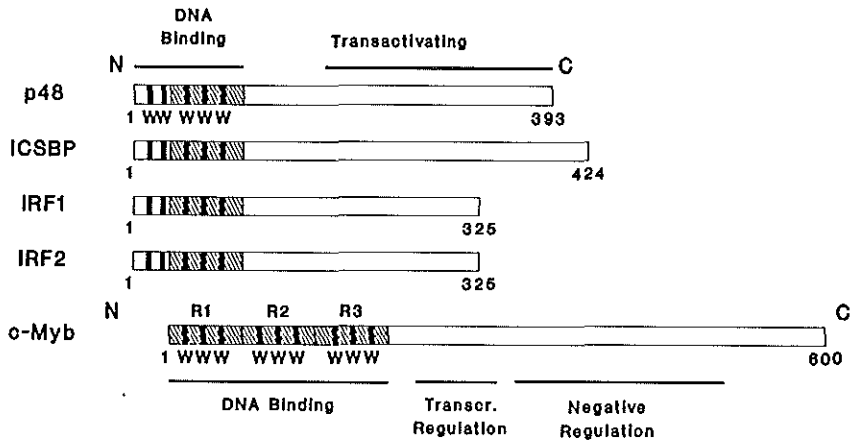

Figure 1. Schematic representation of the structural motifs in ISGF3γ and IRF family members, and c-Myb [containing three imperfect repeats, R1, R2 and R3, of tryptophan (W) clusters].

ISGF3α appears to be composed of three cytoplasmic polypeptides. Activated ISGF3α has no detectable ISRE-binding activity, however, recently it has been suggested that in the active ISGF3 complex, one or more ISGF3α polypeptides may directly contact DNA (Veals et al., 1993). Complete purification of ISGF3α from IFN-treated Hela cell nuclear extracts resulted in the isolation of three proteins (p84, p91 and p113, respectively). Recently, cDNAs encoding these proteins have been cloned (Fu et al., 1992a; Schindler et al., 1992a). Sequence comparison shows that the p113 and p91/p84 proteins have 42% sequence identity. Together, these proteins constitute a novel family of signalling proteins (Fu et al., 1992a; Fu, 1992b). The p84 and p91 proteins are alternatively spliced products of one single gene. P91 contains at its carboxyl terminus 39 additional amino acids to which specific antibodies have been targeted (Schindler et al., 1992a).

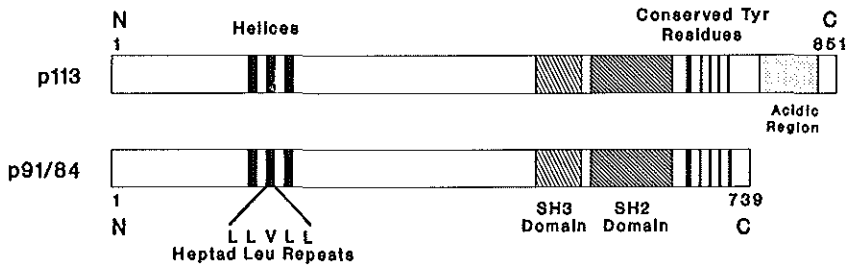

Figure 2. Schematic representation of structural motifs in p113 and p91/84.

The p113 and p91/p84 proteins contain several conserved structural motifs (Figure 2), such as a heptad leucine repeat, a helix-turn-helix; p113 has an acidic carboxy-terminal region (Fu et al., 1992a; Fu, 1992b). These motifs are commonly found in transcription factors (reviewed in Landschultz et al., 1988; Ptashne, 1988; Lewin, 1990). Furthermore, it has been shown that p113 and p91/p84 contain well-conserved SH2 domains (Figure 2) (Fu et al., 1992a; Fu, 1992b), which play important roles in facilitating protein-protein interactions among protein tyrosine kinase (PTK)-regulated proteins (reviewed in Koch et al., 1991). SH3 domains (Mayer et al., 1988; Stahl et al., 1988), which have a possible role in protein traffic and subcellular localization (Koch et al., 1991), have also been identified (Figure 2). In addition, conserved tyrosine residues were observed in p113 and p91/p84 (Figure 2) (Fu et al., 1992a; Fu, 1992b).

Activation of ISGF3 as a nuclear, DNA-binding protein is an early event in IFN-α/β signalling, being detectable within 2 minutes following exposure of cells to IFN-α (Levy et al., 1989). Some protein kinase inhibitors, such as staurosporine and genistein, can prevent both ISGF3 complex formation and ISG expression induced by IFN-α (Reich and Pfeffer, 1990; Fu, 1992b), indicating that activation of ISGF3 may involve protein phosphorylation. ISGF3α proteins, isolated by anti-ISGF3α protein antibodies from cells treated with IFN-γ plus IFN-α, are phosphorylated on tyrosine residues (Schindler et al., 1992b). In fact, a single tyrosine, Tyr 701, is phosphorylated in p91 (Shuai et al., 1993a), and was shown to be important in ISGF3 activation. These events, which are likely to occur close to (or in association with) the cell membrane (David and Larner, 1992; David et al., 1993), trigger the association of these proteins in an immunoprecipitable complex with p48 (that is itself not phosphorylated in response to IFN-α), which translocates to the nucleus (Schindler et al., 1992b) and which has a 20 to 30-fold higher ISRE binding affinity than p48 itself (Levy et al., 1989; Bandyopadhyay et al., 1990; Kessler et al.,

1990).

Immunoprecipitation experiments with p91-specific antibodies provided further insight into the nature of the ISGF3 complex. Only p113, and not p84, co-precipitated with p91, suggesting that two types of ISGF3 complexes could form (i.e. p113+p91+p48 or p113+p84+p48; Schindler et al., 1992b). This finding was extended by the observation that overexpression of either p91 or p84 in a mutant line (U3), which is completely defective in the response to IFN-α (see also Table 4), restored ISGF3 binding activity and ISG induction by IFN-α (Pellegrini et al., 1989; McKandry et al., 1991; Muller et al., 1993a).

IRF-1/IRF-2

In studies of the transcriptional regulation of the human IFN-β gene, two interesting DNA-binding factors, IRF-1 (Miyamoto et al., 1988) and IRF-2 (Harada et al., 1989), were identified (see also Table 3). As mentioned earlier, the amino-terminal regions of these two factors, which confer DNA binding specificity, are structurally conserved (Figure 1) and related to the ISGF3γ binding domain (Harada et al., 1989; Veals et al., 1992). Furthermore, both factors bind the same DNA sequence element, (G/A)(G/C)TTT(G/A)(G/C)TTT(T)G or IRF element, found not only within the promoters of IFN-αs, IFN-β genes, but also in IFN-inducible genes (ISRE core) (Harada et al., 1989; Naf et al., 1991; Tanaka et al., 1993). ISGF3 does not recognize the IRF element, indicating that for initial transcriptional activity in response to IFN-α/β, a slightly longer sequence motif is required (Kessler et al., 1988a; Dale et al., 1989a). Overexpression experiments have shown that IRF-1 functions as an activator on type I IFN genes and ISGs, whereas IRF-2 represses the effect of IRF-1 (Fujita et al., 1989a; Harada et al., 1990). For example, evidence has been presented that IRF-1 appears to play a role in the induction of MHC class I and 2-5AS genes by binding to the ISRE (Harada et al., 1990; Au et al., 1992; Pine, 1992; Reis et al., 1992). In addition, IRF-1 overexpression in some cell lines leads to an antiviral state (Pine, 1992; Matsuyama et al., 1993). On the other hand, kinetic studies for the IFN-inducible genes 2-5AS, PKR, 1-8, and H2-Kb, showed no significant difference between embryonic fibroblasts from IRF-1 deficient and wild-type mice (Matsuyama et al., 1993). So, conflicting data have been published and the physiological role of IRF-1 and IRF-2 in the regulation of ISGs remains presently unclear.

In a variety of cell types, both IRF-1 and IRF-2 mRNAs are constitutively expressed at low levels, however, IRF-2 dominates over IRF-1, binding at approximately 10-fold-higher levels, as a result of its greater protein stability (Watanabe et al., 1991). The IRF-1

gene is efficiently induced in response to virus and both types of IFN, resulting in an increase in IRF-1 activity relative to that of IRF-2 (Fujita et al., 1989b; Harada et al., 1989; Pine et al., 1990; Watanabe et al., 1991). The IRF-1 gene is, however, also induced by other cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), IL-6, and leukemia inhibitory factor (LIF) (Fujita et al., 1989a; Abdollahi et al., 1991; Watanabe et al., 1991), implicating a role for IRF-1 and IRF-2 in determining the cellular response to these cytokines. Furthermore, IRF-binding sequences are seen within promoters of other cytokine and cytokine receptor genes (IL-4, IL-5, IL-7 receptor; Tanaka et al., 1993 and references therein), suggesting their involvement in a complex cytokine network.

IRF-1 may play an inhibitory role in the regulation of cell growth (Yamada et al., 1990; Kirchhoff et al., 1992). In addition, it has been shown that IRF-1 and IRF-2 manifest antioncogenic and oncogenic properties, respectively, in NIH 3T3 cells (Harada et al., 1993). Hence, an imbalance in the IRF-1/IRF-2 ratio may lead to the dysregulation of cell growth, which may be a critical step for oncogenesis. In this regard, it is of interest that the IRF-1 gene, which is mapped to human chromosome 5q31.1, is commonly absent in human leukemia and preleukemic myelodysplasia with deletion or translocations involving 5q31.1 (Willman et al., 1993).

1.1.5b Factors regulated by IFN-γ

An IFN-γ-induced protein factor with affinity for GAS, in the promoter of the GBP gene was named γ-IFN activation factor (GAF). GAF was induced in cytoplast preparations by IFN-γ within 15 minutes, and the kinetics of its activation in cells correlated with the transcriptional activation of GBP (Decker et al., 1991). Thus, GAF may exist in a latent form in the cytoplasm and be rapidly activated following treatment with IFN-γ. Upon activation GAF is translocated to the nucleus and binds GAS. The size of the protein that contacts DNA in the GAF-GAS complex was found to be about 90 kDa (Shuai et al., 1992; Igarishi et al., 1993; Kahn et al., 1993; Pearse et al., 1993; Strehlow et al., 1993), a similar size as one of the ISGF3 proteins (p91) (Fu et al., 1990; Fu et al., 1992a; Schindler et al., 1992a; Schindler et al., 1992b). Experiments with specific antibodies directed against p91, subsequently showed that p91 participates in a GAF gel-shift complex, whereas p84 and p113 do not. It was suggested that p91 alone could be responsible for GAF activity (Shuai et al., 1992). However, Igarashi et al. (1993) showed that the complex (FcRFγ) binding to the FcγRI GAS-like region consisted of at least two protein components (p91 and an unknown 43 kDa protein).

Staurosporine, an inhibitor of protein kinases that blocks the IFN-α-dependent

formation of ISGF3 (Reich et al., 1990) and the IFN-α-dependent phosphorylation of p91 (Schindler et al., 1992b), prevents also the appearance of the GAF DNA binding activity (Shuai et al., 1992) and blocks the IFN-γ-dependent transcription of the GBP gene in isolated nuclei (Shuai et al., 1992). It turned out that GAF (p91) is converted to a form that binds DNA by IFN-γ-induced phosphorylation on the same tyrosine (Tyr701) as in response to IFN-α/β (Schindler et al., 1992b; Shuai et al., 1992). In contrast to p91, p84 cannot activate GAS madiated transcription, although it was phosphorylated and translocated to the nucleus and bound DNA upon IFN-γ treatment (Shuai et al., 1993a). This observation was confirmed in experiments by Muller et al. (1993a), who showed that complementation of U3 mutants, (which lack p91/84) with cDNA constructs expressing p91 at levels comparable to those observed in induced wild-type cells, completely restored the response to both IFN-α and IFN-γ and the ability to form ISGF3 (Muller et al., 1993a). Complementation with p84 similarly restored the ability to form ISGF3 and, albeit to a lower level, the IFN-α response of all genes tested. In contrast, it failed to restore the IFN-γ response of any gene analysed. Thus, p91 mediates activation of transcription in response to IFN-γ.

The transcription factors involved in less well defined pathways (IFN-α via GAS; IFN-γ via ISRE; indirect mechanisms) are less clear. In case of the Ly-6A/E gene, which uses a GAS element for mediating IFN-α and IFN-γ induction, p91 appears to act in the signal transduction pathways of both types of IFN (Khan et al., 1993). The ISGF3 complex is not activated by IFN-γ (Levy et al., 1990b). Therefore, IFN-γ-induced transcription from ISRE-containing promoters is apparently mediated by a different factor(s). The IFN-γ-inducible IP-10 ISRE binding complex, of which the precise protein composition is unknown (Ohmori and Hamilton, 1993), could be detected as early as 30 min. after IFN-γ treatment and was independent of protein synthesis. This indicates that induction of ISRE binding is a primary response to IFN-γ and involves the activation of preexisting cellular factor(s).

From the mutant cell line U2 (see also Table 4), which is unresponsive to IFN-α/β, it has been suggested that ISGF3γ (p48), may play a role in the activation of some but not all IFN-γ-inducible genes. U2 cells express a truncated p48 protein, which is unable to interact with activated ISGF3α. While the IFN-γ-induced activation of early genes is unaltered in U2, the activation of some genes is defective (John et al., 1991; Pellegrini and Schindler, 1993). Interestingly, the antiviral response to IFN-γ is strongly reduced in U2 cells.

1.1.6 Tyrosine kinases involved in the IFN-α/β and IFN-γ signalling pathways

In the previous chapter it has been assigned that the specificity of the cytoplasmic response to IFN-α and IFN-γ, at least partially, results from differential tyrosine phosphorylation of p113, p91 and p84, which have also been termed signal transducers and activators of transcription, or STATs (Shuai et al., 1993a).

The complementation of IFN-resistant mutant cell lines (Table 4) has provided direct evidence for the involvement of the JAK family of non-receptor protein tyrosine kinases (PTK) in the IFN response pathways. This family has so far three identified members, JAK1 (Wilks, 1989; Wilks et al., 1991; Harpur et al., 1992; Howard et al., 1992), JAK2 (Wilks, 1989; Harpur et al., 1992; Silvennoinen et al., 1993a) and Tyk2 (Firmbach-Kraft et al., 1990). Each is about 130 kDa in mass and is characterized by the presence of a classical carboxy-terminal protein tyrosine kinase domain, an adjacent kinase or kinase-related domain and five further domains of substantial amino acid similarity extending towards the amino terminus (Harpur et al., 1992).

Mutant cell line U1 (initially coded 11.1; Pellegrini et al., 1989), which contains functional p113, p91/p84 and p48 genes, is impaired in its ability to bind IFN-αs and activate ISGF3 (Pellegrini et al., 1989). Genetic complementation of this mutant has shown that absence of Tyk2 is responsible for a defectious IFN-α signal transduction pathway (Velazquez et al., 1992). It is conceivable that Tyk2 represents the link between the IFN-α/β receptor and one or more of the ISGF3α proteins (Fu, 1992b). In principle, the SH2 domains of these ISGF3 subunits could bind to phosphotyrosine in Tyk2 itself and/or to phosphotyrosine on one of the other subunits. Both kinase domains appear to be necessary for the biological activity of Tyk2 (Pellegrini and Schindler, 1993). Only a minor fraction of Tyk2 in the cell appears to be associated with the membrane, while a majority is cytosolic (Pellegrini and Schindler, 1993). Although the nature of the association of Tyk2 with the cell membrane has not yet been defined, the lack of functional IFN-α binding sites in Tyk2-deficient cells suggests its interaction with receptor components. This is confirmed by David et al. (1993) who suggested that a membrane-associated tyrosine kinase was necessary for activation of ISGF3 by IFN-α. The normal response of Tyk2-deficient cells to IFN-γ rules out the involvement of this PTK in the IFN-γ pathway.

Development of two other IFN-resistant cell lines, provided direct evidence for the involvement of JAK1 and JAK2 in the IFN response pathways. The U4A mutant cell line, which responds to neither IFN-α nor IFN-γ, expresses a truncated form of JAK1 mRNA and no JAK1 protein, and can be complemented for both IFN-α and IFN-γ responses by

TABLE 4 COMPONENTS OF THE IFN-α/β AND IFN-γ SIGNALLING PATHWAYS

Protein	IFN-α response	IFN-γ response	Mutant cell line
p48	Required	Not required	U2 (IFN-α^-, IFN-γ^+, p48$^-$)
p84	Required/ phosphorylated	Not required	U3 (IFN-α^-, IFN-γ^-, p84/p91$^-$)
p91	Required/ phosphorylated	Required/ phosphorylated (DNA-binding subunit)	U3 (IFN-α^-, IFN-γ^-, p84/p91$^-$)
p113	Required/ phosphorylated	Not required	No mutant known
JAK1	Required/ phosphorylated	Required/ phosphorylated	U4 (IFN-α^-, IFN-γ^-, JAK1$^-$)
JAK2	Not required	Required/ phosphorylated	γ-1 (IFN-α^+, IFN-γ^-, JAK2$^-$)
TYK2	Required/ phosphorylated	Not required	U1 (IFN-α^-, IFN-γ^+, TYK2$^-$)

overexpression of JAK1 (Muller et al., 1993b). The γ1A mutant cell line is unable to respond to IFN-γ (Watling et al., 1993). However, γ1A cells still respond to IFN-α, and have functional p113, p91/p84 and p48 genes. Overexpression of JAK2 in γ1A cells restores IFN-γ responsiveness (Watling et al., 1993).

In parental cells, IFN-γ stimulates both JAK1 and JAK2 tyrosine phosphorylation. In γ1A cells, which lack JAK2, JAK1 is not phosphorylated. Similarly, in U1 cells, which lack Tyk2, IFN-α fails to induce JAK1 phophorylation. In addition, IFN-α or IFN-γ do not induce phosphorylation of either Tyk2 or JAK1 in U4A cells (Muller et al., 1993b). This indicates that JAK PTKs cannot be placed in a linear order where one JAK PTK activates another, because if either JAK PTK in a pair is inactivated the other is not phosphorylated (Silvennoinen et al., 1993b).

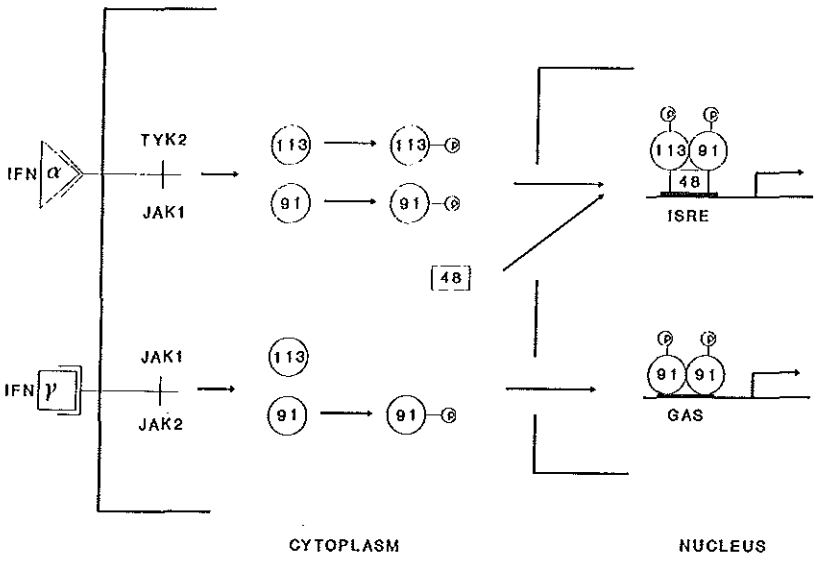

Figure 3. Pathways of signal transduction and transcriptional activation in cells treated with IFN-α and IFN-γ.

Activation of JAK family PTKs by IFN-γ and IFN-α is summarized in Figure 3 (see also Table 4). IFN-γ elicits tyrosine phosphorylation of JAK1 and JAK2, which in turn phosphorylate p91. IFN-α induces phosphorylation of JAK1 and Tyk2, which in turn phosphorylate p113 and p91/p84. Both U1 and U4A cells fail to respond to IFN-α and lack functional Tyk2 and JAK1 respectively, which implies that both of these JAK PTKs are needed for the IFN-α response. Likewise, the failure of U4A and γ1A cells to respond to IFN-γ indicates that both JAK1 and JAK2 are required for the IFN-γ response. The presence of a common substrate, p91, and of a common PTK, JAK1, in the IFN-α and -γ response pathways makes it tempting to speculate that JAK1 might directly phosphorylate p91 (Shuai et al., 1993a; Shuai et al., 1993b) and that p113, being unique to the IFN-α response, might be phosphorylated by Tyk2. The presumed substrate of JAK2 remains to be identified.

A possible clue to the activation mechanism comes from the demonstration that JAK2 directly associates with the erythropoietin receptor and growth hormone receptor and is activated upon ligand binding (Argetsinger et al., 1993; Witthuhn et al., 1993). If the two JAK PTKs required were to interact simultaneously with the cytoplasmic domain of the relevant IFN receptor, this would facilitate mutual activation by transphosphorylation in a manner similar to that in which the two subunits of a ligand-bound receptor PTK dimer transactivate (Schlessinger and Ullrich, 1993). In response to ligand, these receptor PTKs dimerize and become autophosphorylated on multiple tyrosine residues within their cytoplasmic extensions. These phosphotyrosine residues, in turn, allow the activated receptor to associate with other proteins (through SH2 domains) involved in signalling.

JAK PTK activation might require the unusual protein kinase-like domain that lies to the amino-terminal side of the protein tyrosine kinase catalytic domain in these proteins (Wilks et al., 1991). But the exact mechanism involved in JAK PTK activation may be more complex, because a protein-tyrosine phosphatase might also be involved (David et al., 1993; Igarishi et al., 1993).

1.1.7 Crosstalk between IFN and cytokine and growth factor signal transduction pathways

Recently, it has become clear that tyrosine phosphorylation and activation of p91 is not the preserve of the IFNs, and that many cytokines and growth factors, including epidermal growth factor (EGF), platelet derived growth factor (PDGF), colony-stimulating factor-1 (CSF-1), and interleukin-10 (Il-10), can induce tyrosine phosphorylation of p91 (on Tyr 701) or p91-related proteins and activate gene transcription through response elements related to GAS (Fu and Zhang, 1993; Larner et al., 1993; Ruff-Jamison et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993c). For instance, it has been shown that the SIF (sis-inducible factor) response element in the c-fos gene, which has a core sequence related to the GAS element, binds tyrosine-phosphorylated p91 and that this activates transcription (Fu and Zhang, 1993; Sadowski et al., 1993). Activation of p91 by receptor PTKs shows some specificity because other cytokines, like IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) can similarly activate complexes of DNA-binding proteins, but these complexes appear not to contain p91 (Larner et al., 1993).

Binding of ligands, such as EGF or PDGF, to their receptors has already been proven to activate the Ras-pathway (Figure 4), including a series of protein kinases [mitogen-activated protein kinase (MAPK), MAP kinase kinase (MAPKK), and MAP kinase kinase kinase (MAPKKK)], and phosphorylation of the transcription factor AP-1 (Schlessinger et al., 1993). Phosphorylation of the latent cytoplasmic proteins p91 and SIF (possibly by one or more soluble tyrosine kinases associated through SH2 domains with the receptor) indicates a second, more direct pathway to the nucleus in these stimulated cells (Figure 4). A JAK PTK may not be responsible for p91 phosphorylation in each of the cases. For

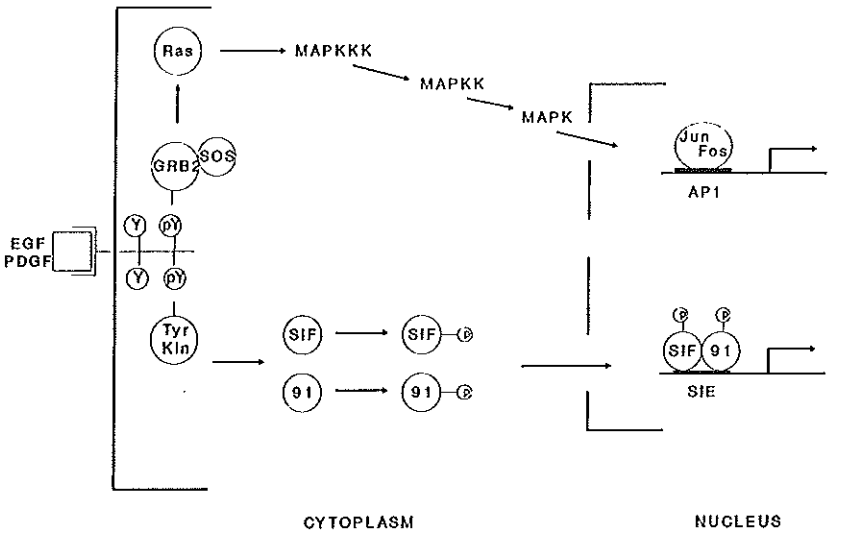

Figure 4. Schematic representation of c-fos promoter activation by EGF/PDGF via the RAS and the STAT pathways.

instance, some of the cytokines, such as EGF, directly activate receptor PTKs. Moreover, p91 binds to the activated EGF receptor, and both p91 binding and its ability to stimulate transcription depend on the integrity of its SH2 domain (Fu and Zhang, 1993), suggesting that the EGF receptor itself phosphorylates p91. However, because EGF induces specific phosphorylation of Tyr 701 (Shuai et al., 1993b), a JAK PTK may prove to be involved.

So, what first appeared to be a membrane-to-nucleus transduction pathway unique to IFNs, became one that is almost universally activated by cytokines. The different cytokines that activate p91 do not all elicit identical cellular responses, so there must be as yet unidentified elements of specificity. This specificity may be determined by whether it is p91 or a p91-related protein that is activated, which in turn may depend on which (JAK family) PTK is activated, and on the binding specificity of the p91 family member SH2 domain. There are three known PTKs of the JAK family, but there are likely to be more that may cooperate with JAK1, JAK2 or TYK2 to phosphorylate individual p91-related proteins. Similarly, it is to be expected that the p113, p91/p84 (ISGF3-α) family is larger than the two members identified so far.

In the case of IFN-α/β and IFN-γ, different sets of genes appear to be activated depending upon the subunit composition of the activated ISGF3 factor. Assembly of activated p91 and p113 into the ISGF3α component after IFN-α treatment allows association of this complex with ISGF3γ (p48), that directs the complex to the ISRE unique to ISGs. In IFN-γ treated cells, a p91 dimer binds to a distinct DNA element, GAS. Other cytokines induce GAS-binding activities with different mobilities (Fu and Zhang, 1993; Larner et al., 1993; Ruff-Jamison et al., 1993; Sadowski et al., 1993; Silvennoinen etal., 1993c), which suggest that there are additional subunits present in these complexes. Ancillary subunits could alter the DNA sequence preference of p91 family members, and it is becoming clear that there are several GAS-related sequences that have different affinities for p91. So, a specificity-determining subunit may be involved in each signalling pathway from a distinct receptor, combining with common p91 subunits and directing the complex to appropriate target genes. In this respect it is also possible that members of the ISGF3α family of proteins associate with various members of the IRF family of DNA-binding proteins, including IRF-1, IRF-2, ICSBP, and c-Myb (Veals et al., 1992).

1.2.1 The ISG-54K/ISG-56K gene family

The human ISG-54K gene belongs to the first series of IFN-α regulated genes detected. Its mRNA synthesis is induced from undetectable levels to maximal rates of transcription within 30-60 min after the addition of IFN-α/β to human fibroblast or to HeLa cells (Larner et al., 1984). After 6 hr of IFN treatment, the level of transcription declines and is undetectable after 24 hr. The accumulation of IFN-induced ISG-54K mRNA (2.8 kb in size) is detectable within 1 hr, lagging about 30 min behind the induction of transcription. The maximum concentration of cytoplasmic mRNA is observed after about 6 hr of IFN treatment. After 24 hr the induced mRNA is present at $<1/10$th of its peak concentration, indicating that turnover reduces the mRNA concentration between 6 and 24 hr.

The ISG-54K gene is composed of two exons, interrupted by a 3.7 kb intron. Use of a putative poly(A) site in the 3' untranslated region and splicing of exons 1 and 2 would lead to production of a mRNA molecule of the observed 2.8 kb size. The open reading frame starts at the most 3'-end of the first exon, which is very short [(approx. 80 bp) and only provides the initiating methionine codon (ATG)], and encodes a protein of 472 amino acids with an approximately Mr of 54 kDa (Levy et al., 1986). The primary translation product would be very hydrophylic, rich in uncharged polar and charged amino acids residues, with the latter displaying a somewhat clustered distribution.
The existence of an ISRE in the ISG-54K promoter (Table 1) and its specific DNA recognition requirements for ISGF3 and IRF1, have been established by extensive mutagenesis studies (Levy et al., 1986; Kessler et al., 1988; Levy et al., 1988).

The human genome contains at least one gene (ISG-56K), but most probably a small gene family, that is structurally related to ISG-54K (Wathelet et al., 1986; Wathelet et al., 1988a). The ISG-56K gene shows an organization identical to that of the ISG-54K gene: two exons, interrupted by an intron of unknown size, of which the first exon is very small (approx. 90 nt) and only provides the initiating methionine codon. Use of a putative poly(A) site in the 3' untranslated region and splicing of exons 1 and 2 would lead to production of a mRNA molecule of 1.9 kb. The open reading frame encodes a protein of 478 amino acids with an approximately Mr of 56 kDa (Wathelet et al., 1986; Wathelet et al., 1988a). The primary translation product, like that of ISG-54K, would be very hydrophylic.

In human fibroblast or in HeLa cells the accumulation of IFN-α/β (and not IFN-γ) - induced ISG-56K mRNA (1.9 kb in size) is detectable within 1 hr (Larner et al., 1984). The

maximum concentration of mRNA is observed after about 6 hr of IFN treatment, while a clear decrease in induced mRNA is seen after 24 hr. Interestingly, in human amniotic cells, the ISG-56K messenger was not only inducible with IFN-α but also with IFN-γ (Wathelet et al., 1986).

So, the ISG-54K and ISG-56K genes are regulated in a coordinate manner by IFN-α/β and (in human fibroblasts or in HeLa cells) their mRNAs have a half-live considerably less than 8-10 hr and perhaps as short as 2-4 hr (Larner et al., 1984). Moreover, both genes are strongly homologous in sequence at the promoter (especially the ISRE region; Table 1), mRNA (60%), and protein (42%) levels. This strongly suggests that the ISG-54K and ISG-56K genes arose through a duplication of an earlier gene and diverged thereafter (Wathelet et al., 1988a). Several observations indicate that the ISG-54K/ISG-56K gene family is not restricted to these two members. The excistance of two pseudogenes homologous to the ISG-56K gene has been described (Wathelet et al., 1988a). Together, the ISG-54K and ISG-56K genes and pseudogenes are mapped to human chromosome 10 (10q23-q24) (Lafage et al., 1992).

The homology between the 54 kDa and 56 kDa putative polypeptides, the similarities in their hydrophobicity and charge profiles, together with the conservation of six cysteine residues, suggest that the two polypeptides may adopt a similar secondary and tertiary structure, and hence might have a common biological activity (Wathelet et al. 1988a). The structure of the most conserved regions does not provide information about a possible function of the proteins encoded by the ISG-54K and ISG-56K genes. Indirect evidence, points to a role of the ISG-54K protein in the growth inhibitory pathway of IFNs (Van Heuvel et al., 1988). It has also been suggested, however, that both proteins are involved in the antiviral effects of IFN (Wathelet et al., 1988b).

Interestingly, the coordenate regulation of the ISG-54K and ISG-56K genes is not restricted to IFN-α/β, but also direct induction by virus and poly(I).poly(C), and other cytokines (like IL-1 or TNF) of both genes is seen (Wathelet et al., 1987; Reich et al., 1988; Wathelet et al., 1988b). This could indicate a common mechanism, using overlapping DNA requirements, involved in regulation of these specific genes. Therefore, these genes may serve as a perfect system to study the mechanisms of signal transduction induced by IFNs and other cytokines.

1.2.2 Scope of the thesis

IFNs mediate a wide variety of effects on target cells. Therefore they are an interesting system to study the mechanisms of action of cytokines. In addition, IFN-regulated gene expression provides a particularly attractive system in which to examine how transcription in the cell nucleus is governed through occupation of a cell-surface receptor by its polypeptide ligand. As part of our ongoing work on IFN-regulated cell growth, this thesis describes the characterization of several hamster IFN-α subspecies and the characterization of IFN-induced transcriptional regulation of the ISG-54K/56K gene family.

In Chapter 2 the structural analysis and chromosomal location of four closely linked hamster IFN-α genes (A1-A4) are described. Specific antiviral activities of the A1 and A3 proteins, on hamster and on mouse cells, are presented.

Chapter 3 describes the molecular cloning and characterization of the hamster ISG-54K gene. Chapter 4 includes the molecular cloning, characterization and chromosomal localization of the mouse ISG-54K/56K gene family. Together, these two chapters identify the presence of an ISRE-doublet in the promoter of these genes, and its functionality upon IFN-α treatment.

Chapter 5 describes the role of the ISRE sequences in the IFN-γ regulation of the ISG-54K promoter. Evidence is presented for a role of the transcription factors p91(ISGF3α) and p48(ISGF3γ), but not p113(ISGF3α), in this process.

REFERENCES

Abdollahi, A., Lord, K.A., Hoffman-Liebermann, and Liebermann, D. (1991) Interferon regulatory factor 1 is a myeloid differentiation primary response gene induced by interleukin 6 and leukemia inhibitory factor: role in growth inhibition. Cell Growth Differ. 2: 401-407.

Adolf, G.R., Fruhbeis, B., Hauptmann, R., Kalsner, I., Maurer-Fogy, I., Ostermann, E., Patzelt, E., Schwendenwein, R., Sommergruber, W., and Zophel A. (1991) Human interferon omega1: isolation of the gene, expression in chinese hamster ovary cells, and characterization of the recombinant protein. Biochim. Biophys. Acta. 1089: 167-174.

Aguet, M., and Merlin, G. (1987) Purification of human γ interferon receptors by sequential affinity chromatography on immobilized monoclonal antireceptor antibodies and human γ interferon. J. Exp. Med. 165: 988-999.

Aguet, M., Dembic, Z., and Merlin, G. (1988) Molecular cloning and expression of human interferon-γ receptor. Cell 55: 273-280.

Amaldi, I., Reith, W., Berte, C., and Mach, B. (1989) Induction of HLA class II genes by IFN-γ is transcriptional and requires a trans-acting protein. J. Immunol. 142: 999-1004.

Argetsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N., and Carter-Su, C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74: 237-244.

Au, W.-C., Raj, N.K.B., Pine, R., and Pitha, P.M. (1992) Distinct activation of murine interferon-α promoter region by IRF-1/ISGF2 and virus infection. Nucl. Acids Res. 20: 2877-2884.

Bandyopadhyay, S.K., Kalvakolanu, D.V.R., and Sen, G.C. (1990) Gene induction by interferons: functional complementation between trans-acting factors induced by alpha interferon and gamma interferon. Mol. Cell. Biol. 10: 5055-5063.

Baron, S., Dianzani, F., Stanton, G.J., and Fleischmann, W.R., Jr., eds. (1987) The interferon system: a current review to 1987. Austin, Tx, University of Texas Press.

Baron, S., Tyring, S.K., Fleischmann, W.R., Jr., Coppenhaver, D.H., Niesel, D.W., Klimpel, G.R., Stanton, G.J., and Hughes, T.K. (1991) Interferons: Mechanism of action and clinical applications. J. Am. Med. Assoc. 266: 1375-1383.

Basta, P.V., Sherman, P.A., and Ting, J.P.-Y. (1988) Detailed delineation of an interferon-γ responsive element important in human HLA-DRA gene expression in a glioblastoma multiform line. Proc. Natl. Acad. Sci. USA 85: 8618-8622.

Bazan, J.F. (1990a) Shared architecture of hormone binding domains in type I and type II interferon receptors. Cell 61: 753-754.

Bazan, J.F. (1990b) Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA 87: 6934-6938.

Benech, P., Vigneron, M., Peretz, D., Revel, M., and Chebath, J. (1987) Interferon-responsive regulatory elements in the promoter of the human 2',5'-oligo(A) synthetase gene. Mol. Cell. Biol. 7: 4498-4504.

Bender,T.P., and Kuehl, W.M. (1986) Murine myb proto-oncogene mRNA: cDNA sequence and evidence for 5' heterogeneity. Proc. Natl. Acad. Sci. USA 83: 3204-3208.

Benoist, C., and Mathis, D. (1990) Regulation of major histocompatibility complex class II genes: X, Y and other letters of the alphabet. Ann. Rev. Immunol. 8: 681-715.

Blanar, M., Boettger, E., and Flavell, R. (1988) Transcriptional activation of HLA-DRα by interferon γ requires a trans-acting protein. Proc. Natl. Acad. Sci. USA 85: 4672-4676.

Blanar, M.A., Baldwin, A.S., Flavell, R.A., and Sharp, P.A. (1989) A gamma-interferon-induced factor that binds the interferon response sequence of the MHC class I gene, H-2Kb. EMBO J. 8: 1139-1144.

Brown, A.M., Barr., C.L., and Ting, J.P.-Y. (1991) Sequences homologous to class II MHC W, X, and Y elements mediate constitutive and IFN γ-induced expression of human class II-associated invariant chain gene. J. Immunol. 146: 3183-3189.

Calderon, J., Sheehan, K.C.F., Chance, C., Thomas, M.L., and Schreiber, R.D. (1988) Purification and characterization of the human interferon-γ receptor from placenta. Proc. Natl. Acad.

Sci. USA 85: 4837-4841.
Canfield, W.M., Johnson, K.F., Ye, R.D., Gregory, W., and Komfeld, S. (1991) Localization of the signal for rapid internalization of the bovine cation-independent mannose 6-phosphate/insuline-like growth factor-II receptor to amino acids 24-29 of the cytoplasmic tail. J. Biol. Chem. 266: 5682-5688.
Capon, D.J., Shepard, H.M., and Goeddel, D.V. (1985) Two distinct families of human and bovine interferon-α genes are coordinately expressed and encode functional polypeptides. Mol. Cell. Biol. 5: 768-779.
Chang, K.C., Hansen, E., Foroni, L., Lida, J., and Goldspink, G. (1991) Molecular and functional analysis of the virus and interferon-inducible human MxA promoter. Arch. Virol. 117: 1-15.
Cohen, B., Peretz, D., Vaiman, D., Benech, P., and Chebath, J. (1988) Enhancer-like interferon responsive sequences of the human and murine (2'-5')oligoadenylate synthetase gene promoters. EMBO J. 7: 1411-1419.
Cohen, B., Vaiman, D., and Chebath, J. (1989) Enhancer functions and in vitro protein binding of native and mutated interferon-responsive sequences. Nucl. Acids Res. 17: 1679-1695.
Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, U., Jing, S., Trowbridge, I.S., and Trainer, J.A. (1990) Transferrin receptor internalization sequence YXRF implicates a tight turn as th structural recognition motif for endocytosis. Cell 63: 1061-1072.
Cross, J.C., and Roberts, R.M. (1991) Constitutive and throphoblast-specific expression of a class of bovine interferon genes. Proc. Natl. Acad. Sci. USA 88: 3817-3821.
Dale, T.C., Rosen, J.M., Guille, M.J., Lewin, A.R., Porter, A.C.G., Kerr, I.M., and Stark, G.R. (1989a) Overlapping sites for constitutive and induced DNA binding factors involved in interferon-stimulated transcription. EMBO J. 8: 831-839.
Dale, T.C., Ali Imam, A.M., Kerr, I.M., and Stark, G.R. (1989b) Rapid activation by interferon α of a latent DNA-binding protein present in the cytoplasm of untreated cells. Proc. Natl. Acad. Sci. USA 86: 1203-1207.
David, M., and Larner, A.C. (1992) Activation of transcription factors by interferon-alpha in a cell-free system. Science 257: 813-815.
David, M., Romero, G., Zhang, Z.-Y., Dixon, J.E., and Larner, A.C. (1993) In vitro activation of the transcription factor ISGF3 by interferon α involves a membrane-associated tyrosine phosphatase and tyrosine kinase. J. Biol. Chem. 268: 6593-6599.
Decker, T., Lew, D.J., Cheng, Y.-S.E., Levy, D.E., and Darnell, J.E., Jr. (1989) Interactions of α- and γ-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. EMBO J. 8: 2009-2014.
Decker, T., Lew, D.J., Mirkovitch, J., and Darnell, J.E., Jr. (1991) Cytoplasmic activation of GAF, an IFN-γ-regulated DNA-binding factor. EMBO J. 10: 927-932.
Dianzani, F., and Antonelli, G. (1989) Physiological mechanisms of production and action of interferons in response to viral infections. Adv. Exp. Med. Biol. 257: 47-60.
Diaz, M.O., Pomykala, H., Bohlander, S., Maltepe,.E., and Olopade, O. (1991) A complete physical map of the type I interferon gene cluster. J. Interferon Res. 11 suppl 1:S85.
Diaz, M.O. (1993) Nomenclature of the human interferon genes. J. Interferon Res. 13: 61-62.
Driggers, P.H., Ennist, D.L., Gleason, S.L., Mak, W.H., Marks, M.S., Levi, B.Z., Flanagan, J.R., Appella, E., and Ozato, K. (1990) An interferon γ-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex I genes. Proc. Natl. Acad. Sci. USA 87: 3743-3747.
Eades, A.M., Litfin, M., and Rahmsdorf, H.J. (1990) The IFN γ response of the murine invariant chain gene is mediated by a complex enhancer that includes several MHC class II consensus elements. J. Immunol. 144: 4399-4409.
Faltynek, C.R., McCandless, S. Chebath, J., and Baglioni, C. (1985) Different mechanisms for activation of gene transcription by interferons alpha and gamma. Virology 144: 173-180.
Farrar, M.A., Fernandez-Luna, J., and Schreiber, R.D. (1991) Identification of two regions within the cytoplasmic domain of the human interferon-γ receptor required for function. J. Biol. Chem. 266: 19626-19635.
Farrar, M.A., Campbell, J.D., and Schreiber, R.D. (1992) Identification of a functionally important sequence in the C terminus of the interferon-γ receptor. Proc. Natl. Acad. Sci. USA 89: 11706-11710.

Feinstein, S.I., Mory, Y., Chernajovsky, Y., Maroteaux, L., Nir, U., Lavie, V., and Revel, M. (1985). Family of human α-interferon-like sequences. Mol. Cell. Biol. 5: 510- 517.

Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J.J. (1990) Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. Oncogene 5: 1329-1336.

Fountoulakis, M., Kania, M., Ozmen, L., Lotscher, H.R., Garotta, G., and van Loon, A.P.G.M. (1989) One single human interferon-gamma receptor can lead to multiple cross-linked products and isolated proteins. Its membrane topology, glycosylation and requirements for binding to interferon-γ. J. Immunol. 143: 3266-3276.

Fountoulakis, M., Juranville, J.F., Stuber, D., Weibel, E.K., and Garotta, G. (1990a) Purification and biochemical characterization of a soluble human interferon-γ receptor expressed in E. coli. J. Biol. Chem. 265: 13268-13275.

Fountoulakis, M., Juranville, J.F., Maris, A., Ozmen, L., and Garotta, G. (1990b) One IFN γ receptor binds one IFN γ dimer. J. Biol. Chem. 265: 19758-19767.

Fountoulakis, M., Schlaeger, E.J., Gentz, R., Juranville, J.F., Manneberg, M., Ozmen, L., and Garotta, G. (1991) Purification and characterization of a soluble mouse interferon-gamma receptor produced in insect cells. Eur. J. Biochem. 198: 441-450.

Friedman, R.L., Manly, S.P., MacMahon, M., Kerr, I.M., and Stark, G.R. (1984) Transcriptional and post-transcriptional regulation of interferon-induced gene expression in human cells. Cell 38: 745-755.

Friedman, R.L., and Stark, G.R. (1985) α-Interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences. Nature 314: 637-639.

Fu, X.-Y., Kessler, D.S., Veals, S.A., Levy, D.E., and Darnell, J.E., Jr. (1990) ISGF3, the transcriptional activator induced by interferon α, consists of multiple interacting polypeptide chains. Proc. Natl. Acad. Sci. USA 87: 8555-8559.

Fu, X.-Y., Schindler, C., Improta, T., Aebersold, R.H., and Darnell, J.E., Jr. (1992a) The proteins of ISGF3, the IFN-α induced activator, define a new gene family of signal transducers. Proc. Natl. Acad. Sci. USA 89: 7840-7843.

Fu, X.-Y. (1992b). A transcription factor with SH2 and SH3 domains is directly activated by an interferon-α-induced cytoplasmic protein tyrosine kinase(s). Cell 70: 323-336.

Fu, X.-Y., and Zhang, J.-J. (1993) Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the c-fos gene promoter. Cell 74: 1135-1145.

Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y., and Taniguchi, T. (1988) Evidence for a nuclear factor(s), IRF1, mediating induction and silencing properties to human IFN-β gene regulatory elements. EMBO J. 7: 3397-3405.

Fujita, T., Kimura, Y., Miyamoto, M., Barsoumian, E.L., and Taniguchi, T. (1989a) Induction of endogenous IFN-α and IFN-β genes by a regulatory transcription factor, IRF-1. Nature 337: 270-272.

Fujita, T., Reis, L.F.L., Watanabe, N., Kimura, Y., Taniguchi, T., and Vilcek, J. (1989b) Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines and activators of second-messenger pathways. Proc. Natl. Acad. Sci. USA 86: 9936-9940.

Gabrielsen, D.S., Sentenac, A., and Fromageot, P. (1990) Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif. Science 253: 1140-1143.

Gray, P.W., and Goeddel, D.V. (1982) Structure of the human immune interferon gene. Nature 298: 859-863.

Hannigan, G.E., and Williams, B.R.G. (1986) Transcriptional regulation of interferon-responsive genes is closely linked to interferon receptor occupancy. EMBO J. 5: 1607-1613.

Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, T. (1989) Structurally similar but functionally distinct factors, IRF1 and IRF2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell 58: 729-739.

Harada, H., Willison, K., Sakakibara, J., Miyamoto, M., Fujita, T., and Taniguchi, T. (1990) Absence of type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. Cell 63: 303-312.

Harada, H., Kitagawa, M., Tanaka, N., Yamamoto, H., Harada, K., Ishihara, M., and Taniguchi, T. (1993) Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. Science 259: 971-974.

Harpur, A.G., Andres, A.-C., Ziemiecki, A., Aston, R.R., and Wilks, A.F. (1992) JAK2, a third

member of the JAK family of protein tyrosine kinases. Oncogene 7: 1347-1353.
Hauptmann, R., and Swetly, P.A. (1985) A novel class of human type I interferons. Nucleic Acids Res. 13: 4739-4749.
Hemmi, S., Merlin, G., and Auget, M. (1992) Functional characterization of mouse-human IFN-γ receptor hybrids: evidence for species specific interaction of the extracellular receptor domain with a putative signal transducer. Proc. Natl. Acad. Sci USA 89: 2737-2741.
Henco, K., Brosius, F.J., Fujisawa, J.I., Haynes, J.R., Hochstadt, J., Kovacic, T., Pasek, M., Schambeck, A., Schmid, J., Todokoro, K., Walchli, M., Nagata, S., and Weissmann, C. (1985) Structural relationship of human interferon alpha genes and pseudogenes. J. Mol. Biol. 185: 227-260.
Van Heuvel, M., Bosveld, I.J., Mooren, A.T.A., Trapman, J., and Zwarthoff, E.C. (1986) Properties of natural and hybrid murine alpha interferons. J. Gen. Virol. 67: 2215-2222.
Van Heuvel, M., Govaert-Siemerink, M., Bosveld, I.J., Zwarthoff, E.C., and Trapman, J. (1988) IFN producing CHO cell lines are resistant to the antiproliferative activity of IFN: A correlation with gene expression. J. Cell. Biochem. 38: 269-278.
Hibino, Y., Kumar, C.S., Mariano, T.M., Lai, D., and Pestka, S. (1992) Chimeric interferon gamma receptors demonstrate that an accessory factor required for activity interacts with the extracellular domain. J. Biol. Chem. 267: 3741-3749.
Himmler, A., Hauptmann, R., Adolf, G.R., and Swetly, P. (1986) Molecular cloning and expression in Escherichia coli of equine type I interferons. DNA 5: 345-356.
Himmler, A., Hauptmann, R., Adolf, G.R., and Swetly, P. (1987) Structure and expression in Escherichia coli of canine IFN-α gene. J. Interferon Res. 7: 173-183.
Howard, O.M., Dean, M., Young, H., Ramsburg, M., Turpin, J.A., Michiel, D.F., Kelvin, D.J., Lee, L., and Farrar, W.L. (1992) Characterization of a class 3 tyrosine kinase. Oncogene 7: 895-900.
Howe, K.M., Reaks, C.F.L., and Watson, R.J. (1990) Characterization of the sequence-specific interaction of mouse c-myb protein with DNA. EMBO J. 9: 161-169.
Hu, R-Q, Gan, Y., Liu, J., Enterline, J., Miller, D., Hayes, M., Bekisz, J., and Zoon, K. (1990) Evidence for multiple binding sites for several species of human lymphoblastoid interferon-alpha. J. Interferon Res. 10 suppl 1:S157.
Hug, H., Costas, P., Staeheli, P., Aebi, M., and Weissmann, C. (1988) Organization of the murine Mx gene and characterization of its interferon- and virus-inducible promoter. Mol. Cell. Biol. 8: 3065-3079.
Igarishi, K.-I., David, M., Larner, A.C., and Finbloom, D.S. (1993) In vitro activation of a transcription factor by gamma interferon requires a membrane-associated tyrosine kinase and is mimicked by vanadate. Mol. Cell. Biol. 13: 3984-3989.
Imam, A.M.A., Ackrill, A.M., Dale, T.C., Kerr, I.M., and Stark, G.R. (1990) Transcription factors induced by interferons α and γ. Nucl. Acids Res. 18: 6573-6580.
Isaacs, A. and Lindenmann, J. (1957) Virus interference. I. The interferon. Proc. R. Soc. Ser. B 147: 258-267.
Israel, A., Kimura, A., Fournier, A., Fellous, M., and Kourilsky, P. (1986) Interferon response sequence potentiates activity of an enhancer in the promoter region of a mouse H-2 gene. Nature 322: 743-746.
John, J., McKandry, R., Pellegrini, S., Flavell, D., Kerr, I.M., and Stark, G.R. (1991) Isolation and characterization of a new mutant human cell line unresponsive to alpha and beta interferons. Mol. Cell. Biol. 11: 4189-4195.
Jung, V. and Pestka, S. (1986) Selection and screening of transformed NIH3T3 cells for enhanced sensitivity to human interferons α and β. Methods Enzymol. 119: 597-611.
Jung, V., Rashidbaigi, A., Jones, C., Tischfield, J., Shows, T.B., and Pestka, S. (1987) Human chromosome 6 and 21 are required for sensitivity to human gamma interferon. Proc. Natl. Acad. Sci. USA 84: 4151-4155.
Kalina, U., Damlin, K., Dembic, Z., Fountoulakis, M., Stuber, D., Ozmen, L., and Garotta, G. (1991) Definition of the human IFN-γ receptor region(s) that interacts with the transducing factor encoded by human chromosome 21. J. Interferon Res. 11, suppl. 1: 82.
Kanei-Ishii, C., Sarai, A., Sawazaki, T., Nakayoshi, H., He, D.-N., Nishimura, Y., and Ishii, S. (1990) The tryptophan cluster: a hypothetical strucuture of the DNA-binding domain of the myb

proto oncogene product. J. Biol. Chem. 265: 19990-19995.
Kappes, D., and Strominger, J.L. (1988) Human class II major histocompatibility complex genes and proteins. Annu. Rev. Biochem. 57: 991-1028.
Kelley, K.A., Kozak, C.A., Dandoy, F., Sor, F., Windass, J.D., DeMaeyer-Guignard, J., Pitha, P.M., and DeMaeyer, E. (1983) Mapping of murine interferon-α genes to chromosome 4. Gene 26: 181-188.
Kessler, D.S., Levy, D.E., and Darnell, J.E., Jr. (1988a) Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. Proc. Natl. Acad. Sci. USA 85: 8521-8525.
Kessler, D.S., Pine, R., Pfeffer, L.M., Levy, D.E., and Darnell, J.E., Jr. (1988b) Cells resistant to interferon are defective in activation of promoter-binding factor. EMBO J. 7: 3779-3783.
Kessler, D.S., Veals, S.A., Fu, X.-Y., and Levy D.E. (1990) IFN-alpha regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. Genes Dev. 4: 1753-1765.
Khan, K.D., Shuai, K., Lindwall, G., Maher, S.E., Darnell, J.E., Jr., and Bothwell, A.L.M. (1993) Induction of the Ly-6A/E gene by interferon α/β and γ requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds. Proc. Natl. Acad. Sci. USA 90: 6806-6810.
Kirchhoff, S., Hoffmann, K., Schaper, F., Hoxter, M., and Hauser, H. (1992) The murine interferon regulatory factor 1 (IRF-1) is a negative regulator of cell proliferation. J. Interferon Res. 12: S102.
Koch, C.A., Moran, M., Sadowski, I., and Pawson, T. (1991) The common src homology region 2 domain of cytoplasmic signalling proteins is a positive effector of v-fps tyrosine kinase function. Mol. Cell. Biol. 9: 4131-4140.
Lafage, M., Clauss, I., Couez, D., Simonetti, J., Wathelet, M.G., and Huez, G. (1992) The interferon- and virus-inducible IFI-56K and IFI-54K genes are located on human chromosome 10 at bands q23-q24. Genomics 13: 458-460.
Landschultz, W.H., Johnson, P.F., and McKnight, S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA-binding proteins. Science 240: 1759-1764.
Langer, J.A., and Pestka, S. (1988) Interferon receptors. Immunol. Today 9: 393-400.
Langer, J.A., Rashidbaigi, A., Lai, L.-W., Patterson, D., and Jones, C. (1990) Sublocalization on chromosome 21 of human interferon-alpha receptor gene and the gene for an interferon-gamma response protein. Som. Cell. Mol. Gen. 16: 231-240.
Larner, A.C., Jonak, G., Cheng, Y.S.E., Korant, B.D., Knight, E., and Darnell, J.E., Jr. (1984) Transcriptional induction of two genes in human cells by β interferon. Proc. Natl. Acad. Sci. USA 81: 6733-6737.
Larner, A.C., Chaudhuri, A., and Darnell, J.E., Jr. (1986) Transcriptional induction by interferon. J. Biol. Chem. 261: 453-459.
Larner, A.C., David, M., Feldman, G.M., Igarashi, K.-I., Hackett, R.H., Webb, D.S.A., Sweitzer, M., Petricoin III, E.F., and Finbloom, D.S. (1993) Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. Science 261: 1730-1733.
Levy, D.E., Larner, A.C., Chaudhuri, A., Babiss, L.E., and Darnell, J.E., Jr. (1986) Interferon-stimulated transcription: Isolation of an inducible gene and identification of its regulatory region. Proc. Natl. Acad. Sci. USA 83: 8929-8933.
Levy, D.E., Kessler, D.S., Pine, R., Reich, N., and Darnell, J.E. (1988) Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative control. Genes Dev. 2: 383-393.
Levy, D.E., Kessler, D.S., Pine, R., and Darnell, J.E., Jr. (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes Dev. 3: 1362-1371.
Levy, D., and Darnell, Jr., J.E. (1990a) Interferon-dependent transcriptional activation: signal transduction without secondary messenger involvement? The New Biologist. 2: 923-928.
Levy, D.E., Lew, D.J., Decker, T., Kessler, D.S., and Darnell, J.E., Jr. (1990b) Synergistic interaction between interferon-α and interferon-γ through induced synthesis of one subunit of the transcription factor ISGF3. EMBO J. 9: 1105-1111.
Lew, D.J., Decker, T., and Darnell, J.E., Jr. (1989) Alpha interferon and gamma interferon stimulate transcription of a single gene through different signal transduction pathways. Mol.

Cell. Biol. 9: 5404-5411.

Lew, D.J., Decker, T., Strehlow, I., and Darnell, J.E., Jr. (1991) Overlapping elements in the guanylate-binding protein gene promoter mediate transcriptional induction by alpha and gamma interferons. Mol. Cell. Biol. 11: 182-191.

Lewin, B. (1990) Commitment and activation at pol II promoters: a tail of protein-protein interactions. Cell 61: 1161-1164.

Van Loon, A.P.G.M., Ozmen, L., Fountoulakis, M., Kania, M., Haiker, M., and Garotta, G. (1991) High affinity receptor for interferon-gamma (IFN-gamma), a ubiquitous protein occuring in different molecular forms on human cells: blood monocytes and eleven different cell lines have the same IFN-gamma receptor protein. J. Leukocyte Biol. 49: 462-473.

Lovett, M., Cox, D.R., Yee, D., Boll, W., Weissmann, C., Epstein, C.J., and Epstein, L.B. (1984) The chromosomal location of mouse interferon-α genes. EMBO J. 3: 1643-1646.

Lutfalla, G., Roeckel, N., Mogensen, K.E., Mattei, M.G., and Uze, G. (1990) Assignment of the human interferon-α receptor gene to chromosome 21q22.1 by in situ hybridization. J. Interferon Res. 10: 515-517.

De Maeyer, E.M., and De Maeyer-Guignard, J. (1988) Interferons and other Regulatory Cytokines. Wiley (Interscience), New York.

Matsuyama, T., Kimura, T., Kitagawa, M., Pfeffer, K., Kawakami, T., Watanabe, N., Kundig, T.M., Amakawa, R., Kishihara, K., Wakeham, A., Potter, J., Furlonger, C.L., Narendran, A., Suzuki, H., Ohashi, P.S., Paige, C.J., Taniguchi, T., and Mak, T.W. (1993) Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. Cell 75: 83-97,

Mayer, B.J., Hamaguchi, M., and Hanafusa, H. (1988) A novel viral oncogene with structural similarity to phospholipase C. Nature 332: 272-275.

McKandry, R., John, J., Flavell, D., Muller, M., Kerr, I.M., Stark, G.R. (1991) High-frequency mutagenesis of human cells and characterization of a mutant unresponsive to both α and β interferons. Proc. Natl. Acad. USA 88: 11455-11459.

Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. (1988) Regulated expression of a gene encoding a nuclear factor, IRF1, that specifically binds to IFN-β gene regulatory elements. Cell 54: 903-913.

Mogensen, K.E., Uze, G., and Eid, P. (1989) The cellular receptor of the alpha-beta interferons. Experientia 45: 500-508.

Muller, M. Laxton, C., Briscoe, J., Schindler, C., Improta, T., Darnell, J.E., Jr., Stark, G.R., and Kerr, I.M. (1993a) Complementation of a mutant cell line: central role of the 91 kD polypeptide of ISGF3 in the interferon-α and -γ signal transduction pathways. EMBO J. 12: 4221-4228.

Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C., Pellegrini, S., Wilks, A.F., Ihle, J.N., Stark, R.S., and Kerr, I.M. (1993b) The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. Nature 366: 129-135.

Naf, D., Hardin, S.E., and Weissmann, C. (1991) Multimerization of AAGTGA and GAAAGT generates sequences that mediate virus inducibility by mimicking an interferon promoter element. Proc. Natl. Acad. Sci. USA 88: 1369-1373.

Nelson, B.E., and Borden, E.C. (1989) Interferons: Biological and clinical effects. Semin. Surg. Oncol. 5: 391-401.

Nelson, N.J., Marks, M.S., Driggers, P.H., and Ozato, K. (1993) ICSBP, a member of the IRF family suppresses IFN-induced gene transcription. Mol. Cell. Biol. 13: 588-599.

Ohmori, Y., and Hamilton T.A. (1993) Cooperative interaction between interferon (IFN) stimulus response element and kB sequence motifs controls IFN-γ and lipopolysaccharide-stimulated transcription from the murine IP-10 promoter. J. Biol. Chem. 268: 6677-6688.

Olopade, O., Jenkins, R.B., Ransom D.T., Malik, K., Pomykala, H., Nobori, T., Cowan, J.M., Rowley, J.D., and Diaz, M.O. (1992) Molecular analysis of deletions of the short arm of chromosome 9 in human gliomas. Cancer Res. 52: 2523-2529.

Pearse, R.N., Feinman, R., and Ravetch, J.V. (1991) Characterization of the promoter of the human gene encoding the high-affinity IgG receptor: Transcriptional induction by γ-interferon is mediated through common DNA response elements. Proc. Natl. Acad. Sci. USA 88: 11305-

11309.
Pearse, R.N., Feinman, R., Shual, K., Darnell, J.E., Jr., and Ravetch, J. (1993) Interferon γ-induced transcription of the high-affinity Fc receptor for IgG requires assembly of a complex that includes the 91-kDa subunit of transcription factor ISGF3. Proc. Natl. Acad. Sci USA 90: 4314-4318.
Pellegrini, S., John, J., Shearer, M., Kerr, I.M., and Stark, G.R. (1989) Use of a selectable marker regulated by alpha interferon to obtain mutations in the signalling pathway. Mol. Cell. Biol. 9: 4605-4612.
Pellegrini, S., and Schindler, C. (1993) Early events in signalling by interferons. TIBS 18: 338-342.
Pestka, S. (1986a) Interferon: A decade of accomplishments, foundations of the future in research and therapy. Semin. Hematol. 23: 27-37.
Pestka, S., ed. (1986b) Interferon, Part C. In: Methods in Enzymology (vol. 119), Academ. press.
Pestka, S., Jung, V., Rashidbaigi, A., Langer, J.A., and Kung, H.F. (1986c) Interferon and their receptors. In: The Interferon System, Vol. 24, Dianzani, F., and Rossi, G.B., eds. Raven Press, pp. 213-220.
Pestka, S., Langer, J.A., Zoon, K.C., and Samual, C.E. (1987) Interferons and their actions. Ann. Rev. Biochem. 56: 727-777.
Pine, R., Decker, T., Kessler, D.S., Levy, D.E., and Darnell, J.E., Jr. (1990) Purification and cloning of interferon-stimulated-gene factor 2 (ISGF2): ISGF2 (IRF1) can bind to the promoters of both beta interferon- and interferon-induced genes but is not a primary transcriptional activator of either. Mol. Cell. Biol. 10: 2448-2457.
Pine, R. (1992) Constitutive expression of an ISGF2/IRF-1 transgene leads to interferon-independent activation of interferon-inducible genes and resistance to virus infection. J. Virol. 7: 4470-4478.
Porter, A.C.G., Chernajovsky, Y., Dale, T.C., Gilbert, C.S., Stark, G.R., and Kerr, I.M. (1988) Interferon response element of the human gene 6-16. EMBO J. 7: 85-92.
Ptashne, M. (1988) How eukaryotic transcriptional activators work. Nature 355: 683-689.
Rashidbaigi, A., Kung, H.F., and Peska, S. (1985) Characterization of receptor for immune interferon in U937 cells with [32P]-labeled human recombinant immune interferon. J. Biol. Chem. 260: 8514-8519.
Rashidbaigi, A., Langer, J.A., Jung, V., Jones, C., Morse, H.G., Tischfield, J., Trill, J.J., Kung, H.F., and Pestka, S. (1986) The gene for the human immune interferon receptor is located on chromosome 6. Proc. Natl. Acad. Sci. USA 83: 384-388.
Revel, M., and Chebath, J. (1986) Interferon-activated genes. Trends Biochem. Sci. 11: 166-170.
Reich, N., Evans, B., Levy, D.E., Fahey, D., Knight, E., and Darnell, J.E., Jr. (1987) Interferon-induced transcription of a gene encoding a 15 kDa protein depends on an upstream enhancer element. Proc. Natl. Acad. Sci. USA 84: 6394-6398.
Reich, N., Pine, R., Levy, D.E, and Darnell, J.E., Jr. (1988) Tanscription of interferon-stimulated genes is induced by adenovirus particles but is suppressed by E1A gene products. J. Virology 62: 114-119.
Reich, N., and Darnell, J.E., Jr. (1989) Differential binding of interferon-induced factors to an oligonucleotide that mediates transcriptional activation. Nucl. Acids Res. 17: 3415-3424.
Reich, N.C., and Pfeffer, L.M. (1990) Evidence for involvement of protein kinase C in the cellular response to interferon α. Proc. Natl. Acad. Sci. USA 87: 8761-8765.
Reid, L.E., Brasnett, A.H., Gilbert, C.S., Porter, A.G.C., Gewert, D.R., Stark, G.R., and Kerr, I.M. (1989) A single DNA response element can confer inducibility by both α- and γ-interferons. Proc. Natl. Acad. Sci. USA 86: 840-844.
Reis, L.F., Harada, H., Wolchok, J.D., Taniguchi, T., and Vilcek, J. (1992) Critical role of a common transcription factor, IRF-1, in the regulation of IFN-β and IFN-inducible genes. EMBO J. 11: 185-193.
Ruff-Jamison, S., Chen, K., and Cohen, S. (1993) Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science 261: 1733-1736.
Rutherford, M.N., Hannigan, G.E., and Williams, B.R.G. (1988) Interferon-induced binding of nuclear factors to promoter elements of the 2-5A synthetase gene. EMBO J. 7: 751-759.
Sadowski, H.B., Shuai, K., Darnell, J.E., Jr., and Gilman, M.Z. (1993) A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 261:

1739-1744.
Schindler, C., Fu, X.-Y., Improta, T., Aebersold, R.H., and Darnell, J.E., Jr. (1992a) The proteins of ISGF3: one gene encodes 91 and 84 kDa ISGF3α proteins. Proc. Natl. Acad. Sci. USA 89: 7836-7839.
Schindler, C., Shuai, K., Prezioso, V.R., and Darnell, J.E., Jr. (1992b) Interferon-dependent tyrosine phophorylation of a latent cytoplasmic transcription factor. Science 257: 809-813.
Schlessinger, J., and Ullrich, A. (1993) Growth factor signalling by receptor tyrosine kinases. Neuron 9: 383-391.
Sen, G.C., and Lengyel, P. (1992) The interferon system. J. Biol. Chem. 267: 5017-5020.
Shuai, K., Schindler, C., Prezioso, V.R., and Darnell, J.E., Jr. (1992) Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91 kDa DNA binding protein. Science 258: 1808-1812.
Shuai, K., Stark, G.R., Kerr, I.M., and Darnell, J.E., Jr. (1993a). A single phosphotyrosine residue of STAT91 required for gene activation by interferon-γ. Science 261: 1744-1746.
Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z., and Darnell, J.E., Jr. (1993b) Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature 366: 580-583.
Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Cleveland, J., Yi, T., and Ihle, J.N. (1993a) Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. Proc. Natl. Acad. Sci. USA 90: 8429-8433.
Silvennoinen, O., Ihle, J.N., Schlessinger, J., and Levy D.E. (1993b) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 366: 583-585.
Silvennoinen, O., Schindler, C., Schlessinger, J., and Levy, D.E. (1993c) Ras-independent growth factor signalling by transcription factor tyrosine phosphorylation. Science 261: 1736-1739.
Soh, J., Donnelly, R.J., Mariano, T.M., Cook, J.R., Schwartz, B., and Pestka, S. (1993) Identification of a yeast artificial chromosome clone encoding an accessory factor for the human interferon γ receptor: Evidence for multiple accessory factors. Proc. Natl. Acad. Sci. USA 90: 8737-8741.
Stahl, M.L., Ferenz, C.R., Kelleher, K.L., Kritz, R.W., and Knopf, J.L. (1988) Sequence similarity of phospholipase C with the non-catalytic region of src. Nature 332: 269-272.
Stefanos, S., Ahn, Y.H., and Pestka, S. (1989) Characterization of human interferon-γ receptor purified from placenta. J. Interferon Res. 9: 719-730.
Stewart, H.J., McCann, S.H., Barker, P.J., Lee, K.E., Lamming, G.E., and Flint, A.P. (1987) Interferon sequence homology and receptor binding activity of ovine throphoblast antiluteolytic protein. J. Endocrinol. 115: R13.
Strehlow, I., Seegert, D., Frick, C., Bange, F.-C., Schindler, C., Bottger, E.C., and Decker, T. (1993) The gene encoding IFP 53/Tryptophanyl-tRNA Synthetase is regulated by the γinterferon activation factor. J. Biol. Chem. 268: 16590-16595.
Sugita, K., Miyozaki, J.I., Apella, E., and Ozato, K. (1987) Interferons increase transcription of a major histocompatibility class I gene via a 5' interferon consensus sequence. Mol. Cell. Biol. 7: 2625-2630.
Tanaka, N., Kawakami, T., and Taniguchi, T. (1993) Recognition DNA sequences of interferon regulatory factor-1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. Mol. Cell. Biol. 13: 4531-4538.
Taylor, J.L., and Grossberg, S.E. (1990) Recent progress in interferon research: molecular mechanisms of regulation, action, and virus circumvention. Virus Res. 15: 1-25.
Trent, J.M., Olson, S., and Lawn, R.M. (1982) Chromosomal localization of human leukocyte, fibroblast and immune interferon genes by means of in situ hybridization. Proc. Natl. Acad. Sci. USA. 79 7809-7813.
Uze, G., Mogensen, K.E., and Aguet, M. (1985) Receptor dynamics of closely related ligands: "fast" and "slow" interferons. EMBO J. 4: 65-70.
Uze, G., Lutfalla, G., and Gresser, I. (1990) Genetic transfer of a functional human interferon α receptor into mouse cells: cloning and expression of its cDNA. Cell 60: 225-234.
Uze, G., Lutfalla, G., Bandu, M.T., Proudhon, D., and Mogensen, K.E. (1992) Behaviour of a cloned murine interferon alpha/beta receptor expressed in homospecific or heterospecific background. Proc. Natl. Acad. Sci. USA 89: 4774-4778.
Veals, S.A., Schindler, C., Leonard, D., Fu, X.-Y., Aebersold, R., Darnell, J.E., Jr., and Levy, D.E.

(1992) Subunit of an alpha-interferon-resposive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. Mol. Cell. Biol. 12: 3315-3324.
Veals, S.A., Santa Maria, T., and Levy, D.E. (1993) Two domains of ISGF3γ that mediate protein-DNA and protein-protein interactions during transcription factor assembly contribute to DNA-binding specificity. Mol. Cell. Biol. 13: 196-206.
Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon α/β signalling pathway. Cell 70: 313-322.
Watanabe, N.J., Sakakibara, A., Hovanessian, T., Taniguchi, T., and Fujita, T. (1991). Activation of IFN-β promoter element by IRF-1 requires a post-transcriptional event in addition to IRF-1 synthesis. Nucl. Acids Res. 16: 4421-4428.
Wathelet, M., Moutschen, S., Defillippi, P., Cravador, A., Collet, M., Huez, G., and Content, J. (1986) Molecular cloning, full length sequence and preliminary characterization of a 56-kDa protein induced by human interferons. Eur. J. Biochem. 155: 11-17.
Wathelet, M.G., Glauss, I.M., Nols, C.B., Content, J., and Huez, G.A. (1987) New inducers revealed by the promoter sequence analysis of two interferon-activated human genes. Eur. J. Biochem. 169: 313-321.
Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988a) The IFI-56K and IFI-54K interferon-inducible human genes belong to the same gene family. FEBS Letters 231, 164-171.
Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988b) Regulation of two interferon-inducible human genes by interferon, poly(rI).poly(rC) and virus. Eur. J. Biochem. 174, 323-329.
Watling, D., Guschin, D., Muller, M., Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Rogers, N.C., Schindler, Stark, G.R., Ihle, J.N., and Kerr, I.M. (1993) Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-γ signal transduction pathway. Nature 366: 166-170.
Weck, P.K., Apperson, S., Stebbing, N., Gray, P.W., Leung, D., Shepard, H.M., and Goeddel, D.V. (1981) Antiviral activities of hybrids of two major human leucocyte interferons. Nucleic Acids Res. 9: 6153-6166.
Weissmann, C., and Weber, H. (1986) The interferon genes. Progress Nucleic Acid Res. Mol. Biol. 33: 251-300.
Weisz, A., Marx, P., Sharf, R., Appella, E., Driggers, P.H., Ozato, K., and Levi, B. (1992) Cloning and characterization of the human ICSBP, a possible negative regulator that binds enhancer elements common to interferon inducible genes. J. Biol. Chem. 26: 25589-25596.
Wilks, A.F. (1989) Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. Proc. Natl. Acad. Sci. USA 86: 1603-1607.
Wilks, A.F., Harpur, A.G., Kurban, R.R., Ralph, S.J., Zurcher, G., and Ziemiecki, A. (1991) Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. Mol. Cell. Biol. 11: 2057-2065.
Williams, B.R.G. (1991) Transcriptional regulation of interferon-stimulated genes. Eur. J. Biochem. 200: 1-11.
Willman, C.L., Sever, C.E., Pallavicini, M.G., Harada, H., Tanaka, N., Slovak, M.L., Yamamoto, H., Harada, K., List, A.F., and Taniguchi, T. (1993). Deletion of IRF-1, mapping to chromosome 5q31.1, in human leukemia and preleukemic myelodysplasia. Science 259: 968-971.
Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J.N. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74: 227-236.
Wu, L.-C., Morely, B.J., and Campbell, R.D. (1987) Cell-specific expression of the human complement protein factor B gene: Evidence for the role of two distinct 5'-flanking elements. Cell 48: 331-342.
Yamada, G., Ogawa, M., Akagi, K., Miyamoto, H., Kakono, N., Itoh, S., Miyazaki, J., Nishikawa, S., Yamamura, K., and Taniguchi, T. (1990) Specific depletion of the B cell population induced by aberrant expression of human IRF-1 (interferon regulatory factor-1) gene in transgenic mice. Proc. Natl. Acad. Sci. USA 88: 532-536.
Yan, C., and Tamm, I. (1991) Molecular cloning and characterization of interferon α/β response element binding factors of the murine (2'-5')oligoadenylate synthetase ME-12 gene. Proc.

Natl. Acad. Sci. USA 88: 144-148.

CHAPTER 2

ISOLATION, PROPERTIES AND CHROMOSOMAL LOCALIZATION OF FOUR CLOSELY LINKED HAMSTER IFN-α GENES

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SUMMARY

Three recombinant phages containing hamster interferon-α genes (Ha IFN-α) were isolated from a hamster genomic library, using a mouse interferon-α (Mu IFN-α) probe. The phage inserts contained overlapping hamster genomic fragments, spanning a total length of approx. 30 kb, and four Ha IFN-α genes (Ha IFN-α A1, -A2, -A3 and -A4). The IFN-α gene cluster could be assigned to hamster chromosome 2. The nucleotide sequences of the four IFN-α genes were determined. Two of the genes are functional (Ha IFN-α A1 and -A3) and two are pseudogenes (-A2 and -A4). The Ha IFN-α A1 and -A3 genes were transiently expressed in COS cells and they gave rise to protein products with antiviral properties on hamster CHO cells. In addition, A1 revealed high antiviral activity on mouse L929 cells.

INTRODUCTION

Interferons (IFNs) are a family of proteins with various biological properties. IFNs are secreted by the producer cells in response to different inducers, and can induce a variety of responses in target cells. These include antiviral, cell growth inhibiting, and immunomodulatory activities (see for reviews Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988; Sen and Lengyel, 1992).

The IFNs have been classified into two types on the basis of their biological and physical properties. IFN-β and IFN-α are members of the Type I IFNs. In all mammalian species studied, the α IFNs are encoded by a family of intronless, closely related genes (Weissmann and Weber, 1986; De Maeyer and De Maeyer-Guignard, 1988; Sen and Lengyel, 1992). In the human genome at least 14 active IFN-α genes (IFNA) (Diaz et al., 1993) and 4 pseudogenes (IFNP) can be discriminated. In addition, the human genome contains a series of IFNA-related genes (IFN-W genes) (Capon et al., 1985; Hauptmann et al., 1985, Diaz et al., 1993). In the mouse genome, twelve different complete IFN-alpha genes have been isolated and characterized. Eleven of these [α1, α2, α4, α5, α6T, α6P, αA, α8, α9, α10(7) and α11] encode biologically active proteins (Shaw et al., 1983; Daugherty et al., 1984; Zwarthoff et al., 1985; Kelley and Pitha, 1985; Dion et al., 1986; Kelley et al., 1986; Seif and De Maeyer-Guignard, 1986; Trapman et al., 1988; Navarro et al., 1989; Coulombel et al., 1991), the other is a pseudo-gene (LeRoscouet et al., 1985).

Human IFN-β (or IFN-B) is encoded by a single copy gene, which is 50% homologous to the IFN-α genes, and which is physically closely linked to the IFN-α gene family. The mouse genome, in common with human, possesses a single IFN-β gene, whereas cows, sheep, and pigs possess multiple IFN-β genes (Weissmann and Weber, 1986; De Maeyer and De Maeyer-Guignard, 1988).

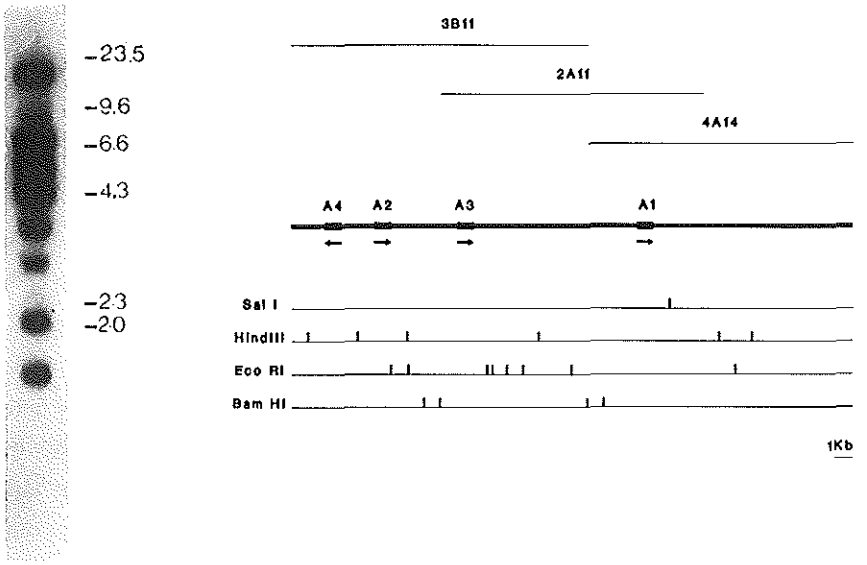
In human, Type I IFN genes are located within the band p22 of the short arm of chromosome 9 (Diaz et al., 1991; Olopade et al., 1992), in mouse, Type 1 IFN genes are clustered on chromosome 4 (Van der Korput et al., 1985). Each member of the Type I IFNs contains 165-170 amino acid residues and binds to the same IFN receptor on the cellular membrane (Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988). It is assumed that the IFN-α gene family arose by repeated duplications of a common ancestral α gene. The IFN-α genes are relatively well conserved. Within a species, the homology between the proteins is 70% or more. Between human and Mu IFN-α proteins the homology is 50 to 60%. Despite these structural similarities, IFNαs are relatively species-specific: most human IFNs have only a low activity on mouse cells and vice versa (Weck et al., 1981). The structural basis for this species specificity is unclear.

Previously, we reported that some Mu IFN-α species are active on hamster cells, whereas others are not (Van Heuvel et al., 1986). To increase our knowledge on the relationship of rodent IFNs we initiated a study on Ha IFNs. Here, we report the molecular characterization of a cluster of four Ha IFN-α genes, and its chromosomal localization. In addition, the antiviral activity of the proteins encoded by Ha IFN-α A1 and -A3 on hamster CHO-12 and mouse L929 cells, is described. The Ha IFN-α genes will form a basis for further comparison of hamster and mouse IFN-α properties.

RESULTS AND DISCUSSION

(A) Organization of the hamster IFN-α gene family.

To isolate IFN-α genes, a hamster genomic library in λEMBL3 was screened according to standard methods (Sambrook et al., 1989). As a probe, a mixture of a 600 bp BamHI/EcoRI Mu IFN-α4 fragment and a 600 bp BamHI/EcoRI Mu IFN-α6 fragment, was used (Zwarthoff et al., 1985). On Southern transfers of genomic DNA from hamster CHO cells, the probe hybridized to eight different fragments (Figure 1A), indicating that the hamster genome harbours an IFN-α gene family containing 8 or more different members.



(A) (B)

FIGURE 1. Isolation and characterization of a genomic cluster of four hamster IFN-α genes. (A) Southern blot analysis of the IFN-α genes in genomic DNA derived from hamster CHO cells. The DNA was digested with EcoRI. For hybridization, a 600 bp BamHI/EcoRI fragment containing the Mu IFN-α6 gene was used. (B) Partial restriction map of the hamster genomic DNA clones 3A11, 2A11, and 4A14, containing the IFN-α genes A1, A3, A2, and A4 (boxed regions). Arrows indicate the direction of transcription. Methods. The hamster genomic library, in λEMBL3, was a kind gift from C. Troelstra (Erasmus University Rotterdam, Dept. of Cell Biology and Genetics). The library was screened according to standard procedures (Sambrook et al., 1989). A mix of a 600 bp Bam HI/Eco RI Mu IFN-α4 fragment and a 600 bp Bam HI/EcoRI Mu IFN-α6 fragment (Zwarthoff et al., 1985) was used as a hybridization probe. After three rounds of plaque purification, DNA was isolated and characterized by restriction mapping and Southern hybridization.

After three rounds of plaque purification, ten recombinant phages were isolated. Five out of these ten phages were randomly chosen and further analyzed. DNA was isolated and characterized by single, double and triple restriction digestion and Southern blotting, using the Mu IFN-α6 gene fragment as a probe. The analysis indicated that the restriction pattern of three phages was identical (phage 3B11), whereas that of the other two inserts was unique (2A11 and 4A14, respectively) (Figure 1B). The 3 different phages, containing inserts of 16-18 kb, were found to contain overlapping hamster genomic DNA fragments, and four regions hybridizing with the Mu IFN-α6 probe. The total size of the overlapping genomic fragments isolated was approx. 30 kb. A physical map of the contig is shown in Figure 1B. The different hybridizing regions correspond to four IFN-α genes, named Ha IFN-α A1, -A2, -A3, and -A4, respectively. The genes A3 and A1 are

separated by 8.4 kb, the distance between genes A3 and A2 is 3.2 kb, that between A2 and A4 is 1.6 kb. The orientation of the A4 gene (as indicated in Figure 1B) is opposite to that of A1, A2 and A3.

(B) Chromosomal localization of the hamster IFN-α gene cluster.

To determine the chromosomal localization of the hamster IFN-α genes, fluorescent in situ hybridization (FISH) on metaphase chromosome spreads from three different hamster cell lines (A23, CHO-12 and DON) was performed (see Legends Figure 2). The biotinylated genomic clones 3B11 and 4A14 (see also Figure 1B), were used as probes for Ha IFN-α A4, -A2, and -A3, and Ha IFN-α A1, respectively. Eight to 15 metaphases were analyzed for the different probes.

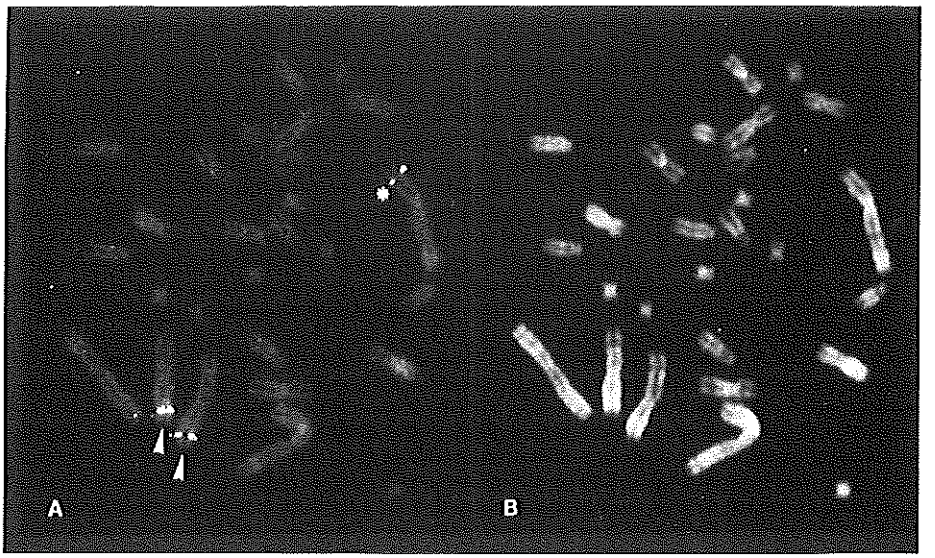


FIGURE 2. Chromosomal localization of the hamster IFN-α gene cluster by in situ hybridization. (A) Metaphase from hamster A23 cell line hybridized with the IFN-α A4, -A2, -A3 genes (probe 3B11), demonstrating signals on two normal chromosomes 2 (indicated by an arrow) and an additional, abnormal chromosome 2. (B) DAPI staining of the same metaphase as (A). Methods, the probes were labeled by standard nick translation with biotin-16-dUTP (Boehringer Manheim). 50-100 ng probe in hybridization mixture (50% formamide, 10% dextransulphate in 2xSSCP) with 5 µg sonicated salm sperm DNA and 5 µg yeast tRNA, was heat denatured and preannealed with 10 µg hamster cot-1 DNA (Gibco BRL, Gaithersberg USA) for 30-60 min. at 37 °C. The target DNA was also heat denatured (70 °C, 2 min.) followed by hybridization overnight at 37 °C. After washing, hybridization sites were visualized by immuno fluorescence using Avidine D-Fitc (Vector, USA). A second round of amplification was usually required. The slides were counterstained with propidium Iodide and 4',6',diamino-2-phenylindole (DAPI) in antifade medium (DABCO).

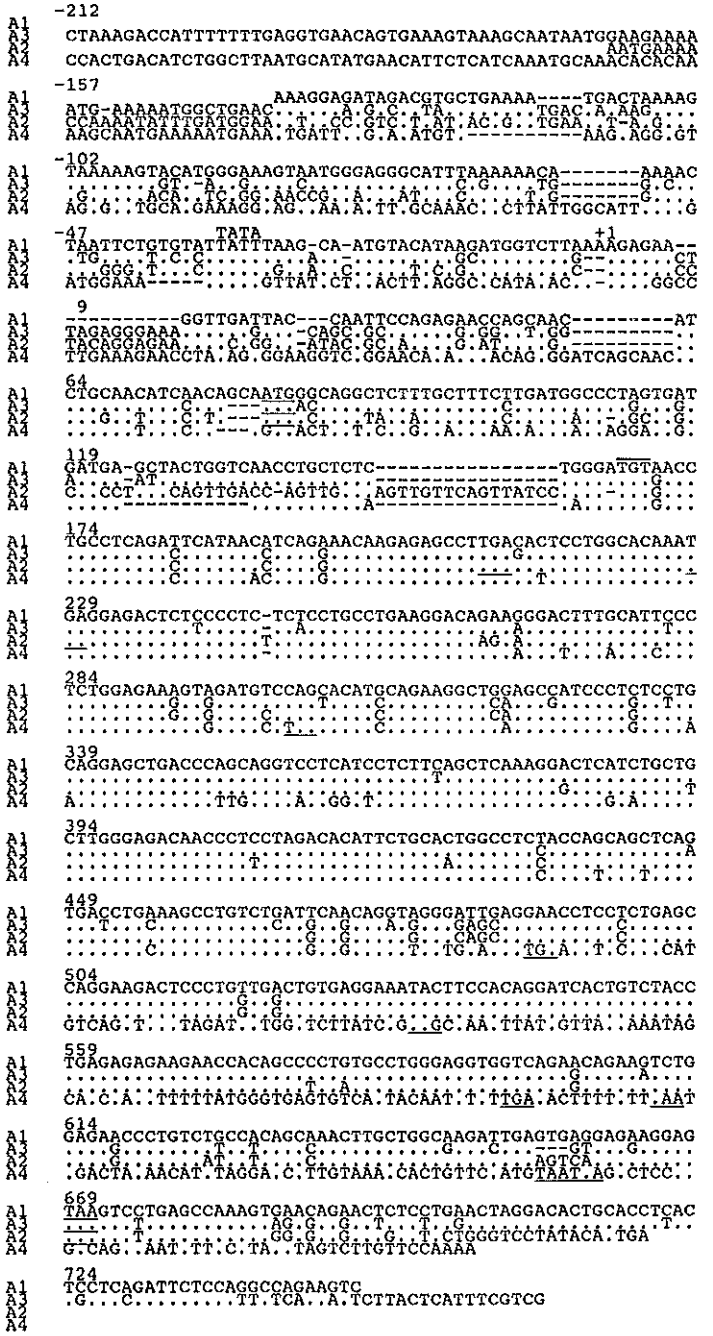
The results of representative experiments are shown in Figure 2. Metaphases from the A23 cell line (Figure 2), hybridized with probe 3B11 (Figure 2) or probe 4A14 (data not shown), clearly demonstrated signals on both chromosomes 2. In 11 out of 15 cells, one additional abnormal chromosome 2 hybridized, whereas in 4 out of 15 cells this was the case for two abnormal chromosomes 2. Metaphases from CHO-12 and DON cell lines hybridized with probe 3B11 or 4A14 (data not shown), showed in all cases signals on one normal and one abnormal chromosome 2. In conclusion, consistent signals for both probes were obtained only with chromosome 2.

(C) The nucleotide sequence of the Ha IFN-α genes.

Appropriate restriction fragments, including the different genes, were subcloned and further characterized by restriction mapping and sequencing. The nucleotide sequences of the different genes are compared in Figure 3. The translation initiation codon (ATG) and presumed stop codons are underlined. In the IFN-α A1 and -A3 genes proper open reading frames (ORF) were found encoding proteins of 190 and 189 amino acids, respectively. The IFN-α A2 and -A4 genes, on the contrary, both showed reading frames, interrupted by stop codons, which would result in the synthesis of truncated IFN proteins.

Allignment of the sequences of Ha IFN-α A1, and -A3 showed 91% homology at the nucleotide level for the region beginning at the ATG start codon and ending at the termination codon TAA at position 669 (Figure 3). The homology between Ha IFN-α A1 and -A2 in this region is 85%. However, because of various insertions and deletions including an insertion of 16 bp, a TGA termination codon in A2 was found at position 200. The Ha IFN-α A4 gene contains a GTG at the position of the A1 and A3 ATG first codon. This, and the further structure of the gene (the first termination codon is reached at position 165; see Figure 3) make it impossible that A4 encodes a functional IFN. The homolgy between the sequenced part of Ha IFN-α A1 and -A4 genes, is only 66%. Starting from position 81 (codon 1 ATG/GTG) until position 487 the homology is still 86%. From position 488 on, the sequence from A4 completely diverges from A1.

FIGURE 3. Homology between the nucleotide sequences of the hamster IFNα-A1, -A3, -A2, and -A4 genes. The transcription start site is indicated as + 1. The initiation codon (ATG) and presumed stop codons (TGA, TAG, TAA) are underlined. The TGT codon, that marks the beginning of the mature protein, is overlined. Dots indicate homology, horizontal bars indicate deletions. A variant TATA-box is indicated by TATA. Methods. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977), using T7 polymerase (Pharmacia, Uppsala, Sweden).



At approximately 30 bp upstream from the presumed transcriptional start site (indicated as +1 in Figure 3; see also Shaw et al, 1983), in A1 and A3 a variant TATA box (TATTTA) is found, which is a characteristic of IFN-α genes. In the small stretch of 3' untranslated region, no polyadenylation signal could be detected.

(D) Structure of the Ha IFN-α A1 and -A3 proteins.

A comparison of the amino acid sequences of the Ha A1 and A3 proteins as deduced from their nucleotide sequences is shown in Figure 4. By comparison with the sequence of selected Mu IFN-α proteins (Mu α1, α4 and α6; Zwarthoff et al., 1985), the first 23 amino acids presumably represent the signal peptide (Figure 4). This sequence is enriched in hydrophobic amino acid residues. Ha-A1 and -A3 show a high homology (87%) in their signal peptide, between hamster and the three mouse species the homology is approx. 80%. The cysteine residue at S21, but not that at S5, is concerved in hamster and mouse sequences.

```
          S1                  S20
                              ▼
A1        MGRLFAFLMALVMMS-YWSTCSLG
A3        .T.........V.N-........
α4        .A..C....I.....Y...A....
α6        .A..C....V.AV..-........
α1        .A..C....V.AV..-..P.....

          1
          ▼                                 ▼
A1        CNLPQIHNIRNKRALTLLAQMRRLSPLSCLKDRRDFAFPLEKVDVQHMQKAGAIPLL
A3        .D...T..L......R........F..T.....K............YI...Q...V.
α4        .D..HTY.LG......V.EE....P........K..G.......N.QI...Q..LV.
α6        .D...T.KL.........I..............K..G..Q....TLKI..EK...V.
α1        .D...T.NL.........V..............K..G..Q....A.QIK..Q...V.

          58                                ▼                 ▼
A1        QELTQQVLILFSSKDSSAAWETTLLDTFCTGLYQQLSDLKACLIQQVGIEEPPLSQE
A3        ............................H...N..Q...M..M.EQ.......
α4        RD....I.N..T...L..T.NA....S..ND.H...N.....VM.-------...T..
α6        S.V...I.NI.T........DA.......ND.....N..Q...V...RL.....T..
α1        S.....I.NI.T........NA....S..ND.H...N..QG..M....VQ.F..T..

          115
                              ▼
A1        DSLLTVRKYFHRITVYLREKNHSPCAWEVVRTEVWRTLSATANLLARLSEEKE*
A3        ...VA..............................A.I..A..SS..........-.*
α4        ....A..T..........K.K..L.....I.A....A..SST...........*
α6        V...A..............K..........A....A..SS..V.G..R...-*
α1        .A..A..............K..........A....A..SS..V.G..R...-*
```

FIGURE 4. Comparison of the amino acid sequence of Ha IFNα-A1 and -A3 with that of the mouse IFN-α1, -α4, and -α6. Conserved cysteines are indicated by arrow heads. Gaps (-) were introduced to maximize allignment. Dots indicate homology and asterisks indicate deduced endpoints. Numbering starts from the mature protein, which is preceeded by the signal peptide.

The mature proteins, encoded by A1 and A3, are 167 and 166 amino acids in length, respectively, and show 85% homology. Between hamster and mouse subspecies this homology varies from 70% (A1/α4) to 80% (A3/α6 and A3/α1). The five cysteine residues present in all Mu IFN-α species at positions 1, 29, 86, 99 and 139 are also found in Ha A1 and Ha A3. Cys1-cys99 and cys29-cys139 can form disulfide bridges (Wetzel et al., 1981). A1 and A3 do not contain the N-glycosylation site (Asn-Ala-Thr) at 79 to 81 found in most mouse IFNs (Trapman et al., 1988).

From site-specific mutagenesis, hybrid scanning, competitions with specific antipeptide antibodies, and the crystal structure of Mu IFN-β, it is believed that three functionally important segments, defined by residues 29-35, 78-95, and 123-140, of the IFN-α molecule are required for receptor binding and activation of specific responses (Nisbet et al., 1985; Eichmann et al., 1990; McMullen et al., 1990; Fish et al., 1992; Senda et al., 1992; Waine et al., 1992). Site-directed mutagenesis of the human IFNs-α has furthermore shown that Leu-30, Arg-33, Phe-36, and Tyr-123, all located in hydrophilic regions, are critical for antiviral activity. These residues are conserved in the Ha and Mu IFN-α sequences, as depicted in Figure 4. The overall comparison of segments 29-35, 78-95, and 123-140, between the different species showed no important sequence differences, indicating conservation of essential domains between IFNs from different species.

(E) Expression and biological activity of the Ha IFN-A1 and -A3 proteins.

The ORFs of Ha IFNα-A1 and -A3 were inserted into the polylinker of pSV328A, thus bringing the genes under the control of the simian virus 40 (SV40) early promoter (van Heuvel et al., 1986). The resulting expression plasmids, pSVA1 and pSVA3, were transiently expressed in COS cells according to the calcium phosphate precipitation method (Chen et al., 1987). IFN activity in the supernatant was subsequently assayed on hamster CHO and mouse L929 cells. Antiviral titers on CHO cells were related to the activity of Mu IFN-α6 (pSVα6) on CHO cells (Van Heuvel et al., 1986). Mu IFN-α4 (pSVα4), which is only active on mouse L929 cells, was taken as a second control (Van Heuvel et al., 1986). Table 1 shows the values found in a representative experiment. It appeared that the Ha IFNα-A1, and -A3 genes coded for IFN subspecies with antiviral activity on hamster cells, as expected Mu IFN-α6 was also active on hamster cells and Mu IFN-α4 was not. The results obtained on mouse cells showed that Ha IFNα-A3 had almost no activity. Interestingly, the activity of Ha IFNα-A1 on mouse cells was even higher than that of α6.

TABLE 1. Antiviral activity produced by expression plasmids as measured on hamster (CHO-12) and mouse (L929) cells.

	CHO (U/ml)	L929 (IU/ml)
pSVA1	6400	12800
pSVA3	12800	16
pSVα4	16	25600
pSVα6	6400	3200

IFN titres on L929 were calculated relative to a NIH reference standard and are presented in IU/ml. Titres on CHO cells were compared to those on L929 cells and given in arbitrary U/ml.
***Methods.** All plasmids used for expression in COS cells were based on the expression plasmid pSV328A (Van Heuvel et al., 1986). For the construction of pSVA3, a XhoII-MseI (nt 50-765 in Figure 3) hamster IFNα-A3 fragment (the MseI site was blunt ended with Klenow DNA polymerase) was subcloned into pGEM7 (BamHI-SmaI) (pGEMA3). The insert was subsequently excised by EcoRI and SacI digestion, and ligated into the EcoRI and SacI site of pSV328A. For the construction of pSVA1 the pGEMA3 construct was used as a basis vector. By digesting pGEMA3 with HincII and Asp718 (the Asp718 site was blunt ended), the complete mature protein coding region and a small part of the signal peptide coding region of A3 was excised. This vector was named pGEM-A3a. Because A1 contains a conserved HincII site at the exact same position as A3 (nt 132 in Figure 3), it was in principle possible to clone in a HincII-EcoNI (nt 132-699) Ha IFNα-A1 fragment (containing the mature protein coding part of A1). First, a HincII-EcoNI (nt 518-699) fragment (the EcoNI site was blunt ended) was cloned into pGEMA3a. Secondly, the HincII-HincII (nt 132-518) fragment of A1 was ligated into this construct using the HincII site. The complete fragment was subsequently excised by EcoRI and SacI and ligated into the EcoRI and SacI site of pSV328A, finally creating pSVA1. In this way a complete Ha IFNα protein could be produced which contained a A3-A1 hybrid signal peptide and a mature A1 protein coding part. COS cells were seeded at 5 x 10^5 cells/6 cm dish. The next day, cells were transfected with the appropriate IFN-α gene construct (5 μg) according to the calcium phosphate precipitation method (Chen et al., 1987); 48 h later the medium was collected. The amount of antiviral activity secreted during this period was assayed on hamster CHO12 and mouse L929 cells in a cytophatic effect reduction assay, using vesicular stomatitus virus (VSV) as a challenge (Van Heuvel et al., 1986). All experiments were carried out at least four times in duplicate with two separate plasmid isolations.*

(F) Comparison of Ha and Mu IFN-α subspecies.

The Mu IFN-α1, -α2, -α4 and -α6 (see also Table 1) differ in their antiviral activity on hamster CHO cells and mouse L929 cells (Van Heuvel et al., 1986). Structure-function analysis of several natural and hybrid Mu IFN-α species implicated the amino-terminal fragment ranging from amino acids 10 to 58 important for the activity on hamster cells (Van Heuvel et al., 1988). A comparison of Ha A1, -A3, α6 and α1 (all active on hamster) with α4 (not active on hamster) (Figure 4), indicates 5 amino acids in this area (positions 10, 19, 20, 45 and 55) in which α4 clearly differs from the other species (also as compared to Mu IFN-α2). This could indicate that amino acid residues at these positions are responsible for the difference in activity on hamster cells.

The carboxy-terminal portion of α4 (from amino acids 130 to 167) was found to be important for its high antiviral activity on mouse L cells (Van Heuvel et al., 1988). Comparison of the carboxy-terminal (130-end) portion of Ha-A1, α4, α6 and α1 (all active on mouse) with Ha-A3 (not active on mouse) (Figure 4) shows no clear signs that could account for the difference in activity. This could indicate that not single amino acid differences, but more subtle differences in amino acids composition between A1 and A3 together, determine the difference in activity on mouse cells.

(G) Comparison of promoter sequences.

The region directly upstream from the TATA box is known to be involved in the virus inducible expression of the IFN-α genes. Studies using deletion mutants have defined a region of the human IFN-α1 gene promoter, known as the virus responsive element (VRE; 46 bp fragment extending from -109 to -64 relative to the cap site), which constitutes the minimum sequence necessary to confer full virus inducibility upon a minimal promoter (Ragg et al., 1983; Weidle et al., 1983; Ryals et al., 1985). Similarly, it was reported that the Inducible Element (IE; 35 bp fragment located between -109 and -75), which overlaps with the VRE, was able to confer inducibility to the Mu IFN-α4 gene (Raj et al., 1989). These elements contain repeats of the AGTGAA motif, or the variants of its permutated form GAAANN, which when multimerized (thereby resembling an IRF-1 binding site) mediate virus inducibility upon a heterologous promoter (Naf et al., 1991). The IE motif contains an almost symmetric sequence GTAAAGAAAGT (αF1, -103 to -94), which partially overlaps with a putative IRF-1 binding site (Au et al., 1993). It has been suggested that the integrity of both of these sites is essential for virus-mediated induction (Raj et al., 1991; Au et al., 1993), and that this requires cooperation between the αF1 binding proteins and IRF-1 or an other protein(s) binding to the IRF-1 site (Au et al., 1993).

Differences in the VRE sequences of individual IFN-α genes in both mice and men, have been suggested to account for differences in the activity of their respective promoters (Bisat et al., 1988; Civas et al., 1991; Raj et al., 1991; Au et al., 1993). In Figure 5 the sequences of the VRE-corresponding region, containing αF1 and the IRF-1 binding site, of the Ha IFN-αA1 and -A3 genes are shown together with the corresponding region of the Mu IFN-α4, -α6 and -α11 genes (Zwarthoff et al., 1985; Raj et al., 1991; Civas et al, 1991) and the Hu IFN-α1 gene (Nagata et al., 1980). Ha IFNα-A1, like Mu IFN-α6, contains a 4 bp deletion in αF1. The finding that α6 is transcriptionally active in mouse L cells (Zwarthoff et al., 1985), indicates that this deletion is not distructive for activity of the Ha A1 promoter.

```
                  -109      VIRUS RESPONSIVE ELEMENT          -64
Hu VRE α1    TAAAGAGTGCATGAAGGAAAGCAAAAACAGAAATGGAAAGTGGCCCAGAAGCATTAA

                                  IRF-1 site
                  -109      αF1                               -64
Mu VRE α4    CAGAGAGTGAAGTAAAGAAAGTGAAAAGAGAATTGGAAAGCAAGGGGAGGGTATTCC
       α6    T.....A.A.GT.----.........G..C.G........TT..A......C....A
      α11    .....................................G.................TA
Ha VRE A1    .T..A.A...CTA----.....A.....TAC..G......T..T.......C...TA
       A3    .T..A.A...CTA.........A.....T.T.-A..G...TC.T.......C...CA
```

FIGURE 5. Comparison of the Virus Responsive Element of IFN-α genes (Ha IFN-α A1 and -A3, Mu IFN-α4, -α6 (Zwarthoff et al., 1985) and -α11 (Civas et al., 1991), and Hu IFN-α1 (Nagata et al., 1980), respectively). Gaps (-) were introduced to maximize allignment. Dots indicate homology. The αF1 and IRF-binding site (Au et al., 1993) are indicated.

Although Ha A1 has an IRF-1-like binding site, variation is seen at positions -98, -92 and -86 as compared to α4. The Ha IFNα-A3 promoter seems to have an intact αF1 together with an IRF-1-like binding site, however, some variations in both elements are seen as compared to Mu IFN-α4. Especially, the T at position -103, instead of a G, in the αF1 element, seems an important difference being responsible for a lower activity (Raj et al., 1991). The IRF-1 site differs at position -92 and -86 as compared to α4. Mutation analysis from the Mu IFN-α11 gene, furthermore, suggested that the -78 A/G substitution (as compared to α4) affects the inducibility by decreasing the affinity of VRE-binding factors (Civas et al., 1991). Interestingly, Ha A3 contains a G at -78, whereas in the other promoters a T residue is conserved at this position. Together, the structural data concerning the Ha A1 and A3 VRE region suggest that both genes can be expressed, although at a low level.

(H) Conclusions

(1) The organization of Ha IFN-α genes as determined here contributes to the establishment of the IFN physical map from different species. The presence of more than one gene within a small piece of hamster DNA shows that the Ha IFNα genes are tightly linked in the genome, as was found to be the case for the human (Diaz et al., 1991; Olopade et al., 1992) and the Mu IFN-α gene family (Kelley et al., 1983; Lovett et al., 1984; Van der Korput et al., 1984). In the human genome, the Type I IFN genes have been assigned to chromosome 9 band p22 (Diaz et al., 1993), in mouse to chromosome 4 (Van der Korput et al., 1985). Our assignment of the hamster IFN-α genes to chromosome 2q is in good agreement with the human-mouse-hamster comparitive gene map (O'Brein and

Greaves, 1991).

(2) Evidence that Ha IFNα-A2 and -A4 are pseudogenes is provided by the finding that although their nucleotide sequence is related to that of Ha IFNα-A1 and -A3, they show many termination codons in the reading frame. The A2 and A4 genes both contain IFNα-like promoter sequences, indicating that a duplication from a functional IFNα gene is the most likely event for the origion of A2 and A4. The nucleotide sequence of A4 diverged completely from A1 at the 3'-terminus, which could mean that only a partial duplication or a DNA integration has taken place.

(3) Ha IFN-αs lack the N-glycosylation site present in most Mu IFN-αs. A similar glycosylation site is found in the Hu and Mu IFN-β proteins, but not in Hu IFN-αs (Higashi et al., 1983; Pestka, 1983). The function of this glycosylation is not understood, but it is not necessary for the biological activity of Mu IFN-αs as demonstrated by the antiviral properties of the Mu IFN-α6 protein.

(4) Presently, we are in the process of constructing mutants, with the objective of making the Mu IFN-α4 protein active on hamster cells. In this way we hope to find out more about the structural requirements of the Ha and Mu IFNα subspecies, important in activity on hamster cells. Similarly, A1-A3 hybrid constructs and A3-MuIFNαs can be prepared for the analysis of the important region for activity on mouse cells.

(5) CHO cells are an excellent system to study the regulation of expression of IFN induced genes (Bluyssen et al., 1994). The availability of A1 and A3 IFN make it possible to investigate IFN-regulated promoters in a homologous system.

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REFERENCES

Au, W.-C., Su, Y., Babu, N., Raj., K., and Petha, P. Virus-mediated induction of interferon A gene requires cooperation between multiple binding factors in the interferon α promoter region. J. Biol. Chem. 268 (1993) 24032-24040.

Adolf, G.R., Fruhbeis, B., Hauptmann, R., Kalsner, I., Maurer-Fogy, I., Ostermann, E., Patzelt, E., Schwendenwein, R., Sommergruber, W., and Zophel A. Human interferon W1: isolation of the gene, expression in chinese hamster ovary cells, and characterization of the recombinant protein. Biochim. Biophys. Acta. 1089 (1991) 167-174.

Bisat, F., Raj, N.B.K., and Pitha, P.M. Differential and cell type specific expression of murine alpha-interferon genes is regulated on the transcriptional level. Nucleic Acids Res. 16, (1988) 6067-6083.

Bluyssen, J.A.R., Vlietstra, R.J., van der Made, A.C.J., and Trapman, J. The ISG-54K promoter contains two adjacent, functional ISREs of different strength, which act synergistically for maximal IFN-α inducibility. Eur. J. Biochem. 220 (1994) 395-402.

Capon, D.J., Shepard, H.M., and Goeddel, D.V. Two distinct families of human and bovine interferon-α genes are coordinately expressed and encode functional polypeptides. Mol. Cell. Biol. 5 (1985) 768-779.

Chen, C., and Okyama, H. High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7 (1987) 2745-2752.

Civas, A., Dion, M., Vodjdani, G., and Doly, J. Repression of the murine interferon α11 gene: identification of negatively acting sequences. Nucleic Acids Res. 19 (1991) 4497-4502.

Coulombel, C., Vodjdani, G., and Doly, J. Isolation and characterization of a novel interferon-α-encoding gene, IFN-α11, within a murine IFN cluster. Gene 104 (1991) 187-195.

Dion, M., Vodjdani, G., and Doly, J. Sequence and expression of a novel murine interferon alpha gene. Homology with enhancer elements in the regulatory region of the gene. Biochem. Biophys. Res. Commun. 138 (1986) 826-834.

Daugherty, B., Martin-Zanca, D., Helder, B., Collier, K., Seamans, T.C., Hotta, K., and Pestka, S. Isolation and bacterial expression of a murine alpha leukocyte interferon gene. J. Interferon Res. 4 (1984) 635-643.

Diaz, M.O., Pomykala, H., Bohlander, S., Maltepe, E., and Olopade, O. A complete physical map of the type I interferon gene cluster. J. Interferon Res. 11 (1991) suppl 1:S85.

Diaz, M.O. Nomenclature of the human interferon genes. J. Interferon Res. 13 (1993) 61-62.

Eichmann, E., Majarov, V.A., Kozhich, A.T., Noll, F., and Zav'yalov, V.P. Biological activities of synthetic peptides of the sequence of human interferon-alpha. Immunol. Letters 24 (1990) 233-236.

Feinstein, S.I., Mory, Y., Chernajovsky, Y., Maroteaux, L., Nir, U., Lavie, V., and Revel, M. Family of human α-interferon-like sequences. Mol. Cell. Biol. 5 (1985) 510-517.

Fish, E.N. Definition of receptor binding domains in interferon-α. J. Interferon Res. 12 (1992) 257-266.

Hauptmann, R., and Swetly, P.A. A novel class of human type I interferons. Nucleic Acids Res. 13 (1985) 4739-4749.

Henco, K., Brosius, F.J., Fujisawa, J.I., Haynes, J.R., Hochstadt, J., Kovacic, T., Pasek, M., Schambeck, A., Schmid, J., Todokoro, K., Walchli, M., Nagata, S., and Weissmann, C. Structural relationship of human interferon alpha genes and pseudogenes. J. Mol. Biol. 185 (1985) 227-260.

Van Heuvel, M., Bosveld, I.J., Mooren, A.T.A., Trapmam, J., and Zwarthoff, E.C. Properties of natural and hybrid murine alpha interferons. J. Gen. Virol. 67 (1986) 2215-2222.

Van Heuvel, M., Bosveld, I.J., Klaassen, P., Zwarthoff, E.C., and Trapman, J. Structure-function analysis of murine interferon-α: Antiviral properties of novel hybrid interferons. J. Interferon Res. 8 (1988) 5-14.

Higashi, Y., Sokawa, Y., Watanabe, Y., Kawadė, Y., Ohno, S., Takaoka, C., and Taniguchi, T. Structure and expression of a cloned cDNA for mouse interferon-beta. J. Biol. Chem. 258 (1983) 9522-9529.

Kelley, K.A., Kozak, C.A., Dandoy, F., Sor, F., Windass, J.D., DeMaeyer-Guignard, J., Pitha, P.M.,

and DeMaeyer, E. Mapping of murine interferon-α genes to chromosome 4. Gene 26 (1983) 181-188.
Kelley, K.A., and Pitha, P.M. Characterization of a mouse interferon locus, I. Isolation of a cluster of four α interferon genes. Nucleic Acids Res. 13 (1985) 805-823.
Korput, J.A.G.M., van der, Hilkens, J., Kroezen, V., Zwarthoff, E.C., and Trapman, J. Mouse interferon alpha and beta genes are linked at the centromere proximal region of chromosome 4. J. Gen. Virol. 66 (1985) 493-502.
Lovett, M., Cox, D.R., Yee, D., Boll, W., Weissmann, C., Epstein, C.J., and Epstein, L.B. The chromosomal location of mouse interferon-α genes. EMBO J. 3 (1984) 1643-1646.
De Maeyer, E.M., and De Maeyer-Guignard, J. Interferons and other Regulatory Cytokines. Wiley (Interscience), (1988) New York.
McMullen, G.L., Sattayasai, N., Hibbs, A.R., and Marzuki, S. Antipeptide antibodies against conserved regions of human interferon-α: Evidence for conformational variations between IFN-α subtypes. Biochem Int. 21 (1990) 261-269.
Naf, D., Hardin, S.E., and Weissmann, C. Multimerization of AAGTGA and GAAAGT generates sequences that mediate virus inducibility by mimicking an interferon promoter element. Proc. Natl. Acad. Sci. USA 88 (1991) 1369-1373.
Nagata, S., Mantei, N., and Weissmann, C. The structure of one of the eight or more distinct chromosomal genes for human interferon-α. Nature 287 (1980) 401-408.
Navarro, S., Dion., M., Vodjdani, G., Berlot-Picard, F., and Doly, J. Isolation and characterization of a functional murine interferon alpha gene which is not expressed in fibroblasts upon virus induction. J. Gen. Virol. 70 (1989) 1831-1839.
Nisbet, I.T., Beilharz, M.W., Hertzog, P.J., Tymms, M.J., and Linnane, A.W. Single amino acid substitutions at conserved residues of human interferon-α can effect antiviral specific activity. Biochem Int. 11 (1985) 301-309.
O'Brein, S.J., and Greaves, J.A.M. Report of the committee of comparitive gene mapping. Cytogen. Cell. Genet. 58 (1991) 1124-1151.
Olopade, O., Jenkins, R.B., Ransom D.T., Malik, K., Pomykala, H., Nobori, T., Cowan, J.M., Rowley, J.D., and Diaz, M.O. Molecular analysis of deletions of the short arm of chromosome 9 in human gliomas. Cancer Res. 52 (1992) 2523-2529.
Pestka, S. The human interferons -from protein purification and sequence to cloning and expression in bacteria: before, between, and beyond. Archiv. Biochem. Biophys. 221 (1983) 1-37.
Pestka, S., Langer, K.A., Zoon, K.C., and Samuel, C.E. Interferons and their actions. Annu. Rev. Biochem. 56 (1987) 727-777.
Ragg, H., and Weissmann, C. Not more than 117 base pairs of 5'-flanking sequence are required for inducible expression of a human IFN-α gene. Nature 303 (1983) 439-442.
Raj, N.B.K., Au, W.-C., and Pitha, P.M. Identification of a novel virus-responsive sequence in the promoter of murine interferon-α genes. J. Biol. Chem. 266 (1991) 11360-11365.
Roscouet, D., le, Vodjdani, G., Lemaigre-Dubreuil, Y., Tovey, M.G., Latta, M., and Doly, J. Structure of a murine alpha interferon pseudogene with a repetitive R-type sequence in the 3' flanking region. Mol. Cell. Biol. 5 (1985) 1343-1348.
Ryals, J., Dierks, P., Ragg, H., and Weissmann, C. A 46-nucleotide promoter segment from an IFN-α gene renders an unrelated promoter inducible to virus. Cell 41 (1985) 497-507.
Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
Sanger, F., Nicklsen, S., and Coulson, A.R. DNA sequencing with chainterminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467.
Seif, I., and De Maeyer-Guignard, J. Structure and expression of a new murine interferon-alpha gene: MuIFN-α9. Gene 43 (1986) 111-121.
Sen, G.C., and Lengyel, P. The interferon system. J. Biol. Chem. 267 (1992) 5017-5020.
Senda, T., Shimazu, T., Matsuda, S., Kawano, G., Shimizu, H., Nakamura, K.T., and Mitsui, Y. Three-dimensional crystal structure of recombinant murine interferon-β. EMBO J. 11 (1992) 3193-3201.
Shaw, G.D., Boll, W., Taira, H., Mantei, N., Lengyel, P., and Weissmann, C. Structure and expression of cloned murine INF-A genes. Nucleic Acids Res. 11 (1983) 555-573.
Trapman, J., van Heuvel, M., de Jonge, P., Bosveld, I.J., Klaassen, P., and Zwarthoff, E.C.

Structure-function analysis of mouse interferon alpha species: MuIFN-α10, a subspecies with low antiviral activity. J. Gen. Virol. 69 (1988) 67-75.
Uze, G., Ltfalla, G., Bandu, M.T., Proudhon, D., and Mogensen, K.E. Behaviour of a cloned murine interferon alpha/beta receptor expressed in homospecific or heterospecific background. Proc. Natl. Acad. Sci. USA 89 (1992) 4774-4778.
Waine, G.J., Tymms, M.J., Brandt, E.R., Cheetham, B.F., and Linnane, A.W. Structure-function study of the region encompassing residues 26-40 of human interferon-α4: Identification of residues important for antiviral and antiproliferative activities. J. Interferon Res. 12 (1992) 43-48.
Weck, P.K., Apperson, S., Stebbing, N., Gray, P.W., Leung, D., Shepard, H.M., and Goeddel, D.V. Antiviral activities of hybrids of two major human leucocyte interferons. Nucleic Acids Res. 9 (1981) 6153-6166.
Weidle, U., and Weissmann, C. The 5'-flanking region of a human IFN-α gene mediates viral induction of transcription. Nature 303 (1983) 442-446.
Weissmann, C., and Weber, H. The interferon genes. in: Progress Nucl. Acid Res. Mol. Biol. 33 (1986) 251-300.
Wetzel, R. Assignment of the disulphide bonds of leukocyte interferon. Nature 289 (1981) 606-607.
Zwarthoff, E.C., Mooren, A.T.A., and Trapman, J. Organization, structure and expression of murine interferon alpha genes. Nucleic Acids Res. 13 (1985) 791-804.

CHAPTER 3

THE INTERFERON STIMULATED GENE 54K PROMOTER CONTAINS TWO ADJACENT, FUNCTIONAL INTERFERON STIMULATED RESPONSE ELEMENTS OF DIFFERENT STRENGTH, WHICH ACT SYNERGISTICALLY FOR MAXIMAL IFN-α INDUCIBILITY

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SUMMARY

The interferon-α (IFN-α) regulated hamster ISG-54K gene, which is activated in hamster CHO-12 cells at least 40-fold, was isolated and the promoter region was characterized in detail. Sequence analysis revealed the presence of two elements, closely related to the Interferon Stimulated Response Element (ISRE) consensus sequence [AGTTTCNNTTTC(C/T)]. The putative ISRE-I sequence (GGTTTCAATTTCT) is located at position -97 to -85; ISRE-II (AGTTTTACTTTCT), which differs at three positions from ISRE-I, is found directly upstream of ISRE-I at position -110 to -98. In a transient transfection assay in CHO-12 cells the wildtype hamster ISG-54K-promoter-CAT reporter construct showed a 40- to 80-fold induction, offering an excellent model to study the functional properties of the two ISREs. To find out whether both elements were functional in IFN regulation of the promoter, selected point mutations were introduced in the -110/-85 region and in flanking sequences. The (mutated) ISG-54K promoter was linked to the CAT reporter gene and transiently expressed in CHO cells in the absence and presence of MuIFN-α6. Transfections showed that both the -97/-85 (ISRE-I) and the -110/-98 (ISRE-II) segment were needed for optimal IFN induction of the ISG-54K promoter. However, ISRE-I has an approx. seven-fold stronger activity as compared to ISRE-II. Sequential substitution of the three ISRE-I bases, which differ in ISRE-II showed that the T at position -105 causes the lower activity of ISRE-II. Transfection of ISG-54K promoter constructs, in which ISRE-I was replaced by ISRE-II, which generates a promoter with two ISRE-II segments, and vice versa (two ISRE-Is), provided further evidence for a role of both elements in IFN-α induction. Importantly, all data obtained in transfection studies show that the two ISREs cooperate synergistically. The mechanism of synergism is most probably an indirect interaction between transcription factors binding to the ISREs, because an increase in the spacial arrangement of the two ISREs with a complete helical turn or half a turn did not result in a substantial decrease of promoter activity.

INTRODUCTION

Type 1 interferons (IFN-α/β) are a family of cytokines, which induce multiple cellular changes. The major biological responses of cells treated with IFNs are the inhibition of viral replication in these cells and a decrease in cell growth rate. During the last few years several steps of the molecular mechanism of the IFN induced signaling pathway have been elucidated, but many aspects remain unclear [see for recent reviews refs. 1,2].

IFNs interact with cells through specific cell surface receptors. The interaction of IFN-α/β with its proper receptor ultimately results in the induction of the expression of a group of IFN-α/β stimulated genes. IFN-α/β induced gene expression is directly regulated on the level of transcription [3,4]. Transcriptional stimulation in response to IFN treatment is at least partially mediated by preexisting cellular proteins, which become activated in response to the signaling pathway. Recently, it has been shown that a cytoplasmic protein tyrosine kinase (tyk2) is involved in the latter process [5].

In general, directly IFN-α/β stimulated genes isolated thusfar are characterized by the presence of a cis-acting DNA sequence [IFN-stimulated response element (ISRE)] in the promoter region. An ISRE consensus sequence AGTTTCNNTTTC(C/T) has been deduced from elements present in the different IFN-α/β regulated promoters [see ref. 6 for examples]. The ISRE specifically binds at least two trans-acting nuclear factors (or transcription factor complexes) in a manner which correlates with transcriptional activation of the target genes [7,8]. However, the involvement of other factors both in upregulation and downregulation of expression, cannot be excluded [7-13]. One of the best characterized positive factors (ISGF2 or IRF-1[9], M[14], or C1-C2 [15]) is induced upon IFN-α/β treatment, but induction requires protein synthesis [7,16]. In cotransfection experiments, ISGF2(IRF-1) can bind to an ISRE and induce transcription from a target promoter [16-18]. A second transcription factor, ISGF3 (E [14] or C3 [15]) is rapidly induced upon treatment of cells with IFN-α/β, even without protein synthesis [7]. Therefore, ISGF3 probably is the major transcription factor involved in the IFN-α/β signal transduction pathway. In the absence of IFN-α/β, ISGF3 is present in an inactive form in the cytoplasm. ISGF3 is composed of two protein components (ISGF3γ and ISGF3α) [19,20]. ISGF3γ binds to the ISRE; the ISGF3α component (which is composed of three related proteins) seems to be activated by phosphorylation. In this way it directs ISGF3γ to the nucleus, resulting in the formation of a more stable DNA-protein complex at the ISRE [20,21].

Surprisingly little information is available about functional (mutational) analyses

of IFN-induced promoters. This seems to be due to absence of reliable transient transfection systems, which provide a high IFN-induction of the promoter. As presented in this study, we found hamster CHO-12 cells to be perfectly suited for these types of experiments.

Expression of the human ISG-54K gene is known to be stimulated by IFN-α/β [22,23]. To investigate the molecular basis of regulation of ISG-54K expression in a homologous system, the hamster ISG-54K gene was isolated and characterized. Subsequently, we explored a detailed functional characterization of the hamster ISG-54K promoter. Our data show that the ISG-54K promoter contains two functional ISREs of different strength, which act synergistically for maximal IFN-inducibility.

MATERIALS AND METHODS

Growth of cells and IFN treatment. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and antibiotics and subcultered by trypsinization. The supernatant of constitutively MuIFN-α6 producing CHO-12 cells (CHO-12 clone 28, which produces MuIFN-α6, a mouse IFN-α species which is active on both mouse and hamster cells, see refs. [24,25]) was used as the IFN source. MuIFN-α6 was purified by affinity chromatography over an anti-MuIFN-α antibody column. For RNA isolation, CHO-12 cells were grown to 50% confluency and for a further 2, 4, 8, 24 or 48 h in the presence (or continued absence) of MuIFN-α6 (50 IU/ml). CHO-12 cells to be used for transfection experiments were seeded at 5×10^5 cells per 6 cm dish. After overnight transfection, cells were incubated for 24 h in the presence of MuIFN-α6 (50 IU/ml) or in the absence of IFN.

Isolation of genomic clones from a hamster genomic DNA library. The hamster genomic library, in λEMBL3, was a kind gift from C. Troelstra (Erasmus University Rotterdam, Dept. of Cell Biology and Genetics). The library was screened according to standard procedures [26]. A 561 bp EcoRI-EcoRI fragment, derived from exon 2 of the human ISG-54K gene [22], was used as a hybridization probe. After three rounds of plaque purification, DNA was isolated and characterized by restriction mapping and Southern hybridization.

RNA analyses. Total cellular RNA was isolated by the guanidinium thiocyanate method [27]. 20 μg RNA was denatured by glyoxal treatment, separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane [Gene Screen, New

England Nuclear (NEN), Boston, Ma], using the method described by the manufacturer. Filters were hybridized with DNA probes labelled as described [28]. Overnight hybridization at 42° C in 50% formamide and washing of the filters were as described by NEN and Sambrook et al. [26]. Filters were exposed to Kodak X-Omat AR film at -70° C using intensifying screens.

Sequence analysis of the hamster ISG-54K gene. Nucleotide sequences were determined by the dideoxy chain termination method [29], using T7 polymerase (Pharmacia, Uppsala, Sweden).

Construction of plasmids. All plasmid constructs were prepared according to standard methods [26]. The promoterless plasmid pCAT-Basic (Promega, Madison, WI) was used as a basis vector to link the ISG-54K promoter fragment to the CAT reporter gene. For pHISG54-1-CAT, a BamHI-PstI (-429/+31) hamster ISG-54K promoter fragment was subcloned into pTZ19 (BamHI/PstI). The insert was subsequently excised by HindIII digestion, blunt ended with Klenow DNA polymerase, followed by SacI digestion. The fragment was then ligated into pCAT-Basic (SacI, XbaI blunt ended).

In vitro mutagenesis by recombinant PCR. Mutations in the ISRE region (-110/-85) in the ISG-54K promoter were introduced according to the method of Higuchi et al. [30]. Standard amplification conditions were: 30 cycles of denaturation for 1 min at 95 °C, annealing for 2 min at 55 °C, and extension for 2 min at 72 °C. Two outer primers were used (indicated below by 'of' and 'or', respectively: f = forward, r = reverse) and two, overlapping, inner primers (denoted by the name of the corresponding mutant: f = forward and r = reverse), which contained the mutation. The oligonucleotides which were used for generation of the different mutants are listed below. Residues in lowercase indicate differences from the wildtype sequence.

```
or (+42 to +21)    5' TCAGTGCAGGCTGCAGCTCGCT
of (-432 to -408)  5' GCGGATCCCTGCCACGCTATGGAGT
pHISG54-2f         5' ATAAAAAGTGAtcaCAGTTTTACTTT
pHISG54-2r         5' AAAGTAAAACTGtgaTCACTTTTTAT
pHISG54-3f         5' AGTGACATCAGgccTACTTTCTGGT
pHISG54-3r         5' ACCAGAAAGTAggcCTGATGTCACT
pHISG54-4f         5' GACATCAGTTTTACcagCTGGTTTCAATTTC
pHISG54-4r         5' GAAATTGAAACCAGctgGTAAAACTGATGTC
pHISG54-5f         5' TTACTTTCTGGccaCAATTTCTCTT
pHISG54-5r         5' AAGAGAAATTGtggCCAGAAAGTAA
pHISG54-6f         5' CTTTCTGGTTTCAAgagCTCTTCTGTGGGGT
pHISG54-6r         5' ACCCCACAGAAGAGctcTTGAAACCAGAAAG
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pHISG54-7f	5' GACATCAGTTTTACCAGCTGGTTTCAAGAGC
pHISG54-7r	5' GCTCTTGAAACCAGCTGGTAAAACTGATGTC
pHISG54-8f	5' TTCAATTTCTCgagTGTGGGGTCAG
pHISG54-8r	5' CTGACCCCACActcGAGAAATTGAA
pHISG54-9f	5' TTACTTTCTaGTTTtAcTTTCTCTTC
pHISG54-9r	5' GAAGAGAAAgTaAAACtAGAAAGTAA
pHISG54-I-If	5' AGTGACATCgGTTTcAaTTTCTGGTT
pHISG54-I-Ir	5' AACCAGAAAtTgAAACgGATGTCACT
pHISG54-9bf	5' TCTcgagtctagtAGTTTTACTTTCTCTTC
pHISG54-9br	5' ACTagtagactcgAGAAAGTAAAACTGATG
pHISG54-10f	5' TCTaGTTTCAATTTCTCTTCT
pHISG54-10r	5' AGAAGAGAAATTGAAACtAGA
pHISG54-11f	5' TCTGGTTTtAATTTCTCTTCT
pHISG54-11r	5' AGAAGAGAAATTaAAACCAGA
pHISG54-12f	5' TCTGGTTTCAcTTTCTCTTCT
pHISG54-12r	5' AGAAGAGAAAgTGAAACCAGA

The PCR products were digested with BamHI/PstI, followed by insertion into pCAT-Basic according to the procedure which was described for the construction of pHISG54-1-CAT. Construct pHISG54-9a-CAT was made from pHISG54-9b-CAT. Digestion of pHISG54-1-CAT with XhoI and SpeI, followed by self ligation, removes 5 bp. The structure of each mutant was confirmed by DNA sequence analysis.

Transfection procedure and CAT assay. CHO-12 cells were seeded at 5 x 10^5 cells/6 cm dish. The next day, cells were transfected with the appropriate pHISG54-CAT reporter gene construct (2.5 μg pHISG54-CAT) according to the calcium phosphate precipitation method [31]. After overnight incubation with the precipitate, incubation was continued in the absence or the presence of MuIFN-α6 (50 IU/ml) for an additional 24 h. CAT activity in cell lysates was determined according to the butyryl CoA assay [32]. All experiments were carried out at least four times in duplicate with two separate plasmid isolations.

RESULTS

Organization of the hamster ISG-54K gene. To isolate the hamster ISG-54K gene, a human ISG-54K exon 2 specific probe (561 bp EcoRI-EcoRI fragment [22]) was used to screen a hamster genomic library in λEMBL3. A positive clone (HG-1) was selected and characterized for BamHI, EcoRI and HindIII cleavage sites (Figure 1A). Exon 2 specific sequences could be localized in a 5 kb EcoRI fragment. The direction of this gene segment

was determined by sequence analysis following comparison to the human homologue [22]. The position of exon 1 was mapped utilizing parts of the 3.4 kb HindIII-EcoRI and the 3.6 kb HindIII-HindIII fragments as hybridization probes on Northern blots of RNA isolated from MuIFN-α6 treated CHO-12 cells (data not shown).

The single copy hamster ISG-54K gene is composed of two exons, the intron has a size of 3.4 kb and the sequences flanking exon 1 and 2 are according to the consensus splice donor/acceptor rules (Figure 1B). The position of the intron was confirmed by sequencing of a cDNA fragment obtained by PCR with exon 1 and exon 2 specific primers (data not shown). The first exon is very small (81 bp), and provides the 5'-untranslated region and the first five nucleotides of the open reading frame (ORF). The second exon contains the remaining part of the ORF and the 3'-untranslated region. The ORF encodes a protein of 468 amino acids. The protein shows no homology to any other known protein sequence, except for the human ISG-54K protein (62%), and, to a lesser extend, the human ISG-56K protein (38%) [33,34]. A putative polyadenylation signal (AATAAA), which will give rise to a mRNA of the correct size is present at +2568.

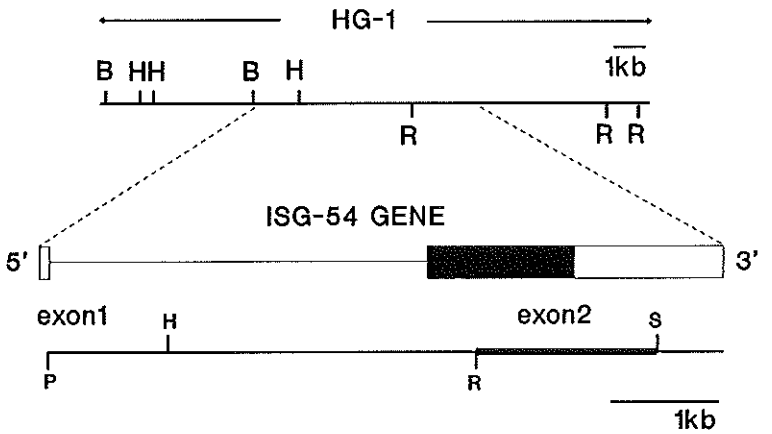

FIGURE 1. Isolation and characterization of the hamster ISG-54K gene. (A) Partial restriction map of the hamster genomic DNA clone HG-1, containing the ISG-54K gene (B:BamHI, R:EcoRI, H:HindIII, P:PstI, S:SacI). Boxed regions indicate the 2 exons, the closed boxes correspond to the open reading frame. The horizontal bar represents the fragment used for hybridisation probe (EcoRI-SacI).

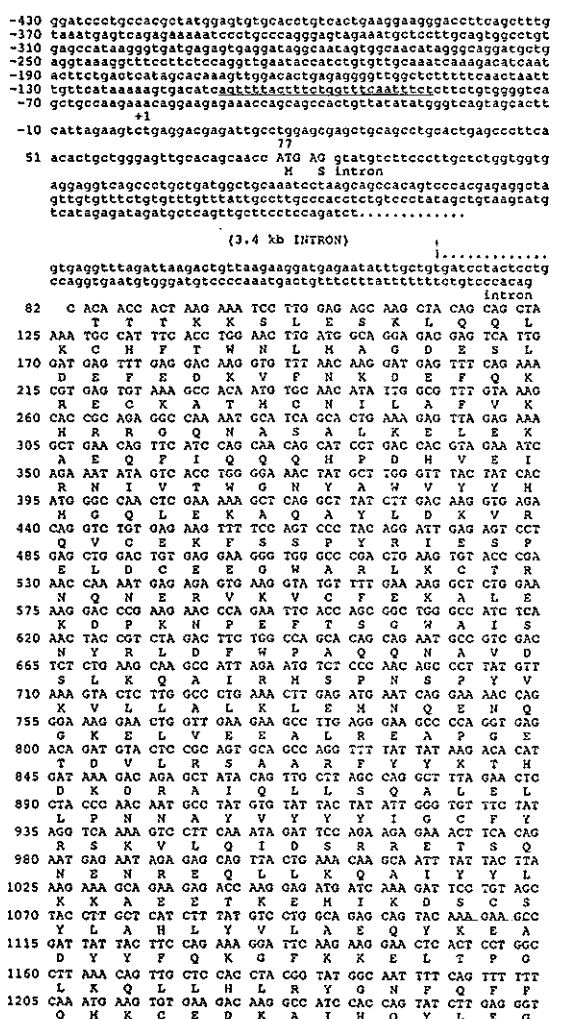

FIGURE 1. Isolation and characterization of the hamster ISG-54K gene. (B) Nucleotide sequence of the hamster ISG-54K gene. The presumed transcription start site is indicated as +1. The two ISRE sequences in the promoter, and the putative polyadenylation signal are underlined. The amino acid sequence of the ISG-54K protein, as deduced from the open reading frame is presented below the nucleotide sequence.

Induction of ISG-54K mRNA expression in hamster CHO-12 cells by MuIFN-α6 treatment. A 1.5 kb EcoRI-SacI probe (Figure 1A) was used to monitor the response of the hamster ISG-54K gene to IFN-α treatment. Figure 2 shows the Northern blot analysis of RNA isolated from IFN-α treated and control CHO-12 cells. The 2.8 kb ISG-54K mRNA, which cannot be detected in the absence of IFN (lane 1; even after overexposure of the autoradiograph, data not shown), is highly induced within 4 h (lane 3) and reaches a maximum level (at least 40-fold induction) approx. 8 h after IFN incubation (lane 4). The mRNA level remains high until at least 48 h postinduction (lane 6).

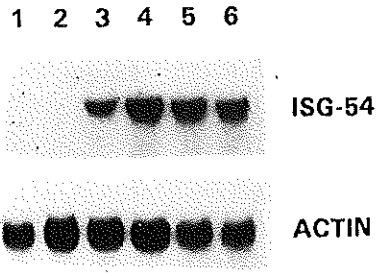

FIGURE 2. Northern blot analysis of IFN-α induced ISG-54K mRNA expression in hamster CHO-12 cells. IFN treatment (50 IU/ml) in CHO-12 cells was for 0, 2, 4, 8, 24, and 48 h (lanes 1-6, respectively). A 1.5 kb EcoRI-SacI fragment, indicated in Figure 1A, was used as a hybridization probe. Hybridization with an actin probe was used as an internal control.

Mutation analysis of the hamster ISG-54K promoter. The presumed transcription start site of the hamster ISG-54K gene is deduced from the corresponding human ISG-54K gene (see Figure 1B and ref. 22). A variant TATA box (TATATA) is found at -32. Figure 3A shows the ISRE consensus sequence AGTTTCNNTTTC(C/T). Comparison of the sequence of the hamster ISG-54K promoter with the ISRE consensus revealed the presence of two structurally closely related segments, one at -97/-85 and a second one, directly upstream of this element at -110/-98 (see Figures 1B and 3A). ISRE-I (-97/-85) deviates at only one position (-97) from the consensus sequence; ISRE-II is different at position -105 (T instead of a highly conserved C). A similar sequence is present in the human ISG-54K promoter (Figure 3A), although previously only the ISRE-I seqence has been recognized as such. In conclusion, structural data suggest that the ISG-54K promoter contains two directly adjacent ISREs.

To test the functional importance of the candidate ISREs, mutations were introduced in the -110/-85 region and in flanking sequences, as indicated in Figure 3B. In

(A)

		ISRE 2	ISRE 1
CONSENSUS ISRE		AGTTTCNYTTTCY	AGTTTCNYTTTCY
ISG-54K	HA	-110 AGTTTTACTTTCT -98	-97 GGTTTCAATTTCT -85
ISG-54K	HU	-112 AATTTCACTTTCT -100	-99 AGTTTCACTTTCC -87

(B)

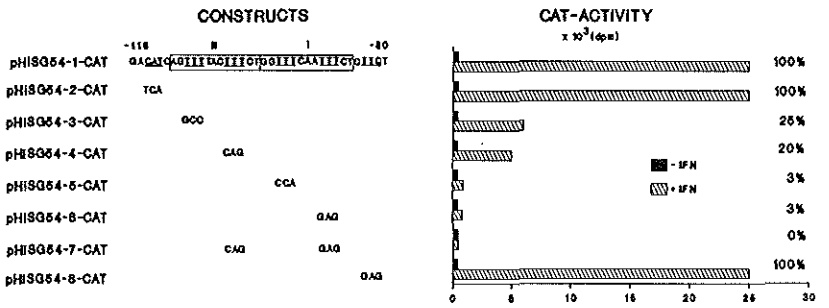

FIGURE 3. Functional analysis of the ISG-54K promoter region. (A) Structural comparison of the ISRE consensus sequence and the hamster and human ISG-54K ISRE regions. Dots indicate identical nucleotides. (N) denotes a random base; (Y) a pyrimidine. (B) Effect of mutation of ISRE and ISRE upstream and downstream sequences on the IFN-α inducibility of the hamster ISG-54K promoter. Mutations are indicated below the wildtype sequence. CHO-12 cells were transfected with wildtype or mutant hamster ISG-54K promoter (BamHI - PstI; -431 to +31) The activity of pHISG54-1-CAT is taken as 100%.

each modified ISRE the three highly conserved adjacent T residues were changed, generating four different "single" mutations (pHISG54-3-CAT to pHISG54-6-CAT in Figure 3B, respectively) and one "double" mutation (pHISG54-7-CAT). Further mutations were made in the regions flanking the ISREs, one 5′ (pHISG54-2-CAT in Figure 3B), the second one 3′ to the ISRE region (pHISG54-8-CAT in Figure 3B). All mutated promoters were used in transient transfection assays in CHO-12 cells. The activities of the mutated promoters were compared with that of the wildtype promoter (pHISG54-1-CAT), which shows a 40- to 80-fold induction upon IFN treatment. The results of a representative experiment are illustrated in Figure 3B. Mutation of the region downstream of the ISRE segment (TTC to GAG at -83/-81) had no effect on the IFN-α response of the ISG-54K promoter (compare the activity of pHISG54-8-CAT and pHISG54-1-CAT). Both mutation of the T triplet at -95/-93 and that at -89/-87 in ISRE-I caused a strong decrease of IFN-α induced promoter

activity, however, the promoter was not completely inactivated. Both pHISG54-5-CAT and pHISG54-6-CAT show a residual activity of 3%. This indicates that ISRE-I alone, although important, is not enough for maximal ISG-54K promoter activity in response to IFN-α. This observation is substantiated by the results obtained with pHISG54-3-CAT and pHISG54-4-CAT, which both disrupt a T-triplet in the ISRE-II region. Interestingly, both mutations show a significant decrease in transcriptional response to IFN-α. Activities of 25% and 20% compared to wildtype were found, respectively. A more upstream mutation (at -114/-112; pHISG54-2-CAT) has no effect on promoter strength. The "double" mutation pHISG54-7-CAT, which has two mutated ISREs shows no response at all to IFN-α, suggesting that the -110/-85 segment determines completely the responsiveness to IFN-α of the ISG-54K promoter.

Properties of the ISRE region. Sequential substitution of the three ISRE-I bases, which differ in ISRE-II, created mutants pHISG54-10-CAT, pHISG54-11-CAT and pHISG54-12-CAT, respectively (Figure 4).

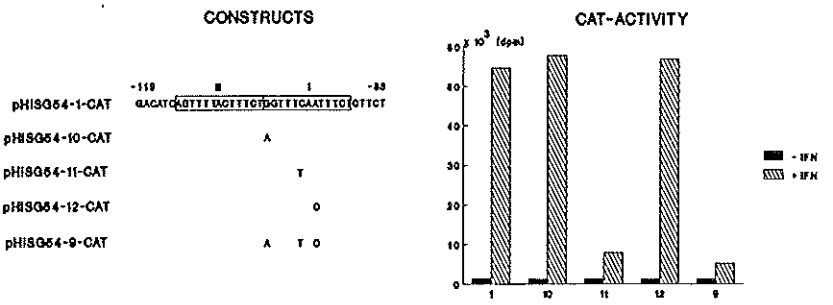

FIGURE 4. Effect of sequential mutation of the three ISRE-I nucleotides, which differ in ISRE-II, on the IFN-α inducibility of the hamster ISG-54K promoter. Mutations are indicated below the wildtype sequence. CHO-12 cells were transfected with wildtype or mutant hamster ISG-54K promoter (BamHI - PstI; -431 to +31) CAT reporter gene constructs as described in Materials and Methods.

When assayed for promoter activity, pHISG54-10-CAT and pHISG54-12-CAT show an activity which is similar to wildtype activity. However, pHISG54-11-CAT has an activity of only 20 % as compared to wildtype, which equals the activity of a promoter that contains two ISRE-II sequences (pHISG54-9-CAT). This indicates that the T at position -105 in ISRE-II is responsible for its lower activity.

The mechanism of ISRE-I and ISRE-II cooperation in ISG-54K promoter activity. Comparison of the hamster ISG-54K wildtype promoter activity (II-I, Figure 5) (100%), containing ISRE-I and ISRE-II, with the activities of a single ISRE-I (I, Figure 5) or ISRE-II (II, Figure 5) (22% and 3%, respectively), indicates synergistic cooperation between ISRE-I and ISRE-II. The same effect is seen if two copies of ISRE-II are present (II-II, Figure 5). Compared to the wildtype promoter, it gives 20% activity. This shows first of all that the combination ISRE-I and ISRE-II functions better then two ISRE-IIs, and, secondly, that two ISRE-IIs are approx. 7 times more active than a single ISRE-II, again pointing to a synergistic cooperation. A promoter with two copies of ISRE-I (I-I, Figure 5), functions slightly better (120%) than the wildtype combination (Figure 5; compare I-I and II-I, respectively), and approx. 5 times better than a single ISRE-I. So, also in this situation a synergistic cooperation between two ISREs is observed.

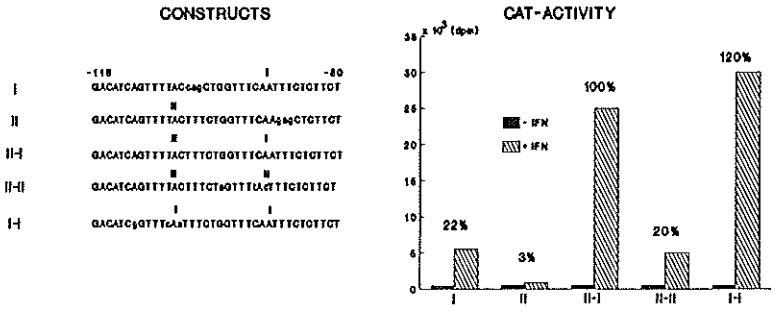

FIGURE 5. The effect of changing the type of ISRE on the IFN-α inducibility of the hamster ISG-54K promoter. Mutations are indicated below the wildtype sequence. CHO-12 cells were transfected with wildtype or mutant hamster ISG-54K promoter (BamHI-PstI; -431 to +31) CAT reporter gene constructs. The activity of pHISG54-1-CAT is taken as 100%.

In the ISG-54K promoter, ISRE-I and ISRE-II are located directly adjacent to each other (Figures 1B and 3A). This led us to investigate the possibility that the synergistic action of the two ISREs could require direct sequence alignment. The effect of varying the spacial arrangement between the ISREs was tested by insertion of two spacer DNAs of different size. Experiments were done with pHISG54-9-CAT, which contains two copies of ISRE-II, as a basis construct. A 5 bp (pHISG54-9a-CAT) and a 10 bp (pHISG54-9b-CAT) insertion mutation were constructed as indicated (see Figure 6 and Materials and Methods). This resulted in the generation of two mutations, in which the two ISREs are

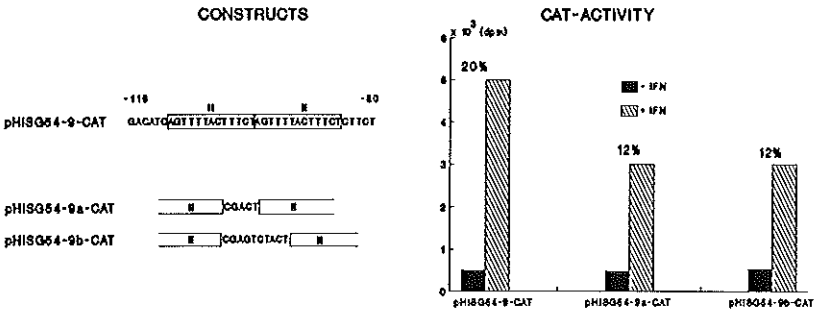

FIGURE 6. Effect of spacing between two ISRE IIs on IFN-α inducibility of the hamster ISG-54K promoter. CHO-12 cells were transfected with wildtype and mutant hamster ISG-54K promoter (BamHI - PstI; -431 to +31) CAT reporter gene constructs. The activity of pHISG54-1-CAT (not indicated in the figure) is taken as 100%.

separated by a half or a complete helical turn, respectively. If transiently expressed in CHO-12 cells in the presence of IFN-α, both pHISG54-9a-CAT and pHISG54-9b-CAT show a slight decrease in activity as compared to the basis construct. However, this small decrease (approx. 40%) does not correspond with a loss of synergistic cooperation between the two ISREs.

DISCUSSION

IFN stimulated genes provide an attractive system to examine in which way transcription regulation in the cell nucleus is mediated through occupation of a cell surface receptor by its polypeptide ligand. IFN stimulated genes respond rapidly to the inductive signal with an increase in transcriptional initiation. Sofar, a dozen directly IFN-α/β regulated target genes has been isolated. These genes contain in the promoter region a sequence closely related to the ISRE consensus sequence AGTTTCNNTTTC(C/T) (see for recent reviews 1,2,6). Although a consensus sequence is established from comparison of target gene promoters, surprisingly little is known about the strength of an ISRE as compared to its sequence. ISRE structure/function analyses have sofar almost exclusively been limited to protein binding studies (7,8). We observed that hamster CHO-12 cells are exceptionally well suited for functional analysis of ISRE sequences in transient transfection assays, using Mu IFN-α6 as inducer.

To study the transcriptional response to IFN-α, we isolated the hamster ISG-54K gene. The hamster ISG-54K promoter contains two functional ISREs [ISRE-I at -97/-85; ISRE-II at -110/-98 (Figures 1B and 3A)]. Previously, ISRE-I has also been identified in the human ISG-54K promoter at a similar position [8]. The hamster and human ISG-54K promoters are highly conserved in the ISRE region. This suggests that the human ISG-54K promoter, also contains two ISREs, which are the major control elements of these promoters. ISRE-I in the human ISG-54K promoter binds ISGF3 and ISGF2, as shown by mutational analysis (8). Functional testing of ISRE-I mutants revealed a slight (50%) decrease in promoter activity.

A detailed analysis of the hamster mutants, as presented in our study, established that both ISRE-I and ISRE-II could independently direct IFN-α regulated transcriptional response, but ISRE-I has an approx. 7-fold higher activity. The T at position -105 in ISRE-II is responsible for the difference in activity between ISRE-I and ISRE-II. The upstream ISRE in the human ISG-54K promoter (human ISRE-II) contains a C at a position comparable to the T in the hamster ISRE-II (Figure 3A), which could render this ISRE more active than ISRE-II in the hamster ISG-54K promoter.

It is of obvious importance to investigate the role of the different ISRE-binding transcription factors in ISG-54K expression. The set of mutants generated in this study together with the hamster CHO-12 transfection system, will form an excellent model to study in detail the interaction of ISREs with purified ISGF2(IRF-1) [9], ISGF3 (components) [34-37] and other factors [10-13, 15]. Co-transfection experiments with expression plasmids encoding the various factors and the different ISRE-CAT reporter gene constructs will provide further information on their role in IFN mediated regulation of gene expression.

The synergistic cooperativity of two ISREs did not strictly depend on the combination of ISREs, but for maximum activity the ISRE doublet had to contain at least one ISRE-I. Variation of the spacial arrangement between two ISRE-IIs did not drastically affect promoter activity. This points to a mechanism of synergism between two ISREs through an indirect interaction between IFN-stimulated transcription factors binding to the different ISREs. It can be hypothesized that, upon IFN-α induction, IFN-stimulated transcription factors, when bound to the two different ISREs, interact simultaneously with the same or different components of the preinitiation complex at the transcriptional start site (Figure 7). Therefore, it would be interesting to study the interaction of the different IFN-α induced factors with general transcription factors and/or presumed co-activators [38].

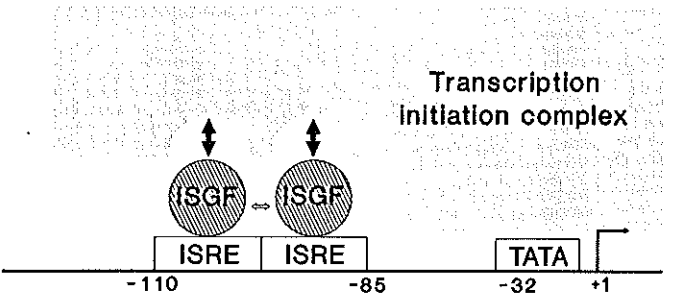

FIGURE 7. Schematical representation of the interaction of ISRE-binding transcription factors (ISGFs) with the transcription initiation complex at the ISG-54K promoter.

Structural comparison reveals that, in addition to the ISG-54K gene, the related ISG-56K gene, and the ISG-15K gene are candidate genes with two adjacent ISREs in the promoter region (ISG-56K: AGTTTCACTTTCCCCTTTCGGTTTCC [23]; ISG-15K: GGCTTCAGTTTCGGTTTCCCTTTCC [39]). ISG-54K, ISG-56K and ISG-15K are not only induced by IFN-α/β, but also directly by virus and poly(I).poly(C) [23,40,41]. This could indicate a common mechanism involved in regulation of these specific genes. Detailed analysis of the ISRE region of these genes further shows striking structural homology to the -96/-64 fragment, including the PRDI and PRDIII region, of the IFN-β promoter (-64 CACTTTCACTTCTCCCTTTCACTTTTCCTAT -96), which is necessary for virus induction of the IFN-β gene [9, 42-45]. Virus induction of the IFN-β promoter is at least partially mediated by ISGF2(IRF-1). Whether it also involves activation of ISGF3 has not been clearly established. So, it is possible that in response to viruses or other cytokines (like IL-1 or TNF) [23,40,45], the ISRE region functions as a PRDI/III like region. In this situation the direct adjacent sequence alignment of the two ISREs could be important to reach maximum induction. Experiments to test this possibility are in progress.

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REFERENCES

1. Williams, B.R.G. (1991) Transcriptional regulation of interferon-stimulated genes. Eur. J. Biochem. 200, 1-11.
2. Sen, G.C., and Lengyel, P. (1992) The interferon system. J. Biol. Chem. 267, 5017-5020.
3. Friedman, R.L., Manly, R.P., Macmahon, M., Kerr, I.M., and Stark, G.R. (1984) Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell 38, 745-755.
4. Larner, A.C., Jonak, G., Cheng, Y.S.E., Korant, B., Knight, E., Jr., and Darnell, J.E., Jr. (1984) Transcriptional induction of two genes in human cells by ß-interferon. Proc. Natl. Acad. Sci. USA 81, 6733-6737.
5. Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon α/ß signalling pathway. Cell 70, 313-322.
6. Pellegrini, S. and Schindler, C. (1993) Early events in signalling by interferons. Trends Biochem. Sci. 18, 338-342.
7. Levy, D.E., Kessler, D.S., Pine, R., Reich, N., and Darnell, J.E., Jr. (1988) Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. Genes Dev. 2, 383-393.
8. Kessler, D.S., Levy, D.E., and Darnell, J.E., Jr. (1988) Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. Proc. Natl. Acad. Sci. USA 85, 8521-8525.
9. Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, T. (1989) Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind the same regulatory elements of IFN and IFN-indicible genes. Cell 58, 729-739.
10. Yan, G., and Tamm, I. (1991) Molecular cloning and characterization of interferon α/ß response element binding factors of the murine (2'-5')oligo adenylate synthetase ME-12 gene. Proc. Natl. Acad. Sci. USA 88, 144-148.
11. Wedrychowski, A., Henzel, W., Huston, L., Paslidis, N., Ellerson, D., McRae, M., Seong, D., Howard, O.M.Z., and Deisseroth, A. (1992) Identification of proteins binding to interferon-inducible transcriptional enhancers in hematopoietic cells. J. Biol. Chem. 267, 4533-4540.
12. Yan, G., and Tamm, I. (1992) Molecular cloning and characterization of additional factors that bind the interferon-α/ß response element B of the murine (2'-5')oligoadenylate synthetase ME-12 gene. Proc. Natl. Acad. Sci. USA 89, 8859-8863.
13. Nelson, N., Marks, M.S., Driggers, P.H., and Ozato, K. (1993) Interferon consensus sequence-binding protein, a member of the interferon regulatory factor family, supresses interferon-induced gene transcription. Mol. Cell. Biol. 13, 588-599.
14. Dale, T.C., Rosen, J.F., Guille, M.J., Lewin, A.R., Porter, A.G.C., Kerr, I.M., and Stark, G.R. (1989) Overlapping sites for constitutive and induced DNA binding factors involved in interferon-stimulated transcription. EMBO J. 8, 831-839.
15. Porter, A.C., Chernajovsky, Y., Dale, T.C., Gilbert, C.S., Stark, G.R., and Kerr, I.M. (1988) Interferon response element of the human gene 6-16. EMBO J. 7, 85-92.
16. Pine, R., Decker, T., Kessler, D.S., Levy, D.E., and Darnell, J.E., Jr. (1990) Purification and cloning of interferon-stimulated gene factor 2 (ISGF2): ISGF2 (IRF-1) can bind to the promoters of both beta interferon- and interferon- stimulated genes but is not a primary transcriptional activator of either. Mol. Cell. Biol. 10, 2448-2457.
17. Pine R. (1992) Constitutive expression of an ISGF2/IRF1 transgene leads to interferon-independent activation of interferon-inducible genes and resistance to virus infection. J. Virol. 66, 4470-4478.
18. Reis, L.F.L., Harada, H., Wolchok, J.D., Taniguchi, T., and Vilcek, J. (1992) Critical role of a common transcription factor, IRF-1, in the regulation of IFN-beta and IFN-inducible genes. EMBO J. 11, 185-193.
19. Levy, D.E., Kessler, D.S., Pine, R., and Darnell, J.E., Jr. (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-α-stimulated transcription, reconstituted in vitro. Genes Dev. 3, 1362-1371.
20. Kessler, D.S., Veals, S.A., Fu, X.-Y., and Levy, D.E. (1990) IFN-alpha regulates nuclear

translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. Genes Dev. 4, 1753-1765.
21. Schindler, C., Shuai, K., Prezioso, V.R., and Darnell, J.E., Jr. (1992) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science **257**, 809-812.
22. Levy, D.E., Larner, A.C., Chaudhuri, A., Babiss, L.E., and Darnell, J.E., Jr. (1986) Interferon-stimulated transcription: isolation of an inducible gene and identification of its regulatory region. Proc. Natl. Acad. Sci. USA **83**, 8929-8933.
23. Wathelet, M.G., Clauss, I.M., Nols, C.B., Content, J., and Huez, G.A. (1987) New inducers revealed by the promoter sequence analysis of two interferon-activated human genes. Eur. J. Biochem. **169**, 313-321.
24. Heuvel, M. van, Bosveld, I.J., Mooren, A.T.A., Trapman, J., and Zwarthoff, E.C. (1986) Properties of natural and hybrid Murine Alpha Interferons. J. Gen. Virol. **67**, 2215-2222.
25. Heuvel, M. van, Govaert-Siemerink, M., Bosveld, I.J., Zwarthoff, E.C., and Trapman, J. (1988) IFN producing CHO cell lines are resistant to the antiproliverative activity of IFN: A correlation with gene expression. J. Cell. Biochem. **38**, 269-278.
26. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
27. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**, 5294-5299.
28. Feinberg, A.P., and Vogelstein, P. (1983) A technique for radiolabeling DNA restriction endinuclease fragments to high specific activity. Anal. Biochem. **132**, 6-13.
29. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463-5467.
30. Higuchi, R., Kruvuel, B., and Saiki, R. (1989) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucl. Acids Res. **16**, 7351-7367.
31. Chen, C., and Okyama, H. (1987) High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**, 2745-2752.
32. Seed, B., Sheen, J-Y. (1988) A simple phase-extraction assay for chloramphenicol acetyltransferase activity. Gene **67**, 271-277.
33. Wathelet, M., Moutschen, S., Defilippi, P., Cravador, A., Collet, M., Huez, G., and Content, J. (1986) Molecular cloning, full length sequence and preliminary characterization of a 56-kDa protein induced by interferons. Eur. J. Biochem. **155**, 11-17.
34. Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988) The IFI-56K and IFI-54K interferon-inducible human genes belong to the same gene family. FEBS Letters **231**, 164-171.
35. Fu, X.-Y., Schindler, C., Improta, T., Aebersold, R., and Darnell, J.E., Jr. (1992) The proteins of ISGF-3, the interferon α-induced transcriptional activator, define a gene family involved in signal transduction. Proc. Natl. Acad. Sci. USA **89**, 7840-7843.
36. Schindler, C., Fu, X.-Y., Improta, T., Aebersold, R., and Darnell, J.E., Jr. (1992) Proteins of transcription factor ISGF-3: One gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon α. Proc. Natl. Acad. Sci. USA **89**, 7836-7839.
37. Veals, S.A., Schindler, C., Leonard, D., Fu, X.-Y., Aebersold, R., Darnell, J.E., Jr., and Levy, D.E. (1992) Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and myb families of DNA-binding proteins. Mol. Cell. Biol. **12**, 3315-3324.
38. Pugh, B.F., and Tjian, R. (1992) Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. J. Biol. Chem. **267**, 679-682.
39. Reich, N., Evans, B., Levy, D.E., Fahey, D., Knight, E., Jr and Darnell, J.E., Jr. (1987) Interferon-induced transcription of a gene encoding a 15 kDa protein depends on an upstream enhancer element. Proc. Natl. Acad. Sci. USA **84**, 6394-6398.
40. Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988) Regulation of two interferon-inducible human genes by interferon, poly(ri).poly(rC) and virus. Eur. J. Biochem. **174**, 323-329.

41. Reich, N., Pine, R., Levy, D.E, and Darnell, J.E., Jr. (1988) Tanscription of interferon-stimulated genes is induced by adenovirus particles but is suppressed by E1A gene products. J. Virology 62, 114-119.
42. Fujita, T., Shibuya, H., Hotta, H., Yamanishi, K., and Taniguchi, T. (1987) Interferon-β gene regulation: Tandemly repeated sequences of a synthetic 6 bp oligomer function as a virus-inducible enhancer. Cell 49, 357-367.
43. Goodbourn, S., and Maniatis, T. (1988) Overlapping positive and negative regulatory domains of the human ß-interferon gene. Proc. Natl. Acad. Sci. USA 85, 1447-1451.
44. Fan, C.M., and Maniatis, T. (1989) Two different virus-inducible elements are required for human ß-IFN gene regulation. EMBO J. 6, 101-110.
45. LeBlanc, J.-F., Cohen, L., Rodrigues, M., and Hiscott, J. (1990) Synergism between distinct enhanson domains in viral induction of the human beta interferon gene. Mol. Cell. Biol. 10, 3987-3993.

CHAPTER 4

STRUCTURE, CHROMOSOME LOCALIZATION AND REGULATION OF EXPRESSION OF THE INTERFERON-REGULATED MOUSE ISG-54K/ISG-56K GENE FAMILY

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SUMMARY

The interferon-α (IFN-α) regulated mouse ISG-54K/-56K gene family, which is composed of at least four members (ISG-54K, ISG-56K1, K2 and K3), was isolated and characterized. In addition, the chromosomal localization of the four genes was determined. The ISG-54K and ISG-56K1 genes show an identical organization. Both are composed of a very small first exon and a second exon, which contains the complete open reading frame, except for the ATG start codon and the first two nucleotides of the second codon. In both genes, the two exons are separated by a small intron (5 kb and 2.5 kb, respectively). Expression of both genes is rapidly induced by IFN-α (within 2 h). The ISG-54K promoter region contains two sequences, which are closely related to the Interferon Stimulated response Element (ISRE) consensus sequence (ISRE-I: GGTTTCAATTTCT, and ISRE-II: AGTGTTACTTTCT). The two elements are located directly adjacent to each other. A similar organization was recently established for the hamster ISG-54K promoter (Bluyssen et al., 1994). However, the mouse promoter is 70% less active than the hamster promoter. It turned out that ISRE-II is hardly active, due to the G at position 4, which is a T in the hamster ISG-54K ISRE 2 and in the ISRE consensus sequence. The ISG-56K1 promoter region contains at a similar position two functional ISREs of identical strength (ISRE-I: AGTTTCAGTTTCT, and ISRE-II: AGTTTCACTTTCC). In the ISG-56K1 promoter, the two ISRE motifs are separated by six bp. In addition to the ISG-56K1 gene, (parts) of two closely related genes (56K2 and 56K3) were isolated. Both fragments contain an ISG-56K1-related open reading frame. However, we were unable to isolate the presumed first exon of 56K2 and 56K3, neither could we show expression of the genes, sofar. The ISG-54K, -56K1, -K2 and -K3 genes could all be assigned to the D1 region of mouse chromosome 19, suggesting a tight clustering.

INTRODUCTION

Type I interferons (IFN-α/β) are cytokines that exert many effects on a wide variety of target cells. These include the establishment of an antiviral state, inhibition of cell growth, and modulation of the immune system (see for reviews De Maeyer and De Maeyer-Guignard, 1988; Williams, 1991; Sen and Lengyel, 1992; Stark and Kerr, 1992).

Type I IFNs interact with a specific cell surface receptor to trigger its biological response (Uze et al., 1990). Recently, the signal transduction mechanism has been

elucidated to a large extend. Upon binding of the ligand, the IFN receptor activates at least two cytoplasmic tyrosine kinases (JAK1 and Tyk2) (Velazquez et al., 1992; Muller et al., 1993; Shuai et al., 1993; Silvenoinnen et al., 1993). This leads to the phosphorylation of the structurally related transcription factors STAT p113 and p91/p84 (Fu et al., 1992a; Fu et al., 1992b; Schindler et al., 1992a; Schindler et al., 1992b). Together with the p48 DNA-binding subunit (Levy et al., 1989) they form the multiprotein complex ISGF3, which interacts with specific sequences in the DNA (Dale et al., 1989; Levy et al., 1989; Kessler et al., 1990). This subsequently leads to the induction of expression of a group of cellular genes (for review see Pellegrini et al., 1993); the products of these IFN-stimulated genes (ISGs) either singly or coordinately mediate the biological activities of IFNs.

Functional and structural analyses of the promoter of IFN-α/β target genes resulted in the identification of a minimal response element needed for IFN-induction (referred to as Interferon Stimulated Response Element or ISRE; consensus sequence AGTTTCNNTTTCY) (Levy et al., 1986; Reich et al., 1987; Cohen et al., 1988; Porter et al., 1988; Pellegrini and Schindler, 1993).

Sofar, a dozen IFN-regulated genes have been identified (Pellegrini and Schindler, 1993). Among these are the human (Larner et al., 1984; Levy et al., 1986) and hamster ISG-54K gene (Bluyssen et al., 1994) and the human ISG-56K gene (Larner et al., 1984; Wathelet et al., 1986), which are structurally related, indicating that they are derivatives from a common ancestor. The function of these genes is unknown. Both the ISG-54K and ISG-56K gene map to human chromosome 10 (10q23-q24) (Lafage et al., 1992). To be able to investigate in more detail the function of the ISG-54K and ISG-56K genes, and analyze the regulation of their in vivo expression, we initiated the analysis of the mouse homologs. In this study we present the structure, chromosomal localization and regulation of expression of the mouse ISG-54K/56K gene family.

MATERIALS AND METHODS

Growth of cells and IFN treatment. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and antibiotics. The supernatant of constitutively mouse IFN producing CHO cells [CHO-12 clone 28, which produces MuIFN-α6 was used as the IFN source (van Heuvel et al., 1988). MuIFN-α6, which is active on both hamster and mouse cells (van Heuvel et al., 1986), was purified by affinity chromatography over a MuIFN-α specific antibody column.

For RNA isolation, L929 cells were grown to 50% confluency and for a further 2,

8 and 24 h in the presence (or continued absence) of MuIFN-α6 (50 IU/ml). For transfection experiments, CHO cells were seeded at 5 x 10^5 cells per 6 cm dish. After overnight transfection, cells were incubated for 24 h in the presence of MuIFN-α6 (50 IU/ml) or in the absence of IFN.

Isolation of ISG-54K/56K genes from a mouse genomic DNA library. The mouse genomic library, in λEMBL3 SP6/T7, was purchased from Clontech (Palo Alto, CA). The library was screened according to standard procedures (Sambrook et al., 1989). A 1.5 kb EcoRI-SacI fragment, derived from exon 2 of the hamster ISG-54K gene (Bluyssen et al., 1994), was used as a hybridization probe in order to screen for the mouse ISG-54K gene. To identify phages containing the mouse ISG-56K genes, a 380 bp fragment, obtained by amplification on mouse L929 genomic DNA using specific (degenerate) primers (5'-AGTGATCAGGGGCAACTTTGCTTGG-3' and 5'-TGTTCGAAGTCCCGTTCCTC(C/T)TG(A/G)AA-3'; see also Figure 2B), was used as a hybridization probe. After three rounds of plaque purification, DNA was isolated and characterized by restriction mapping and Southern hybridization.

DNA and RNA analyses. Genomic DNA (20 μg), isolated from L929 cells, was digested with the proper restriction enzymes and the fragments were separated over a 0.8% agarose gel. Southern blotting and hybridization were essentially as described (Sambrook et al., 1989).

Total cellular RNA was isolated by the guanidinium thiocyanate method (Chirgwin et al., 1979). 20 μg RNA was denatured by glyoxal treatment, separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (GeneScreen, New England Nuclear (NEN), Boston, Ma), using the method described by the supplier. Filters were hybridized with DNA probes labelled as described (Feinberg and Vogelstein, 1983). Overnight hybridization at 42°C in 50% formamide and washing of the filters were as described by NEN and Sambrook et al. (1989). Filters were exposed to Kodak X-Omat AR film at -70°C using intensifying screens.

S1-nuclease-protection assay, RACE and RT-PCR. For the S1-nuclease protection assay, a 330 bp EcoRI-RcaI ISG-54K and a 320 bp BamHI-NcoI ISG-56K DNA fragment (see Figures 2A and 2B, respectively) were used as a probe. The fragments were end-labeled with T4 polynucleotide kinase (Gibco BRL, Grand Island, NY), and approx. 100.000 cpm radiolabelled fragment were annealed overnight at 55°C to 40 ug L929 RNA in 80% formamide/40 mM-Pipes (pH 6.5), 0.4 M NaCl, 1 mM EDTA (Favoloro et al., 1980). S1-nuclease (Boehringer, Mannheim, Germany) digestions were carried out for 1 h at 37°C, and the resulting protected fragments were separated on a 6% denaturing polyacrylamide

gel.

To obtain ISG-56K exon 1 sequences, we used the RACE (Rapid Amplification of cDNA Ends) protocol according to Frohman et al. (1988). Primer 5'-CTTCTCTTGCTCAGCTGCTCGCT-3' (conserved between ISG-56K1, -2, and -3) was applied to synthesize first strand cDNA on RNA isolated from IFN-α treated mouse L929 cells. For RT-PCR (reverse-transcriptase - polymerase chain reaction) of ISG-54K2 and ISG-54K3, primer 5'ATGACAGCCTGGATG-3' (conserved between ISG-56K2 and ISG-56K3) was used in combination with the above mentioned RT-primer on L929 cDNA. PCR conditions: 30 cycles of 1 min 94°C, 1 min 55°C and 2 min 72°C.

Sequence analysis of the mouse ISG-54K and ISG-56K genes. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1974), using T7 polymerase (Pharmacia, Uppsala, Sweden).

Construction of plasmids. All plasmid constructs were prepared according to standard methods (Sambrook et al., 1989). The promoterless plasmid pCAT-Basic (Promega, Madison, WI) was used as a basis vector to link a ISG-54K or ISG-56K promoter fragment to the CAT reporter gene. For pMISG54-1-CAT, an EcoRI-PstI (-252/+32) mouse ISG-54K promoter fragment, with a blunt ended EcoRI site, was subcloned into pTZ19 (SmaI/PstI). The insert was subsequently excised by HindIII digestion (the HindIII site was blunted with Klenow DNA polymerase), followed by SacI digestion. Next, the fragment was ligated into the SacI and blunt ended XbaI sites of pCAT-Basic. To construct the pMISG56-1-CAT plasmid, a BamHI-HindIII (-223/+299) mouse ISG-56K1 promoter fragment was subcloned into pGEM7 (BamHI-HindIII). Using this construct as template DNA in PCR, the m13 forward and reverse primers were applied to amplify the ISG-56K1 promoter fragment. This PCR product was subsequently digested with HinfI, blunted, followed by SacI digestion. The fragment was then ligated into pCAT-Basic (SacI/XbaI blunt ended).

In vitro mutagenesis. Mutations in the ISRE region (-110/-85 in the ISG-54K promoter; -100/-69 in the ISG-56K1 promoter) were introduced according to the method of Higuchi et al. (1989). Standard conditions were: 30 cycles of denaturation for 1 min at 95 °C, annealing for 2 min at 55 °C, and extension for 2 min at 72 °C. Two outer primers were used (indicated below by 'of' and 'or', respectively: f = forward, r = reverse) and two, overlapping inner primers (denoted by the name of the corresponding mutant: f = forward and r = reverse), with the mutation. The oligonucleotides which were used for generation of the different mutants are listed below. Residues in lower case indicate differences from the wildtype sequence.

ISG-54K:		
of	5' GAGCTCGGTACCGAATTCAG	3'
or	5' TCAGGAGCTAAGGAAGCTAA	3'
pMISG54-2f	5' GTGACATACGTtTTACTTTCTGG	3'
pMISG54-2r	5' CCAGAAAGTAAaACTGATGTCAC	3'
ISG-56K:		
of	m13 forward primer	
or	m13 reverse primer	
pMISG56-2f	5' CTTCAGTTTCACggaCCAGTCTCAGTTTCA	3'
pMISG56-2r	5' TGAAACTGAGACTGGtccGTGAAACTGAAG	3'
pMISG56-3f	5' TTTCACTTTCCAGgacCAGTTTCAGTTTCT	3'
pMISG56-3r	5' AGAAACTGAAACTGgtcCTGGAAAGTGAAA	3'
pMISG56-4f	5' TCTCAGTTTCAGgacCTCACTGCTGAC	3'
pMISG56-4r	5' GTCAGCAGTGAGgtcCTGAAACTGAGA	3'

The amplified ISG-54K fragment was digested with SacI and PstI, and subcloned into pTZ19 SacI/PstI, followed by insertion into pCAT-Basic according to the procedure which was described for the construction of pMISG54-1-CAT. The ISG-56K1 PCR products were digested with HinfI, which was blunt ended with Klenow DNA polymerase, and SacI, following inserting in pCAT-Basic by the procedure which was described for the construction of pMISG56-1-CAT. The structure of each mutant was confirmed by sequencing.

Transfection procedure and CAT assay. CHO cells were seeded at 5 x 10^5 cells/6 cm dish. The next day, cells were transfected with the appropriate promoter-CAT reporter gene construct (5 μg) according to the calcium phosphate precipitation method (Chen and Okyama, 1987). After overnight incubation with the precipitate, incubation was continued in the absence or the presence of MuIFN-α6 (50 IU/ml) for an additional 24 h. Subsequently cells were harvested, CAT activity in cell lysates was determined according to the butyryl CoA assay (Seed and Sheen, 1988). All experiments were carried out at least four times in duplicate with two different plasmid isolates. In contrast to observations in HeLa cells (Pine et al., 1988), transfection of CHO cells with the calcium phosphate precipitation method did not give rise to high background levels.

Fluorescent in situ hybridization analysis (FISH). The probes were labeled by standard nick translation with biotin-16-dUTP (Boehringer Manheim). 50-100 ng probe in hybridization mixture (50% formamide, 10% dextransulphate in 2xSSCP) with 5 μg sonicated salm sperm DNA and 5 μg yeast tRNA, was heat denatured and preannealed with 10 μg mouse cot-1 DNA (Gibco BRL, Gaithersberg USA) for 30-60 min. at 37 °C. The

target DNA was also heat denatured (70 °C, 2 min.) followed by hybridization overnight at 37 °C. After washing, hybridization sites were visualized by immunofluorescence using Avidine D-Fitc (Vector, USA). A second round of amplification was usually required. The slides were counterstained with propidium iodide and 4',6',diamino-2-phenylindole (DAPI) in antifade medium (DABCO).

RESULTS

Organization of the mouse ISG-54K and ISG-56K genes.

To isolate the mouse ISG-54K gene, a hamster ISG-54K exon 2 specific probe [1.5 kb SacI-EcoRI fragment (Bluyssen et al., 1994)] was used to screen a mouse genomic library in λEMBL3. This resulted in the isolation of two overlapping clones (MG-1 and MG-2), which were characterized for BamHI, EcoRI and HindIII sites (Fig. 1A). Exon 2 specific sequences could be localized on a 2.8 kb EcoRI-EcoRI fragment. The direction of this gene segment was determined by sequence analysis, and comparison to the hamster and the human ISG-54K gene structures (Levy et al., 1986; Bluyssen et al., 1994). By

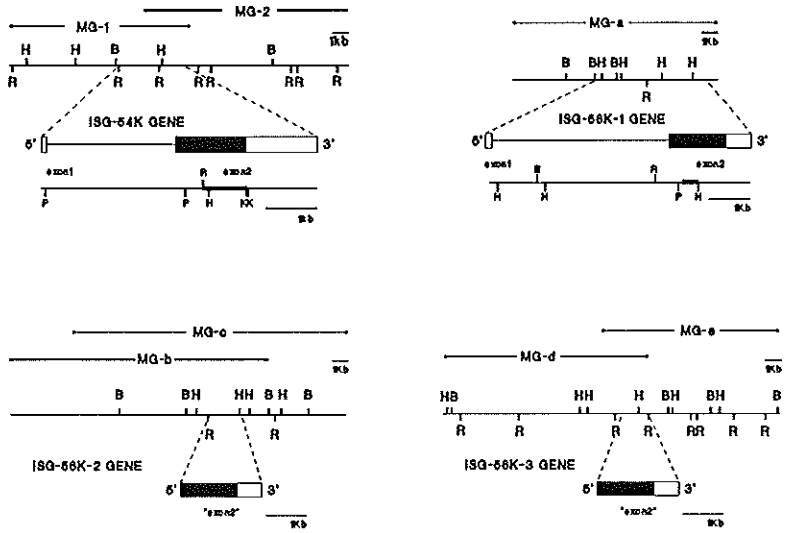

Figure 1. Isolation and characterization of the mouse ISG-54K/ISG-56K gene family. (A) Partial restriction map of the mouse genomic DNA clones MG-1 and -2, MG-a and, MG-b and -c, and MG-d and -e, containing the ISG-54K and ISG-56K1, -56K2, and -56K3 gene, respectively (B:BamHI, R:EcoRI, H:HindIII, K:KpnI, P:PstI, S:SacI). Boxed regions indicate the 2 exons, the closed boxes correspond to the open reading frame. The horizontal bars below the ISG-54k and -56K1 genes, represent the fragment used as hybridization probe (EcoRI-KpnI and PCR-amplified fragment, respectively).

Southern blot hybridization of phage MG-1 DNA, digested with different enzymes, with a hamster ISG-54K exon 1 specific probe, exon 1 of the mouse ISG-54K gene could be localized. Southern hybridization of mouse genomic DNA with an ISG-54K 0.9 kb EcoRI-KpnI exon 2 specific probe (indicated in Figure 1A below the ISG-54K gene) showed that the mouse ISG-54K gene is single-copy (Fig. 1B) .

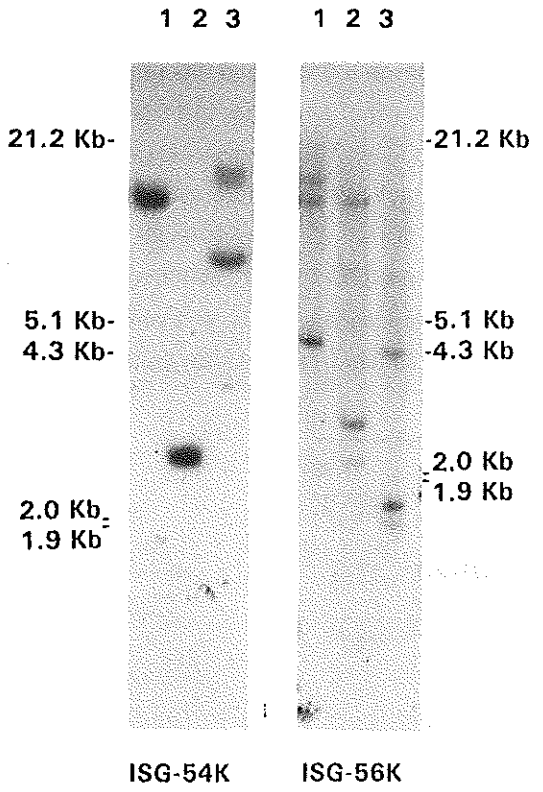

Figure 1. Isolation and characterization of the mouse ISG-54K/ISG-56K gene family. (B) Southern blot analysis of the ISG-54K and ISG-56K genes in genomic DNA derived from mouse L929 cells. The DNA was digested with BamHI (lane 1), EcoRI (lane 2), and HindIII (lane 3). For hybridization a mouse ISG-54K 0.9 kb EcoRI-KpnI and a 0.4 kb mouse ISG-56K, exon 2 specific probe were used, respectively.

In order to isolate the mouse ISG-56K gene(s), we first generated a probe by amplification of an ISG-56K specific fragment on genomic DNA from mouse L929 cells, using degenerate primers (see Material and Methods; indicated in Figure 2B). The primer sequences were deduced from conserved regions of human ISG-56K gene (Wathelet et al., 1986) and hamster (Bluyssen et al., 1994) and human (Levy et al., 1986) ISG-54K genes.

The resulting 0.4 kb exon 2 mouse ISG-56K probe (indicated in Figure 1A, below the ISG-56K1 gene), hybridized with three different fragments on a Southern Blot of mouse genomic DNA (Figure 1B), suggesting the existance of at least three different ISG-56K-like genes. The probe did not cross-hybridize with the mouse ISG-54K gene.

By screening a mouse genomic library in λEMBL3, using the 0.4 kb mouse ISG-56K PCR fragment as a probe, we isolated three different sets of phages (MG-a; MG-b, and -c; MG-d, and -e, respectively), according to the BamHI, EcoRI, and HindIII restriction maps (Figure 1A). ISG-56K exon 2 specific sequences could be localized on a 1 kb EcoRI-HindIII fragment in MG-a, on a 2 kb BamHI-BamHI fragment in MG-b, MG-c and on a 0.8 kb EcoRI-HindIII fragment in MG-d, MG-e, respectively. The direction of the open reading frame in each gene (named ISG-56K1, ISG-56K2 and ISG-56K3, respectively) was determined by sequence analysis, and comparison to the human ISG-56K gene structure (Wathelet et al., 1986). With the RACE protocol (using a primer which is conserved between ISG-56K1, -2, and -3 to make first strand cDNA; indicated in Figure 2B) on RNA isolated from IFN-α treated mouse L929 cells, cDNA fragments were isolated, which after sequencing all turned out to contain exon 1 sequences of the ISG-56K1 gene. By hybridization of such a probe with phage MG-a DNA, digested with different enzymes, exon 1 of ISG-56K1 was localized on a 0.5 kb BamHI-HindIII fragment approx. 5 kb upstream of exon 2 (see also Figure 1A). Hybridization with phage MG-b and MG-d, using the 0.5 kb BamHI-HindIII fragment, however, did not provide a positive fragment. In addition, for RT-PCR of ISG-54K2 and ISG-54K3, a conserved primer between ISG-56K2 and ISG-56K3 was used in combination with the above mentioned RT-primer on L929 cDNA. However, this did not result in identification of exon 1 sequences specific for these genes. Trying several other approaches, including restriction enzyme analysis of L929 RT-PCR cDNA, and screening of another mouse genomic library with an ISG-56KI exon I fragment, we were unable to identify ISG-56K2 and ISG-56K3 cDNA and exon 1 sequences.

Response of mouse ISG-54K and ISG-56K gene expression to IFN-α. The 0.9 kb EcoRI-HindIII fragment was used as a hybridization probe to monitor the response of the mouse ISG-54K gene to IFN-α treatment. Figure 1C shows the Northern blot analysis of RNA isolated from IFN-α treated and control L929 cells, actin mRNA is used as an internal control. The ISG-54K mRNA (~2.8 kb), not present without IFN (lane 1), even after overexposure of the autoradiograph (not shown), is highly induced within 2 h (lane 2) reaching the maximum level around 8 h after IFN addition (lane 3). The ISG-54K mRNA concentration remains high until at least 24 h postinduction (lane 4).

To determine the mouse ISG-56K1 mRNA expression pattern, following IFN induction in L929 cells (Figure 1C), a similar experiment was done (using the 0.4 kb PCR amplified fragment as a probe). The mouse ISG-56K1 messenger (~ 1.9 Kb) is present at a very low basal level in untreated L929 cells (lane 1), significant induction already occurs after 2 h of IFN treatment (lane 2). Maximum level is reached after 8 h of incubation (lane 3); expression remains high for at least 24 h (lane 4).

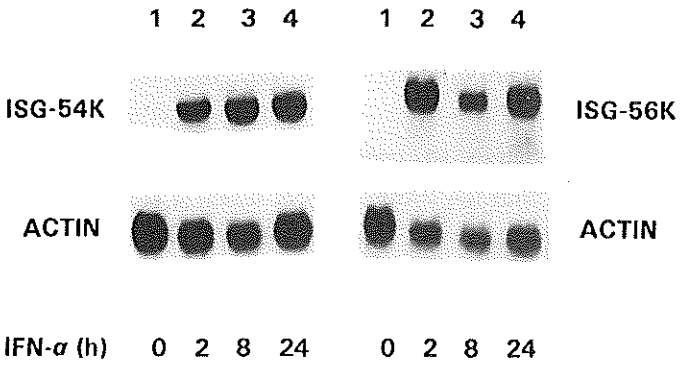

Figure 1. Isolation and characterization of the mouse ISG-54K/ISG-56K gene family. (C) Northern blot analysis of IFN-α induced ISG-54K and ISG-56K1 mRNA expression in mouse L929 cells. IFN treatment (50 IU/ml) in L929 cells was for 0, 2, 8, and 24h (lanes 1-4, respectively). The same probes as in (B) were used for hybridization. An actin probe was used as an internal control.

Analysis of the mouse ISG-54K and ISG-56K1 gene: nucleotide and predicted amino acid sequence. The ISG-54K and ISG-56K1 genes show an identical organization (Figures 1 and 2). In both genes, the open reading frame is separated over two exons. The intron, of which the position was confirmed by PCR on cDNA using exon 1 and exon 2 specific primers (data not shown), has a size of 2.5 and 5 kb, respectively. In both genes, the first exon provides 5'-untranslated sequences and the first five nucleotides of the open reading frame. The second exon contains the remaining part of the open reading frame and 3'-untranslated sequences. The ISG-54K open reading frame encodes a protein of 473 amino acids; the ISG-56K1 open reading frame encodes 464 amino acids. The proteins are structurally related (40% homologous). Both proteins are very hydrophilic, rich in polar (28.5 % and 30 %, respectively) and charged (31.7% and 29.5%, respectively) amino acid residues. The search for sequence homology in data bases did not result in detection of any related gene, except for the human ISG-54K and ISG-56K genes (see also Levy et al., 1986; Wathelet et al., 1986; Wathelet et al., 1988a).

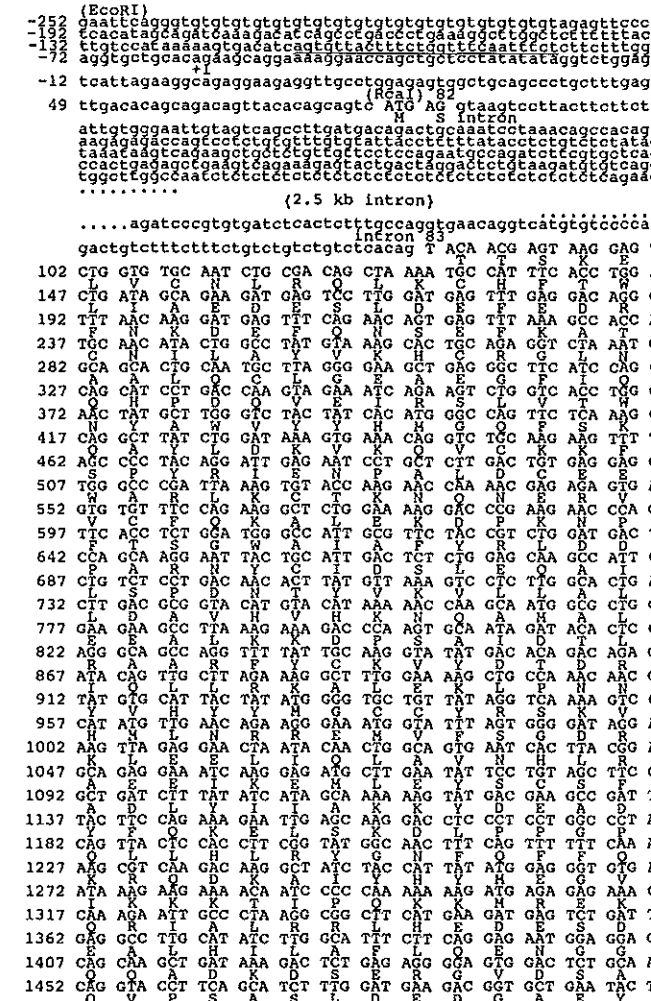

Figure 2. Structural analysis of the mouse ISG-54K gene and ISG-56K1 gene. (A) Nucleotide sequence of the mouse ISG-54K gene. The transcription start site is indicated as +1. The EcoRI-ScaI fragment was used as a probe for S1 analysis. The ISRE motifs in the promoter are underlined. The amino acid sequence of the ISG-54K protein, as deduced from the open reading frame is presented below the nucleotide sequence.

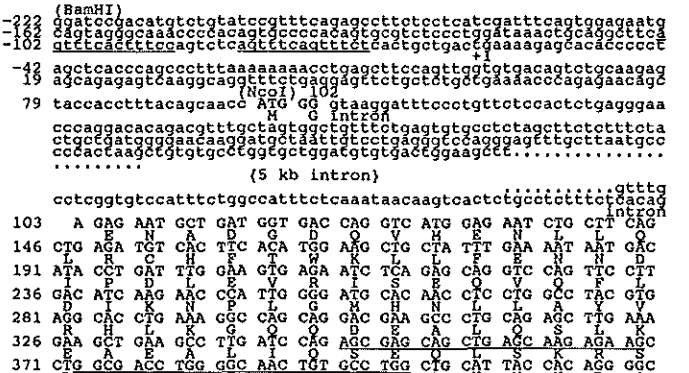

Figure 2. Structural analysis of the mouse ISG-54K gene and ISG-56K1 gene. (B) Nucleotide sequence of the mouse ISG-56K1 gene. The transcription start site is indicated as +1. The BamHI-NcoI fragment was used as a probe for S1 analysis. The ISRE motifs in the promoter are underlined. The amino acid sequence of the ISG-56K1 protein, as deduced from the open reading frame is presented below the nucleotide sequence. Primers used for construction of the PCR probe (5'-AGTGATCAGGGGCAACTTTGCTTGG-3' and 5'-TGTTCGAAGTCCCGTTCCTC(C/T)TG(A/G)AA-3') and for RACE protocol (5'-CTTCTCTTGCTCAGCTGCTCGCT-3') (see also Materials and Methods) are underlined.

Determination of the transcription start sites of the mouse ISG-54K and -56K1 genes. In order to identify the transcription initiation site(s) of the mouse ISG-54K and ISG-56K1 genes, an S1 nuclease protection experiment was performed on RNA isolated from L929 cells, which were treated with IFN for 8 h. RNA from untreated cells was used as a control. Hybridizing probes were chosen in the region spanning the longest cDNA fragments, and deduced from the known structure of the human ISG-54K and ISG-56K genes (Levy et al., 1986; Wathelet et al., 1986). The results of the experiments are depicted in Figure 3. In case of the ISG-54K gene, protected fragments (lane 2), mapped in a region between 77 and 82 bp upstream of the ATG translation start codon (see also Figure 2A).

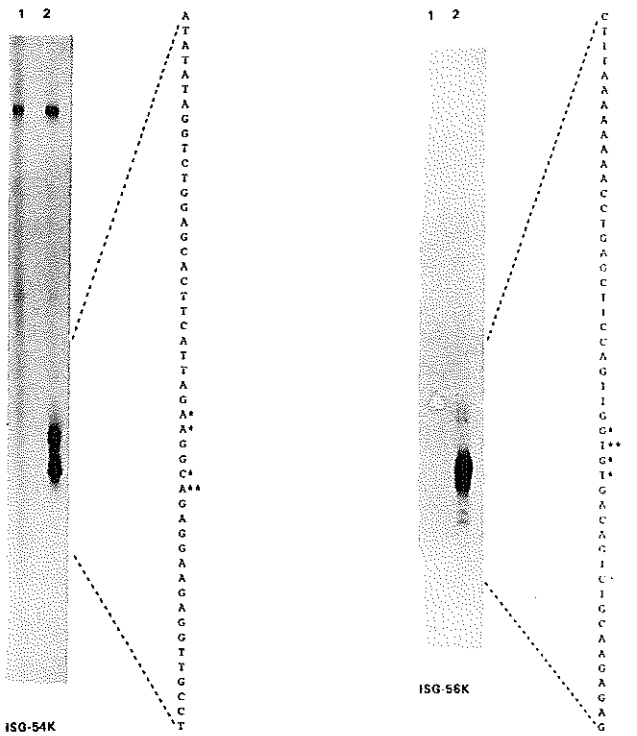

Figure 3. Determination of the transcription initiation sites of the mouse ISG-54K gene and ISG-56K1 gene by S1 nuclease mapping. A 330 bp EcoRI-RsaI ISG-54K and a 320 bp BamHI-NcoI ISG-56K1 probe were used, respectively (see also Figures 2A and B). Lane1, L929 RNA (untreated); lane 2, L929 RNA (treated for 8h with IFN-α). The end points of the major protected fragments are indicated with an asterisks alongside a sequence containing part of the ISG-54K and ISG-56K1 promoter respectively.

The most prominent signal (+1 in Figure 2A) co-located with the position of the transcription initiation site as defined in the human ISG-54K gene (Levy et al., 1986). RNA from untreated cells did not give protected fragments (lane 1), indicating again that ISG-54K mRNA is not expressed in cells grown in the absence of IFN.

For the ISG-56K1 gene, protected fragments were identified, which map in a region between 95 and 98 bp upstream of the ATG (see also Figure 2B). The most prominent signal was found at a T, 97 bp upstream of the ATG (+1 in Figure 2B).

Chromosomal localization of the mouse ISG-54K/ISG-56K gene family. To determine the chromosomal localization of the mouse ISG-54K and ISG-56K genes, FISH on metaphase chromosome spreads of two different mouse leukemia cell lines (RED-8 and RMB-2) was performed (see Material and Methods). The biotinylated genomic clones MG-1 and MG-2, MG-a, MG-c, and MG-e (see Figure 1A), were used as probes for ISG-54K, ISG-56K1, ISG-56K2, and ISG-56K3, respectively.

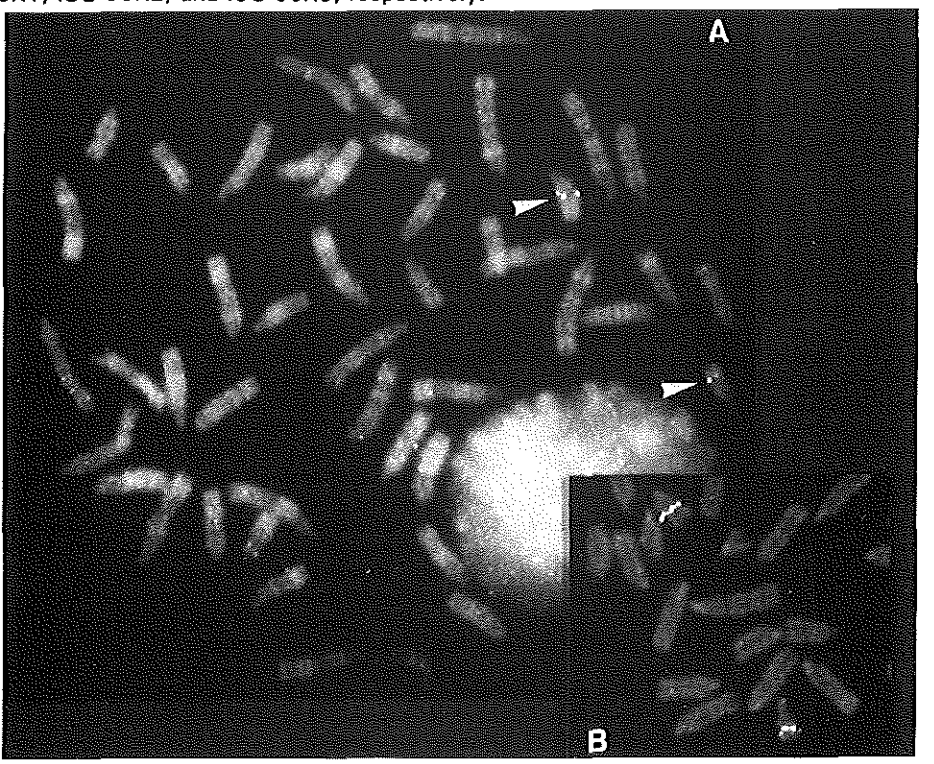

Figure 4. Chromosomal localization of the mouse ISG-54K gene by in situ hybridization. (A) Metaphase from Red-8 cell line hybridized with the ISG-54K gene (probe MG-2), demonstrating signals on chromosome 19 and 19q+ (indicated by an arrow). R-banding was done with propidium iodide after Brdu incorporation in late-S phase. (B) Partial metaphase of Red-8 cells with clear signals on chromosome 19 and 19q+ using probe MG-2.

Eleven to 45 metaphases were analyzed for the different probes. The results of representative experiments are shown in Figures 4 and 5. Metaphases from the Red-8 cell line (Figure 4A and B), hybridized with probe MG-2 (ISG-54K), clearly demonstrated signals on chromosome 19 and 19q+ (Figure 4).

Figure 5. Chromosomal localization of the mouse ISG-56K genes by in situ hybridization. Partial metaphase of mouse leukemia cell line RMB-2, hybridized with probes for different ISG-56K genes. (A) Probe for the ISG-56K1 gene (MG-a) maps on both chromosomes 19 (indicated by an arrow) and in one third of the cells on an unidentified marker chromosome that presumably contains chromosome 19 sequences (indicated by an asterisk). (A') DAPI staining of the same metaphase as (A). (B) Hybridization with the ISG-56K2 gene probe (MG-c), clearly labeling both chromosomes 19. (B') DAPI staining of the same metaphase as (B). (C)/(D) Hybridization with the ISG-56K3 gene probe (MG-e), showing clear signals on both chromosome 19 and in one third of the cells (D) on the proximal part of a large chromosome. (C') DAPI staining of the same metaphase as (C).

On the RMB-2 cell line (data not shown) signals on both chromosomes 19 were seen. Using MG-1 (only done on RED-8 cells), appart from signals on 19 an 19q+ an additional signal was seen (in 100% of the cells) on the proximal part of a large chromosome (data not shown). Metaphases from RMB-2 cell line, hybridized with MG-a (ISG-56K1), MG-c (-56K2) or MG-e (-56K3) showed in all cases signals on both chromosomes 19 and in one third of the cells on the proximal part of un unidentified marker chromosome (Figures 5A, B, C and D). Using metaphase spreads from Red-8 cells, Mg-a, MG-c and MG-e all hybridized with chromosome 19 and 19q+. MG-e (-56K3) showed in one third of these cells an additional signal, located on the same large chromosome as seen after MG-1 hybridization (data not shown). In conclusion, consistent hybridization signals for all probes used were only obtained with chromosome 19.

Structural and functional analysis of the mouse ISG-54K and ISG-56K1 gene promoter. Previously, we showed that the hamster ISG-54K promoter contains two adjacent, functional ISREs (ISRE-I and ISRE-II) of different strength, which act synergistically for maximal IFN-α inducibility (Bluyssen et al., 1994). The mouse ISG-54K promoter is structurally closely related to that of the corresponding hamster gene, and the mouse ISG-54K ISRE-region shows a comparable organization (indicated with ISRE-II and ISRE-I) (Figures 2A and 6A). However, using a -290 to +31 mouse ISG-54K promoter-CAT reporter construct in a transient transfection assay, a consistently lower activity as compared to the hamster wildtype promoter (approx. 30%) was observed (shown in Figure 6A, denoted with pMISG54-1-CAT and pHISG54-1-CAT, respectively). Structural analysis of the mouse ISG-54K ISRE-region (AGTGTTACTTTCTGGTTTCAATTTCT), shows that it is completely identical to that of the hamster promoter (AGTTTTACTTTCTGGTTTCAATTTCT), except for a G at position 4 in the presumed ISRE-II sequence, instead of a T in the hamster promoter at this position (Figure 6A). To test whether the G in the mouse ISG-54K ISRE-II was responsible for the lower activity, a G to T substitution was introduced at this position, thereby creating mutant pMISG54-2-CAT (Figure 6A). If transfected in CHO cells this mutant has the same maximum promoter activity in the presence of IFN as the hamster wildtype promoter (pHISG54-1-CAT) (Figure 6A).

Structural analysis of the mouse ISG-56K1 promoter shows the presence of two segments, which completely fit with he ISRE consensus sequence AGTTTCNNTTTCY (ISRE-II: AGTTTCACTTTCC, and ISRE-I: AGTTTCAGTTTCT in Figures 2B and 6B). However, in contrast to the ISG-54K ISREs, the ISG-56K1 ISRE motifs are not adjacent, but separated by 6 bp. To test the functional importance of ISRE-II, ISRE-I, and the

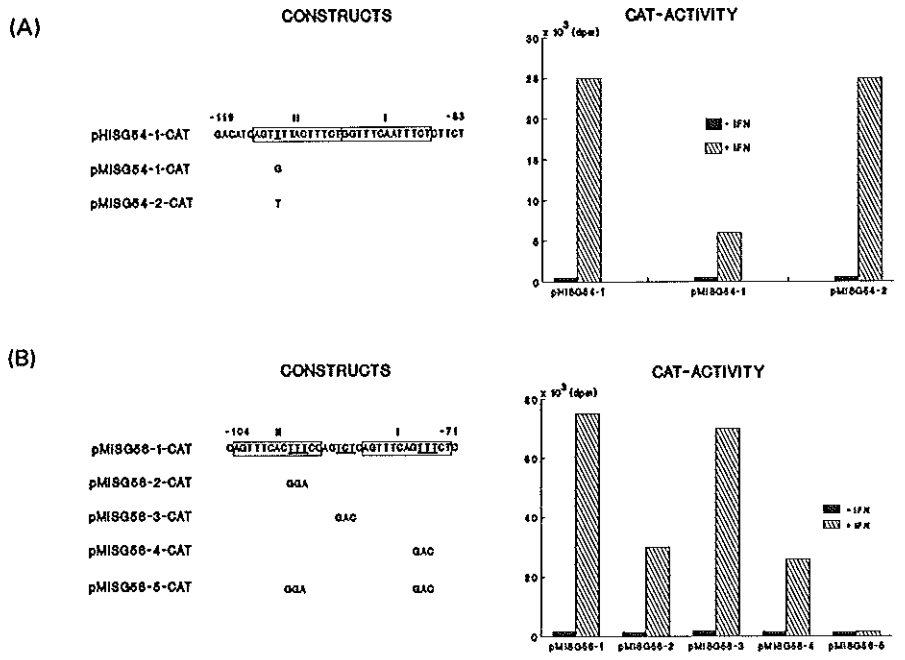

Figure 6. Characterization of the mouse ISG-54K and ISG-56K1 promoter. (A) Interferon-α inducibility of wild type and mutant mouse ISG-54K promoter CAT constructs. Mutations are indicated below the wildtype sequence. CHO cells were transfected with promoter CAT reporter gene constructs as described in Materials and Methods. After transfection, cells were grown with IFN (50 IU/ml; 24 h) or without IFN. Quantitation of CAT activity was achieved as described in the Materials and Methods section. The activity of pHISG54-1-CAT is taken as 100%. (B) Interferon-α inducibility of wild type and mutant mouse ISG-56K1 promoter CAT constructs. For details see (A and Materials and Methods). The activity of pMISG56-1-CAT is taken as 100%.

intervening sequence, several mutations were introduced into the mouse ISG-56K1 promoter as indicated (see Figure 6B). In this way three different "single" mutants (denoted pMISG56-2-CAT to pMISG56-4-CAT in Figure 6B) and one "double" mutant (pMISG56-5-CAT) were constructed. Each of these mutants was used in transient transfection assays in CHO cells and compared with the wildtype ISG-56K1 promoter (pMISG56-1-CAT). The results of a representative experiment are shown in Figure 6B. Single ISRE-II mutation or single ISRE-I mutation caused a decrease of IFN-α induced promoter activity of about 70%, as compared to that of the wildtype promoter (Figure 6B). This indicates that both ISREs are equally important for maximal ISG-56K promoter

activity. Mutation of the sequence between ISRE-II and ISRE-II (pMISG56-3-CAT) had no effect on promoter activity. The "double" mutant pMISG56-5-CAT, with a mutation of both ISRE sequences, shows no response at all to IFN-α, indicating that ISRE-II and ISRE-I of the mouse ISG-56K1 promoter are essential for maximal response to IFN.

DISCUSSION

In order to be able to study the function of the ISG-54K and ISG-56K genes, and to investigate in vitro and in vivo regulation of expression, we isolated the mouse ISG-54K/ISG-56K gene family. The sequences of the complete ISG-54K and ISG-56K1 genes are closely related, especially in the first half of the open reading frame. This is in agreement with the structural homology between the human ISG-54K and ISG-56K proteins (Levy et al., 1986; Wathelet et al., 1986; Wathelet et al., 1988a). The expression in response to IFN-α showed a similar pattern (Figure 1C). Previously, we showed that the hamster ISG-54K promoter contains two adjacent, functional ISREs (ISRE-I and ISRE-II) of different strength, which act synergistically for maximal IFN-α inducibility (Bluyssen et al., 1994). We postulated an identical mechanism for the human ISG-54K and the human ISG-56K promoter, which show a similar structural organization of two adjacent candidate ISREs (Bluyssen et al., 1994). The mouse ISG-54K promoter showed a structural ISRE organization (ISRE-II: AGTGTTACTTTCT and ISRE-I: GGTTTCAATTTCT, adjacently located) which was almost identical to that of the hamster ISG-54K promoter (Bluyssen et al., 1994). However, the mouse ISG-54K promoter was 70% less active than the hamster promoter, which was caused by the G at position 4 in ISRE-II. To determine whether this G was mouse strain specific or not, we amplified and sequenced the corresponding fragment from different mouse sources (P919, NIH/Swiss, BALB/c, C57Bl). In all DNA preparations a G was found in ISRE-II instead of a T (data not shown), so, the mouse ISG-54K promoter contains one strong ISRE (ISRE-I) and a related sequence (ISRE-II), which is not or hardly active. This is in contrast to the human (Wathelet et al., 1988b), monkey (Bluyssen, to be published) and hamster (Bluyssen et al., 1994) ISRE-II. Because of the synergistic interaction, even the weak ISRE-II in the hamster promoter is of importance for high induction levels.

The mouse ISG-56K1 promoter contains two functional ISRE motifs (ISRE-II: AGTTTCACTTTCC, and ISRE-I: AGTTTCAGTTTCT; Figure 6A). Both ISREs are needed for maximum activity upon IFN-α treatment. Individually they show a similar activity (30-40

% of the wildtype promoter). Compared to the ISRE consensus (AGTTTCNNTTTCY), ISRE-II and ISRE-I of the mouse ISG-56K1 promoter are "perfect" ISREs. In contrast to the human ISG-56K promoter, the ISREs are not localized adjacently, but separated by 6 bp. Although not tested in this study, it is to be expected that the 6 bp distance hardly affects promoter strength [compare the studies on the hamster ISG-54K promoter (Bluyssen et al., 1994)]. Although less clear than in the hamster ISG-54K promoter (Bluyssen et al., 1994), functional studies again point to a mechanism of cooperativity between two ISREs through transcription factors binding to the two motifs.

Additional to the ISG-54K and ISG-56K1 gene, we identified two other mouse ISG56K genes (ISG-56K2, and ISG-56K3). The genes are structurally closely related to each other and also to ISG-56K1 exon 2 sequences. Although a long open reading frame and a perfect splice acceptor site were found (Figure 7 and data not shown), we were unable to locate the presumed exon 1 of ISG-56K2 and ISG-56K3, indicating the possibility that we are dealing with two pseudogenes. The finding that we were unable sofar to identify transcripts from ISG-56K2 and ISG-56K3 supports this hypothesis. The occurrence of human ISG-56K pseudogenes has also been suggested (Wathelet et al., 1988; Lafage et al., 1992). On the other hand it is very well possible that the 56K2 and 56K3 regions both are part of a larger, functional gene from which the more upstream exon(s) remains to be isolated. If there is such an exon, its structure must deviate considerably from ISG-56K1 exon 1. It would be of high interest to further investigate different tissues for ISG-56K2 and ISG-56K3 expression. However, because the promoter region is unknown, their regulated expression might be completely different from that of ISG-56K1 (and ISG-54K).

In Figure 7 a comparison is presented between the deduced amino acid sequences of the mouse ISG-56K1, ISG-56K2 and ISG-56K3, and ISG-54K genes. Especially the N-terminal half of the various proteins is highly conserved. All cysteine residues in this region in ISG-56K1 (seven) are present in 56K2 and 56K3; five of them can also be found in ISG-54K, a sixth overall conserved cysteine is present at position 290. This suggests that the different polypeptides can adopt a similar secundary and tertiary structure, and might have a common biological function. It can be speculated that well conserved motifs are of high importance for this function. Unfortunately, the amino acid sequences do not provide a clue in this respect.

```
        1
MO56-1  MGENADGDQVMENLLQLRCHFTWKLLFENNDIPDLEVRISEQVQFLDIKNPLGM
MO56-2    AKSHSCLIYDS.VE.........VI.KV.M.........TEF.-.ASYSI..
MO56-3    .ESH.SHIHDS.DE.......E.DIK.KH.H...IK...TEFR-.PIYSI..
MO54    .STT-SKESLES..R..K.....N.IA.DESLDEF.D.VFNKDE.QNSEFKAT.

        55
MO56-1  HNLLAYVRHLKGQQDEALQSLKEAEALIQSE---QLSKRSLATWGNCAWLHYHR
MO56-2  .......G.....................---.....R..............
MO56-3  ..............................---.....................
MO54    C.I....K.CR.LNEA...C.G...GF..QQHPD.VEI...V....Y..VY..M

        109
MO56-1  GSLAEAQVYLDKVEKVCKEFSSPFRYRLECAEMDCEEGWALLKCGGGNYKQAMA
MO56-2  ........................Q.....................IQ...G...
MO56-3  ........................................R...SQ..T....
MO54    .QFSK..A.....KQ...K....--..I.NPAL.......R...TKNQNERVKV

        163
MO56-1  CFAKALKVEPENPEYNTGYAVVAYRQD-LDDNFISLEPLRKAVRLNPEDPYLKV
MO56-2  ...............A..........--H..GT..QH.Q...SV.........
MO56-3  ..E............A...D...H..--YY.GN..Q..K...SVK........
MO54    ..Q...EKD.K...FTS.W.IAF..L.DWPARNYCIDS.EQ.IQ.S.DNT.V..

        217
MO56-1  LLALKL-QDLGEHVEAEAHIEEALSSTSCQSYVIRYAAKYFRRKHRVDKALHLL
MO56-2  ......-...HKLE...K....T.PRI...P..FGYV.......GL..E..EF.
MO56-3  ......-...RKTD...K..K..TLTI..QNNIFG.V.......GC..E..GF.
MO54    ......DAVHVHKNQ.M.LV....KKDPSAIDTLLR..RFYCKVYDT.R.IQ..

        271
MO56-1  NRALQASPSSGYLHYQKGLC-YKQQISQLRTSRNRQPRRQDNVQELAQQ--AIH
MO56-2  G.....Q.C.TF..F.I...-H.KRLI.IKKAS.....GE...RADQS.HL..C
MO56-3  K....TK...P...F.I...-H.T.FF..KKA----T..EN..RADQSCHL..C
MO54    RK..EKL.NNA.V..YM.-.C.RSKVHHMLNR.EMVFS-G.RKKLEELIQL.VN

        325
MO56-1  EFQETLKLRPTFEMAYVCMAEVQAEIHQYEEAERNFQKALNNKTLVAHIEQDIH
MO56-2  ..KR.......YV....T...M...KN.LK...D....L...SN.ED..Q.E..
MO56-3  ..K........YDR..ID......KN..QK...D...EV..MSN.GDYMQ.E..
MO54    HLRKAEEIKEML.YSCSFL.DLYIIAKK.D..DYY...E.S-.D.PPGPK.LL.

        379
MO56-1  LRYGRFLQFHKQSEDKAITLYLKGLKVEEKSFAWRKLLTALEKVAERRVCQNVH
MO56-2  F.......YY....EA............VT.HYRD.P.K..AKL.K..-K..-.
MO56-3  F.......YY....EA............VT.HYRD...K...EL..G.-K..-.
MO54    ....N.QF.QMKRQ....YH.ME.V.IKK.TIPQK.MREK.QRI.L..LHEDES

        433
MO56-1  LVESTSLLGLVYKLKGQEKNALFYYEKALRLTGEMNPAF*
MO56-2  VL.NLG.........NTSE.MSC.........A...E.*
MO56-3  VL.........CR.R.DTSE.MSC.........A...E.*
MO54    DS.ALHI.AFLQENG.GQQ-.DKDS.RGVDSANQVPS.SLDEDGAEY*
```

Figure 7. Homology between the predicted amino acid sequences of the mouse ISG-56K, and ISG-54K genes. Conserved Cysteines are indicated by arrow heads, homology is indicated by dots; stripes indicate deletions. Asterisks indicate the deduced end-points. The ISG-56K2/-K3 conserved primer, used for race protocol (5'-ATGACAGCCTGGATG-3'), is underlined in the -56K2 sequence.

The ISG-54K gene and ISG-56K1, -56K2 and -56K3 genes are all assigned to the D1 region of chromosome 19. Although no overlapping genomic fragments were found, this strongly suggests that the four genes are tightly clustered.

The structural homology indicates that the genes arose through duplications of a common ancestral gene (named X in Fig. 8). On the basis of the structural homology an "evolutionary pedigree", as shown in Figure 8, can be proposed. Because the homology is much higher between ISG-56K1 and ISG-56K2 and -56K3 64 and 62%, respectively) than between ISG-54K and ISG-56K1, -56K2, and -56K3 sequences (40% in all cases), it can be suggested that the first gene duplication resulted in ISG-54K and a common ISG-56K1,2,3 ancestor (ISG-56K in Figure 8). As a next duplication, the evolution of a ISG-56K2,3 precursor gene (ISG-56K-1A in Figure 8) and ISG-56K1 can be proposed. This ISG-56K1A gene may not have contained ISG-56K1-like exon 1 sequences. The most recent duplication event could result in the generation of ISG-56K2 and -56K3, which are highly homologous to each other (80%).

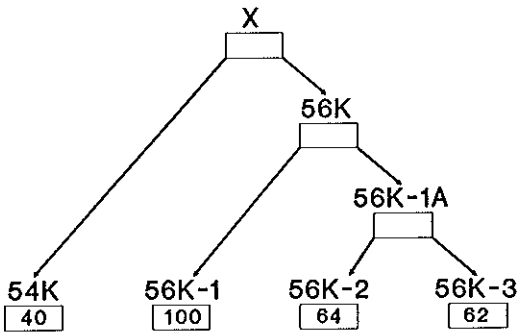

Figure 8. Evolutionary origin of the mouse ISG-54K/ISG-56K gene family (X, ancestrol gene). Numbers in the open boxes represent homology with ISG-56K1.

In the human genome, the ISG-54K gene and the ISG-56K gene have been assigned to chromosome 10 at bands q23-q24 (Lafage et al., 1992). Presumed pseudogenes were found at chromosome 10 and 13 (Wathelet et al., 1988a; Lafage et al., 1992). Our assignment of the mouse ISG-54K/56K genes to chromosome 19D1 is in good agreement with the human-mouse comparitive gene map (Davisson et al., 1991; Lyon and Kirby, 1993). Human chromosome 10q homologous loci are present on mouse chromosome 14 and 19. However, most loci on human 10q23-q24 (RBp4, DNTT and others) have their corresponding mouse homolog on mouse chromosome 19D1.

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REFERENCES

Bluyssen, J.A.R., Vlietstra, R.J., van der Made, A.C.J., and Trapman, J. (1994) The ISG-54K promoter contains two adjacent, functional ISREs of different strength, which act synergistically for maximal IFN-α inducibility. Eur. J. Biochem. 220, 395-402.

Chen, C., and Okyama, H. (1987) High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7: 2745-2752.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299.

Cohen, B., Peretz, D., Vaiman, D., Benech, P., and Chebath, J. (1988) Enhancer-like interferon responsive sequences of the human and murine (2'-5') oligoadenylate synthetase gene promoters. EMBO J. 7: 1411-1419.

Dale, T.C., Rosen, J.F., Guille, M.J., Lewin, A.R., Porter, A.G.C., Kerr, I.M., and Stark, G.R. (1989) Overlapping sites for constitutive and induced DNA binding factors involved in interferon-stimulated transcription. EMBO J. 8: 831-839.

Davisson, M.T., Lalley, P.A., Peters, J., Doolittle, D.P., Hillyard, A.L., and Searle, A.G. (1991) Report of the comparitive commitee for human, mouse and other rodents. Cytogenet. Cell. Genet. 58: 1152-1189.

Favoloro, J., Treisman, R., and Kamen, R. (1980) Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. 65: 718-749.

Feinberg, A.P., and Vogelstein, P. (1983) A technique for radiolabeling DNA restriction endinuclease fragments to high specific activity. Anal. Biochem. 132: 6-13.

Frohman, M.A., Dush, M.K., and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85: 8998-9002.

Fu, X.-Y., Schindler, C., Improta, T., Aebersold, R.H., and Darnell, J.E., Jr. (1992a) The proteins of ISGF3, the IFN-α induced activator, define a new gene family of signal transducers. Proc. Natl. Acad. Sci. USA 89: 7840-7843.

Fu, X.-Y. (1992b). A transcription factor with SH2 and SH3 domains is directly activated by an interferon-α-induced cytoplasmic protein tyrosine kinase(s). Cell 70: 323-336.

Heuvel, M. van, Bosveld, I.J., Mooren, A.T.A., Trapman, J., and Zwarthoff, E.C. (1986) Properties of natural and hybrid Murine Alpha Interferons. J. Gen. Virol. 67: 2215-2222.

Heuvel, M. van, Govaert-Siemerink, M., Bosveld, I.J., Zwarthoff, E.C., and Trapman, J. (1988) IFN producing CHO cell lines are resistant to the antiproliverative activity of IFN: A correlation with gene expression. J. Cell. Biochem. 38: 269-278.

Higuchi, R., Kruvuel, B., and Saiki, R. (1989) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucl. Acids Res. 16: 7351-7367.

Kessler, D.S., Veals, S.A., Fu, X.-Y., and Levy, D.E. (1990) IFN-alpha regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. Genes Dev. 4: 1753-1765.

Lafage, M., Clauss, I., Couez, D., Simonetti, J., Wathelet, M.G., and Huez, G. (1992) The interferon- and virus-inducible IFI-56K and IFI-54K genes are located on human chromosome 10 at bands q23-q24. Genomics 13: 458-460.

Larner, A.C., Jonak, G., Cheng, Y.S.E., Korant, B., Knight, E., Jr., and Darnell, J.E., Jr. (1984) Transcriptional induction of two genes in human cells by ß-interferon. Proc. Natl. Acad. Sci. USA 81: 6733-6737.

Levy, D.E., Larner, A.C., Chaudhuri, A., Babiss, L.E., and Darnell, J.E., Jr. (1986) Interferon-stimulated transcription: isolation of an inducible gene and identification of its regulatory region. Proc. Natl. Acad. Sci. USA 83: 8929-8933.

Levy, D.E., Kessler, D.S., Pine, R., and Darnell, J.E., Jr. (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-α-stimulated transcription, reconstituted in vitro. Genes Dev. 3: 1362-1371.

Lyon, M.F., and Kirby, M.C. (1993) Mouse genome atlas. Mouse Genome 91: 40-80.
De Maeyer, E., and De Maeyer-Guignard, J. (1988) Interferons and other regulatory cytokines. John Wiley & Sons, New York.
Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C., Pellegrini, S., Wilks, A.F., Ihle, J.N., Stark, R.S., and Kerr, I.M. (1993) The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. Nature 366: 129-135.
Pellegrini, S., and Schindler, C. (1993) Early events in signalling by interferons. TIBS 18: 338-342.
Pine, R., Levy, D.E., Reich, N., and Darnell, J.E., Jr. (1988) Transcriptional stimulation by $CaPO_4$-DNA precipitates. Nucl. Acids Res. 16: 1371-1378.
Porter, A.C., Chernajovsky, Y., Dale, T.C., Gilbert, C.S., Stark, G.R., and Kerr, I.M. (1988) Interferon response element of the human gene 6-16. EMBO J. 7: 85-92.
Reich, N., Evans, B., Levy, D.E., Fahey, D., Knight, E., Jr and Darnell, J.E., Jr. (1987) Interferon-induced transcription of a gene encoding a 15 kDa protein depends on an upstream enhancer element. Proc. Natl. Acad. Sci. USA 84: 6394-6398.
Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.
Schindler, C., Fu, X.-Y., Improta, T., Aebersold, R.H., and Darnell, J.E., Jr. (1992a) The proteins of ISGF3: one gene encodes 91 and 84 kDa ISGF3α proteins. Proc. Natl. Acad. Sci. USA 89: 7836-7839.
Schindler, C., Shuai, K., Prezioso, V.R., and Darnell, J.E., Jr. (1992b) Interferon-dependent tyrosine phophorylation of a latent cytoplasmic transcription factor. Science 257: 809-813.
Seed, B., Sheen, J-Y. (1988) A simple phase-extraction assay for chloramphenicol acetyltransferase activity. Gene 67: 271-277.
Sen, G.C., and Lengyel, P. (1992) The interferon system. J. Biol. Chem. 267: 5017-5020.
Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z., and Darnell, J.E., Jr. (1993b) Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature 366: 580-583.
Silvennoinen, O., Ihle, J.N., Schlessinger, J., and Levy D.E. (1993) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 366: 583-585.
Stark, G.R., and Kerr, I.M. (1992) Interferon-dependent signalling pathways: DNA elements, transcription factors, mutations, and effects of viral proteins. J. Interferon Res. 12: 147-151.
Uze, G., Lutfalla, G., and Gresser, I. (1990) Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. Cell 60: 225-234.
Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon α/β signalling pathway. Cell 70: 313-322.
Wathelet, M., Moutschen, S., Defilippi, P., Cravador, A., Collet, M., Huez, G., and Content, J. (1986) Molecular cloning, full length sequence and preliminary characterization of a 56-kDa protein induced by human interferons. Eur. J. Biochem. 155: 11-17.
Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988a) The IFI-56K and IFI-54K interferon-inducible human genes belong to the same gene family. FEBS Letters 231: 164-171.
Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988b) Regulation of two interferon-inducible human genes by interferon, poly(rI).poly(rC) and virus. Eur. J. Biochem. 174: 323-329.
Williams, B.R.G. (1991) Transcriptional regulation of interferon-stimulated genes. Eur. J. Biochem. 200: 1-11.

CHAPTER 5

IFN-γ INDUCTION OF THE ISG-54K PROMOTER THROUGH ISRE SEQUENCES; ROLE OF P48(ISGF3γ) AND P91(ISGF3α) BUT NOT P113(ISGF3α)

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Submitted for publication.

SUMMARY

Interferon stimulated gene-54K (ISG-54K) gene expression was found to be interferon-γ (IFN-γ) regulated in Vero cells, which lack the IFN-α/β genes. This IFN-γ induction was shown to be mediated through the interferon stimulated response element (ISRE) sequence. Co-transfection of Vero cells with expression vectors for the 48-kilodalton interferon stimulated gene factor3 (ISGF3) protein (p48), the 91-kilodalton (ISGF3) protein (p91) and the 113-kilodalton ISGF3 protein (p113) in the presence of IFN-γ, showed a role of p48 and p91, but not p113 in ISG-54K promoter activation in the IFN-γ response pathway. In contrast, IFN-α stimulation was maximal in the presence of all three factors. The results obtained implicate that, in addition to the IFN-γ activated sequence (GAS) mediated pathway, IFN-γ can regulate gene expression via activation of p48, p91 and a proposed third factor, which interact in an ISGF3-like complex with the ISRE sequence. Our findings could explain the overlapping set of genes regulated by both IFN-α/β and IFN-γ.

INTRODUCTION

Interferons (IFNs) can induce an antiviral state in target cells. In addition, IFNs are known to affect many other cell functions including cellular differentiation, growth and the immune response. IFN-α/β (Type I IFNs) and IFN-γ (Type II IFN) interact with cells through distinct receptors (1). Receptor-specific ligand binding in either case rapidly induces tyrosine phosphorylation of latent cytoplasmic transcription factors (2).

Transcription factors active in the IFN-α/β signal transduction pathway have been extensively documented. Most important, IFN-α/β binding leads to phosphorylation on tyrosine of the structurally related 113K (p113) and 91K (p91) [or the alternatively spliced 84K (p84)] components of the α-subunit of ISGF3 (interferon-stimulated gene factor 3) (3,4). Subsequently, activated ISGF3α together with the 48K (p48) DNA-binding γ-subunit (5), join in the multiprotein complex ISGF3 and migrate to the nucleus (6). Here the ISGF3 complex binds to the interferon-stimulated response element (ISRE) sequence in the regulatory region of target genes, to initiate transcription (6,7). The two related cytoplasmic protein tyrosine kinases TYK2 and JAK1 have been shown to be essential in the IFN-α/β signal transduction pathway (8-10).

Although it has recently drawn much attention, the IFN-γ signal transduction pathway is less well understood. Interestingly, IFN-γ can activate a set of genes, which is partially overlapping with the IFN-α/β target genes (1). Recently, it has been shown that the 91 kDa component of the α subunit of ISGF3 (p91) is phosphorylated on the same tyrosine (Tyr 701) in response to IFN-γ as in response to IFN-α/β (11,12). Activated p91 translocates to the nucleus, where it binds to the GAS (gamma activating sequence) element to initiate transcription of target genes (2,13). The protein tyrosine kinases JAK1 and JAK2 are involved in the IFN-γ signal transduction pathway (9,10,14).

Sofar, the mechanism of overlapping IFN-α/β and IFN-γ target gene expression is only partly resolved (both involve JAK1 and p91). Using the interferon-stimulated gene-54K (ISG-54K) gene as a model, we provide evidence that the ISRE sequence can not only be a target for IFN-α/β activated transcription factors, but also for IFN-γ activated factors. Both p48 and p91, but not p113 are important for IFN-γ induced initiation of ISG-54K transcription. We presume, that in case of IFN-γ, in addition to p48 and p91, a third factor is involved in optimal activation of the ISG-54K promoter through the ISRE sequence.

RESULTS AND DISCUSSION

Figure 1A shows a Northern blot analysis of RNA isolated from IFN-α and IFN-γ treated (for 24 h) and control Vero cells, hybridized with an ISG-54K specific probe (15). Both IFN-α (lane 2) and IFN-γ (lane 3) are able to induce ISG-54K mRNA expression. Because Vero cells lack IFN-α/β genes (16), the IFN-γ induction is not indirect via IFN-α/β. Previously, it was assumed that ISG-54K induction was IFN-α specific (17). Our data show that, at least in Vero cells, and in a HeLa cell subline grown in Rotterdam (data not shown), ISG-54K expression can also be induced by IFN-γ.

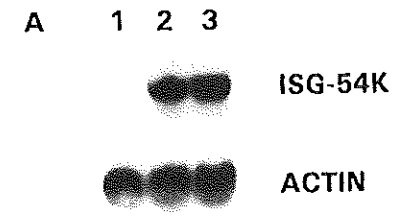

Fig 1. Regulation of the monkey ISG-54K gene. (A) Northern blot analysis of IFN-α and IFN-γ-induced ISG-54K mRNA expression in monkey Vero cells. IFN treatment (500 IU/ml) in Vero cells was for 24 h (lane 1, no treatment; lane 2, IFN-α; lane 3, IFN-γ). A 561 bp EcoRI fragment (15), was used as a hybridization probe. Hybridization with an actin probe was used as an internal control.

To study the IFN-γ regulation of the ISG-54K promoter in more detail, the monkey ISG-54K promoter was isolated (18), and the sequence was determined (Fig. 1B). The promoter is highly homologous to the previously isolated human and hamster counterparts (17,19). Two identical, adjacent ISRE sequences (AGTTTCACTTTCT) are present in the -88 to -113 region. To find out whether the monkey ISG-54K promoter was able to respond directly to IFNs, it was cloned in front of the CAT reporter gene (18) (pAISG54-1-CAT). Lysates prepared from transiently tranfected Vero cells, with or without IFN-α or IFN-γ treatment, were assayed for CAT activity (20) (Fig. 1C). A response to both IFN-α and IFN-γ is seen. The relative responses are in good agreement with the levels of mRNA induction of the endogenous gene (Fig. 1A). So, the promoter fragment used, containing the double ISRE region, is sufficient to confer inducibility to each type of IFN.

(B)

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-388  CCTTAGCAGGAAGTGGGCTTTGCTATTCTGTGTATATCTTTAACAATACC
-338  TGCTGAAAAGAACCCTTTTGTTAAATAATTGTATTACGAGTGCCCAACTT
-288  AGTCCTTTCGAATATGAGATAAGAGAGGGACCGTGCACAAGAGCAATGCC
-238  CCCAGACACATCTTGAAGTGAAGCACGGGGCGAATGAAACATCCCTCTCT
-188  GCTGCCTTCTTTCTCTGATACGCCTCTGATTCCCGAGGAAAAAGAGTGCT
-138  TCATTTCCCTGTAAAGAGTCCTGCCAGTTTCACTTTCTAGTTTCACTTTC
 -88  TCTTTTGCGACGTCAGCTGAAGGGAAACAAGCGGAAAGGAACCAGAGGCC
                                                    +1
 -38  AGTTGTGTATATAGGTCTTTTCAGCGTTTATTGGTGGCAGAAGAGGAA
```

(C)

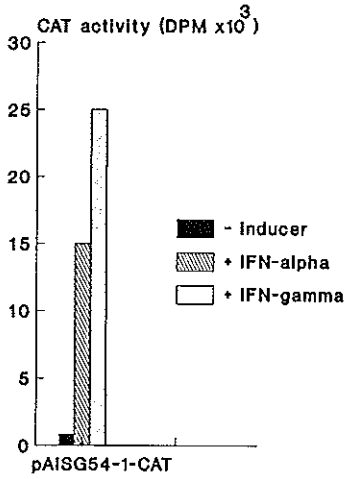

Fig 1. Regulation of the monkey ISG-54K gene. (B) Nucleotide sequence of the monkey ISG-54K promoter. The presumed transcription start site is indicated as + 1. The duplicated ISRE sequence and a variant TATA-box are underlined. (C) IFN-α and IFN-γ-inducibility of the monkey ISG-54K promoter. Vero cells were transfected with a monkey ISG-54K promoter-CAT reporter gene construct (18). CAT-activity (DPM x10³) in cells treated with IFN-α and IFN-γ was compared to that from untreated cells.

Previously, we generated a large series of mutants in the ISRE region of the hamster ISG-54K promoter, in order to establish the role of the two ISRE sequences in IFN-α induction in hamster CHO cells (19). Our data showed that the two ISREs are both necessary and sufficient for maximum activation of the ISG-54K gene upon IFN-α treatment. To study a presumed role of the double ISRE sequence in relation to IFN-γ, a set of hamster (wild type and mutant) ISG-54K promoter CAT constructs was used in transient transfection assays in Vero cells (20). For IFN-α and IFN-γ, an identical induction pattern emerged (Fig. 2). Mutations in the regions flanking the ISREs had no effect on promoter activity (pHISG54-2-CAT and pHISG54-8-CAT); mutation of the T triplet at -95/-93 and that at -89/-87 in ISRE-I caused a strong decrease of both IFN-α and IFN-γ induced promoter activity (pHISG54-5-CAT and pHISG54-6-CAT); mutants pHISG54-3-CAT and pHISG54-4-CAT, which both disrupt a T-triplet in the ISRE-II region (-108/-106 and -102/-100, respectively), showed a partial decrease in activity in response to IFN-α or IFN-γ. Importantly, the "double" mutation (pHISG54-7-CAT) completely knocked out IFN-α and IFN-γ inducibility. Constructs containing two ISRE-Is (pHISG54-I/I-CAT in Fig. 2) or two ISRE-IIs (pHISG54-II/II-CAT in Fig. 2), show a similar IFN-α and IFN-γ induction. Together, these results point not only to an essential role of the ISRE sequences in IFN-γ activation, but also to a similar mechanism of IFN-γ and IFN-α induction, involving identical or similar transcription factors.

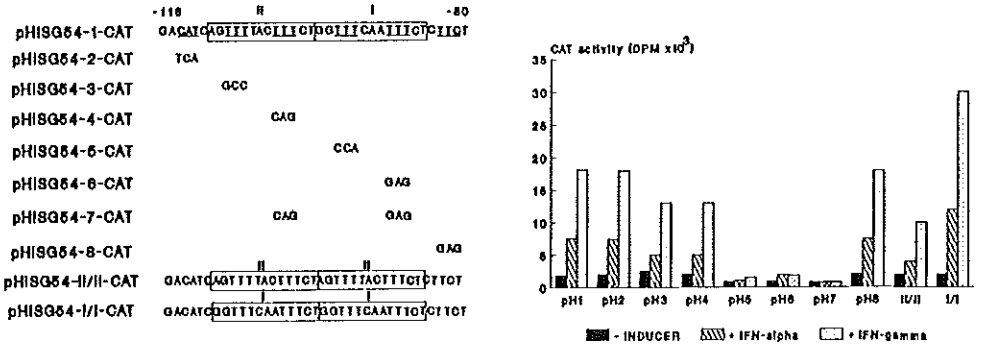

Fig. 2. Effect of mutation of ISRE I and ISRE II and ISRE upstream and downstream sequences on IFN-α and IFN-γ inducibility of the hamster ISG-54K promoter. Mutations are indicated below the wildtype sequence. Vero cells were transfected with wildtype or mutant hamster ISG-54K promoter (BamHI - PstI; -431 to +31) CAT reporter gene constructs as described (19,20). CAT-activity (DPM x10³) in cells treated with IFN-α and IFN-γ was compared to that from untreated cells.

Using the ISG-15K ISRE region as a model system, the ISGF3 complex has shown to be the transcriptional activator that mediates IFN-α-induced activation (6,21). To investigate the role of this complex in relation to ISG-54K activation upon IFN-α treatment, electrophoresis mobility shift assays (EMSA) were performed (22). Unfortunately, for unknown reasons, the ISG-54K ISRE region (even a mutant with two strong ISREs) provides much weaker in vitro protein-DNA interactions than the ISG-15K ISRE region. Therefore, we were unable to obtain reproducible band shifts with IFN-γ sensitive Vero and HeLa cell extracts (data not shown). Figure 3 show results obtained with extracts from IFN-α treated hamster CHO cells and the ISG-15K and ISG-54K ISRE regions, respectively (22; there is no IFN-γ, which is active on CHO cells). ISGF3, which cannot be detected in extracts of untreated cells (lane 1), is induced within 2 h (lane 2) and reaches a maximum level approximately 5 h after IFN-α treatment (lane 3). The ISGF3 level remains high until at least 24 h postinduction (lane 4), which correlates with the kinetics of appearence of the ISG-15K and ISG-54K messenger (19,23) in IFN-α treated CHO-12 cells. These data, combined with the transfection experiments presented in Fig. 2 make it very likely that ISGF3 or ISGF3-like complexes are involved in IFN-α and IFN-γ activation of the ISG-54K promoter.

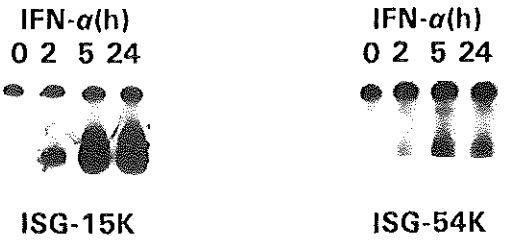

Fig. 3. ISGF3 DNA-binding activity in response to IFN-α treatment. CHO cells were untreated or treated with IFN-α (as indicated) before detergent lysis and analysis of extracts by gel band shift with labeled oligonucleotide (22). Cytoplasmic extracts (5 μg) were added to DNA binding reactions containing an ISG-15K ISRE oligonucleotide or an ISG-54K ISRE oligonucleotide (22), respectively.

To study in more detail the role of ISGF3 constituents (p113, p91, and p48) in relation to IFN-α- and IFN-γ-induced activation of the ISG-54K promoter, co-transfection experiments were performed in Vero cells, using the hamster ISG-54K wild type promoter-CAT construct (pHISG54-1-CAT in Fig. 4) and different combinations of expression plasmids containing cDNAs of the various ISGF3 transcription factors (p48, p91, p113; p84 was not investigated) (24). A representative experiment is shown in Figure 4. In the absence of IFN, overexpression of two factors increased measurable basal activity of the

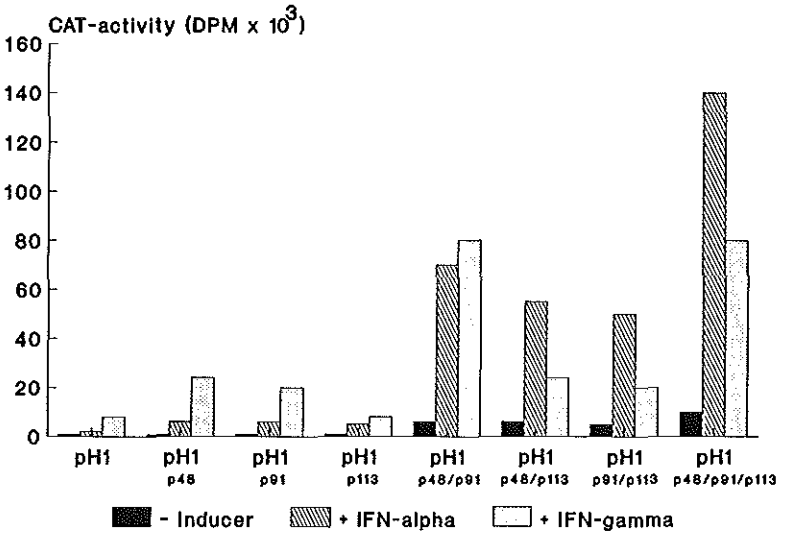

Fig. 4. Effect of co-transfection of different ISGF3-components on ISG-54K IFN-α and IFN-γ induction. Vero cells were transfected with a wildtype hamster ISG-54K promoter-CAT reporter gene constructs (pH1) alone or together with expression plasmids, directing the synthesis of p48, p91, and p113 (24), as indicated. CAT-activity (DPM x10³) in cells treated with IFN-α and IFN-γ was compared to that from untreated cells.

ISG-54K promoter, possibly due to a low level of phosphorylation by endogenous JAK1/JAK2/TYK2. In the presence of IFN-α, any combination of two overexpressed factors highly increases ISG-54K promoter activity. We presume that the two overexpressed factors form complexes with the endogenous third component, which is present at a lower level. As expected, maximal activity is observed when all three components of the ISGF3 complex (p48, p91, p113) are overexpressed. In contrast, in none of the (co)transfection experiments, overexpressed p113 affected ISG-54K promoter induction in the presence of IFN-γ, extending the observation that p113 cannot be activated in the IFN-γ pathway (11,12). However, separate p48 or p91 overexpression in the presence of IFN-γ increases promoter activity in Vero cells; p48/p91 cotransfection provides an even higher activation of the ISG-54K promoter. Interestingly, this maximum level is lower than that of IFN-α induction in the presence of p48/p91/p113. Our data show that, in addition to p91 important in the IFN-γ pathway via binding to the GAS as has been well documented (2,11-13), there is a second IFN-γ response pathway, which includes p91, and also p48. We presume that p48 and p91 can bind as a complex to the ISRE sequence. Our results presented here for the ISG-54K promoter, were also found to be true for the ISG-56K promoter (data not shown), and might be extended to other

promoters which contain ISRE sequences, and which can be induced by both IFN-α and IFN-γ.

Although we cannot completely exclude the possibility that p48 and p91 are sufficient for optimal activation of the ISG-54K promoter in the presence of IFN-γ, we find it more likely that a third, so far unidentified factor is involved. Several data support this hypothesis. (i) In the co-transfection experiments we observed that maximal activity in the presence of IFN-γ was lower than in the presence of IFN-α (Fig. 4). This is in contrast with the expression level of the endogenous gene (Fig. 1A) and the activity of the ISG-54K promoter, without overexpression of the ISGF3 components (Fig. 1C, 2 and 4). (ii) In some cell lines the ISG-54K gene can be activated by IFN-α, but not by IFN-γ (earlier reported HeLa cells (16); and 2fTGH cells, in which the ISG-54K (data not shown) and the highly homologous ISG-56K promoter (25) have been tested). If the p48/p91 complex, which can be formed in these cells because IFN-α is active, is sufficient for activation, IFN-γ would also be able to do so. (iii) The observation that in mutant cell lines, which lack p91(p84) and contain p48 and p113 (25), IFN-α induction is knocked out, strongly suggests that a complex of at least three factors is needed in the IFN-α pathway (4,11,25). Because the same ISRE element can be involved in the IFN-γ pathway and because the mutations in the ISREs has such a similar effect in both IFN-α and IFN-γ pathways (Fig. 2), it would be surprising if two of the three components, which are also involved in the IFN-α pathway would be sufficient for IFN-γ-induced activation.

In Fig. 5 we propose an extension of the original Darnell model of IFN regulated gene expression (11). The IFN-γ response pathway can be split into two directions: one via the GAS element and a second route, which is via the ISRE. The GAS element binds p91 and possibly a different (IFN-γ specific) member of the p91/p113 family; the ISRE binds the ISGF3 complex, containing p48/p91/p113, in case of IFN-α activation and an ISGF3-like complex, consisting of p48/p91 plus a third component (X), in case of IFN-γ activation. It is a higly attractive hypothesis to presume that this third component is a novel (IFN-γ specific) member of the p113/p91 gene family, which may or may not be identical to p91-like components binding to the GAS element. While JAK1 most certainly is the enzyme that phosphorylates Tyr 701 in p91 (10), component X may therefore be a specific substrate for JAK2. The model, as presented here, could cover the observation of regulation by IFN-α/β and IFN-γ of partially overlapping sets of genes.

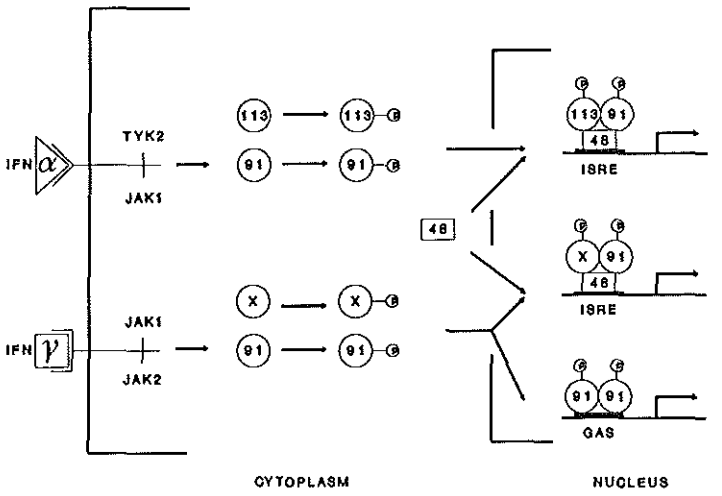

Fig. 5. Pathways of signal transduction and transcriptional activation in cells treated with IFN-α or IFN-γ.

REFERENCES AND NOTES

1. S. Pestka, T.A. Langer, K.C. Zoon, C.E. Samual, Annu. Rev. Biochem. 56, 727 (1987); P. Staeheli, Adv. Virus Res. 38, 147 (1990); G.C. Sen and R.M. Ransohoff, Adv. Virus Res. 42, 57 (1993).
2. S. Pellegrini and C. Schindler, Trends Biochem. Sci. 18, 338 (1993).
3. X.-Y. Fu, C. Schindler, T. Improta, R. Aebersold, J.E. Darnell Jr., Proc. Natl. Acad. Sci. USA 89, 7840 (1992); M.J. Gutch, C. Daly, N. Reich, ibid. p. 11411; C. Schindler, X.-Y. Fu, T. Improta, R. Aebersold, J.E. Darnell Jr., ibid. p. 7836;
4. C. Schindler, K. Shuai, V.R. Prezioso, J.E. Darnell Jr. Science 257, 809 (1992).
5. S.A. Veals et al., Mol. Cell. Biol. 12, 3315 (1992); S.A. Veals, T. Santa Maria, D.E. Levy, ibid. 13, p. 196 (1993).
6. D.E. Levy, D.S. Kessler, R. Pine, J.E. Darnell Jr., Genes Dev. 3, 1362 (1989).
7. T.C. Dale, A.M.A. Imam, I.M. Kerr, G.R. Stark, Proc. Natl. Acad. Sci. USA 86, 1203 (1989); D.S. Kessler, S.A. Veals, X.-Y. Fu, D.E. Levy, Genes Dev. 4, 1753 (1990).
8. L. Velazquez, M. Fellous, G.R. Stark, S. Pellegrini, Cell 70, 313 (1992)
9. M. Muller et al., Nature 366, 129 (1993); O. Silvennoinen, J.N. Ihle, J. Schlessinger, D.E. Levy, ibid. p. 583.
10. K. Shuai et al., ibid. p. 580.
11. K. Shuai, C. Schindler, V. Prezioso, J.E. Darnell Jr., Science 258, 1808 (1992).
12. K. Shuai, G.R. Stark, I.M. Kerr, J.E. Darnell Jr., ibid. 261, 1744 (1993).
13. T. Decker, D.J. Lew, J. Mirkovitch, J.E. Darnell Jr., EMBO J. 10, 927 (1991); D.J. Lew, T. Decker, I. Strelow, J.E. Darnell Jr., Mol. Cell. Biol. 11, 182 (1991).
14. D. Watling et al., Nature 366, 166 (1993).
15. We prepared and analyzed RNA isolated from HuIFN-α (500 IU/ml) or HuIFN-γ (500 IU/ml) treated (for 24 h) or control Vero cells as described (19). As a probe, a 561 bp EcoRI-EcoRI fragment, derived from exon 2 of the human ISG-54K gene (17), was used.
16. J. Desmyter, J.L., Melnick, W.E. Rawls, J. Virol. 2, 955 (1986).
17. D.E. Levy, A.C. Larner, A. Chaudhuri, L.E. Babiss, J.E. Darnell Jr., Proc. Natl. Acad. Sci. USA 83, 8929 (1986).
18. Using human ISG-54K derived primers (5'CAGGCAGCAGTACTCTTCAGAAATC3' and 5'TCTGAGCTCAAGGACTACATTAAAAG3'), we amplified, on monkey Vero genomic DNA, a 400 bp monkey ISG-54K promoter fragment (see also Figure 1B). For pAISG54-1-CAT, this SacI-XbaI monkey ISG-54K promoter PCR fragment was subcloned into pCAT-Basic (SacI, XbaI; Promega, Madison, WI).
19. J.A.R. Bluyssen, R.J. Vlietstra, A. van der Made, J. Trapman, Eur. J. Biochem. 220, 395-402 (1994).
20. We prepared and analyzed lysates as described (19). The appropriate pHISG54-CAT or pAISG54-CAT reporter gene construct as well as the expression plasmids for p48, p91, and p113 (24), were used in 5 μg/dish. After overnight incubation with the precipitate, incubation was continued in the absence or the presence of HuIFN-α or HuIFN-γ (500 IU/ml) for an additional 24 h. Each transfection (in duplicate) was independently done at least three times.
21. D.S. Kessler, D.E. Levy, J.E. Darnell Jr., Proc. Natl. Acad. Sci. USA 85, 8521 (1988).
22. We prepared and analyzed protein extracts from CHO cells (treated for 0, 2, 5, and 24 h with MuIFN-α6; 50 IU/ml) in gel band shift assays as described (6,7) using as probe a ^{32}P-labeled, double stranded oligonucleotide: ISG-54K (ISRE I-ISRE I)-oligo (5'GATCATCGGTTTCAATTTCTGGTTTCAATTTCTCTT3'), ISG-15K (ISRE)-oligo (5'GATCGGCTTCAGTTTCGGTTTCCCTTTCCCGAG3').
23. M. van Heuvel et al., J. Cell. Biochem. 38, 269 (1988).
24. Full-length cDNAs encoding p48, p91 and p113 were cloned into the mammalian expression vector pRc/CMV (Invitrogen).
25. M. Muller et al., EMBO J. 12, 4221 (1993).

CHAPTER 6

CONCLUDING REMARKS

CONCLUDING REMARKS

IFN stimulated genes provide an attractive system to examine in which way transcription regulation in the cell nucleus is mediated through occupation of a cell surface receptor by its polypeptide ligand. In addition, because of the wide variety of biological effects of IFNs it is interesting to identify the function of IFN-regulated genes. Sofar, a dozen directly IFN-α/β regulated target genes have been identified (reviewed by Pellegrini and Schindler, 1993). All these genes respond rapidly to the inductive signal with an increase in transcriptional initiation. In the promoter region they contain at least one sequence closely related to the ISRE consensus sequence AGTTTCNNTTTC(C/T).

In this thesis, studies on the regulation of expression of the IFN-stimulated ISG-54K/ISG-56K gene family are reported. The hamster ISG-54K gene and the mouse ISG-54K and ISG-56K1 genes were characterized. The genes are identically organized: two exons interrupted by one intron (varying from 2.5 to 5 kb in length). The first exon is very small (80-90 bp) and only provides the initiating ATG codon of the open reading frame. The transcriptional response of the genes to IFN-α/β is shows a comparable pattern and is very rapid. In addition to the mouse ISG-56K1 gene, (parts of) two closely related genes (ISG-56K2 and -56K3) were isolated. Both fragments contain an ISG-56K1-related open reading frame. However, as yet we were unable to isolate the presumed first exon of -56K2 and -56K3, and neither could we detect expression of the genes.

6.1.1 Function of the 54K/56K proteins

The structure of the hamster and mouse ISG-54K and ISG-56K polypeptides is quite similar (Chapter 3 and 4), especially in the amino-terminal half of the molecules (50 % homology at the amino acid level). This is also true for the human ISG-54K and the related ISG-56K protein (see Chapter 1.2.1). The structural similarity suggests that they might have a comparable biological activity. Concerning the function of these proteins no direct clues have been found. Indirect evidence points to a role in the growth inhibitory pathway of IFNs (van Heuvel et al., 1988), as well as in the antiviral effects of IFNs (Wathelet et al., 1988b). To obtain better insight in the physiological role of ISG-54K and ISG-56K, several experimental approaches can be proposed. A first approach would be to study the cellular and tissue localization of the proteins. In a first attempt to do so (in coolaboration with Dr. Boersma (MBL/TNO, Rijswijk) rabbit polyclonal antibodies against three ISG-54K peptides with predicted immunogenic potential and specificity were developed. Two out of three antisera obtained recognized an ISG-54K bacterial read

through product containing the peptide sequence. These antisera were used in Western blotting and immunocytochemical experiments on COS cells transiently transfected with a mouse ISG-54K eukaryotic expression vector. On this material specific immuno reactivity was not obtained, however (data not shown).

Several possibilities might be considered to further elucidate the functions of the ISG-54K and -56K proteins. First of all, it would be of interest to construct cell lines, which produce the ISG-54K or ISG-56K protein constitutively or in a regulated fashion, and to monitor the growth rate and resistance to viral infection of these cell lines.

To investigate general ISG-54K and ISG-56K1 function in vivo, it can be considered to generate knock-out mouse strains by homologous recombination. If we asume that the ISG-56K2 and -56K3 are pseudogenes, two different situations can occur. (i) ISG-54K and ISG-56K1 have a similar function and expression pattern, implying that a double knock-out mouse would be necessary to result in a phenotype. (ii) ISG-54K and ISG-56K1 have a different function or expression pattern, implying that single knock-out mice might be informative. Because the ISG-54K/56K genes constitute only one set of a multitude of IFN-regulated genes, it is quite well possible that the reulting phenotype will not be abnormal. In that case, crossing with mouse strains, in which other IFN-regulated genes are inactivated, might provide additional answers.

The situation would be even more complex if ISG-56K2 and 56K3 are both functionally identical to ISG-54K and ISG-56K1. Because it will be impossible to generate mice in which all four genes are inactivated, an in vivo approach would only be feasable if ISG-56K2 and ISG-56K3 have an expression pattern which is completely different from that of ISG-54K and ISG-56K1 (which is not impossible).

6.1.2 Cooperativity between two ISREs

In the promoters of the ISG-54K and ISG-56K genes, approximately 100 bp upstream of the transcriptional start site, two ISRE sequences can be found (ISRE-I and ISRE-II). In Figure 1 the ISRE consensus sequence and the ISRE regions of the ISG-54K/ISG-56K genes from different species, as presently known, are compared (Chapters 3, 4 and 5: Bluyssen et al., 1994; Bluyssen, unpublished). In all species examined, the organization is similar, although there are small differences. Mutation analysis of the hamster ISG-54K (Chapter 3) and mouse ISG-56K1 (Chapter 4) promoters, indicates that the two ISREs can function separately when induced by IFN-α, however, for a maximal response both elements are necessary. The monkey and human ISG-54K/56K promoter both contain two strong ISREs, as is the case for the mouse ISG-56K1 promoter.

		ISRE-II		ISRE-I	
CONSENSUS ISRE		AGTTTCNNTTTCY		AGTTTCNNTTTCY	
ISG-54K	HA	-110 AGTTTTACTTTCT -98	-97	GGTTTCAATTTCT	-85
ISG-54K	MU	-110 AGTGTTACTTTCT -98	-97	GGTTTCAATTTCT	-85
ISG-54K	HU	-112 AATTTCACTTTCT -100	-99	AGTTTCACTTTCC	-87
ISG-54K	MO	-113 AGTTTCACTTTCT -101	-100	AGTTTCACTTTCT	-88
ISG-56K1	MU	-103 AGTTTCACTTTCC -91	-84	AGTTTCAGTTTCT	-72
ISG-56K	HU	-117 AGTTTCACTTTCC -105	-104	CCTTTCGGTTTCC	-92
ISG-56K	MO	-117 ACTTTCACTTTCT -105	-104	CGTTTCGGTTTCC	-92

Figure 1. Structural comparison of the ISRE consensus sequence with the ISRE regions of the ISG-54K/ISG56K genes from different species. HA, Hamster; HU, Human; MO, Monkey; MU, Murine. (N) denotes a random base; (Y) a pyrimidine.

However, the hamster ISG-54K ISRE-II is weak. The structure of the mouse ISG-54K ISRE region differs at only one position from that of the hamster homolog, which results in an (almost) completely inactive ISRE-II. Nevertheless, the mouse ISG-54K gene is still highly inducible with IFN (Chapter 4). This indicates that, in order to obtain physiologically relevant ISG-54K levels, one (strong) ISRE can be sufficient.

When we increased the spacing between the two ISREs in the hamster ISG-54K promoter to 5 to 10 bp only a small effect on the cooperativity between the two elements was observed (Chapter 3). This finding indicates that the 6 bp distance between the ISREs in the mouse ISG-56K1 promoter is of minor importance. To complete these studies, it would be of interest to increase the distance between the ISREs even further, and then investigate the effect on promoter activity. Furthermore, inserting fragments of different length between the two ISREs and the transcription start site might indicate whether or not the ISREs indeed need to be positioned in the proximal promoter region, as almost without exception is observed in IFN-regulated genes. Sofar, this subject has not been studied in detail. In a pilot study we observed that insertion of a 750 bp fragment in the hamster ISG-54K wt promoter, downstream of the ISREs resulted in its almost complete

inactivation (Bluyssen, unpublished).

6.1.3 IFN-α/β-regulated DNA-binding factors

Many proteins interact with ISRE-like sequences (Chapter 1). Some of these factors (like IRF-1) stimulate transcription, whereas others inhibit or do not affect at all the transcription rate of ISRE-containing promoters. For the ISGF3 complex an increasing amount of evidence is provided that it mediates the IFN-α-induced expression of target genes (Kessler et al., 1988; Levy et al., 1989). Several recently performed experiments support this point. In CHO cells ISGF3 was found to be activated within 2 h, reaching a maximum level approx. 5 h after IFN-α treatment and remaining high for at least 24 h (Chapter 5). In contrast, in one of the used HeLa cell strain, although rapid activation occurred, ISGF3 was no longer present 8 h after initiation of IFN-α treatment (Levy et al., 1988; data not shown). Remarkably, in both cell types ISGF3 presence correlated exactly with the kinetics of appearance of the ISG-54K messenger (Chapter 3 and Larner et al., 1984). Because sofar only for the ISGF3 complex such a correlation was observed, it is even possible that ISGF3 might be the sole factor in IFN-α/β regulation of ISGs.

It seems likely, that in case of one functional ISRE the same factors are involved as in the case of two cooperating ISREs, but this needs to be further substantiated. Therefore, it is of importance to study in more detail the role of different ISRE-binding transcription factors in ISG-54K/ISG-56K expression upon IFN-α treatment. However, a drawback of the ISG-54K/ISG-56K system is that it is very difficult to show ISRE-protein interactions in standard in vitro bandshift assays (Chapter 5, and data not shown). The ISG-15K ISRE (AGTTTCGGTTTCC), on the contrary, has a very high affinity for these factors (Chapter 5, and data not shown). By changing the two central nucleotides, AA (in hamster ISG-54K ISRE-I) to GG (as in the ISG-15K ISRE), which are supposedly of minor importance for ISRE activity, the stability of the in vitro protein-DNA complexes was increased. Although not optimal, this seems to be the only possibility to find out more about the proteins interacting with ISG-54K/56K ISRE sequences.

Numerous recent publications clearly show that ISGF3 activity can be regulated by specific protein tyrosine kinases (Tyk2, JAK1) (Velazquez et al., 1992; Muller et al., 1993; Shuai et al., 1993b; Silvennoinen et al., 1993b). Much less is known about protein tyrosine phosphatases, which obviously must be involved in the inactivation of the ISGF3 complex. The prolonged expression of the active ISGF3 complex in CHO cells indicates that important tyrosine phosphatases, which are able to inactivate the complex, are lacking in these cells. Therefore, the CHO system would serve as an excelent model to study

these specific phosphatases. cDNAs encoding phosphatases can be transfected into CHO cells and, in co-transfection experiments, their effect on ISG-54K promoter activity can be monitored.

6.1.4 Cross-talk between IFN-α/β and IFN-γ signaltransduction pathways

In Chapter 5 we studied the role of the ISRE sequences in IFN-γ regulation of the ISG-54K gene. Evidence was presented, showing that the ISRE sequence is not only a target for IFN-α/β activated transcription factors. In case of IFN-γ activation, we proposed that an ISGF3-like complex binds to the ISRE, containing p48, p91 and a third component X (possibly a novel, IFN-γ specific member of the p113/p91 gene family). To obtain more evidence for the IFN-γ induced, ISRE-mediated pathway, a system has to be developed in which binding of this IFN-γ-specific complex to the ISRE and/or gene activating via the complex can be monitored.

For binding studies, either the ISG-54K/ISG-56K system has to be optimized, as discussed above, or ISRE sequences of other genes, which are similarly activated by IFN-γ have to be identified. In this respect, studying the role of IFN-γ on ISG-15K expression might be a possibility. Another candidate gene would be the IP-10 gene. An IFN-γ-inducible IP-10 unidentified ISRE binding complex, which involves activation of preexisting cellular factor(s) (Ohmori and Hamilton, 1993), can be detected as early as 30 min after IFN-γ treatment. Eventually, factor X has to be isolated, which will enable analysis of its precise role.

An alternative approach to identify "factor X" would be to isolate cDNAs which encode proteins structurally related to the p84/p91/p113 gene family. It is expected that this family is larger than the two members identified sofar. If new cDNAs become available, their expression can be tested in cell lines responsive to IFN-γ via the ISRE. In addition, they can be used in co-transfection studies, similarly as presented here in Chapter 5.

We have not as yet studied the role of p84 in the IFN-γ-induced ISRE-mediated pathway. Although phosphorylated by IFN-γ, p84 is not involved in the IFN-γ signal transduction pathway via the GAS element (Shuai et al., 1993a). However, p84 might play a role in the IFN-γ-ISRE signalling route. In the model proposed, a prerequisite would be that it can interact with factor X and take over the function of p91.

Further complexity of the functional overlap between IFN-α/β and IFN-γ regulated genes comes from evidence that GAS elements cannot only be used in genes inducible by IFN-γ, but also in genes induced by IFN-α (Khan et al., 1993). Therefore, in Figure 2 we

propose a further extension of the model of IFN-regulated gene expression we presented in Chapter 5. Assembly of activated p91 (or p84) and p113 (IFN-α-specific) into the ISGF3α component after IFN-α treatment, allows association with ISGF3γ (p48), which directs the complex to the ISRE. In case of IFN-γ activation of the ISRE route, activated p91 (or possibly p84) assembles with X (IFN-γ-specific, possibly p113-related), allowing subsequent association with p48. In this way the complex is directed to the ISRE.

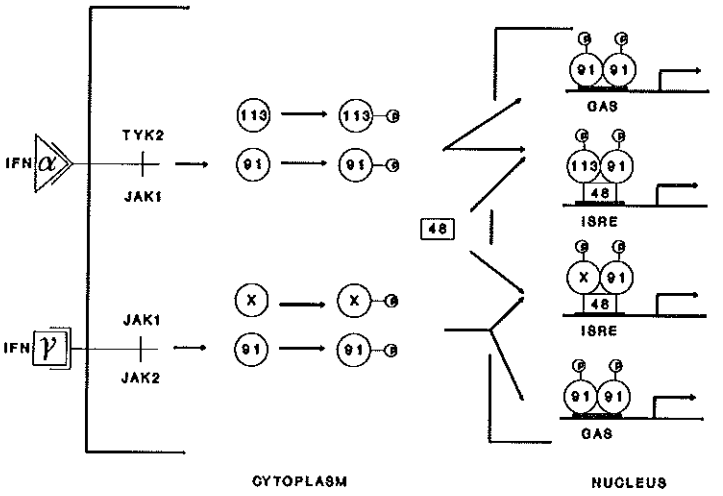

Figure 2. Pathways of signal transduction and transcriptional activation in cells treated with IFN-α/β or IFN-γ.

In IFN-γ as well as in IFN-α/β-treated cells, activated p91 can bind directly to the GAS (Shuai et al., 1992; Khan et al., 1993). Similar to the ISRE binding complexes, additional specificity-determining subunits are thought to be present in these GAS-binding complexes (Fu and Zhang, 1993; Larner et al., 1993; Ruff-Jamison et al., 1993; Sadowski et al., 1993; Silvennoinen etal., 1993a). This model supports the idea that a specificity-determining subunit combined with a common p91 subunit directs the complex to the appropiate target genes. Identification of these specificity-determining subunits, which could belong to the same family, should shed more light on the more and more complex IFN signal transduction pathways.

REFERENCES

Bluyssen, H.A.R., Vlietstra, R.J., van der Made, A.C.J., and Trapman, J. (1994) The ISG-54K promoter contains two adjacent, functional ISREs of different strength, which act synergistically for maximal IFN-α inducibility. Eur. J. Biochem. 220, 395-402.

Fu, X.-Y., and Zhang, J.-J. (1993) Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the c-fos gene promoter. Cell 74: 1135-1145.

Van Heuvel, M., Govaert-Siemerink, M., Bosveld, I.J., Zwarthoff, E.C., and Trapman, J. (1988) IFN producing CHO cell lines are resistant to the antiproliferative activity of IFN: A correlation with gene expression. J. Cell. Biochem. 38: 269-278.

Kessler, D.S., Levy, D.E., and Darnell, J.E., Jr. (1988) Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. Proc. Natl. Acad. Sci. USA 85: 8521-8525.

Khan, K.D., Shuai, K., Lindwall, G., Maher, S.E., Darnell, J.E., Jr., and Bothwell, A.L.M. (1993) Induction of the Ly-6A/E gene by interferon α/β and γ requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds. Proc. Natl. Acad. Sci. USA 90: 6806-6810.

Larner, A.C., Jonak, G., Cheng, Y.S.E., Korant, B.D., Knight, E., and Darnell, J.E., Jr. (1984) Transcriptional induction of two genes in human cells by β interferon. Proc. Natl. Acad. Sci. USA 81: 6733-6737.

Larner, A.C., David, M., Feldman, G.M., Igarashi, K.-I., Hackett, R.H., Webb, D.S.A., Sweitzer, M., Petricoin III, E.F., and Finbloom, D.S. (1993) Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. Science 261: 1730-1733.

Levy, D.E., Kessler, D.S., Pine, R., Reich, N., and Darnell, J.E. (1988) Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative control. Genes Dev. 2: 383-393.

Levy, D.E., Kessler, D.S., Pine, R., and Darnell, J.E., Jr. (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes Dev. 3: 1362-1371.

Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C., Pellegrini, S., Wilks, A.F., Ihle, J.N., Stark, R.S., and Kerr, I.M. (1993) The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. Nature 366: 129-135.

Ohmori, Y., and Hamilton T.A. (1993) Cooperative interaction between interferon (IFN) stimulus response element and kB sequence motifs controls IFN-γ and lipopolysaccharide-stimulated transcription from the murine IP-10 promoter. J. Biol. Chem. 268: 6677-6688.

Pellegrini, S., and Schindler, C. (1993) Early events in signalling by interferons. TIBS 18: 338-342.

Ruff-Jamison, S., Chen, K., and Cohen, S. (1993) Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science 261: 1733-1736.

Sadowski, H.B., Shuai, K., Darnell, J.E., Jr., and Gilman, M.Z. (1993) A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 261: 1739-1744.

Shuai, K., Schindler, C., Prezioso, V.R., and Darnell, J.E., Jr. (1992) Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91 kDa DNA binding protein. Science 258: 1808-1812.

Shuai, K., Stark, G.R., Kerr, I.M., and Darnell, J.E., Jr. (1993a). A single phosphotyrosine residue of STAT91 required for gene activation by interferon-γ. Science 261: 1744-1746.

Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z., and Darnell, J.E., Jr. (1993b) Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature 366: 580-583.

Silvennoinen, O., Schindler, C., Schlessinger, J., and Levy, D.E. (1993a) Ras-independent growth factor signalling by transcription factor tyrosine phosphorylation. Science 261: 1736-1739.

Silvennoinen, O., Ihle, J.N., Schlessinger, J., and Levy D.E. (1993b) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 366: 583-585.

Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon α/β signalling pathway. Cell 70: 313-322.

Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988) Regulation of two interferon-inducible human genes by interferon, poly(rI).poly(rC) and virus. Eur. J. Biochem. 174, 323-329.

SUMMARY

IFNs mediate a wide variety of effects on target cells. Therefore, they are an interesting system to study the mechanisms of action of cytokines. Chapter 2 describes the molecular characterization of a cluster of four Ha IFN-α genes (Ha IFNα-A1, -A2, -A3 and -A4), which are all located on a 30 kb genomic segment. This IFN-α gene cluster could be assigned to hamster chromosome 2q, indicating that the Ha IFN-α genes, like the Hu and MuIFN-α gene family, are tightly linked in the genome. The Ha IFNα-A1 and -A3 genes both contain proper open reading frames (ORF), encoding proteins of 190 and 189 amino acids (including a signal peptide of 23 amino acids), respectively. A1 and A3 showed 90% homology in this region. The IFNα-A2 and -A4 genes both showed reading frames interrupted by stop codons, which would result in the synthesis of truncated IFN proteins. Structural comparison of Ha-A1 and A3, Mu and Hu IFN-α proteins, indicated conservation of essential domains. The same was true for the promoter region of the gene, involved in the virus inducible expression (virus responsive element or VRE; a 46 bp fragment extending from -109 to -64 relative to the cap site) of IFN-α genes. However, the VRE sequence suggests that the A1 and A3 genes are expressed at a low level. Transient expression of the Ha IFN-α A1 and -A3 genes in COS cells showed that both genes gave rise to protein products with antiviral properties on hamster CHO cells. In addition, A1 revealed high antiviral activity on mouse L929 cells.

To study the transcriptional response to IFN-α, the IFN-α regulated ISG-54K/ISG-56K gene family was characterized. In Chapter 3 the hamster ISG-54K gene (which is activated in hamster CHO-12 cells by IFN-α at least 40-fold) was isolated and the promoter region was characterized in detail. Sequence analysis revealed the presence of two motifs, closely related to the Interferon Stimulated Response Element (ISRE) consensus sequence [AGTTTCNNTTTC(C/T)]. The putative ISRE-I sequence (GGTTTCAATTTCT) is located at position -97 to -85; ISRE-II (AGTTTTACTTTCT), which differs at three positions from ISRE-I, is found directly upstream of ISRE-I at position -110 to -98. In a transient transfection assay in CHO cells it was shown that both the -97/-85 (ISRE-I) and the -110/-98 (ISRE-II) segment were needed for optimal IFN induction of the ISG-54K promoter. ISRE-I is seven-fold more active than ISRE-II; ISRE-I and ISRE-II act synergistically for maximal IFN-inducibility. The mechanism of synergism is most probably an indirect interaction between transcription factors binding to the ISREs, because an increase in the spacial arrangement of the two ISREs did not result in a substantial decrease of promoter activity.

In Chapter 4 the mouse ISG-54K/-56K gene family, which is composed of at least four members (ISG-54K, ISG-56K1, -56K2 and -56K3), was isolated and characterized. The ISG-54K and ISG-56K1 genes show a similar organization. Both are composed of a very small first exon and a second exon, which contains the complete open reading frame, except for the ATG start codon and the first two nucleotides of the second codon. In both genes, the two exons are separated by a small intron (2.5 kb and 5 kb, respectively). The ISG-54K open reading frame encodes a protein of 473 amino acids; the ISG-56K1 open reading frame encodes 464 amino acids. The proteins are structuraly related (40%), however a possible function until now is unknown. Expression of both genes is rapidly induced by IFN-α (within 2 h). The mouse ISG-54K promoter region shows a similar ISRE organization as compared to the hamster ISG-54K promoter (ISRE-I: GGTTTCAATTTCT, and ISRE-II: AGTGTTACTTTCT). However, the mouse promoter is 70% less active than the hamster promoter. It turned out that ISRE-II is hardly active, due to the G at position 4, which is a T in the hamster ISG-54K ISRE-II and in the ISRE consensus sequence. The ISG-56K1 promoter region contains at a similar position two functional ISREs of identical strength (ISRE-I: AGTTTCAGTTTCT, and ISRE-II: AGTTTCACTTTCC). In the ISG-56K1 promoter, the two ISRE sequences are separated by six bp. In addition to the ISG-56K1 gene, (parts) of two closely related genes (K2 and K3) were isolated. Both isolated fragments contain an ISG-56K1-related open reading frame. However, we were unable to isolate the presumed first exon of K2 and K3, neither could we show expression of the genes, sofar. Together, the ISG-54, 56K1, K2 and K3 genes could all be assigned to the D1 region of mouse chromosome 19, suggesting a tight clustering.

In Chapter 5 the ISG-54K gene expression was shown to be IFN-γ regulated. This IFN-γ induction was found to be mediated through the ISRE sequence. Co-transfection of Vero cells with expression vectors for the 48-kDa interferon stimulated gene factor3 (ISGF3) protein (p48), the 91-kDa (ISGF3) protein (p91) and the 113-kDa ISGF3 protein (p113) in the presence of IFN-γ, showed a role of p48 and p91, but not p113 in ISG-54K promoter activation in the IFN-γ response pathway. In contrast, IFN-α stimulation was maximal in the presence of all three factors. The results obtained implicate that, in addition to the IFN-γ activated sequence (GAS) mediated pathway, IFN-γ can regulate gene expression via activation of p48, p91 and a proposed third factor, which interact in an ISGF3-like complex with the ISRE sequence. Our findings could explain the mechanism of regulation of expression of overlapping set of genes by IFN-α/β and IFN-γ.

SAMENVATTING

Interactie van IFN met de cel resulteert in een breed scala van biologische effecten. Om die reden is IFN een geschikt systeem voor het bestuderen van het werkingsmechanisme van cytokines in het algemeen. Hoofdstuk 2 beschrijft de molekulaire karakterisering van een kluster van vier hamster IFN-α genen (Ha IFNα-A1, -A2, -A3 en -A4), gezamelijk gelegen op een genomisch segment ter grootte van 30 kilobasen. Dit cluster van IFN-α genen kon gelocaliseerd worden op hamster chromosoom 2q. Dit houdt in dat, net als de humane en muize IFN-α genen, de hamster IFN-α genen dicht bij elkaar liggen in het genoom. De Ha IFNα-A1 en -A3 genen bevatten beiden een volledig open leesraam, respectievelijk koderend voor een eiwit van 190 en 189 aminozuren (een signaalpeptide van 23 aminozuren meegerekend). A1 en A3 vertonen 90% homologie in dit gebied. De IFNα-A2 en -A4 genen bevatten beiden een open leesraam dat onderbroken wordt door stopkodons, wat de synthese van getrunceerde IFN eiwitten tot gevolg heeft. Uit de structurele vergelijking van Ha -A1 en A3 met muize en humane IFN-α eiwitten, blijkt dat essentiele domeinen geconserveerd zijn. Ditzelfde geldt voor het bij de virus-geinduceerde expressie betrokken deel van de IFN-α promoter (virus responsief element of VRE; een 46 baseparen fragment gelegen op positie -109 tot -64 ten opzichte van de transcriptie start site). Structureel gezien doet de VRE van -A1 en -A3 echter vermoeden dat beide genen laag tot expressie komen. Kortdurende expressie van de hamster -A1 en -A3 genen in COS cellen liet verder zien dat deze beide genen eiwitten kunnen produceren met antivirale activiteiten op hamster CHO cellen. Daarnaast bleek het -A1 eiwit ook een hoge antivirale activiteit te hebben op muize L929 cellen.

Om de door IFN-α-geinduceerde gen transcriptie te kunnen bestuderen werd de door IFN-α-gereguleerde ISG-54K/ISG-56K genfamilie geisoleerd en gekarakteriseerd. In Hoofdstuk 3 wordt de isolatie van het hamster ISG-54K gen (dat in hamster CHO-12 cellen, behandeld met IFN-α, tenminste 40-maal geactiveerd wordt) en een gedetaileerde karakterizatie van de promoter van dit gen beschreven. Structuuranalyse onthulde de aanwezigheid van twee motieven, die sterk lijken op de consensus sekwentie van het Interferon-gestimuleerde Responsieve Element [ISRE; AGTTTCNNTTTC(C/T)]. De vermeende ISRE-I (GGTTTCAATTTCT) sekwentie ligt in het gebied van -97 to -85; direct aansluitend, van -110 tot -98, is de ISRE-II (AGTTTTACTTTCT) sekwentie gelegen. Deze wijkt op drie posities af van ISRE-I. Met behulp van een kortdurende expressie methode in CHO cellen werd aangetoond dat zowel het -97/-85 (ISRE-I) als het -110/-98 (ISRE-II)

segment noodzakelijk zijn voor optimale IFN inductie van de hamster ISG-54K promoter. ISRE-I is daarbij 7-maal actiever dan ISRE-II; ISRE-I en ISRE-II werken synergistisch voor een maximale inductie door IFN. Omdat een vergroting in afstand tussen de twee ISRE segmenten niet leidde tot een substantiele daling van de activiteit van de promoter, omvat het mechanisme van synergisme hoogstwaarschijnlijk een indirecte interactie tussen transcriptiefactoren die binden aan beide ISRE elementen.

In Hoofdstuk 4 wordt de isolatie en karakterisering van de muize ISG-54K/ISG-56K genfamilie beschreven. Deze familie bestaant uit tenminste vier leden (ISG-54K, ISG-56K1, -56K2 en -56K3). De ISG-54K en ISG-56K1 genen vertonen een vergelijkbare opbouw. Beiden bestaan uit een klein eerste exon en een tweede exon, dat vrijwel het gehele open leesraam bevat (op het ATG startkodon en de eerste 2 nucleotiden van het tweede kodon na). De exonen worden gescheiden door een klein intron (respektievelijk 2.5 en 5 kilobasen groot). Het ISG-54K open leesraam kodeert voor een eiwit van 473 aminozuren; het ISG-56K1 open leesraam kodeert voor een eiwit van 464 aminozuren. Beide eiwitten zijn structureel verwant (40%), hun functie echter is onbekend. De expressie van beide genen wordt snel geinduceerd door IFN-α (binnen 2 uur). De muize ISG-54K promoter vertoont een ISRE opbouw die vergelijkbaar is met die van de hamster ISG-54K promoter (ISRE-I: GGTTTCAATTTCT, en ISRE-II: AGTGTTACTTTCT). De muize promoter is echter 70% minder actief dan de hamster promoter. Het bleek dat ISRE-II vrijwel niet actief was, als gevolg van de G op positie 4. Op deze plaats is in zowel de hamster ISG-54K ISRE-II als in de ISRE consensus sekwentie een T aanwezig. Het ISG-56K1 promoter gebied bevat twee functionele ISRE segmenten (ISRE-I: AGTTTCAGTTTCT, en ISRE-II: AGTTTCACTTTCC) van gelijke sterkte, op een overeenkomstige positie als in de ISG-54K promoter. In de ISG-56K1 promoter zijn echter de twee ISRE elementen gescheiden van elkaar door zes baseparen. Naast het ISG-56K1 gen zijn tevens (delen van) twee verwante genen (ISG-56K2 en -56K3) geisoleerd. De beide geisoleerde fragmenten bevatten een aan ISG-56K1 verwant open leesraam. Tot nu toe waren we echter niet in staat om het vermeende eerste exon te isoleren van -56K2 en -56K3, evenmin konden we expressie van de genen aantonen. De ISG-54K, ISG-56K1, -56K2 en -56K3 genen konden allen gelocaliseerd worden op muize chromosoom 19D1. Dit suggereert dat ze geclusterd voorkomen op het genoom.

In Hoofdstuk 5 wordt beschreven dat de expressie van ISG-54K gereguleerd wordt door IFN-γ. Voor deze inductie door IFN-γ bleek de ISRE sekwentie van belang te zijn. Co-transfectie van Vero cellen met expressievectoren voor het 48-kiloDalton (kDa) interferon gestimuleerde gen factor 3 (ISGF3) eiwit (p48), het 91-kDa (ISGF3) eiwit (p91) en het

113-kDa (ISGF3) eiwit (p113) in aanwezigheid van IFN-γ, liet een duidelijke rol voor p48 en p91, maar niet voor p113, zien in de activiteit van de ISG-54K promoter in de IFN-γ signaaltransductie route. Voor maximale stimulatie door IFN-α was de aanwezigheid van alle drie de factoren noodzakelijk. Deze resultaten suggereren dat naast de route die gebruik maakt van de IFN-γ geactiveerde sekwentie (GAS), er een tweede route is waarin IFN-γ gen expressie kan reguleren via activatie van p48, p91 en een vermeende derde factor, die als een ISGF3-achtig complex binden aan de ISRE. Onze bevindingen kunnen het mechanisme van regulatie van overlappende genen door IFN-α/β en IFN-γ verklaren.

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