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# Regulation of IFN-α Expression

by

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# Regulation of IFN-α Expression

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## Abstract

The biology of interferon-alpha (IFN-α) proteins has best been studied for their induced expression in response to virus infections and their antiviral activities. IFN-α proteins also have potent antiproliferative activities and as such, they are involved in normal cell growth regulation. There is mounting evidence that loss of function or dysregulation of the IFN system may contribute to the pathogenesis of a number of human illnesses. In order to better study the expression of IFN-α genes, a PCR-based approach using consensus IFN-α primers was developed which can detect all 14 members of the IFN-α gene family. Using this approach, IFN-α subtypes were observed to be differentially regulated in response to particular forms of stimuli and in different cell types. For example, three IFN-α subtypes, IFNA1, IFNA2 and IFNA8, accounted for over 80% of the IFN-α mRNA induced in the promonocytic cell line, U937, but less than 50% of the IFN-α mRNA in peripheral blood mononuclear cells (PBMCs). Further studies on the regulation of IFN-α expression in U937 cells revealed that there are distinct forms of IFN-α inducibility in response to particular combinations of "priming" agents and specific inducers. The induced production of IFN-α proteins in U937 cells stimulated with lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) was strictly dependent on pretreatment or "priming" of the cells with IFN-α or IFN-γ. Finally, IFN-α induction in U937 cells was determined to require the activity of the dsRNA-dependent protein kinase, PKR. Loss-of-function phenotypes in U937 stable transformant cell clones were generated by overexpression of an antisense PKR transcript or a trans-dominant negative PKR gene. Both types of PKR-deficient cells exhibited impaired induction of IFN-α and IFN-β genes, as well as reduced abilities to restrict virus replication. In summary, these results indicate that IFN-α genes are differentially responsive to particular forms of stimuli and that PKR plays a central role in regulating the expression of the IFN-α gene family.

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# List of Abbreviations

| | |
|---|---|
| 2-AP | 2-aminopurine |
| AIDS | acquired immunodeficiency syndrome |
| CMV | cytomegalovirus |
| cpm | counts per minute |
| DNA | deoxyribonucleic acid |
| dsRNA | double-stranded RNA |
| eIF-2α | eukaryotic initiation factor-2-alpha |
| EBV | Epstein-Barr virus |
| EMCV | encephalomyocarditis virus |
| FCS | fetal calf serum |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| HIV | human immunodeficiency virus |
| HSV | herpes simplex virus |
| IFN | interferon |
| Ig | immunoglobulin |
| IFNAR | interferon-alpha receptor |
| IκB | inhibitor of NF-κB |
| IL | interleukin |
| IRF | interferon regulatory factor |
| ISRE | interferon-stimulated response element |
| ISG | interferon-stimulated gene |
| ISGF3 | interferon-stimulated gene factor-3 |
| LPS | lipopolysaccharide |
| M-CSF | macrophage colony-stimulating factor |
| MHC | major histocompatibility antigen |
| mRNA | messenger ribonucleic acid |
| NF-κB | nuclear factor-kappa B |
| NIPC | natural interferon-producing cell |
| NRD | negative regulatory domain |
| PBS | phosphate buffered saline |
| PBMC | peripheral blood mononuclear cells |

# List of Abbreviations

| | |
|---|---|
| PDGF | platelet-derived growth factor |
| PCR | polymerase chain reaction |
| PKR | dsRNA-dependent protein kinase |
| PMA | phorbol 12-myristate 13-acetate |
| PRD | positive regulatory domain |
| RNA | ribonucleic acid |
| STAT | signal transducer and activator of transcription |
| SV40 | simian virus 40 |
| TCID$_{50}$ | median tissue culture infective dose |
| TGF-$\beta$ | transforming growth factor-beta |
| TNF | tumour necrosis factor |
| VRE | virus responsive element |
| VSV | vesicular stomatitis virus |

# Acknowledgements

# Chapter One

## Introduction

# Interferons and Their Actions

Interferons (IFNs) were named for their ability as soluble factors to inhibit or "interfere" with viral replication (89). Studies on IFNs over the decades have led to their characterization as prototypical molecules for the large group of secreted proteins referred to as cytokines (143, 172). Cytokines facilitate much of the cell-to-cell communication required for regulating diverse biological processes including embryogenesis, haematopoiesis, immunity, inflammation and homeostasis, all of which also involve the actions of IFNs (99, 172). IFN actions are initiated by their binding to specific membrane receptors, which stimulates a signaling cascade and leads to the induced expression of IFN-stimulated genes (ISGs) (30, 191). It is widely believed that most IFN actions are mediated through the biochemical activities of ISG-encoded proteins. However, the production of IFN proteins themselves is tightly regulated, such that high levels of IFN expression only occurs in response to appropriate stimuli such as virus infections. Therefore, IFN-specific actions rely as much on the factors which regulate the expression of IFN genes themselves, as it does on the subsequent cellular responses to IFN proteins. This introductory chapter focuses on providing an overview of the biological significance and regulation of Type I IFNs, with particular emphasis on the IFN-α genes.

## A. The IFN Gene Family

Prior to the cloning of their genes, IFN proteins were classified on the basis of their primary cellular source. Accordingly, they were described as leukocyte (IFN-α), fibroblast (IFN-β), or immune (IFN-γ) IFNs. Currently, IFN-α and IFN-β are grouped together as Type I IFNs, while IFN-γ is designated as a Type II IFN (32). This nomenclature was adopted to indicate the binding specificity of each class of proteins for their respective cell surface receptors, the Type I IFN and Type II IFN receptors (42, 183). In addition, the standardized nomenclature distinguishes IFN genes by using the form IFNX, where X is an upper case letter

or number, while IFN proteins are designated with IFN-x, where x is a lower case, Greek symbol (32). For example, IFNA1 and IFNB genes encode for IFN-α1 and IFN-β proteins.

**Type I IFNs.** The human Type I IFNs comprise of over 20 genes belonging to three structurally related gene families, IFN-alpha (α), IFN-beta (β), and IFN-omega (ω). These genes share several similarities: they contain a high level of sequence homology to each other (78), they are among the few mammalian genes that lack introns, they all reside within a gene cluster on human chromosome 9 at band 9p22 (a similar Type I IFN gene cluster exists on chromosome 21 in mouse)(33, 139), and the encoded proteins of all three classes bind competitively to a common Type I IFN receptor (13). Human IFN-α1 was the first of the cytokine genes to be cloned and expressed as a recombinant protein product (134). Shortly thereafter, the existence of multiple IFN-α gene family members was revealed by analysis of additional, cross-hybridizing, complementary DNA and genomic DNA clones (60, 133). Currently, 14 functional IFN-α genes have been identified in humans, along with several pseudogenes, which encode for mature proteins 166 amino acids in length, except IFN-α2 which encodes for 165 amino acids (32, 78). The shared homologies of IFN-α subtype proteins range upwards from 70%, while the level of nucleotide sequence homology between IFN-α genes ranges between 80-100%. Two genes, IFN-α1 and IFN-α13, appear to have identical coding sequences but differ in their flanking extragenic regions. The single IFN-β gene encodes a protein of the same 166 amino acids in length but it only exhibits 15-30% amino acid sequence homology with the IFN-α proteins (173). IFN-β has a potential N-glycosylation site at position 80 and mature proteins have been shown to be glycosylated (101). In contrast, IFN-α proteins lack N-glycosylation sites but it has been proposed that O-glycosylation may account for the glycosylation detected on a subset of purified, natural IFN-α species (143). The IFN-ω gene family in humans consists of one functional gene (and seven pseudogenes), that encodes for a 172 amino acid protein with approximately 60% homology to IFN-α proteins (16). Lastly, Type I IFN genes are strongly conserved among all mammalian species examined so far. Generally, they all possess multiple IFN-α and IFN-ω genes, and a single IFN-β gene, but with

the exception of the ungulates (e.g. horses, pigs, and cattle) which possess multiple IFN-β genes (189).

**Type II IFN.** Human Type II IFN is represented by a single gene, IFN-γ, that is different from Type I IFNs in many respects. IFN-γ is a 146 amino acid protein encoded by four exons, unlike the intronless Type I IFNs. Distinct from the Type I IFN gene clusters, Type II IFN resides on chromosome 12 in humans and on chromosome 10 in mice. IFN-γ protein has no significant sequence homology with any of the Type I IFN genes. It binds only to its own cognate Type II IFN receptor (IFN-γ receptor), and exhibits no competitive binding for Type I IFN receptors (42). In addition, IFN-γ proteins exist naturally as homodimers and are only active in this form, while Type I IFN proteins exist as functional monomers (42).

The biological functions of IFN-γ are also very different from Type I IFNs. IFN-γ is primarily produced by T-lymphocytes and natural killer cells in response to antigenic or mitogenic stimuli. Unlike the Type I IFNs, IFN-γ expression is not directly inducible by virus infection or dsRNA. While IFN-γ does possess direct antiviral functions, its specific activities are generally lower than that of IFN-α or IFN-β. The best known biological roles of IFN-γ involve mediation of a wide range of immune functions that are not regulated by Type I IFNs. These characteristics have led to the generalization that IFN-α and IFN-β function more as antiviral agents in the context of innate immunity, while IFN-γ acts primarily in modulating the specific, cell-mediated immune responses.

## B. Biological activities of Type I IFNs

**Antiviral activities.** Type I IFNs are produced by cells in response to virus infection. The primary function of these secreted IFNs is to contain viral spread by protecting neighbouring cells against further infection. Type I IFNs are effective in inhibiting many types of RNA and DNA viruses and their antiviral effects can be manifested at a number of different steps in a viral life cycle including penetration, proviral integration by retroviruses, transcription, translation, viral RNA stability, assembly of progeny virus, and budding (96,

161). The antiviral activity of IFNs results from the induction of a transient but potent "antiviral state" that is mostly mediated by the *de novo* synthesis of ISG proteins. As a result, the replication of any subsequently invading viruses in IFN-treated cells is greatly inhibited or in some cases, completely abrogated. Of the numerous ISG proteins that have so far been identified, only a few have been studied in detail to elucidate the biochemical basis of their antiviral actions. A general theme that has emerged is that given ISG proteins are typically effective against only certain types of viruses. For example, three of the best-studied ISG proteins are the IFN-induced, dsRNA-dependent protein kinase (PKR), the 2'-5' oligoadenylate synthetases (2-5A synthetases), and the 2'-5' oligoadenylate-dependent ribonuclease (2-5A-dependent RNase) (their activities are discussed later, Mechanisms of IFN Action). All three enzymes are effective in inhibiting the replication of picornaviruses such as Mengo virus and encephalomyocarditis virus (EMCV) but, they seem to be ineffective against vesicular stomatitis virus (VSV) or herpes simplex virus (HSV)(20, 76, 125). Alternatively, the Mx family of ISGs are effective at inhibiting VSV, influenza, and measles viruses but, not the picornaviruses (167, 168). It would seem logical that antiviral mechanisms which target a certain aspect of a viral life cycle would only be effective against those viruses sharing that replicative step. Owing to the broad range of viruses that IFNs can inhibit, there likely exists additional antiviral pathways, possibly mediated by some of the ISG proteins whose genes have been cloned but whose biological activities are currently unknown.

The importance of IFNs in defending against viral infections *in vivo* has been demonstrated using mouse models. Inhibiting the function of different components of the IFN system in mice, typically results in increased susceptibility to infection by viruses or other microbial pathogens, as well. Earlier studies had reported that injection of neutralizing antibodies against IFNs into mice resulted in a higher replication rate of challenge virus (67). More recently, "gene knockout" mouse models have been generated containing targeted homozygous disruptions of specific genes from the IFN system. Mice lacking a functional IFN-γ receptor display increased susceptibility to vaccinia virus and lymphocytic

choriomeningitis virus as compared to wild-type mice but, they have normal susceptibility to VSV and Semliki Forest virus (86). However, mice lacking a functional Type I IFN (IFN-α/β) receptor display increased susceptibility to infection by a much wider range of viruses, including all four of the above mentioned viruses (130). Finally, mice with homozygous deletions of the IFN regulatory factor-1 (IRF-1) gene, an IFN system-associated transcription factor (discussed further in later sections), exhibit increased susceptibility to EMCV, but not to VSV or HSV (98, 118). These examples further affirm the notion that distinct antiviral pathways exist with selective efficacy for particular types of viruses.

The significance of the antiviral role of IFNs is also indicated by the many strategies which viruses possess for specifically evading IFN actions (63, 96, 174). Virus encoded mechanisms have been described which target every major regulatory step along the entire IFN pathway, starting from inhibition of IFN production, to blocking ISG transcription, and finally, to inhibiting the biochemical activities of ISG proteins (96, 174). Infection of primary monocytes with human immunodeficiency virus (HIV) results in a selective loss of IFN-α inducibility, while the inducibility of IFN-β or IFN-ω was unaltered (56). Infection by hepatitis B virus is associated with deficiencies in IFN-β production (190) and in the generation of IFN-stimulated responses (51). IFN induction of ISG transcription is also inhibited by adenovirus infection, through a mechanism that relies on the E1A viral oncoprotein (1, 69). Adenovirus, HIV, vaccinia virus, and rotavirus, each encode for unique mechanisms which suppress the kinase function of PKR (18, 31, 100, 104, 155). In addition, 2-5A synthetase activity is inhibited in HSV- and SV-40-infected cells (174). Many examples of viral strategies have also been described for evasion of other cytokine systems and host immune mechanisms (63).

**Antitumour activities.** One of the most significant discoveries in basic biology research is that certain viruses are capable of causing tumours (14). Given its known antiviral properties, it was logical to investigate whether IFNs are effective against virus-induced tumours. Indeed, IFNs were found to be highly effective in inhibiting both the formation and the growth of cancers in mice caused by oncogenic viruses such as leukemia-inducing Friend

and Rauscher viruses, polyoma virus, and Rous sarcoma virus (65, 66). In follow-up experiments designed to elucidate the mechanisms of their antitumour activities, IFNs were found to be also highly effective in treating transplantable tumours and spontaneously occurring tumours, cancers with clearly no viral component. Several distinct mechanisms have now been identified which likely mediate the antitumour activities of IFNs *in vivo* (70). Two of the better studied mechanisms include the direct growth inhibitory effects of IFNs on the tumour cells and, the enhancement of host immune functions for eliminating tumour cells (see Antiproliferative and Immunomodulatory Activities). Additional antitumour properties of Type I IFNs include their inhibitory effects on cell motility, an increasingly important aspect in the biology of cancer metastasis. As well, IFN-α has been shown to have antiangiogenic properties, that is, it can inhibit the new formation of supply blood vessels which are critically needed by a growing tumour to increase in size. It is likely that several if not all of these mechanisms work in concert to mediate the full range of antitumour actions by IFNs *in vivo*.

**Antiproliferative activities.** IFNs, along with cytokines such as transforming growth factor-beta (TGF-β) and interleukin-6 (IL-6), are among the few well-known mammalian proteins with potent antiproliferative properties (97). Reasons for studying the antigrowth activities of IFNs are not only to advance their possible therapeutic usefulness but also, to understand the normal homeostatic mechanisms involved in cell growth regulation. IFNs are effective in inhibiting the growth of transformed and nontransformed cells from many cellular backgrounds. When added to cells in tissue culture, IFN treatment typically does not result in lysis or killing of the cells but rather, it tends to reduce their overall rate of cell division. These growth inhibitory effects are usually reversible since upon removal of the IFN from the medium, the cells generally resume multiplying at their original growth rate. The antigrowth effects of Type I IFNs can be manifest at any of several steps in the cell cycle including, inhibiting the progression from $G_0/G_1$ to S phase, prolonging of the phases of the cell cycle, decreasing the rate of DNA synthesis, and lengthening of the period between mitoses (154). Some specific molecular mechanisms implicated in mediating the cell growth inhibition by

IFNs include downregulation of c-*myc* expression, a proto-oncogene (38), suppression of E2F, a cell cycle-regulator (122), and decreased phosphorylation of the retinoblastoma susceptibility gene product, a known cell-cycle regulator which also regulates E2F activity (151).

The significance of IFNs' antiproliferative actions extends beyond merely limiting tumour cell growth but also includes regulating normal growth processes for many cell types. Several models of cytokine-induced proliferation have been studied which involve a coordinate induction of Type I IFNs. In many of these cases, the induced IFNs appear to function as part of a negative feedback mechanism for limiting cell growth. For example, mitogenic stimulation of BALB/c 3T3 cells by PDGF induces IFN-β expression (54, 200). Similar induction of IFN-β occurs during terminal differentiation of human promonocytic U937 cells or mouse myeloid M1 cells (152, 175, 195). The evidence to support a negative regulatory role for the endogenously produced IFN is indicated by the further increased proliferation of the U937 and M1 cells when neutralizing antibodies against IFN-α or IFN-β are included during their differentiation period. In addition, the induction of IFN-α and IFN-β has been implicated in restricting cell growth during the differentiation of primary cultures of monocytes or bone marrow precursor cells, from mice and humans (127, 199). Alternatively, IFNs can exert the opposite effect in different cell types since the proliferation of B lymphocytes or Friend leukemic cells in response to mitogenic stimuli is enhanced by the addition of IFNs (4, 128, 175).

Owing to the many varied regulatory effects that IFNs have on cell growth, it has been hypothesized that dysregulation of the IFN system may contribute to tumorigenesis (111, 145). Consistent with this hypothesis, certain cancers are associated with chromosomal abnormalities resulting in the loss of IFN-α and IFN-β genes, or IFN-stimulated genes such as IRF-1 (34, 139, 192). This hypothesis is further supported by direct studies indicating that certain components of the IFN system may function in tumour suppressor roles, that is, inactivation of their function is associated with a tumorigenic phenotype (111). Such a role has been suggested for PKR from experiments using trans-dominant negative PKR mutant proteins which can suppress the normal function of the endogenous wildtype PKR. In two separate studies, overexpression of

different trans-dominant negative PKR genes caused similar malignant transformation of NIH 3T3 cells, and tumour formation when these cells were injected into mice (102, 124). While these experiments do not definitively prove a tumour suppressor role for PKR, they at least indicate its oncogenic potential. IRF-1 and IRF-2 are related transcription factors which have been implicated with regulating the expression of Type I IFN genes and ISGs (94, 126, 150). A tumour suppressor function for IRF-1 was suggested by its ability to suppress the oncogenesis mediated by overexpression of IRF-2, which is believed to be a specific repressor for the transcriptional activity of IRF-1 (73). Furthermore, cellular transformation by oncogenes is enhanced in cells from mice with homozygous deletions for IRF-1 (171). Lastly, an anti-oncogenic activity has been described for the Type I IFN receptor gene, IFNAR-1, since its overexpression inhibits the tumorigenic potential of the leukemic cell line, K-562 (26).

**Immunomodulatory activities.** In addition to their direct antiviral activities, the production of Type I IFNs following viral infections also modulates the functions of both arms of the immune system, the innate or non-specific immunity, and the T- and B-lymphocyte-mediated specific immunity. The major cell types that mediate innate immunity include monocytes and natural killer cells. IFN-α has long been known to enhance the cytolytic activities of both of these cell types in eliminating virally-infected cells or tumour cells (66). Type I IFNs also upregulate the expression of Major Histocompatibility (MHC) Class I antigens, which are involved in the mechanisms by which virus-infected or tumour cells are recognized by cytotoxic T-cells and natural killer cells (143). The importance of Type I IFNs in regulating natural killer cell activity is indicated by gene knockout mice which lack functional Type I IFN receptors. These mice are deficient in their IFN-α-inducible antiviral responses and they display significantly reduced natural killer activity during acute viral infections (130).

The modulation of specific immunity by Type I IFNs affects both B- and T-lymphocytes. The regulatory effects of IFNs can be manifested on the lymphocytes' immediate cellular functions or on their subsequent differentiation into cell types with specialized functions. Addition of IFN-α to bulk cultures of human PBMCs results in increased

immunoglobulin (Ig) production, specifically the IgG and IgM classes (135). Similarly, injection of mice with IFN-α results in enhanced IgG2a production, but it also leads to suppression of IgE production (47). The mechanisms underlying these actions are complex, since they represent both direct actions by IFN on B cells, and indirect actions on T cells which are important regulators of B cell functions. Enhancement of IgG2a production by IFN-α was associated with an increase in IFN-γ production by T cells, which is known to stimulate IgG2a secretion. Also, the suppression of IgE secretion by IFN-α was associated with diminished interleukin-4 (IL-4) production by T cells, known to be required for IgE production. The effects of IFN-α on the regulation of IFN-γ and IL-4 are further complicated since it may represent direct effects on their gene transcription or, on the development of the specialized T cell subsets which produce either IFN-γ or IL-4, but not both. In a separate study, IFN-α was found to favour the development of T cells with Th0 or Th1 phenotypes, which are characterized by the ability to produce IFN-γ, as opposed to a Th2 phenotype, which is associated with IL-4 production (141).

**Interaction of IFNs with other cytokine systems.** A common feature of cytokines is their propensity for crosstalk, that is, cytokine systems exert positive or negative influences on each other resulting in complex regulatory networks. Many forms of crosstalk have been described involving interactions among the different IFNs themselves and, between IFNs and other cytokine systems. Crosstalk between two cytokines can occur in a variety of ways such as by regulating each other's expression (e.g. the effects of IFN-α on IFN-γ and IL-4 expression in T-cells) or by modulating each other's biological activities. Firstly, Type I IFNs have long been known to enhance their own production in a positive feedback phenomenon generally referred to as "priming" (see later, Regulation of Type I IFN Expression). Type I IFNs can cooperate with Type II IFNs when used together to treat certain cells, resulting in a synergistic enhancement of their antiviral and antiproliferative activities (48). The basis behind this synergism likely results from a combination of factors including the synergistic stimulation of common signaling components, increased de novo synthesis of signaling components, and the

induction of overlapping sets of ISGs (87). However, the relationship between Type I and Type II IFNs is complex since IFN-γ pretreatment of other cell types results in the inhibition of IFN-α induced activities, through downregulation of Type I IFN receptors (71). With regards to other cytokines, IFNs can synergize with tumour necrosis factor (TNF) for enhanced antiviral activities (193). IFN-α or IFN-γ treatment of cells upregulates the surface expression of TNF receptors (106) and also, IFN treatment primes cells for enhanced TNF production in response to stimulation by LPS (107). Alternatively, IFNs can inhibit the expression of other cytokines. Type I IFNs inhibit interleukin-8 inducibility in hematopoietic cells (6) and in fibroblasts (138), but not in all cell types. IFN-β can inhibit IFN-γ and TNF production which may represent a mechanism underlying IFN-β's anti-inflammatory properties (7). Lastly, IFNs can modulate the activities of other cytokines indirectly through the induction of specific cytokine inhibitors. IFN-α induces the expression of the soluble interleukin-1 receptor antagonist (IL-1ra) which functionally blocks IL-1 activity (178). Also, treatment of patients and healthy volunteers with IFN-α results in an increase of soluble TNF-receptor p55 in the serum (179). Therefore, through their participation in cytokine networks, IFN actions are extended beyond their immediate cellular effects by modulating the expression and biological activities of other cytokines.

**Therapeutic use of Type I IFNs.** The diversity and potency of their biological activities suggests there is considerable potential in using IFNs to treat human illnesses. Indeed, clinical trials have demonstrated the efficacy of IFNs in the management of several disease conditions (70). These include acute viral infections, for instance, by hepatitis B and C viruses, and virus-related cancers, such as the many papillomavirus-associated neoplasms and Kaposi's sarcoma in HIV-infected patients. IFN-α has also been effective in treating several types of cancers including hematological malignancies, like hairy cell leukemia and chronic myelogenous leukemia, and solid tumours like renal cell carcinoma and, squamous and basal cell carcinomas of the skin. Significantly, IFN-β has been recently shown to be effective in treating multiple sclerosis, for which no effective treatments had previously been available (7). However,

aberrant expression of IFN-α is associated with a variety of human illnesses including Type I diabetes (52, 170), certain autoimmune diseases (147, 159), and the progression of AIDS (39, 164). In addition, some of the more severe forms of side effects resulting from IFN therapy have included autoimmune disease-like symptoms (70, 159). Therefore, the clinical usefulness of IFNs must be balanced against their potentially deleterious effects.

## C. Do the different Type I IFN proteins have distinct functions?

The different IFN-α subtypes and IFN-β proteins utilize the same Type I IFN receptor complex and induce many of the same cellular functions (e.g. antiviral and antiproliferative activities)(5, 13). It would appear, at least superficially, that there is little functional discrimination between the different Type I IFNs. However, the fact that large IFN-α and IFN-β gene families have been evolutionarily conserved in all mammalian species, would suggest that there are important biological reasons for maintaining multiple Type I IFN genes. Indeed, there is mounting experimental evidence to support the notion that the different Type I IFN proteins are functionally distinct in their actions.

**IFN-α subtypes.** Given that there are at least fourteen human IFN-α genes, it is clearly a challenge to study all of them for their biological properties. Several reports have studied sets of IFN-α subtype proteins by performing relatively straightforward side-by-side comparisons of their functional activities. While the quantitative differences between them are sometimes subtle, IFN-α subtypes are distinguishable by their overall profiles of biological characteristics for parameters such as receptor binding affinity, antiviral activity, antiproliferative activity and ability to stimulate natural killer cell activity. In addition, it quickly became apparent that the relative ratios between these biological activities were not always constant and could vary by several orders of magnitude for different IFN-αs (143). For example, while recombinant IFN-αD and IFN-αJ proteins (currently named IFN-α1 and IFN-α7, respectively) were comparable to other IFN-α proteins in their antiviral and antiproliferative activities, they were both deficient in their ability to stimulate tumour antigen expression (64). IFN-αJ was also

deficient for stimulating natural killer cell activity but interestingly, it was able to block stimulation by other IFN-α subtypes, thus demonstrating its competency in receptor binding (140). These examples of discordant biological activities may facilitate distinguishing between different IFN-α proteins, but more importantly, they suggest that distinct biological responses are signaled by particular forms of interaction between an IFN protein and the Type I IFN receptor. Consistent with this, Zoon and colleagues have studied 20 purified fractions of naturally produced human lymphoblastoid IFN-α subtype proteins. They observed that while most IFN-α components exhibited good correlation between their antiproliferative activities and receptor binding affinities, anomalous groups of IFN-α components were also identified (84). Specifically, members of the Group 3 IFN-α proteins were defined on the basis of possessing high antiproliferative activity but weak receptor binding affinity, while Group 4 IFN-α proteins possessed strong receptor binding affinity and yet, exhibited low biological activity.

There is also indirect evidence that IFN-α proteins may differ in their antiviral activities for particular viruses. A purified mixture of leukocyte-derived natural IFN-α species, IFN-αn3, was determined to possess similar specific antiviral activity as recombinant IFN-α2b (2 X $10^8$ antiviral units/mg) when titrated against VSV in a standard IFN biological assay. However, IFN-αn3 was found to be 10- to 100-fold more effective in inhibiting HIV replication than recombinant IFN-α2b, recombinant IFN-α2a, or purified natural IFN-α2 (41). The identities of the IFN-α subtypes with the greater efficacy against HIV have not yet been determined. Alternatively, some IFN-α proteins may be particularly ineffective against HIV. PBMCs produce IFN-α in response to HIV-infected cells. Interestingly, HIV-induced IFN-α activity was observed to be 20-fold less effective than equal amounts of recombinant IFN-α2b in inhibiting HIV replication (57). Furthermore, low concentrations of the HIV-induced IFN-α even enhanced HIV replication. Although the identities of the HIV-induced IFN-α subtypes are not yet known, it is conceivable that IFN-α proteins with low antiviral activity but strong receptor binding affinity can outcompete those IFN-αs with effective antiviral activity but weak

binding affinity. It would certainly be advantageous for HIV, or any other virus, to preferentially induce IFN-α subtypes which are less effective at inhibiting its own replication.

**IFN-β.** It is not unexpected that IFN-β exerts many of the same biological activities as IFN-α proteins since they utilize a common receptor. However, the coding sequence of IFN-β is only 15-30% homologous with the IFN-α genes, which alone would suggest that IFN-β has some unique biological properties. In support of this, certain IFN-β actions have been found to be clearly distinguishable from that of IFN-α proteins. For example, B cell proliferation in response to *Staphylococcus* was enhanced by IFN-β, but in contrast, it was inhibited by IFN-α (74). Similarly, differentiation of Friend leukemia cells by dimethylsulfoxide (DMSO) was enhanced by IFN-β but inhibited by IFN-α (4, 154). In an unusual example, overexpression of IFN-β in transgenic mice or treatment of normal mice with IFN-β protein, both resulted in the induction of endogenous IFN-α proteins. However, treatment of the mice with IFN-α protein did not induce the expression of endogenous IFN-α or IFN-β genes, indicating that this phenomenon was not reciprocal and thus, clearly distinguishing IFN-β from IFN-α actions. Finally, a mutant cell line, U1A, has been characterized which is deficient in its response to IFN-α but remains partially responsive to IFN-β, suggesting that IFN-β interacts differently with the Type I IFN receptor than IFN-α (93, 142). While these examples clearly illustrate functional differences between IFN-α and IFN-β, the distinction between their biological roles *in vivo* still remains poorly understood.

**IFN-ω.** The biological functions of IFN-ω have been least studied among the IFNs. IFN-ω is coordinately induced along with IFN-α genes in response to viral infection. It has antiviral activities similar to IFN-α protein, but its range of antiproliferative or immunomodulatory activities remains to be determined (2). Perhaps the most intriguing aspect of IFN-ω lies in its strong sequence homology to a family of IFN-related genes called trophoblast IFNs present in ruminant ungulate species such as cattle, sheep and goats. Both the bovine and ovine trophoblast IFNs are secreted in large quantities by the embryonic trophectoderm during the critical period of maternal recognition in pregnancy.

Trophoblast IFNs can inhibit the production or release of the luteolysin, prostaglandin $F_2\alpha$, and thus, this may a mechanisms for extending the lifespan of the corpus luteum (11). While ovine trophoblast IFNs can exert antiviral and antiproliferative activities on cultured cell lines (112), it is unclear whether these properties are significant in their roles during pregnancy. In addition, it is unknown whether IFN-ω plays a similar role in humans or in other non-ruminant mammals, although constitutive production of Type I and Type II IFNs has also been described in the pig trophoblast during pregnancy (109).

## Mechanisms of IFN Action

### A. Signaling pathways of Type I IFNs

**Type I IFN receptor complexes.** The biological actions of Type I IFNs are mediated by signaling cascades initiated by their binding to the Type I IFN receptor complex. Competitive binding studies have indicated that all classes of Type I IFN proteins, IFN-α, IFN-β and IFN-ω, utilize a single, shared, membrane receptor (13). It is now known that Type I IFN receptors are composed of a number of subunits and that multiple forms exist due to the differential usage of subunits (24, 25, 35).

The genes encoding for two of the Type I IFN receptor subunits have so far been isolated. IFNAR-1 was the first isolated using an expression cloning strategy. Mouse cells are normally insensitive to human IFN-α, but stable transfection of such cells with the human IFNAR-1 cDNA conferred partial antiviral responses to human IFN-αB protein (182). However, the sensitivity of the transfected mouse cells for human IFN-αB was 100-fold less than that of human cells. Furthermore, IFNAR-1 expression alone was incapable of providing binding or functional responsiveness to human IFN-β or other IFN-α subtype proteins. These results affirmed the necessity of additional components for reconstituting a complete Type I IFN receptor complex. The second Type I IFN receptor gene, IFNAR-2, was initially characterized as an IFN-α-binding protein present in serum and urine (137). Cloning of different IFNAR-2 cDNAs has revealed that alternative gene splicing generates at least three forms, the soluble receptor form (IFNAR-2a)(136), and two membrane-bound forms which are distinguished by containing either a truncated (IFNAR-2b)(23, 136) or full-length intracellular tail (IFNAR-2c)(36, 113). The lone expression of the IFNAR-2 membrane forms in mouse cells confers binding ability for all classes of human Type I IFNs, but with only low or intermediate levels of binding affinity. Biological responsiveness and reconstitution of high affinity binding requires both IFNAR-1 and IFNAR-2c to be expressed together. Thus, the current model for the architecture of the Type I IFN receptor specifies IFNAR-2 as the primary ligand binding

subunit while both IFNAR-1 and IFNAR-2 are required for transmembrane signaling. It is presently unknown how exactly do Type I IFN proteins physically interact with these two subunits. Furthermore, it remains to be determined how the differential responses induced by IFN-α subtypes and IFN-β proteins are mediated at the receptor level. It is possible that additional subunits, such as those identified by biochemical cross-linking studies (25), are required to further specify cell type-specific responses or to distinguish between signaling by different IFN-α subtypes or IFN-β proteins.

**Janus family of tyrosine kinases.** Like most other cytokine systems, signaling from the Type I IFN receptor involves the activation of kinases that in turn, leads to the phosphorylation of downstream effector proteins. Both of the Type I IFN receptor chains possess short intracellular domains lacking intrinsic kinase activity. It would be predicted that the Type I IFN receptor must associate with cytoplasmic kinases, as is typical for a number of other cytokine receptors which also lack intrinsic kinase domains (e.g. IFN-γ, IL-2)(99, 169).

The identification of a critically necessary kinase in the IFN-α signaling pathway was made possible by the isolation of a mutant cell line U1A that was selected for its unresponsiveness to IFN-α (142). By performing genetic complementation, a protein tyrosine kinase, Tyk2, was identified which was sufficient to restore the IFN-α signaling defect in U1A cells (185). However, the IFN-α deficiency in a different mutant cell line named U4A was complemented, not by Tyk2, but by a related tyrosine kinase, Jak1 (93, 119, 129). Jak1 and Tyk2 are members of the growing Janus family of tyrosine kinases whose members have now been implicated in mediating signal transduction for a majority of the known cytokine systems (30). While many of the detailed molecular mechanisms remain to be resolved, the current model for Type I IFN signaling proposes that Tyk2 and Jak1 are associated with IFNAR-1 and IFNAR-2c, respectively. Ligand binding is required in order to bring together both receptor chains and their associated kinases. Once Jak1 and Tyk2 become activated, likely through inter-molecular phosphorylation, they are then capable of mediating further downstream

signaling by phosphorylating their substrates, such as the Signal Transducers and Activators of Transcription (STAT) proteins.

**Family of STAT factors.** The induction of genes in response to IFN-α/β is regulated primarily by the activation of an essential transcription factor, ISGF3, and its resultant binding to the Interferon-Stimulated Response Elements (ISREs), contained in the promoter regions of most ISGs. ISGF3 consists of three subunits, STAT1, STAT2, and ISGF3γ/p48, each of which resides in the cytoplasm of resting cells in a latent form. Again, the exact biochemical details still need to be resolved, but it is thought that following binding of Type I IFNs, the activated Tyk2 and Jak1 kinases phosphorylate the Type I IFN receptor chains at specific tyrosine residues which become docking sites for STAT1 and STAT2 proteins. Once recruited to the receptor, STAT1 and STAT2 become tyrosine phosphorylated by Tyk2 or Jak1, which confers upon them the ability to associate with ISGF3γ/p48 and form the functionally active ISGF3 transcription factor (30). The evidence to date indicates essential roles for Janus kinases and STAT proteins in Type I IFN signal transduction, but there is also compelling evidence for the involvement of protein kinase C (149) and phospholipase $A_2$ activation (72). These enzymatic activities may supply costimulatory types of signals that could contribute to the specificity of the signaling cascade or to modulating the duration of the signal.

## B. ISG proteins and their regulation by dsRNA.

Upon infection by viruses, a typical cellular response includes a sharp reduction in overall RNA and protein synthesis. Earlier studies had observed that the degree of inhibition for both of these processes was even greater in cells which had been treated with IFN prior to infection by virus (68). Thus, it seemed that a mode of action for IFNs involved *de novo* synthesis of proteins which can better sense the presence of a virus infection and then act to limit its replication by inhibiting viral RNA and protein synthesis. Presently, the mechanisms reponsible for the recognition of viral infections in mammalian cells are poorly understood. While it is likely that many forms of recognition exists for different viruses and their unique life

cycles, one component that appears to be common to infections by several RNA and DNA viruses, involves the detection of viral dsRNA (15, 29, 110). Many RNA virus genomes contain a dsRNA component and in addition, dsRNA-containing transcripts are often generated as intermediates during the replicative life cycle of both RNA and DNA viruses. Among the best studied ISG proteins are the dsRNA-dependent protein kinase, PKR (also known previously as the interferon-induced p68 kinase or DAI), and the 2-5A synthetase gene family. Interestingly, these represent the only mammalian proteins known so far which require binding to dsRNA for activation of their enzymatic functions.

**PKR.** The existence of a dsRNA-activated protein kinase was first indicated by studies on regulation of protein synthesis in rabbit reticulocyte lysates. The addition of dsRNA to these lysates greatly inhibited the rate of protein translation, thus mirroring the condition in virus-infected cells (43). The mechanism responsible for this process was determined to involve the phosphorylation of eukaryotic initiation factor-2-alpha (eIF-2$\alpha$) by a latent kinase that was activated by dsRNA (80). Subsequent studies in eukaryotic cells indicated that increased phosphorylation of eIF-2$\alpha$ by only 10-20% in the cell was sufficient to sequester virtually all of the limiting factor eIF-2B and halt protein translation (79). The identity of this dsRNA-dependent kinase has been determined following the cloning of the human and mouse PKR genes (45, 123). PKR is a serine/threonine kinase which is present in most cell types at low levels and in an inactive form. PKR binds to dsRNA by virtue of two conserved dsRNA-binding domains, motifs which are also contained in other dsRNA-binding proteins from eukaryotes and prokaryotes (166). Upon binding dsRNA, PKR manifests two distinct kinase activities. PKR initially undergoes autophosphorylation, likely through an inter-molecular mechanism as a homodimer. As a result, the phosphorylated PKR becomes activated and is then capable of phosphorylating exogenous substrates, such as eIF-2$\alpha$ or histone proteins (177).

Several lines of evidence support a role for PKR in antiviral functions. IFN treatment of cells induced transcription of the PKR gene and led to elevated levels of PKR protein (123). Activated PKR and increased eIF-2$\alpha$ phosphorylation were detected in lysates from virus-

infected cells (153, 158). Overexpression of the human PKR gene in mouse cells was shown to confer increased resistance to EMCV replication (125). Also, the importance of PKR's antiviral role is supported by the large number of viruses which can inhibit PKR function and the diversity of mechanisms by which they do so: adenovirus directs the transcription of short RNAs (VA RNAs), which bind PKR but prevent its activation (100); HIV encodes an essential transcriptional transactivator, tat, which directly binds PKR and inhibits its kinase activity (121, 155); rotavirus encodes an inhibitor protein, NSP3, which contains a dsRNA-binding domain and prevents PKR activation by competing for binding of dsRNA (105); and finally, vaccinia virus encodes for two PKR-inhibitory proteins, one which acts similarly as NSP3 by encoding a dsRNA-binding motif (E3L (18)), and the other, a viral homologue of eIF-2α, which prevents phosphorylation of the authentic eIF-2α (K3L (31)). Lastly, the importance of these viral mechanisms is indicated by the observations that mutant variants of adenovirus (100) and vaccinia virus (31), deleted of their respective PKR-inhibitory genes, replicate to lower titers and are more sensitive to inhibition by IFN.

PKR has been implicated in additional biological activities apart from its antiviral roles. A long-suggested function for PKR in regulating cell growth was supported by experiments in which the expression of human PKR in yeast produced a dramatic, slow growth phenotype, reminiscent of the cell growth arrest in IFN-treated mammalian cells (21). This antigrowth effect was believed to be due to hyperphosphorylation of the endogenous yeast eIF-2α by PKR since the slow growth phenotype could be reverted by cotransfection with a mutant eIF-2α gene altered at the single, PKR phosphorylation site, serine[51]. PKR has also been suggested to function in a tumour suppressor role since overexpression of trans-dominant negative PKR proteins leads to malignant transformation of fibroblasts (discussed earlier, Antiproliferative Activities)(102, 124). The mechanisms responsible for this transformation process has yet to be fully understood but it could be related to recently identified functions of PKR as an important signal transducer (22, 111). Many laboratories have provided indirect evidence supporting a role for PKR in regulating IFN-α and IFN-β gene expression (see Regulation of Type I IFN

Genes). Others have also indicated potential involvement of PKR in the signaling pathways of cytokines such as interleukin-3 (IL-3) (91) and platelet-derived growth factor (PDGF) (131). Given that PKR may be central to so many regulatory pathways, it becomes more readily apparent how perturbation of PKR function could lead to growth dysfunction or tumorigenesis.

**2-5A synthetase and 2-5A-dependent RNase.** The discovery of the 2-5A system originated from efforts to understand how pretreatment of cells with IFN causes the reduced accumulation of viral RNAs following infection. Using reovirus RNA as the target, a latent ribonuclease activity was identified in IFN-treated cell extracts which only became activated in the presence of dsRNA (160). Biochemical fractionation of this ribonuclease activity led to the identification of two complementary parts representing different phases of action. In the first phase, the addition of dsRNA activated an enzyme which catalyzes the conversion of ATP into short, oligoadenylate chains joined by an atypical 2'-5' phosphodiester linkage; hence, the enzyme was named 2'-5' oligoadenylate synthetase. These 2-5A molecules then bind and activate a latent 2-5A-dependent RNase (also known as RNase L) in the second fraction. Studies involving the overexpression of 2-5 synthetase (20) and a trans-dominant negative 2-5A-dependent RNase mutant (76) have implicated their involvement in mediating IFN-α's antiviral and antiproliferative activities. However, alternative biological functions for these two enzymes have also been suggested. The biology of the 2-5A synthetases is complex since multiple isoforms exist in both humans and in mice. Some are encoded by separate genes while others are generated by alternative gene splicing (117, 156). It has been suggested that the smaller 40- and 46-kDa isoforms of 2-5A synthetase mediate antiviral activities (20) while the larger 100-kDa isoform may be involved with pre-mRNA splicing, a process which involves the formation of an RNA lariat structure containing a 2'-5' phosphodiester bond (165). Only one form of the 2-5A-dependent RNase has been detected and studies have suggested its possible involvement in the cleavage of cellular mRNAs and ribosomal RNAs during apoptosis (19).

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# Regulation of Type I IFN Genes

## A. Type I IFN expression

**IFN-producing cells.** IFN is normally not synthesized by cells in their resting state but, all classes of Type I IFNs can be strongly induced in response to stimuli such as viral infections or dsRNA. Various cell types, however, differ in their capacity for expressing certain IFNs. IFN-$\alpha$ expression seems to be mostly confined to cells of hematopoietic origin such as PBMCs and cell lines, like the lymphoblastoid Namalwa cells or the promonocytic U937 cells (27, 81). In PBMCs, two cell populations have been identified as the major IFN-$\alpha$ producers, monocytes and a rare, HLA-DR+ cell type called a "natural IFN-$\alpha$ producing cell" (NIPC), which is likely a dendritic cell (46, 50). While it is unknown whether the IFN-$\alpha$ synthesized from these two cell types may fulfill different biological functions, monocytes and NIPCs are readily distinguishable by their IFN production characteristics. For example, Sendai virus elicits IFN-$\alpha$ production primarily from the monocytes, while HSV preferentially stimulates the NIPCs (44). In addition, NIPCs can produce up to 100-fold more IFN-$\alpha$ protein than an equal number of monocytes (46). IFN-$\beta$, in contrast, appears to be inducible in almost every cell type examined, although its expression has most commonly been studied in fibroblasts (143). One notable exception involves undifferentiated embryonal carcinoma cells which are deficient in producing either IFN-$\alpha$ or IFN-$\beta$ (12, 53). However, these cells become competent for Type I IFN production following their induced differentiation (75), thus mirroring the developmental regulation of the IFN system during embryogenesis (10).

**Inducers of Type I IFNs.** The best known inducers of Type I IFNs are viruses and dsRNA. A wide range of both DNA viruses (e.g. HSV, cytomegalovirus) and RNA viruses (e.g. Sendai virus, influenza virus, EMCV) are efficient IFN inducers (44). However, there are some DNA viruses such as SV40 and adenovirus which do not seem to stimulate any IFN production. As mentioned earlier, a long-standing hypothesis proposes that mammalian cells respond to virus infections by detecting the presence of viral dsRNA. The basis for this

hypothesis stems from early studies which determined that purified viral dsRNA (e.g. reovirus genomic RNA or bacteriophage MS2 replicative form RNA) or synthetically produced dsRNA (e.g. poly [A]·poly[U] or poly [I]·poly[C]) were similarly capable of inducing IFN synthesis when injected into animals or added to cultured cells (110). As a result, viral infection and poly [I]·poly[C] have been used as the standard inducers in the majority of studies on IFN expression. However, poly [I]·poly[C] is generally a less potent IFN inducer than an active virus infection, which could indicate that virus infections are simply more efficient at presenting or maintaining dsRNA within the cell, or that viral infections provide alternative IFN-inducing signals in addition to dsRNA.

Many examples of IFN induction have been described that do not involve viral replication or any obvious dsRNA component. Several laboratories have used glutaraldehyde-fixed HSV-infected fibroblasts as IFN inducers, since this preferentially stimulates NIPCs but not monocytes (49, 50, 59). Viral replication is clearly not involved in this situation but physical contact between the fixed HSV-infected cells and the NIPCs is required. Thus, this form of IFN induction appears to result from the generation of a stimulatory signal at the surface of the IFN-producing cell. Potential candidates for such a stimulatory signal might include membrane-bound viral proteins or cellular membrane proteins whose expression becomes upregulated or otherwise altered due to the viral infection. In support of the former, antibodies against the HSV-1 glycoprotein D were observed to inhibit IFN production by HSV-infected cells (108). Alternatively, support for the latter possibility was provided by findings that antibodies against the $\beta_2$ integrins (which are leukocyte-specific cell membrane adhesion molecules) also inhibited IFN induction by the fixed HSV-infected cells (17). In a somewhat related example, certain tumour cell lines, that are free of any infection by viruses or mycoplasma, have been observed to induce high levels of IFN-α from PBMCs when co-cultured together (181). The authors have suggested that tumour antigens or otherwise dysregulated cell surface antigens may be responsible for stimulating IFN-α expression, possibly in a manner analogous to the recognition of tumour and virus-infected cells by natural killer cells. Also, the

IFN-α induction by tumour cells was distinct from a mixed leukocyte reaction which produces IFN activity but, at much lower levels and only after several days longer of incubation.

Several cytokines have been identified which can induce IFN production, further supporting the general notion that IFN genes can be regulated by cell surface-mediated signaling. IFN-β is induced in fibroblasts in response to the inflammatory cytokines, IL-1 (184) and TNF-α (92). IFN-α is induced in neutrophils by granulocyte colony-stimulating factor (G-CSF) but not in bulk PBMCs (162). Low levels of both IFN-α and IFN-β have been detected during the differentiation of murine bone marrow cells (127), M1 myeloid cells (152), or human promonocytic U937 cells (175), in response to macrophage colony-stimulating factor (M-CSF) or hydroxyvitamin D3. In these circumstances, however, it is unclear whether the IFN induction represents a direct response to the particular extracellular stimuli used or, an indirect response to some undefined signal generated later during the differentiation process. Finally, LPS can stimulate IFN-α production in mouse bone marrow cells and peritoneal macrophages, and in cytokine-primed human blood monocytes (77). LPS, which is a cell wall component of Gram-negative bacteria, elicits its cellular responses through stimulation of the CD14 transmembrane receptor on mammalian cells (194).

**Effects of priming on IFN regulation.** It has long been known that the quantity of IFN protein synthesized in response to virus infection can be enhanced by pretreatment or "priming" of the producer cells with even small amounts of IFN (88). The biological significance of this phenomenon is thought to represent an amplification mechanism in order to generate a rapid and systemic IFN response shortly following a virus infection. The mechanisms of priming have yet to be elucidated, perhaps partly because this effect is manifest differently in particular cell-inducer combinations. Some studies have reported that priming results in earlier IFN production but with no net change in IFN mRNA, while others have reported that priming has no effect on the kinetics of IFN induction but it increases IFN mRNA levels (163). Priming is dependent on cellular protein synthesis, which might suggest that priming serves to increase the cellular levels of protein components required for IFN gene transcription. Alternatively,

priming may work by inducing cellular factors which increase IFN mRNA stability or its translational efficiency. Lastly, IFN expression can also be downregulated by pretreatment with cytokines such as IL-4, which has been shown to inhibit IFN-α and IFN-β production in PBMCs induced with Sendai virus (58).

**Constitutive IFN production.** As stated earlier, most cells in their resting state do not synthesize IFN. However, spontaneous IFN production has been described in certain cells and physiological conditions in the apparent absence of any viral replication. For example, many human B-lymphoblastoid cell lines, arising from transformation by Epstein-Barr virus (EBV), secrete low levels of IFN-α constitutively despite the lack of active EBV replication (144). In one particular cell line, LuKII, the constitutively produced IFN was determined to consist almost entirely (>90%) of a single subtype, IFN-α2 (3). This was contrasted by the heterogeneous mixture of IFN-α subtypes that was induced upon infection of LuKII cells with exogenous virus. Since IFN-α can stimulate proliferation of B-cells, the authors have suggested that successful transformation of B-cells may require concomitant expression of IFN-α as an autocrine growth factor. In a different context, mutant cell clones which constitutively produce IFN-α or IFN-β, have been isolated using a strategy that involves several rounds of chemical mutagenesis and an enforced selection procedure (120). This type of strategy was successfully used to identify the components of the Jak-STAT pathway in Type I IFN downstream signaling (30). Therefore, this set of IFN-producing mutant cells could represent a very useful tool to elucidate the factors responsible for the regulation of Type I IFN genes.

**Aberrant IFN-α expression in human diseases.** Constitutive IFN-α expression has been associated with a number of human diseases. In HIV-infected patients, the presence of IFN-α in serum is a prognostic indicator for disease progression towards endstage AIDS, while there is no detectable IFN activity in serum from uninfected or healthy, HIV-infected individuals (39). High levels of serum IFN-α is also associated with a number of diverse, nonviral diseases including aplastic anemia (199), systemic lupus erythematosus (147), and rheumatoid arthritis (159). In all of these cases, however, the cellular sources of the aberrant

IFN-α production and the inducing stimuli are not known. While PBMCs are the best studied IFN producers in humans, IFN-α expression has been detected in certain organs as well. For example, IFN-α expression in pancreatic β-islet cells was determined to be associated with patients suffering from Type I diabetes mellitus (52). Furthermore, the causal nature of this association is supported by a transgenic mouse model whereby the directed expression of IFN-α to pancreatic β-islet cells resulted in the development of Type I diabetes in the mice (170). Conversely, low levels of IFN-α have been detected in the spleen and bone marrow from humans and mice, in the absence of any disease conditions (67, 90, 180, 199). It is possible that a regulated, basal level of IFN-α synthesis may constitute a normal part of homeostatic mechanisms in mammals.

**Differential expression of IFN-α subtypes.** The existence of multiple IFN-α subtypes makes it difficult to comprehensively study their individual expression. Distinguishing between IFN-α subtypes at the level of mRNA or protein is confounded by their high degree of sequence homology and the large number of different subtypes. Consequently, Northern blot analysis can detect the overall mRNA levels of the entire IFN-α family but this technique cannot distinguish between subtypes. Only a few studies, by using S1 nuclease and a panel of subtype-specific probes, have demonstrated the detection of mRNAs for individual IFN-α subtypes. In the first of such studies, 8 IFN-α subtypes were analyzed for their induced expression in PBMCs or cell lines (81). IFN-α1, -α2, and -α4 constituted the major fraction of IFN-α transcripts measured, while only low level expression of the others could be detected. Similar differential expression of IFN-αs has also been observed in mouse cells induced by virus infection (83, 95). Detection of IFN-α protein levels is typically performed using biological assays to measure antiviral activity. Discriminating between IFN-α and IFN-β in a mixture is possible by using neutralizing antibodies specific for IFN-β, or cross-reactive for IFN-α subtype proteins. However, currently, no reliable method are available for discriminating between different IFN-α subtype proteins on a small scale. For such purposes, it has been necessary to prepare large IFN preparations for biochemical fractionation and protein sequencing (198).

## B. Type I IFN promoter elements

Given their distinct patterns of cell type-specific expression, it is obvious that IFN-α and IFN-β gene expression are governed by different regulatory mechanisms. However, their coordinate induction in certain permissive cell types, by virus infection or dsRNA, implies that these two gene systems share some common regulators. Some conserved sequences have been noted between the human IFN-α1 and IFN-β gene promoters, but it is unknown whether these similarities may account for the overlap in their regulation. The IFN-β promoter has been more extensively studied with regards to defining important regulatory cis-elements and identifying the transcription factors which act upon these sequences (82). Briefly, the essential IFN-β promoter is contained within approximately 100 basepairs upstream of the transcription start site. Extensive mutational analysis of this promoter region has identified four distinct Positive Regulatory Domains, PRDI through PRDIV (40, 62, 196), and two Negative Regulatory Domains, NRDI and NRDII (61, 62). The transcription factors which bind to the PRD elements and regulate IFN-β gene expression include NF-κB, IRF-1, ATF-2, and HMG I(Y) (37, 55, 176). Of the several IFN-α subtypes, only the promoter of human IFN-α1 gene has been studied in detail (146). Deletional analyses have identified a 46-basepair promoter fragment, from positions -109 to -64 of the IFN-α1 gene, that is capable of mediating virus-inducible transcriptional activity (148, 157, 188). However, finer mapping of regulatory elements in the IFN-α1 promoter is currently lacking since it has not been subjected to extensive mutational analysis like the IFN-β promoter. By directly comparing their sequences, the IFN-α1 promoter does not contain any elements which directly correspond to PRDI-PRDIV of the IFN-β promoter (Fig.1). Specifically, it lacks NF-κB binding sites and while it does contain putative IRF-1 binding sites, it is unknown whether these sites are functionally important and whether IRF-1 binds to them *in vivo* (8). The major similarities between the IFN-α1 and IFN-β promoters are somewhat subtle in that they exist in the form of conserved hexamer repeats with the general configuration of GAAANN (where NN can represent combinations such as GT, GC, CT or CC). Multimerization of certain GAAANN forms functional virus-inducible enhancer

Fig. 1. Organization of the IFN-α1 and IFN-β promoters. The 46-basepair VREα1 in the IFN-α1 promoter is a functional cis-element which can confer virus-inducible transcriptional activity to a heterologous promoter. The "TG" and IRF-like boxes designated in the IFN-α1 promoter only represent putative sites for transcription factor binding (114). The IFN-β promoter contains the four positive regulatory domains, PRDI through PRDIV, which were functionally defined by mutational analyses. The PRD sites bind a variety of transcription factors including IRF-1, NF-κB, HMG I(Y), and ATF-2 (37, 176).

**IFN-α1**

VREα1

repA

TGTAAAGGTGCATGAGGAAGCAAAGC GAATGGAAGT GCCCAGAGCATTA GAAGTGGAAAT CGTATGTCCTATTTAAGGC

GAAGCGAAGC   GAATGGAAGTG

| | | |
-115 -109 -64 -32

IRF-like   "TG"   IRF-like

**IFN-β**

-104   -99   -91   -77   -50

PRDIV   PRDIII   PRDI   PRDII

CTAAAATGTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCTGA

**Consensus**

☐ hexamer motif   A A G T G A

▨ IRF-1 like site   A G A A G T G A A A G T

▥ Oct motif   A T G C A A A T

■ ATF/CREB site   T G A C G T C/A A/G

**Fig. 1.**

elements (114, 132). A number of factors have been described which bind constitutively to these artificial GAAANN elements or the IFN-α1 promoter *in vitro*. They include IRF-1, the IEFga factor, and a novel "TG" protein (114). However, the functional significance of these factors to IFN-α regulation is uncertain. It is important to note that the binding of these factors was not increased in extracts from virus-infected cells, whereas inducer-dependent activation of factor binding is characteristic of most inducible transcription regulatory elements. For example, virus-inducible binding of factors, such as NF-κB and ATF-2, to PRDII and PRDIV, respectively, has been well characterized. Furthermore, studies on IFN-α gene regulation in murine cells have described virus-inducible binding of novel but as yet, unidentified, factors to elements in the mouse IFN-α4 promoter (8).

## C. Signaling aspects in regulating Type I IFN induction

As mentioned earlier, a likely mechanism by which cells recognize virus infection is the detection of viral dsRNA. Consequently, it is reasonable to infer that cellular factors exist for recognizing dsRNA and, that some of these factors are connected to the signaling pathways responsible for regulating IFN gene transcription (110, 116, 197). Taking into account two aspects, the importance of phosphorylation events in signal transduction and the unique status of PKR as the only known dsRNA-dependent protein kinase, have led many researchers to hypothesize that PKR acts as a signal transducer in regulating IFN gene induction. Early biochemical studies had determined that the kinase activity of PKR could be inhibited by a purine analogue, 2-aminopurine (43). The mechanism for this inhibition may involve competitive binding for the ATP-binding site on PKR since the optimal inhibitory concentration for 2-aminopurine is approximately 100-fold higher than the concentration of ATP required for PKR's kinase activity. Furthermore, the inhibitory properties of 2-aminopurine can be overcome by simply using high concentrations of ATP in *in vitro* kinase assays (85). If it does indeed compete with ATP for binding, this property of 2-aminopurine appears to be relatively specific for PKR since it does not globally alter cellular phosphorylation patterns nor does it

inhibit protein kinase A or protein kinase C activities (115, 187). By taking advantage of its PKR-inhibitory ability, several groups have reported that 2-aminopurine selectively inhibits the induction of IFN-β by poly [I]· poly[C] in human fibroblasts (197), and it inhibits both IFN-α and IFN-β induction by virus infection in mouse fibroblasts and chick embryo cells (28, 116).

These findings provided indirect evidence to support the model of PKR functioning as a signal transducer for virus- and dsRNA-mediated transcriptional activation. Given such a role, PKR presumably must function by phosphorylating cellular substrates which are responsible for initiating IFN gene transcription. In the IFN-β promoter, a major point of regulation lies with PRDII, a well-characterized binding site for the transcription factor NF-κB. NF-κB exists in a latent cytoplasmic form and is held in check by association with an inhibitory protein, IκB (9). Activation of NF-κB occurs following appropriate stimuli, such as virus infection or dsRNA treatment (186), which results in phosphorylation of IκB and its subsequent dissociation from NF-κB. Recently, it has been demonstrated that PKR can activate latent, cytoplasmic NF-κB by specifically phosphorylating IκB (103). This identified IκB as a additional substrate for PKR and provided the first direct evidence linking PKR activities to IFN-β induction. It is yet unknown whether PKR may also regulate the activities of IRF-1 and ATF-2 on the IFN-β promoter's PRDI and PRDIV elements, respectively. Once the essential IFN-α-specific transcription factors are identified, it will be of interest to investigate whether or not PKR is involved in regulating their actions. Lastly, it is likely that Type I IFN expression is also regulated by pathways which are independent of PKR actions. For example, virus induction of IFN-α and IFN-β is not inhibited by 2-aminopurine in primary mouse spleen cells, although IFN induction in mouse L929 fibroblasts is inhibited by the same concentration of 2-aminopurine (28). Also, as described earlier, several forms of Type I IFN induction do not require viral replication or a dsRNA component and as such, these signaling pathways may not require PKR. However, PKR involvement in these cases have yet to be studied.

## <u>Outline of Thesis</u>

IFN-α proteins are involved in regulating multiple biological activities but their own expression is also stringently regulated. To better understand the biological roles of IFN-α genes, it is necessary to study the characteristics of their induced expression and the mechanisms involved in mediating IFN-α gene transcription. Distinguishing between IFN-α subtypes is problematic owing to the high degree of homology within the IFN-α gene family. Chapter Two describes a PCR-based approach which I have developed in order to detect and discriminate between the mRNA of different IFN-α subtypes. Using this system, the expression of IFN-α subtypes in response to different inducers and by different cell types was investigated. IFNs are usually not synthesized in the absence of stimuli, and yet, there is a need for a vigorous induction of IFN production upon appropriate stimulation in order to be able to deal effectively with a virus infection, for example. Chapter 3 describes the effects of priming monocytic cells with IFNs or other reagents which enable a more rapid induction of IFN-α expression and responsiveness to a wider range of inducing stimuli. Despite the importance of IFN-α regulation to antiviral defenses, presently, little is known about the signaling pathways which regulate IFN-α expression. In Chapter Four, the role of the dsRNA-dependent kinase, PKR, in IFN-α regulation was studied using mutant cell lines which were generated that are functionally deficient for PKR activity. We have observed that PKR was required not only for IFN gene induction, but for cellular antiviral responses as well. Finally, in Chapter Five, I will summarize this work and discuss its implications on our understanding of the workings of the IFN system.

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# Chapter Two

## Transcriptional Expression of
## Human Interferon-α Subtype Genes

# Abstract

Interferon-α (IFN-α) proteins contribute in the immune response against pathogens and in the regulation of cell growth. However, studying their biological roles is complex since the IFN-α gene family encodes for at least 14 closely related subtype proteins with similar but, subtly distinctive, functional profiles. In order to investigate the expression of IFN-α genes at the mRNA level, a reverse-transcription-PCR-based strategy was developed that uses consensus PCR primers capable of amplifying all known IFN-α sequences. The composition of IFN-α subtypes from a sample was determined by subcloning the resultant PCR product and sequencing random clones. Alternatively, the PCR product was digested with restriction enzymes that can uniquely identify a particular IFN-α subtype. By using genomic DNA samples to test this system, amplification of individual IFN-α subtypes appeared to be relatively consistent and without overt signs of preferential amplification for one subtype over another. These methods were then applied to determine the composition of induced IFN-α subtype mRNAs. Following stimulation of promonocytic U937 cells by inducers including virus, double-stranded RNA, lipopolysaccharide, or phorbol ester, IFNA8 was the most prevalent subtype induced, followed by IFNA1 or IFNA2. In both U937 and lymphoblastoid Namalwa cells, these three subtypes together accounted for over 80% of the total pool of IFN-α mRNA. In contrast, the pattern of IFN-α subtypes induced in peripheral blood mononuclear cells (PBMCs) appeared to be more diverse as IFNA1, IFNA2, and IFNA8, collectively accounted for only 40-50% of total IFN-α mRNA. In summary, the expression of IFN-α subtypes is differentially regulated and these patterns are influenced by the cellular background of the IFN-producing cell and the induction conditions used.

# Introduction

Large IFN-α gene families are present in humans and in all other mammalian species studied to date. but the biological significance for maintaining so many subtypes is poorly understood. Weissmann and Weber had posed the question of whether multiple IFN-α genes only represent "an evolutionary accident" with no selective advantage or rather. that distinct IFN-α species have evolved to exercise specific functions (35). In support of the latter. studies of recombinant or purified. natural IFN-α proteins have shown that particular subtypes can be distinguished by their profile of specific biological activities (17. 26. 27). It is possible that these unique characteristics fulfill specialized biological roles *in vivo* and in accord with this. it seems reasonable to hypothesize that the expression of IFN-α subtypes may be differentially regulated in response to distinct environmental signals or in different cell types.

Currently. it is largely unknown whether the functional characteristics attributed to given IFN-α subtypes by *in vitro* means, correlate to their actual functions in *in vivo* settings. A major obstacle in furthering this area of investigation lies in the lack of means for detecting and distinguishing different IFN-α subtype proteins. Monoclonal and polyclonal antibodies have been used which are cross-reactive against most IFN-α proteins but none have been shown to be specific for only one subtype. It has been possible to study the expression of IFN-α subtypes at the mRNA level despite the technical difficulties arising from the large number of family members and their high degree of sequence homology. Previous studies have employed modified S1 nuclease mapping or RNase protection techniques in order to detect the mRNA for specific IFN-α subtypes (15, 16, 19, 24).

For this study. we have designed consensus PCR primers which can amplify the known IFN-α subtypes in a single reaction. The need for a panel of subtype-specific probes was obviated by using sequencing or restriction enzyme digest analyses to identify IFN-α sequences in the resultant PCR product. These methods were then applied to study the levels of individual IFN-α subtype mRNAs induced following viral infection or by other stimuli.

## Materials and Methods

**Cell culture and inductions.** U937 cells and Namalwa cells were cultured at 37°C in 5% $CO_2$ with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). L929 cells were cultured similarly except using Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS. Peripheral blood mononuclear cells were isolated from healthy laboratory volunteers using Ficoll-Hypaque gradient and subsequently cultured in RPMI-1640 with 10% FCS.

Stock solutions of poly [I]·poly [C] (in physiological salt, Pharmacia) were prepared in sterile $H_2O$. EMCV stocks were prepared by passage in murine L929 cells and the viral titer was determined in terms of $TCID_{50}$ using L929 cells. Lipopolysaccharide (LPS, Sigma) stocks were prepared in sterile PBS. Phorbol 12-myristate 13-acetate (PMA, Sigma) stocks were prepared by first dissolving in DMSO before further dilution in PBS. Inductions with poly [I]·poly [C] (100 μg/ml), EMCV (10 $TCID_{50}$/ cell), LPS (5 ng/ml) or PMA (5 nM) were performed by direct addition of the inducer into the cell culture media to yield the indicated final concentrations. Cells were IFN-primed for 18 hours by the addition of either recombinant human IFN-α2 (Schering) or IFN-γ (Amgen), yielding a final concentration of 200 U/ml.

**Reverse-transcriptase-polymerase chain reaction (RT-PCR).** Total cellular RNA was isolated from cells using a modified acid guanidinium thiocynate procedure (4). Briefly, cell pellets were lysed with Solution D (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl) and extracted twice with phenol-chloroform, before isopropanol precipitation of the total RNA. We have found that the additional extraction step greatly reduces carryover contamination by genomic DNA. First strand cDNA synthesis was performed by first annealing 2 μg of total RNA from each sample with 0.4 μg of random hexamer (Pharmacia) in 10 μl total volume. A final reaction mix, containing 200U MMLV reverse transcriptase (Gibco-BRL), 1 mM each dNTP (Pharmacia), 10 mM DTT, and 5X RT

reaction buffer (Gibco-BRL), was made up to 25 µl and incubated at 37°C for 1 hour. 2 µl portions of each cDNA mix were subsequently used for PCR amplification.

PCR amplification reactions were performed in a 50 µl reaction volume containing 50 pmol each of the upstream and downstream primers, 2 units Taq DNA polymerase (Gibco-BRL), 0.2 mM of each dNTP, 2.5 mM MgCl$_2$ and 10X PCR reaction buffer (Gibco-BRL). The cycling conditions for IFN-α PCR consisted of denaturation at 94°C for 5 min during the initial cycle, otherwise 94°C for 30 sec during subsequent cycles, annealing at 60°C for 30 sec. and extension at 72°C for 1 min, for 40 total cycles. PCR amplification for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for equivalent RNA loading and general integrity. Primers for GAPDH PCR consisted of 5'-CCATGGAGAAGGCTGGGG (upstream) and 5'-CAAAGTTGTCATGGATGACC (downstream) (5). The cycling conditions involved denaturation at 94°C for 5 min during the initial cycle, otherwise 94°C for 30 sec during subsequent cycles, annealing at 60°C for 30 sec. and extension at 72°C for 30 sec, for a total of 32 cycles. PCR products were analyzed by gel electrophoresis in 1.5% agarose containing ethidium bromide.

**Subcloning of PCR products.** The 372 basepair (bp) IFN-α PCR products were isolated by 1% agarose gel electrophoresis and purified using GeneClean (BIO 101). The recovered PCR-DNA was then digested overnight with *Bam*HI and *Hind*III and subsequently, purified again using GeneClean. The replicative form of the M13 vector, MP10, was digested with *Bam*HI and *Hind*III and treated with calf intestinal alkaline phosphatase (Gibco-BRL). Equimolar amounts of the digested IFN-α-PCR-DNA and the linearized MP10 were ligated using T4 DNA ligase and transformed into E. Coli, DH5αF' strain (Gibco-BRL). M13 plaque clones were selected at random, small scale ssDNA was prepared, and clones were sequenced by the dideoxynucleotide chain termination method (31).

**Quantification of IFN-α subtypes by restriction digest analysis of PCR products.** IFN-α PCR products were quantified by performing the PCR reaction using an unlabeled upstream and a radioactively labeled downstream IFN-α consensus primers. The downstream

primer was endlabeled using [γ-$^{32}$P]ATP and T4 polynucleotide kinase. Following amplification, the radiolabeled IFN-α PCR product was precipitated using sodium acetate and ethanol, and resuspended in H$_2$O. Equal aliquots of the radiolabeled IFN-α PCR product were digested separately with the restriction enzymes, *AvaI*, *AvaII*, *MaeIII*, and *TaqI*, overnight. Each of the digested samples were then analyzed alongside an equal aliquot of undigested sample by 15% native polyacrylamide gel electrophoresis and visualized by autoradiography. Uncut PCR products and restriction fragments were excised from the dried gels and the radioactivity for each was determined by scintillation counting. Since endlabeling attaches a single $^{32}$P-phosphate group to each downstream primer molecule, the counts per minute (CPMs) originating from the uncut PCR-DNA products or restriction fragments are directly proportional to their molar quantity. Therefore, the proportional representation of an IFN-α subtype in a PCR sample was determined by calculating the ratio in CPMs between the subtype-specific restriction fragment and the original amount of uncut IFN-α PCR DNA:

$$\text{Proportion of IFN-}\alpha\text{ Subtype} = \frac{\text{CPM(restriction fragment)}}{\text{CPM(total)}},$$

whereby, CPM(total) was calculated as the sum of CPM(restriction fragment) and the CPMs for the uncut (372 bp) IFN-α PCR DNA species remaining in the same sample after complete digestion. Performing the calculations in this manner excludes incorporation of the error factor arising from variations in loading between samples. Background CPM values were subtracted from the measurements for each DNA band.

# Results

**Strategy for detection of all IFN-α subtypes.** The high degree of sequence homology among IFN-α genes makes it difficult for probe hybridization methods to achieve the high stringency conditions needed in order to discriminate between different IFN-α subtypes. To develop an efficient method for detecting IFN-α transcripts, we devised a strategy that takes advantage of their shared homology. We reasoned that if it was possible to design PCR primers that are capable of amplifying all known IFN-α genes, they could then be used for detecting expression of IFN-α subtypes at the mRNA level with the incorporation of a reverse-transcriptase step. Furthermore, the composition of IFN-α sequences in these samples could be determined by analysis of the resultant DNA-PCR products with conventional molecular biology techniques. The usefulness of such a strategy depended on two important factors. Firstly, it would be necessary to directly show that such PCR primers can amplify all of the known IFN-α subtypes. Secondly, the proportional composition of different subtypes from the original sample should be faithfully represented in the final PCR product after amplification. In this study, we provide evidence supporting the first consideration by using a "clone and sequence" type of analysis, and for the second consideration by using a "restriction enzyme digest" analysis.

**Design of consensus IFN-α PCR primers.** Two guidelines were used in selecting the primer sequences. Firstly, the sequences should derive from the more highly conserved regions of the IFN-α gene family. Thus, by minimizing the degree of degeneracy needed to account for non-conserved positions, the specificity of the consensus IFN-α primers should be better maintained. Secondly, there should be sufficient sequence variation over the length of the expected PCR product to be able to distinguish between IFN-α subtypes. We identified two blocks of sequence, spanning roughly two-thirds of the IFN-α coding region, which met these criteria (Fig. 1a). Based on the published sequence for this region (14), it is possible to distinguish between all known IFN-α subtypes but for two exceptions. Firstly, IFNA1 and

IFNA13 are believed to be separate genes although they share identical coding regions. For convenience, they will be referred to together as IFNA1. Secondly, IFNA5 and IFNAP22 (also known as GX-1 (14)) are indistinguishable within the regions bound by these primers. IFNAP22 is believed to be transcriptionally expressed since it was isolated from a cDNA library. However, it is considered a pseudogene since it contains a deleted "G" in its ATG start codon and hence, no protein is thought to be made. For convenience, we will only refer to IFNA5. Having selected the IFN-α-specific sequences, additional sequences were incorporated into the consensus primers to facilitate cloning of the PCR products (Fig. 1b). HindIII and BamHI restriction enzyme sites were added onto the 5' ends of the upstream and downstream IFN-α primers, respectively. These particular restriction sites were selected since they are not present in any of the known IFN-α genes. Also, since the endonuclease efficiency of restriction enzymes is generally diminished at sites closer to the ends of a DNA duplex, extra "G" bases were added 5' of both sites in order to increase their distance from the ends.

**Consensus IFN-α primers enable PCR amplification of all known IFN-α genes.**
Genomic DNA contains all IFN-α subtypes represented in equimolar amounts within a highly complex DNA mixture. As such, genomic DNA appeared to represent an ideal template with which to test both the sensitivity and specificity of PCR amplification for the entire IFN-α gene family. Southern blot analyses have indicated that HeLa cells appear to have a normal representation of the IFN gene cluster (8). PCR performed on 1 nanogram of HeLa cell genomic DNA using the consensus IFN-α primers, yielded a single PCR product of the expected size, 372 basepairs (Fig. 2a, lane 8). To determine which IFN-α sequences had been amplified, the PCR product was cloned and the subtypes contained in randomly selected clones were identified by dideoxy-sequencing. Fig. 2b summarizes the results from analysis of clones derived from two independent PCR amplifications of HeLa genomic DNA. The results show that sequences for all of the known IFN-α subtypes were represented. Neither IFN-β, IFN-ω, nor any novel IFN-α-related sequences were identified among the clones analyzed. Therefore,

the consensus IFN-α PCR primers are capable of specifically amplifying the entire family of IFN-α subtype genes.

**Investigation of IFN-α subtype mRNA expression by sequence analysis.** PBMCs have been commonly used for studying IFN-α expression (1, 15, 25). Using reverse transcriptase-linked PCR (RT-PCR), IFN-α mRNA was undetectable in uninduced cells, while in PBMCs induced with poly [I]-poly [C] or EMCV, IFN-α mRNA accumulation was detectable at 3 and 6 hours (Fig. 2a, lanes 1-6). It is important to note that since all IFN-α genes lack introns, the presence of contaminating genomic DNA in the RNA samples could generate a positive signal. We have determined in preliminary experiments that by performing a second phenol-chloroform extraction during the RNA isolation procedure, contamination of this type was substantially reduced. To test this, RNA samples were subjected to PCR amplification with the IFN-α consensus primers to verify the absence of genomic DNA before cDNA synthesis was performed. The PCR products from the 6 hour timepoints for both inducers were subcloned and the sequence analyses of randomly selected clones are summarized in Fig. 2c. Several subtypes were represented among these clones suggesting that these IFN-α genes were coordinately induced. Although there were no obvious differences between the patterns of IFN-α's induced by poly [I]-poly [C] and EMCV, certain subtypes, such as IFNA1, IFNA5, and IFNA14, were detected with greater frequency than others. It is possible that these frequencies reflect roughly the relative mRNA levels of each IFN-α gene but, the use of sequence analysis here was intended primarily to provide a qualitative determination of IFN-α subtype expression. A more quantitative approach using restriction digest analysis of the IFN-α PCR product will be discussed in the following section.

IFN-α mRNA expression was next studied in two established cell lines with different cellular backgrounds, the promonocytic U937 cells and the lymphoblastoid Namalwa cells. These particular cell lines have also been commonly used to study IFN gene regulation (5, 15, 17). Since we had earlier investigated the IFN-α genes that were rapidly induced in PBMCs, we wanted similarly to determine which represented the rapid response IFN-α subtypes expressed

in these cell lines. Our concurrent studies have determined that the kinetics of IFN-α induction in both U937 and Namalwa cells are significantly delayed as compared to PBMCs. Induction of IFN-α mRNA by poly [I]-poly [C] or EMCV is not detectable in these cell lines until after 12-16 hours (see Chapter 3, (7)), in contrast to the rapid induction detected in PBMCs within the first 6 hours. However, we have also determined that a rapid IFN-α response in either cell line is possible once they have been pretreated with low concentrations of IFN-α or IFN-γ proteins, a phenomenon known generally as priming (18, 29). Specifically, in IFN-primed U937 and Namalwa cells, IFN-α mRNA was detected by RT-PCR 4 hours following stimulation with poly [I]-poly [C] or EMCV (Fig. 3a, results not shown). These samples were selected for analysis by the "clone and sequence" method and the results are summarized in Figs. 3b and 3c. Again, several different IFN-α genes were coordinately induced in both U937 and Namalwa cells but, there were no obvious differences in the IFN-α subtype patterns induced by poly [I]-poly [C] or EMCV. However, the overall IFN-α expression patterns for the two cell lines appeared to be more similar to each other than to PBMCs. Firstly, the most prevalent subtypes detected in both cell lines were IFNA2 and IFNA8, and with higher frequency than in PBMCs. Secondly, while IFNA1 was the most prevalent subtype detected in PBMCs, it was less frequently detected in U937 and Namalwa cells. Thirdly, the expression of IFNA7, IFNA10, and IFNA17 was detected in both cell lines but not in PBMCs. Lastly, it is interesting to note that IFNA6 was the only subtype which was amplified from genomic DNA but whose mRNA was not detected from any of the cell types. Hiscott et al. were also unable to detect transcripts for this subtype and noted that owing to a partial deletion in its presumed promoter region, IFNA6 may represent a transcriptional pseudogene (15).

**Detection of IFN-α subtype genes by restriction digest analysis.** Having shown that the consensus IFN-α primers can simultaneously amplify the IFN-α gene family, we were interested next in determining whether the amplification of different subtypes was uniform and consistent from one PCR reaction to another. If the relative proportions of IFN-α subtypes from a sample are faithfully reproduced after PCR amplification, then this PCR reaction could be

further developed as a means for quantifying the levels of different IFN-α subtypes in a sample. For this purpose, it would be necessary to be able to efficiently detect individual subtype from a mixed IFN-α PCR product. Examination of the restriction maps for the IFN-α genes indicated that certain subtypes can be distinguished by unique restriction enzyme sites. Thus, the generation of an appropriately sized restriction fragment following digestion of a PCR sample with a subtype-specific restriction enzyme, would indicate the presence of that IFN-α sequence. Moreover, if amplification of the IFN-α sequences was uniform, the ratio of the restriction fragment to the initial amount of uncut PCR product should reflect the proportion of that subtype in the original template sample. Lastly, quantification of the PCR product and the restriction fragments could be carried out by using one radioactively endlabeled primer in the PCR reaction and measuring the radioactivity of the respective DNA bands after their separation by gel electrophoresis.

Genomic DNA was used again as a template to test for uniformity of subtype amplification, since each IFN-α gene is expected to be naturally represented with equimolar ratios. The resultant IFN-α PCR products were digested with one of four restriction enzymes: 1) AvaII, which only cuts IFNA1 to give a 205 bp 3'-fragment; 2) MaeIII, which only cuts IFNA2 (at +1316) and IFNA16 (at +1298) to give 127 bp and 145 bp 3'-fragments, respectively; 3) AvaI, which only cuts IFNA4 to give a 327 bp 3'-fragment; and 4) TaqI, which only cuts IFNA8 to give a 182 bp 3'-fragment (Table 1). We had specifically searched for unique restriction sites that could identify IFNA1, IFNA2, IFNA4, and IFNA8, in attempt to corroborate our results from using the clone and sequence approach, and to compare with the results reported by Hiscott et al. (15), suggesting that these subtypes were among the more highly expressed IFN-α genes. Samples of genomic DNA from three cell lines, U937, Namalwa and HeLa cells, were amplified using an unlabeled upstream and a radiolabeled downstream consensus IFN-α primer. As shown in Fig. 4, each subtype-specific enzyme generated restriction fragments of the expected sizes. Moreover, the percentage representation for the five subtypes examined approximated the expected frequency of 7.1%, based on

calculating one of fourteen functional subtypes (Table 2). Also, the relative amplification of each of these subtypes remained consistent despite changing two variables, increasing the amount of input template DNA to 5 or 25 ng, or decreasing the number of amplification cycles (Table 2).

**Quantitative determination of IFN-α subtype mRNA levels by restriction digest analysis.** The restriction digest method was applied to re-evaluate the induction of IFN-α subtype mRNAs. PBMCs induced with poly [I]-poly [C] or EMCV were observed to express all five of the subtypes examined (Table 3). While there were differences in the levels for each induced subtypes, both inducers elicited similar patterns. IFNA2 and IFNA8 accounted for the highest levels ranging between 8% and 16% of the total IFN-α pool. The levels of IFNA1, IFNA4 and IFNA16 were all lower, each accounting for less than 8%. Although IFN-α genes are rapidly inducible in freshly isolated PBMCs without priming, we were nevertheless interested in whether IFN pretreatment would affect the pattern of IFN-α subtypes induced. PBMCs were primed with IFN-α (200U/ml) for 18 hours before receiving the same stimulation with poly [I]-poly [C] or EMCV. As a result, IFN priming did appear to cause some subtle changes (Table 3, rows 3 and 4). While the induction of IFNA4 and IFNA16 genes was not appreciably different, the levels of IFNA1, IFNA2 and IFNA8 were each moderately increased by approximately twofold following priming, in response to either inducer. It should be noted that complete restriction enzyme digestion of amplified cDNA samples was monitered by simultaneous digestion of control genomic DNA-derived PCR samples.

Next, examination of the IFN-α genes induced in U937 cells revealed some interesting differences, as compared to PBMCs. Following stimulation by any of the inducers used, the expression of either IFNA4 or IFNA16 was not detectable in U937 cells (Fig. 5, note absence of a 327 bp-IFNA4 fragment in lanes 2 and 7, and absence of a 145 bp-IFNA16 fragment in lanes 4 and 9). With stimulation by poly [I]-poly [C] or EMCV, the expression of IFNA2 and IFNA1 in these cells each accounted for approximately 20% and 10%, respectively, while IFNA8 levels accounted for over 50% (Fig. 5 and Table 4). Nearly identical patterns were also observed for

IFN-primed U937 cells, induced with poly [I]-poly [C] or EMCV (Table 4). The similarities in the patterns of subtypes induced by poly [I]-poly [C] or virus infection may not be surprising considering the results of several studies which suggest that these inducers regulate IFN expression through a common signaling pathway involving the dsRNA-dependent kinase, PKR (20, 22, 23). We were then interested in examining IFN-α expression in response to atypical inducers which do not involve an intrinsic double-stranded RNA component. In concurrent studies, we have determined that LPS or PMA can induce IFN-α in U937 cells, but only after these cells had been IFN-primed (Chapter 3, (7)). Analysis of the subtypes induced in these samples again provided a familiar pattern: IFNA4 and IFNA16 were not detected, while IFNA8 and IFNA2 accounted for approximately 50% and 20%, respectively, and IFNA1 levels at roughly 20% were modestly higher than observed for EMCV or poly [I]-poly [C] (Table 4, rows 5 and 6). It is also interesting to note that the five subtypes detected in PBMCs collectively accounted for only 35%-60% of the total IFN-α pool, depending on the induction conditions. In contrast, the three subtypes detected in U937 cells, IFNA1, IFNA2 and IFNA8, typically accounted for 75%-90% of the total IFN-α pool.

Lastly, studying Namalwa cells revealed yet further, distinct patterns of IFN-α subtype expression. Infection of Namalwa cells by EMCV alone induced IFNA1, IFNA2 and IFNA8, but not IFNA4 nor IFNA16 (Table 5). While this subset of expressed subtypes resembles that of U937 cells, the relatively low levels for each subtype more resembles the IFN-α profile of PBMCs. However, a substantive change results from IFN-priming of the Namalwa cells. The proportional levels of IFNA2 and IFNA8 each increased by twofold. As a result, while IFNA2 and IFNA8 constituted approximately 50% of the IFN-α pool in unprimed cells induced with EMCV, these two subtypes together accounted for over 90% of IFN-α mRNA in IFN-primed Namalwa cells following stimulation.

**Figure 1. Consensus IFN-α primers.** (A) The two highly conserved regions of the IFN-α gene family used to design the consensus IFN-α PCR primers, are designated with nucleotide numbering from Henco et al. (19). IFN-α genes containing non-conserved nucleotides (bold) are shown, while all other IFN-α genes (not shown) have sequences identical to the IFNA-consensus. (B) IFN-α-specific sequences in the upstream and downstream consensus IFN-α PCR primers are represented in uppercase with arrowheads indicating their nucleotide positions. Positions with degeneracy are indicated by square brackets containing the choice of nucleotides present. Extraneously added nucleotides with no correspondence to IFN-α sequences are represented in lowercase. These include the "GG" clamps, for enhanced 5'-end annealing, and *Hind*III and *Bam*HI restriction sites (underlined), for facilitating the cloning of the PCR product.

# A

## For Upstream Primer:

5' (+1078)        3' (+1099)

     ↓          ↓

| | |
|---|---|
| IFNA1 | TTCCTCCTGTCTGATGGACAGA |
| IFNA2 | TTTCTCCTGCTTGAAGGACAGA |
| IFNA6 | TTTCTCCTGTCTGAAGGACAGA |
| IFNA7 | TTTCTCCTGCTTGAAGGACAGA |
| IFNA-concensus | TTTCTCCTGCCTGAAGGACAGA |

## For Downstream Primer:

5' (+1415)        3' (+1435)

     ↓          ↓

| | |
|---|---|
| IFNA5 | TGTGCATGGGAGGTTGTCAGA |
| IFNA21 | TGTGCTTGGGAGGTTGTCAGA |
| IFNA-concensus | TGTGCCTGGGAGGTTGTCAGA |

# B

    (+1078)              (+1099)

      ↓               ↓

Up:  5' ggaagcTT[T/C]CTCCTG[T/C][T/C]TGA[A/T]GGACAGA 3'

Down: 5' gggatccTCTGACAACCTCCCA[A/G/T]GCACA 3'

      ↑           ↑

    (+1435)         (+1415)

Fig. 1.

**Figure 2.** **(A) Amplification of IFN-α genes from cDNA and genomic DNA samples.** Following stimulation of PBMCs with the indicated inducers, cDNA was prepared and amplified using the consensus IFN-α (upper panel) or GAPDH PCR primers (lower panel). Lanes 1 and 2 represent controls in which PBMCs receiving no treatments were harvested at 0 and 6 hour timepoints. Lanes 3 and 5 correspond to induction with EMCV induction (V) while lanes 4 and 6 correspond to poly [I]·poly [C] induction (IC), for 3 or 6 hours as indicated. Lanes 7 and 8 represent the negative and positive PCR controls, respectively. **(B) IFN-α subtype identification by sequence analysis.** The IFN-α PCR products from two independent amplification reactions using HeLa cell genomic DNA, were subcloned into M13 vector and random clones were sequenced to identify the IFN-α subtype present. **(C)** The clone and sequence approach was applied to the IFN-α PCR products derived from PBMCs induced with EMCV or poly [I]·poly [C] for 6 hours.

**A**

| Inducer | | - | - | V | IC | V | IC | (-) | (+) |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | | 0 | 6 | 3 | 3 | 6 | 6 | | |
| | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |



←— IFN-α

←— GAPDH
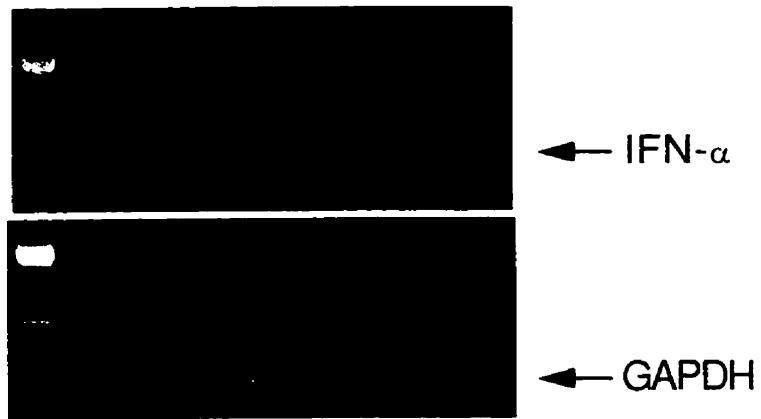
**Fig. 2a.**

**B**
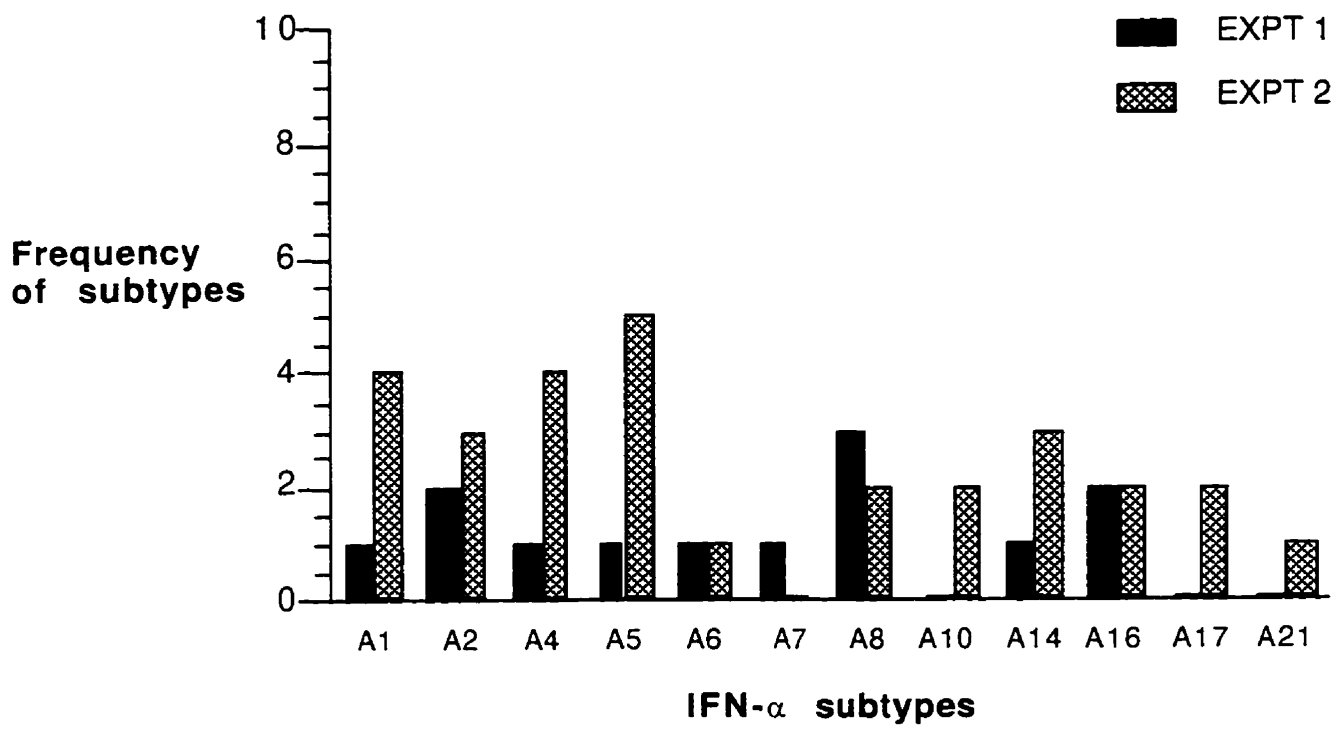


Fig. 2b.

# C
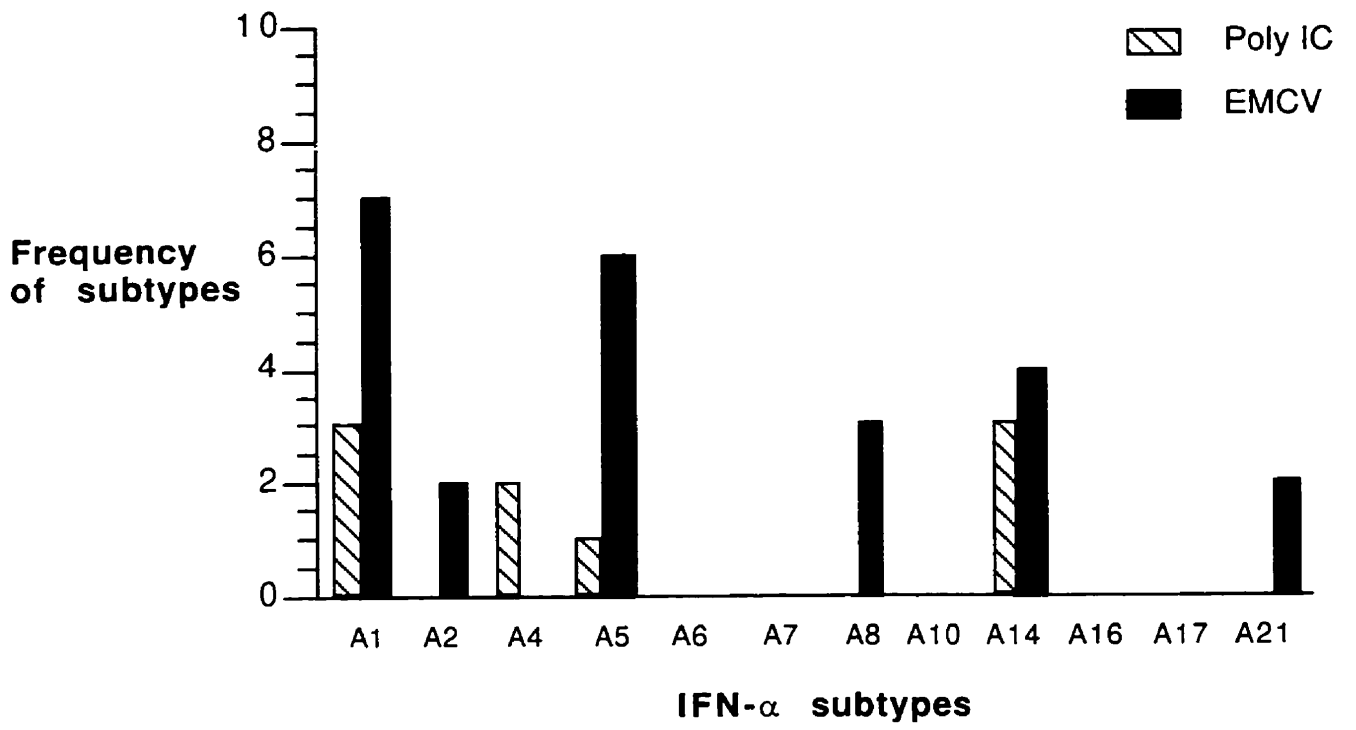


Fig. 2c.

**Figure 3. Determination of IFN-α subtype expression by RT-PCR and sequence analysis.**
(A) U937 cells, stimulated with IFN inducers, were harvested at different timepoints and RT-PCR analysis was performed to detect IFN-α and GAPDH mRNA. Lanes 1-5 show the kinetics of IFN-α induction by poly [I]·poly [C] or EMCV in unprimed U937 cells. Lanes 6-8 show IFN-α induction by the same inducers but in IFN-primed U937 cells. (B), (C) The clone and sequence approach was applied to the IFN-α PCR products derived from IFN-primed U937 and Namalwa cells which were induced with EMCV or poly [I]·poly [C] for 4 hours.

**A**

Time (h)  0  4  8 16 24 0  2  4
          1  2  3  4  5  6  7  8

Inducer:



Poly I:C

⟵ IFN-α

⟵ GAPDH

Time (h)  0  4  8 16 24 0  2  4
          1  2  3  4  5  6  7  8

EMCV



⟵ IFN-α

⟵ GAPDH
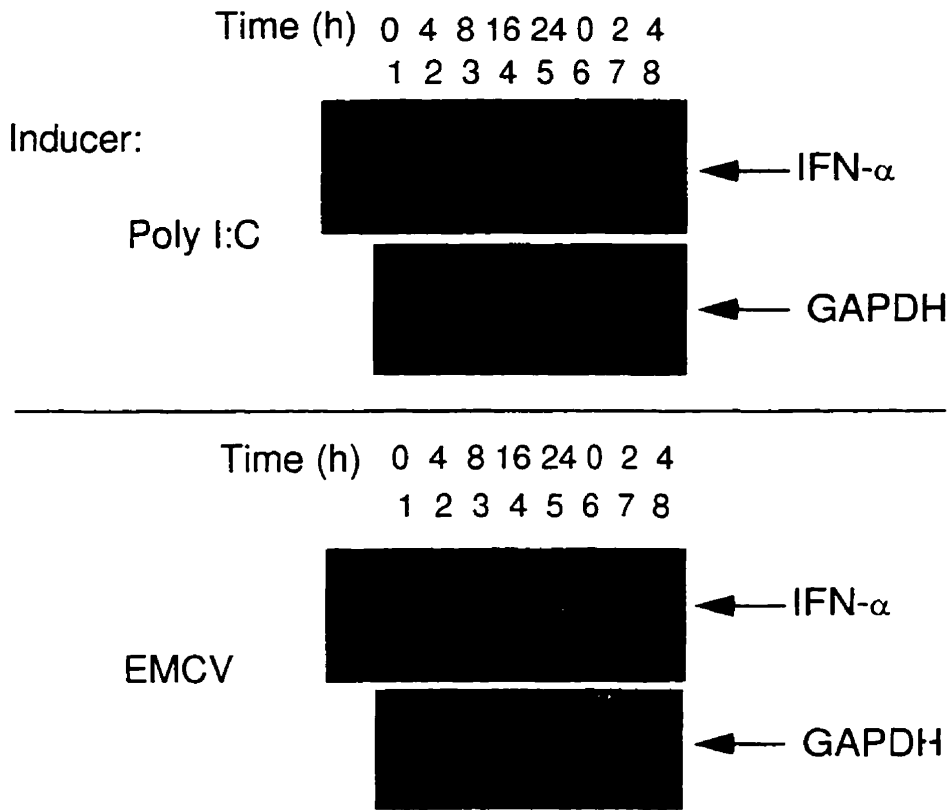
**Fig. 3a.**

**B**
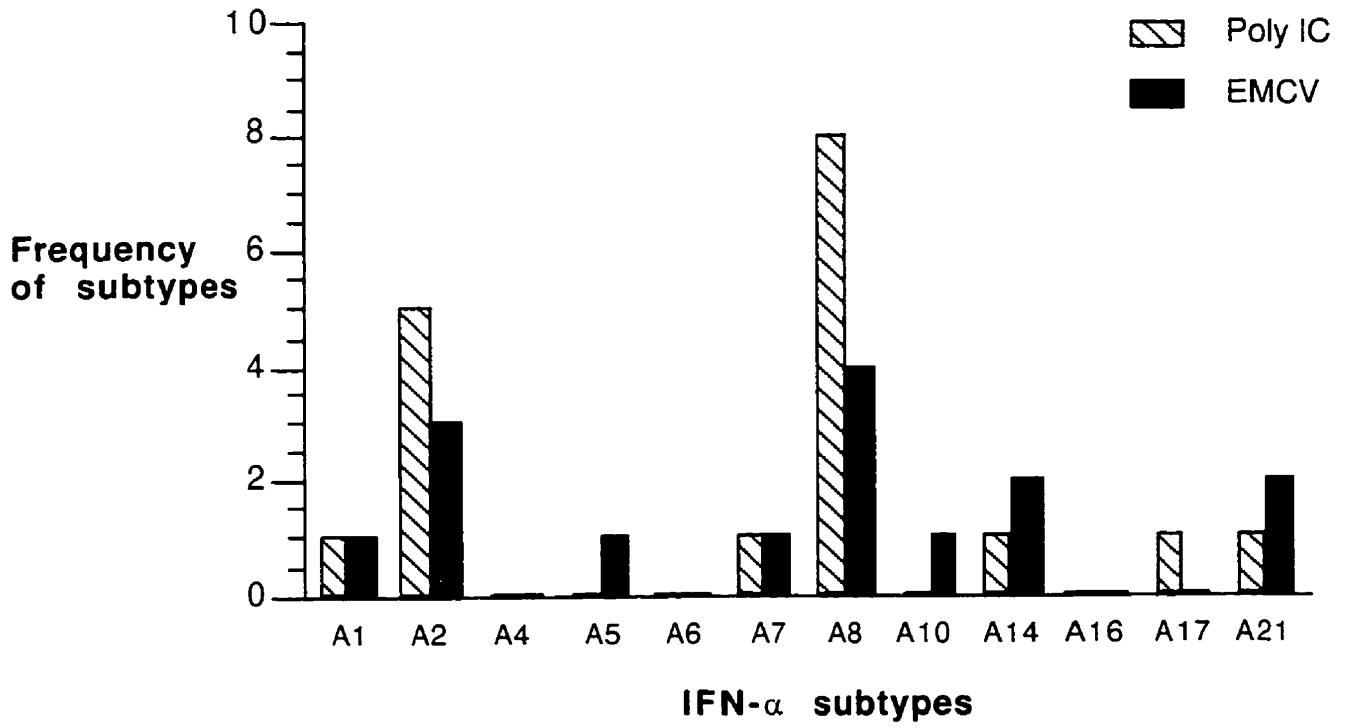


Fig. 3b.

**C**
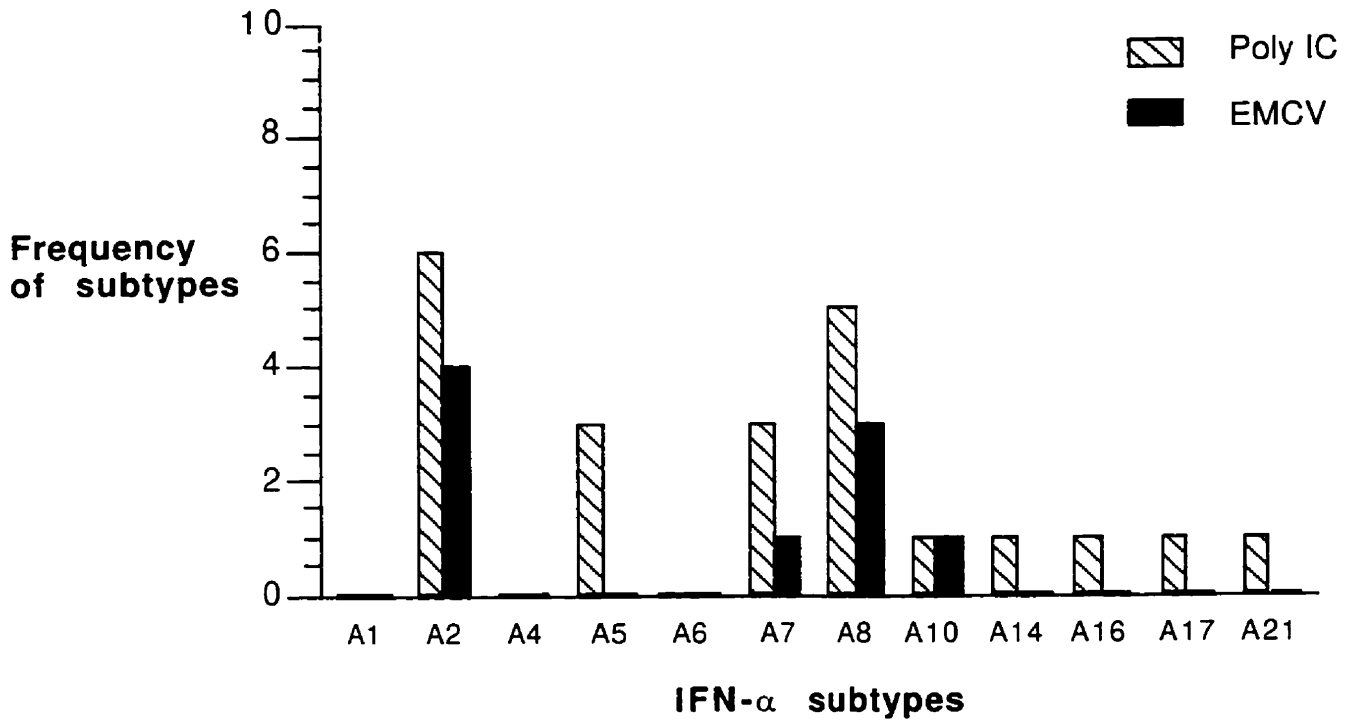


**Fig. 3c.**

**Figure 4. Identification of IFN-α subtypes using restriction digest analysis.** Genomic DNA from U937 and Namalwa cells were amplified using unlabelled upstream and radio-endlabelled downstream consensus IFN-α primers. The uncut IFN-α PCR products are contained in lanes 1 and 6 while the remaining lanes contain equivalent aliquots from either sample after digestion by the indicated restriction enzymes. Uncut and digested products were separated by gel electrophoresis using 15% native polyacrylamide gels. Subtype-specific restriction fragments were produced with the expected sizes as described in Table 1. The DNA markers represent increasing increments of 100 bp.

**A**



U937 (genDNA)    Namalwa (genDNA)

uncut  AvaI  AvaII  MaeIII  TaqI    uncut  AvaI  AvaII  MaeIII  TaqI
M    1     2     3      4       5       6      7     8      9       10    M

uncut IFNA4
IFNA1 IFNA8
IFNA16 IFNA2
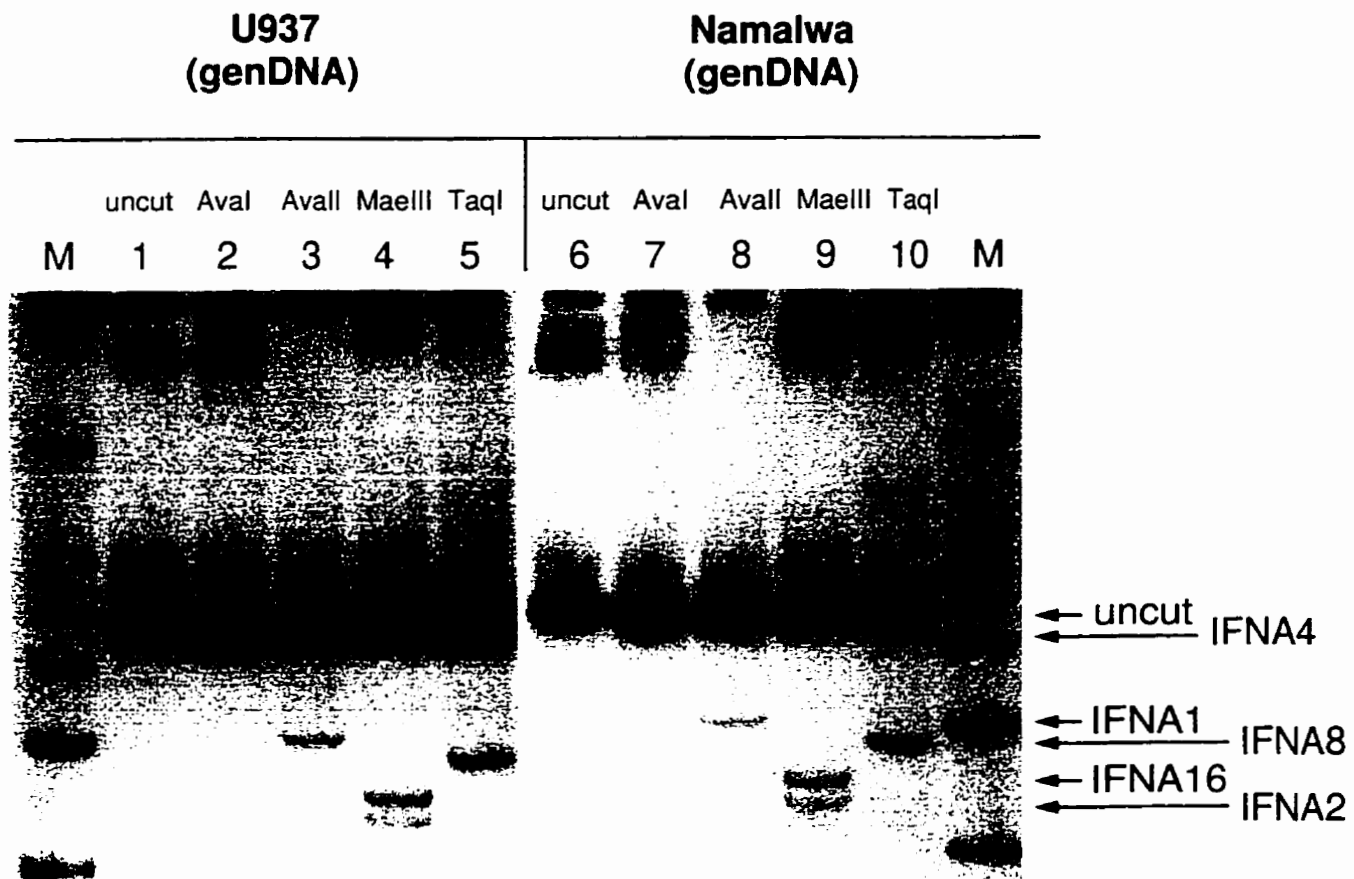
**Fig. 4.**

**Figure 5. Determination of IFN-α subtype expression using restriction digest analysis.**
cDNA samples, prepared from U937 cells induced with EMCV or poly [I]-poly [C], were amplified using unlabelled upstream and radio-endlabelled downstream consensus IFN-α primers. The uncut IFN-α PCR products are contained in lanes 1 and 6 while the remaining lanes contain equivalent aliquots from either sample after digestion by the indicated restriction enzymes. Uncut and digested products were separated by gel electrophoresis using 15% native polyacrylamide gels.

**A**
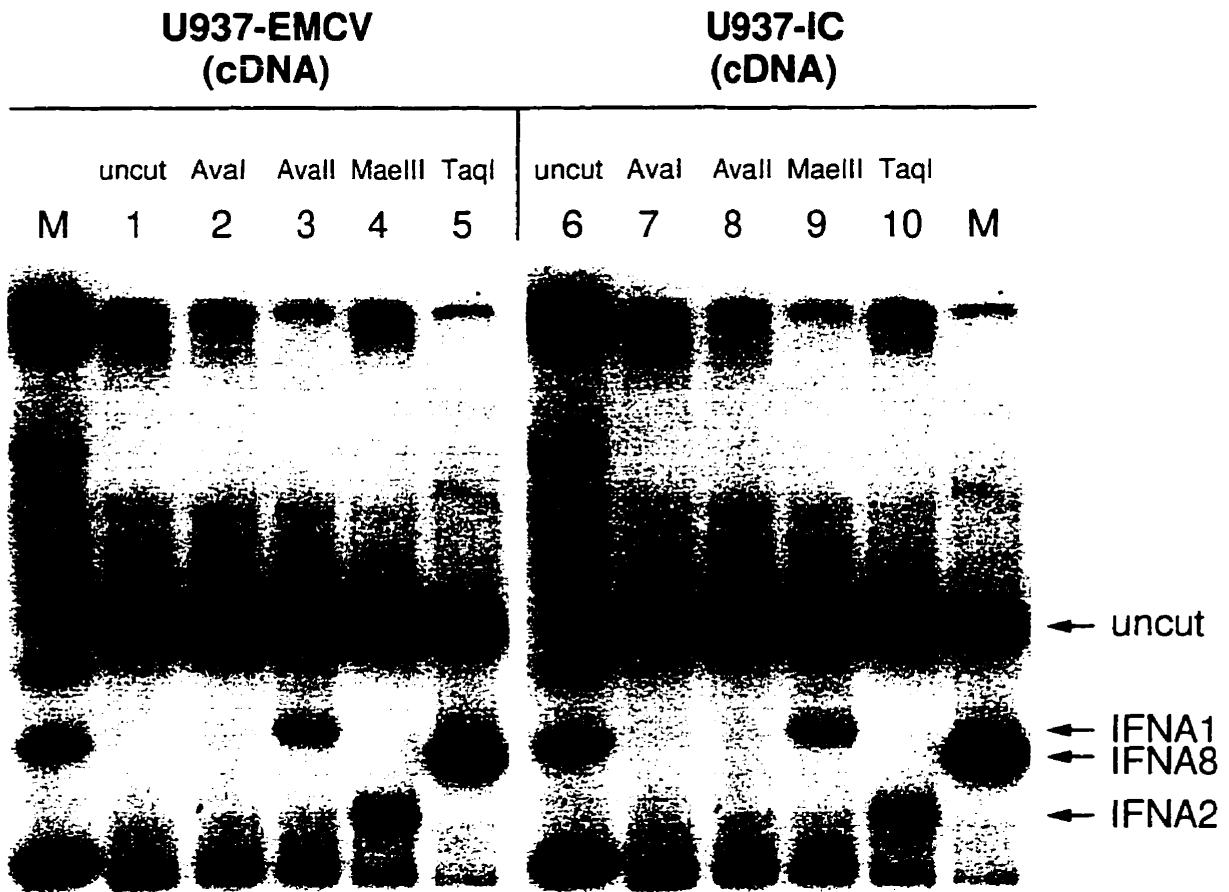


Fig. 5.

**Table 1. Unique restriction enzyme sites contained in IFN-α genes.** Gene sequences were obtained from Henco et al. (20) and unique restriction sites were identified for individual IFN-α subtype genes. Digestion of the respective IFN-α subtype sequences with the indicated restriction enzymes are expected to yield two restriction fragments of the indicated sizes. The 3' fragments (in bold) were detected in this study by using 3' radio-endlabelled PCR primers.

| IFN-α subtype | Restriction site positions | Restriction fragments (bp) | |
|---|---|---|---|
| | | 5' primer | **3' primer** |
| IFNA1 | Ava II, 1237 | 167 | **205** |
| IFNA2 | Mae III, 1316 | 245 | **127** |
| IFNA4 | Ava I, 1115 | 45 | **327** |
| IFNA8 | Taq I, 1260 | 190 | **182** |
| IFNA16 | Mae III, 1298 | 227 | **145** |

**Table 1.**

**Table 2. Quantification of IFN-α subtypes from genomic DNA.** Subtype-specific fragments, as represented in Fig.4, were excised and quantitified, as described in Methods. The representation of each subtype from the different templates used is expressed as a percentage, from the average of three samples (standard deviation in brackets). The amount of DNA used for each amplification reaction is indicated. 40 amplification cycles were used for all reaction except in Row 5 (35 cycles), as indicated.

| Genomic DNA | IFNA1 | IFNA2 | IFNA4 | IFNA8 | IFNA16 |
|---|---|---|---|---|---|
| U937 1 ng | 5.9 (0.3) | 5.6 (0.6) | 6.9 (2.4) | 9.1 (1.0) | 5.9 (0.7) |
| Namalwa 1 ng | 4.9 (0.1) | 5.7 (1.0) | 5.1 (1.0) | 9.3 (0.6) | 7.0 (2.5) |
| U937 5 ng | 5.2 (0.1) | 5.4 (1.5) | 7.6 (3.0) | 11.5 (1.0) | 8.2 (1.0) |
| U937 25 ng | 5.7 (1.4) | 6.0 (1.0) | 4.4 (1.8) | 11.0 (2.2) | 6.3 (1.6) |
| U937 1 ng- 35 cycles | 5.0 (1.1) | 5.6 (0.4) | 6.9 (2.8) | 11.0 (0.6) | 7.4 (0.4) |
| HeLa 2.5 ng | 4.2 (0.9) | 5.2 (1.6) | 7.7 (0.7) | 9.1 (1.7) | 7.3 (0.4) |

**Table 2.**

**Tables 3-5. Quantification of IFN-α expression from PBMCs, U937 and Namalva cells.**

The representation of subtypes from the different cDNA sources, indicated, is presented as a

percentage from the average of three samples (standard deviation in brackets). Subtypes which

were not detected are designated (**n.d**). 40 amplification cycles were used throughout.

| PBMC-cDNA | IFNA1 | IFNA2 | IFNA4 | IFNA8 | IFNA16 |
|---|---|---|---|---|---|
| Poly IC | 7.3 (0.6) | 12.9 (3.5) | 5.6 (2.1) | 14.6 (0.5) | 4.2 (1.7) |
| EMCV | 2.4 (0.3) | 8.1 (0.7) | 3.9 (1.7) | 16.3 (2.7) | 4.2 (1.2) |
| IFN-α + IC | 16.2 (6.7) | 14.9 (1.0) | 5.0 (4.3) | 24.9 (1.8) | 0.7 (0.6) |
| IFN-α + EMCV | 7.1 (1.0) | 13.1 (0.3) | 8.2 (3.3) | 21.3 (2.3) | 3.3 (1.8) |

**Table 3.**

| U937-cDNA | IFNA1 | IFNA2 | IFNA4 | IFNA8 | IFNA16 |
|---|---|---|---|---|---|
| Poly IC | 6.6 (1.2) | 16.4 (1.0) | n.d. | 55.7 (2.3) | n.d. |
| EMCV | 8.0 (1.7) | 22.1 (2.1) | n.d. | 57.3 (5.2) | n.d. |
| IFN-α + IC | 13.1 (1.0) | 16.6 (1.2) | n.d. | 44.0 (4.3) | n.d. |
| IFN-α + EMCV | 11.1 (1.2) | 20.0 (1.1) | n.d. | 42.5 (1.4) | n.d. |
| IFN-γ + LPS | 20.3 (1.4) | 16.8 (1.1) | n.d. | 51.9 (2.6) | n.d. |
| IFN-γ + PMA | 21.9 (1.9) | 16.1 (0.4) | n.d. | 48.9 (2.2) | n.d. |

**Table 4.**

| Namalwa-cDNA | IFNA1 | IFNA2 | IFNA4 | IFNA8 | IFNA16 |
|:---:|:---:|:---:|:---:|:---:|:---:|
| EMCV | 2.5 (0.6) | 20.2 (0.8) | n.d. | 29.7 (1.7) | n.d. |
| IFN-α + IC | 3.7 (1.2) | 41.5 (2.6) | n.d. | 55.2 (3.4) | n.d. |
| IFN-α + EMCV | 4.6 (1.9) | 44.7 (3.6) | n.d. | 57.3 (4.0) | n.d. |

**Table 5.**

## Discussion

In this study, we have shown that consensus IFN-α PCR primers are capable of simultaneously amplifying the entire IFN-α gene family. Furthermore, their use permitted quantitative analysis of IFN-α subtype expression at the mRNA level. In order to test the utility of these consensus primers, they were first used to amplify IFN-α sequences from genomic DNA. After cloning the resultant PCR products, sequence analysis of random clones revealed that all known IFN-α sequences were represented (Fig. 2). For a more efficient means of identifying IFN-α subtypes, we took advantage of restriction enzyme sites which are unique to certain IFN-α genes (Table 1). At the outset, it was uncertain whether each subtype was being amplified uniformly, as PCR reactions are intrinsically somewhat unpredictable. However, analysis by the restriction digest approach indicated that the proportional amplification of the five selected subtypes (IFNA1, -A2, -A4, -A8, -A16) was reasonably consistent among several different samples. These samples included genomic DNA from HeLa, U937 and Namalwa cells, and also involved changing variables such as the input amount of template DNA and the number of amplification cycles (Table 2). The representation of each subtype in most cases approximated the expected frequency of 7.1 % (calculated on the basis of fourteen functional subtypes) but, two exceptions should be noted. Firstly, since IFNA1 and IFNA13 are believed to represent two distinct genes but with identical coding regions, the frequency of the *Ava*II digestion product was expected to approximate 14%; instead, its observed frequency in our assay was closer to 7%. While we do not have a definitive explanation to account for this, some possibilities include: 1) IFNA1 and IFNA13 sequences are both consistently under-amplified at a one-half rate by the PCR reaction; 2) IFNA1 and IFNA13 are actually allelic versions of the same gene; or 3) IFNA1 and IFNA13 are distinct genes but one has a slightly different sequence from that which is published and consequently lacks the *Ava*II site at position +1237. Secondly, the frequency of the IFNA8-specific *Taq*I restriction fragments ranged between 9% and 11%. Although this could indicate a small degree of

preferential amplification. IFNA8 sequences did not appear over-represented among the sequenced clones derived from genomic DNA (Fig. 2b). By identifying additional unique restriction sites. it would be possible to examine the amplification characteristics of other IFN-α subtypes. This would help to further assess the uniformity in amplification of IFN-α sequences by this method. For this purpose, it would also be informative to examine the distribution of IFN-α subtypes from a panel of human genomic DNA samples.

The use of the above mentioned methods then permitted the detection of individual IFN-α subtypes expressed in response to varied stimuli and from different cell types. For PBMCs stimulated with poly [I]-poly [C] or EMCV, we observed using restriction digest analyses that IFNA2 and IFNA8 expression was highest among the five subtypes detected. By comparison, Hiscott et al. used S1 nuclease protection assays and reported that in PBMCs induced with Sendai virus, IFNA1 mRNA expression was highest, followed by IFNA2 and IFNA4, while IFNA8 mRNA levels were barely detectable (21). The differences between our two studies may result from inherently distinct characteristics between Sendai virus as an inducer, in their study, and EMCV or poly [I]-poly [C] as inducers, in this study. Alternatively, it may reflect differences between PBMCs of individual donors or between the experimental assay systems. With particular regards to the IFNA1 levels measured by Hiscott et al., the authors had noted that the probe used likely detected both IFNA1 and IFNA13 transcripts (15). Considering the possibility that our detection method for IFNA1/A13 using *Ava*II is identifying only one of these genes, this could explain the differences in the measured IFNA1 levels between our studies. Lastly, we have also observed that IFN priming of the PBMCs seems to selectively increase the induced mRNA levels for certain subtypes (IFNA1, IFNA2, and IFNA8), but not for others (IFNA4 and IFNA16) (Table 3).

Stimulation of the U937 and Namalwa cell lines yielded distinct patterns of IFN-α subtype expression (Table 4). IFNA2 and IFNA8 were also the most highly expressed subtypes in both cell lines but in contrast to PBMCs, the levels of these two subtypes were substantially higher, accounting for 50% to over 90% of the total IFN-α pool. However, the two cell lines

could be distinguished on the basis that IFNA8 levels were consistently at least double that of IFNA2 in U937 cells. while the expression of either subtype was always nearly equivalent in Namalwa cells. In addition. IFN priming seemed to influence subtype expression in Namalwa. by selectively increasing IFNA2 and IFNA8 levels. but not in U937 cells. We wish to point out that while there was possibly some indication of preferential amplification for IFNA8 sequences. evident by its frequency from genomic DNA. this factor amounted to approximately 1.5-fold over the expected frequency. Therefore. even if the IFNA8 mRNA levels detected by restriction analysis are reduced by twofold. IFNA8 still represents one of the principal induced subtypes. Others have also described PCR-based approaches for studying the expression of human IFN-α genes. These methods involved using subtype-specific PCR primers (1) or general primers containing highly conserved IFN-α sequences (25), but none to our knowledge have demonstrated a capability to detect all known IFN-α nor to quantify IFN-α subtype levels.

The results of this study are consistent with the general conclusions of several studies that IFN-α subtypes are differentially expressed in human and mouse cells (15, 16, 19). Our data further proposes that the cellular background of the IFN producing cell is an important factor in determining particular patterns of IFN-α subtype expression. In addition. IFN-priming appears to enhance the induced expression of certain select IFN-α genes. However, no obvious differences were noted in the patterns of subtype expression as induced by either poly [I]-poly [C] or EMCV in all the cell types studied. Moreover. the IFN-α subtypes induced by LPS or PMA in U937 cells were also very similar to that by poly [I]-poly [C] or EMCV. As a possible explanation. we have recently reported that suppression of the dsRNA-dependent kinase. PKR. in U937 results in impaired IFN-α inducibility by any of these four inducers (6). Thus. despite their disparate nature. it is conceivable that they would induce IFN-α genes similarly given that their actions are mediated by a common signaling component, PKR.

The advantages of our PCR strategy, that it requires only small amounts of RNA sample and precludes the need for a large panel of IFN-α probes, may facilitate studying IFN-α subtype expression in other biological contexts. For example, two major cell types have been identified

as the primary IFN-α producers in PBMCs, monocytes and a rare, HLA-DR+ "null" cell type that resembles a dendritic cell (11). Depending on the type of inducer, dendritic cells may produce over 50-fold more IFN-α activity than similar numbers of monocytes (10). As they also differ in other aspects of IFN production, such as differential responses to certain inducers (11), it is conceivable that monocytes and HLA-DR+ cells may express distinctive subsets of IFN-α subtypes. Alternatively, many varied forms of IFN induction which do not involve virus infection have been described (2, 25, 34), and it is possible that these forms of stimuli may induce yet other novel patterns of IFN-α expression. One interesting category involves glutaraldehyde-fixed virus-infected cells which appear to stimulate IFN-producing cells via a cell surface interaction; that is, viral replication in the producer cell is not required for IFN induction. Examples of this have been described for stimulation of PBMCs with fixed cells previously infected by herpes simplex virus (30), coronavirus (21), or HIV (3, 13) Interestingly, it has been reported for the IFN-α activity induced from PBMCs by HIV-infected cells, that not only did it have 20-fold lower activity than recombinant IFN-α2 in inhibiting HIV replication, this IFN-α preparation could even enhance HIV growth at low concentrations (13). Therefore, a better understanding of the IFN-α species induced by a given virus could help in elucidating which IFN-α subtypes are more or less effective against that particular virus.

Finally, PCR using the consensus IFN-α primers could be useful for investigating the role of aberrant IFN-α expression that has been associated with several human diseases. Notably, chronically high serum levels of an unusual "acid-labile IFN-α" have been described in patients with AIDS (9) or autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (28, 32). It is unknown what commonality, if any, could be responsible for the presence of IFN-α in these diseases. Determining the cellular source and identity of the IFN-α subtypes in such patients may help to understand the pathogenesis of these diseases. Another important example involves type 1 diabetes in which the localized expression of IFN-α in pancreatic, insulin-secreting β cells has been strongly correlated with this disease in humans (12). Furthermore, the causal nature of this correlation is supported by the finding that diabetes

develops in transgenic mice bearing an IFN-α transgene that is only expressed in pancreatic β cells (33). To fully understand the role of IFN-α genes in the pathogenesis of these diseases, it may be as important to study the consequences of aberrant IFN-α expression as it is to determine whether only particular IFN-α subtypes are responsible for manifestation of disease.

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# Chapter Three

**Priming enhances the kinetics of IFN-$\alpha$ induction and responsiveness to LPS and phorbol ester**

# Abstract

The expression of interferon-alpha (IFN-α) genes is strongly induced by virus infection or double-stranded RNA (dsRNA). Priming or pretreatment of cells with certain cytokines has been shown to modulate the characteristics of IFN gene induction in response to stimuli. Since a major cellular source of IFN-α production *in vivo* are monocytes, the promonocytic U937 cell line has commonly been used to study IFN expression. U937 cells stimulated with encephalomyocarditis virus (EMCV) or synthetic dsRNA, poly [I]·poly [C], resulted in the induced accumulation of IFN-α mRNA but, with relatively delayed kinetics as compared with IFN-β mRNA induction. However, the kinetics of IFN-α induction were accelerated in U937 cells following priming by IFN-α, IFN-γ or PMA. Furthermore, priming of the U937 cells was strictly required for enabling the induction of IFN-α mRNA in response to lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA). At the protein level, the induced synthesis of IFN-α by poly [I]·poly [C], LPS or PMA was also strictly dependent on priming. Lastly, IFN production in response to all inducers tested was most markedly reduced by 2-aminopurine, a known inhibitor of the dsRNA-dependent kinase, PKR, whereas the protein kinase C-specific inhibitor, calphostin C, was only partially inhibitory for IFN production. These results indicate that priming enhances IFN-α gene regulation on several levels including the kinetics of induction, range of responsiveness to particular stimuli, and IFN protein synthesis. In addition, the signaling pathways regulating IFN-α expression in U937 cells appear to involve the activities of PKR and to a lesser extent, protein kinase C.

# Introduction

The Type I IFN genes are normally transcriptionally silent but they are strongly activated in response to virus infection or dsRNA. In addition, the amount of IFN proteins synthesized can be appreciably increased by pretreatment of the producer cells with low concentrations of IFN prior to their stimulation, a phenomenon generally referred to as priming (7, 15, 21). This phenomenon is believed to be biologically relevant as an amplification mechanism which enables a rapid and widespread IFN-mediated antiviral response following the initial recognition of a virus infection. The effects of IFN-priming on IFN-$\alpha$ and IFN-$\beta$ gene regulation has been studied in a variety of cell types from human and mouse backgrounds. The biochemical mechanisms underlying the priming phenomenon remain to be elucidated and likely, there are different proteins involved in mediating the priming effects for IFN-$\alpha$ gene regulation as opposed to IFN-$\beta$ regulation. In mouse L929 fibroblast cells, virus infection induces IFN-$\alpha$ and IFN-$\beta$ gene transcription proficiently but this induction is further increased in primed cells (21). Alternatively, IFN-$\beta$ is poorly inducible in human HeLa and 143 tk-fibroblast cells, but priming enables efficient IFN-$\beta$ induction in response to virus or poly [I]·poly [C]. Furthermore, cell fusion experiments between the poorly inducible human fibroblasts and highly inducible mouse C-127 cells, suggested that the human cells are deficient in a trans-acting factor required for IFN-$\beta$ gene activation, which is presumably induced following IFN-priming (4).

The expression of human IFN-$\alpha$ genes appears to be mostly restricted to hematopoietic cells. Since a primary source of IFN-$\alpha$ production in peripheral blood mononuclear cells (PBMCs) are the monocytes, the promonocytic cell line, U937, has been extensively studied for its monocyte-like qualities, including IFN-$\alpha$ gene expression. In this study, we have observed that the induction of IFN-$\alpha$ genes in U937 is enhanced in many aspects by the priming effects of exogenous IFNs and phorbol ester.

## Materials and Methods

**Cell culture and inductions.** U937 cells were cultured at $37^{\circ}C$ in 5% $CO_2$ with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). T98G and L929 cells were cultured similarly except using Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS. EMCV stocks were prepared by passage in murine L929 cells and the viral titer was determined in terms of $TCID_{50}$ using L929 cells. Poly [I]·poly [C] (in physiological salt, Pharmacia) stock solutions of 2 μg/ml were prepared in sterile $H_2O$. Lipopolysaccharide (LPS, Sigma) stocks of 1 μg ml were prepared in sterile PBS. Phorbol 12-myristate 13-acetate (PMA, Sigma) stocks of 1 mM were prepared by dissolving in DMSO before further dilution in phosphate-buffered saline (PBS). 2-aminopurine (Sigma) was dissolved in PBS containing glacial acetic acid (1:200) to prepare a 150 mM stock solution. Calphostin C (Calbiochem) stocks of 50 uM were prepared by dissolving in DMSO before further dilution in PBS. Treatments of cells with the abovementioned inducers and kinase inhibitors were performed by direct addition into the cell culture media to yield the indicated final concentrations. U937 cells were primed by culturing for 18 hours in the presence of recombinant human IFN-α2 (Schering) (200 U ml), IFN-γ (Amgen) (200 U/ml) or PMA (50 nM). Supernatants for assaying IFN production were collected 24 hours following induction.

**Reverse-transcriptase-polymerase chain reaction (RT-PCR).** RNA extraction, cDNA synthesis, and PCR to detect IFN-α, IFN-β, GAPDH were performed identically as described in Chapter 2.

**Antiviral assay for IFN activity.** IFN activity was measured by a bioassay as in a previous report (17). In brief, samples were serially diluted in cell culture medium and added to T98G monolayers, plated in 96-well microtiter plates. After incubation for 16-18 hours, this medium was removed and the T98G cells were challenged with EMCV at $10^6$ $TCID_{50}$/ml for 24 hours. Cytopathic effects were determined by staining with 0.05% crystal violet. IFN titers were defined on the basis in which one unit of antiviral activity represents the amount of IFN required to confer 50% protection against cytopathic effects.

# Results

**Delayed IFN-α inducibility in U937 cells.** Previously, we have observed that stimulation of freshly isolated PBMCs with EMCV or poly [I]·poly [C] results in a rapid and coordinate induction of IFN-α and IFN-β mRNA accumulation, within 3-6 hours (Chapter Two). When these same inducers were used to stimulate U937 cells, the induction of IFN-α mRNA accumulation by EMCV or poly [I]·poly [C] was substantially delayed in comparison with PBMCs, being detectable by 16 hours (Fig. 1a and 1b). Initially, it was unclear whether this delayed response may have been due to inefficient uptake or recognition of the inducers by U937 cells. To address this possibility, the induction of IFN-β mRNA was assayed in the same set of samples. As shown in Figs. 1a and 1b, U937 cells produced detectable IFN-β mRNA levels in response to EMCV or poly [I]·poly [C] within the first 4 hours which remained detectable through to 24 hours later. These observations indicated that not only were U937 cells capable of responding rapidly to these stimuli, but that the mechanisms which regulate IFN-α and IFN-β expression are clearly distinct.

**Priming enables rapid IFN-α induction.** Priming is a well known phenomenon whereby pretreatment of cells with IFN results in enhanced IFN production following subsequent stimulation. In order to determine whether priming would alter the kinetics of IFN-α induction, U937 cells were pretreated with recombinant IFN-α2 (100 U/ml) for 18 hours. Upon stimulation with poly [I]·poly [C], IFN-α mRNA was detected in the primed U937 cells after as early as 1 hour (Fig. 2a, lanes 2-5). Also, the induction of IFN-α by EMCV was similarly rapid, being detectable by 4 hours (Fig 2a, lanes 6-9). We were next interested in determining whether IFN-γ can function similarly as IFN-α for priming, since IFN-α and IFN-γ share some common biological activities and both are well known inducers of macrophage and monocyte activation (10). As indicated in Fig. 2b, IFN-γ primed cells were also competent for a rapid IFN-α response upon stimulation with EMCV or poly [I]·poly [C] (lanes 1-3). Lastly, we investigated whether PMA was effective as a priming agent. PMA is well studied as an inducer

of acute inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α). interleukin-1 (IL-1) and interleukin-6 (IL-6) and as well. PMA serves as a general activator of macrophage/monoctyes (1). Given this. modulation of IFN-α expression by PMA would not be unexpected. However. a previous report has indicated that pretreatment of human PBMCs with PMA results in inhibition of IFN-α induction (22). To investigate its effects. U937 cells were treated with PMA for 18 hours but this did not induce detectable IFN-α expression. Unexpectedly. priming by PMA did facilitate the rapid induction of IFN-α in response to EMCV or poly [I]·poly [C] in a manner similar to IFN-priming (Fig. 2b, lanes 4-6). It should be noted that PMA treatment alone for shorter periods of 3-6 hours (see later, Fig. 4a) or longer periods of 24-48 hours did not induce IFN-α mRNA (data not shown).

**Priming enhances IFN-α protein synthesis.** Having determined that priming accelerates the kinetics of IFN-α mRNA induction. we next examined the effects of priming on the production of IFN-α proteins. By using an EMCV dose of 1.0 $TCID_{50}$/cell. IFN production was increased between two- and eightfold in U937 cells primed with IFN-α. IFN-γ. or PMA. as compared to cells which had not been primed (Fig. 3a). The effects of priming were more pronounced when a suboptimal EMCV dose of 0.1 $TCID_{50}$/cell was used. whereby primed U937 cells produced between 16 and 64 U/ml of IFN activity while unprimed cells produced no detectable IFN at all. The effects of priming were similarly pronounced using poly [I]·poly [C] as the inducer. Stimulation of unprimed U937 cells with 100 μg/ml of poly [I]·poly [C] yielded no detectable levels of IFN protein. despite the presence of detectable IFN-α mRNA in such cells (Fig. 1b. 16h and 24h). In contrast. cells primed with IFNs or PMA produced between 32 and 128 U/ml of IFN activity in response to poly [I]·poly [C] (Fig. 3b). Interestingly. PMA-priming resulted consistently in higher levels of IFN production than priming by either of the IFNs. It should be noted that IFN-α constitutes 80-100% of the IFN activity in these samples. as determined by neutralization with specific antibodies against IFN-α or IFN-β (data not shown). Also. U937 cells do not produce IFN-γ mRNA or protein in response to EMCV. as determined by RT-PCR and immunodetection methods (data not shown).

**IFN-α induction by LPS or PMA requires priming.** Given that priming appears to generally increase the sensitivity of U937 cells to stimuli such as virus or dsRNA. it is possible that primed U937 cells are more sensitive to other forms of stimuli. as well. LPS is a potent activator of macrophages and monocytes. and LPS can induce IFN production in mouse peritoneal macrophages (8). Also. as mentioned earlier. PMA is a potent inducer of several cytokine genes. The roles of LPS and PMA as IFN inducers were studied and as indicated in Fig. 4a (lanes 1-3). stimulation of U937 cells with either inducer alone had no effect on IFN-α mRNA expression. However. both LPS and PMA were efficient inducers of IFN-α mRNA in IFN-primed cells (Fig. 4a. lanes 4-9). These patterns were also consistent at the level of IFN-α protein synthesis. IFN production in U937 cells primed with IFN-α. IFN-γ or PMA was induced by LPS in a dose-dependent manner (Fig. 4b). IFN induction in primed cells was evident at LPS doses as low as 0.1 ng/ml. whereas in unprimed cells. LPS stimulation alone at concentration up to 500 ng/ml had no effects (Fig. 4b. data not shown). Similarly. IFN production in primed U937 cells was induced by PMA treatment at a concentration as low as 1.0 nM (Fig. 4c). PMA treatment alone at higher concentrations (up to 1 μM) and for longer periods (up to 48 hours) did not induce any detectable IFN activity.

**Effect of kinase inhibitors on IFN induction.** In order to investigate the signaling pathways involved for these different forms of IFN-α induction. two specific kinase inhibitors were studied for their effects on IFN induction. 2-aminopurine has been used as inhibitor of the PKR. while calphostin C is a potent and highly specific inhibitor of protein kinase C. The addition of either kinase inhibitors caused a dose-dependent reduction of EMCV-induced IFN-α production in unprimed U937 cells (Fig. 5a). Furthermore, the two kinase inhibitors can act synergistically. Whereas virus-induced IFN production was reduced from 1280 U/ml, down to 64 U/ml by 1.0 mM 2-aminopurine and. down to 256 U/ml by 25 nM calphostin C, the combination of both inhibitors at these doses resulted in the complete abrogation of IFN synthesis (Fig. 5a).

Lastly. the two kinase inhibitors were compared for their effects on IFN induction in response to poly [I]·poly [C]. EMCV. LPS or PMA. For this purpose. U937 cells were primed by IFN-$\gamma$ and then stimulated with these inducers in absence or presence of 5 mM 2-aminopurine or 100 nM calphostin C. These concentrations were used since they nearly abrogated IFN production by induction with EMCV alone. The inhibition of IFN production by calphostin C in all cases was only partial. although IFN titers were consistently reduced four- to eightfold. 2-aminopurine completely inhibited IFN production in all cases except for only partial reduction in response to EMCV.

Fig. 1. **Kinetics of IFN-α and IFN-β mRNA accumulation in U937 cells.** U937 cells were stimulated (A) with EMCV (10 $TCID_{50}$/cells) or (B) poly [I]·poly [C] (100 μg/ml) and harvested at the indicated times for RNA extraction. Samples were subjected to RT-PCR with primer specific for IFN-α, IFN-β, and GAPDH. Negative controls (-) represent PCR performed on RT reagents without sample RNA. Positive controls (+) represent PCR amplification of 1.0 ng human genomic DNA (for IFN-α or IFN-β) or a known positive cDNA sample (GAPDH). The DNA markers (M) represent a ladder of increasing increments of 100-basepairs.
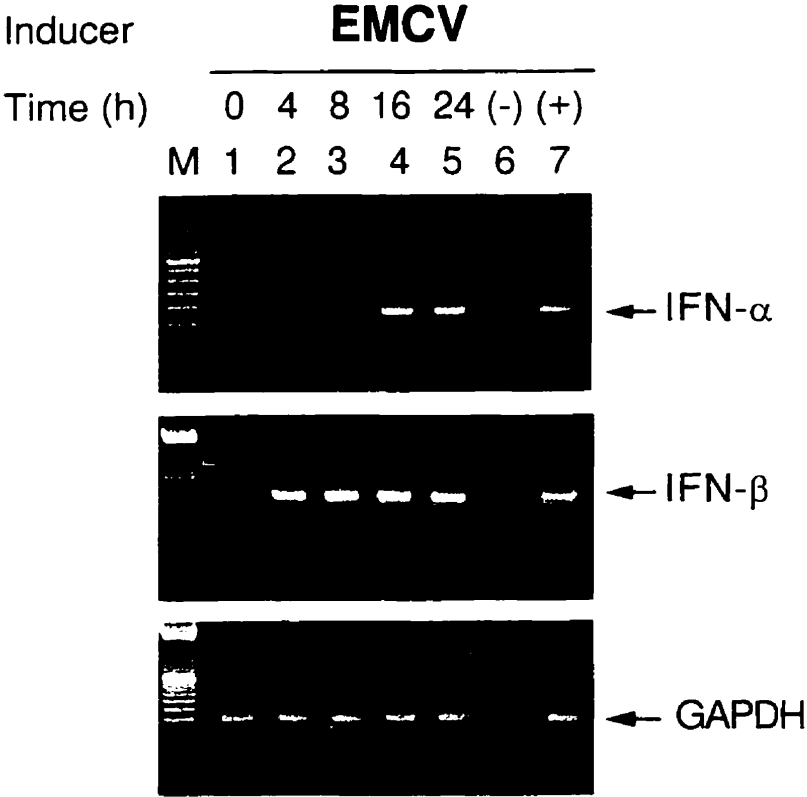
**A**



Fig. 1a.

**B**

| Inducer | **poly I:C** | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (h) | 0 | 4 | 8 | 16 | 24 | (-) | (+) |
| M | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

←IFN-α

←IFN-β

← GAPDH
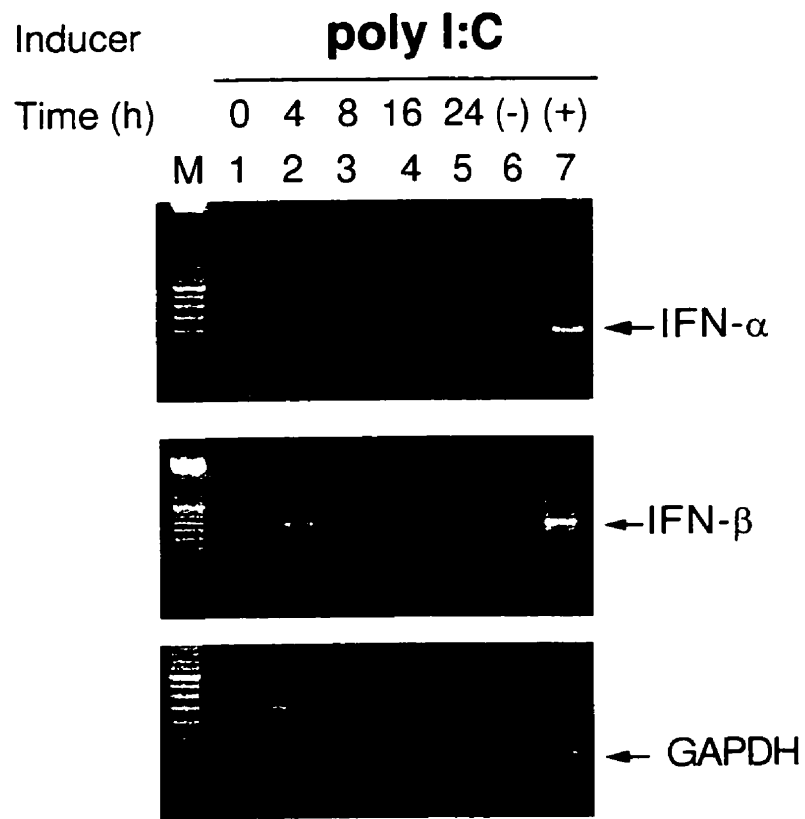
Fig. 1b.

Fig. 2. Priming enhances IFN-α mRNA induction by EMCV or poly [I]·poly [C]. U937

cells were primed by pretreatment with (A) IFN-α (100 U/ml), (B) IFN-γ (100 U/ml) or PMA

(50 nM) for 18 hours. Primed cells were then stimulated with EMCV (V) or poly [I]·poly [C]

(IC) for the times indicated in (A) or for 4 hours in (B). Cells were harvested to extract total

RNA and RT-PCR was performed to detect IFN-α and GAPDH mRNA.

**A**

Priming with:                        **IFN-α**

Inducer              **Poly I:C**          **EMCV**

Time (h)      0  1  2  4  6 | 1  2  4  6

M   1  2  3  4  5  6  7  8  9  (-)  (+)



← IFN-α

← GAPDH

Fig. 2a.

**B**

Priming with:  **IFN-γ** | **PMA**

Inducer  -  V  IC | -  V  IC

M  1  2  3  4  5  6  (-)  (+)

←IFN-α

←GAPDH

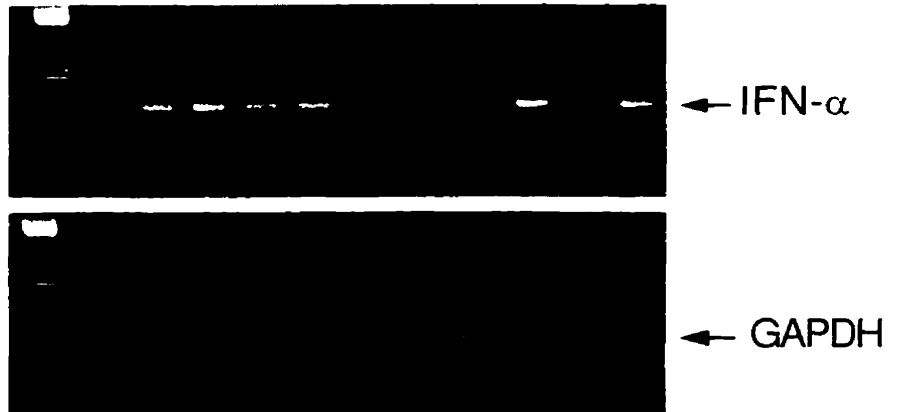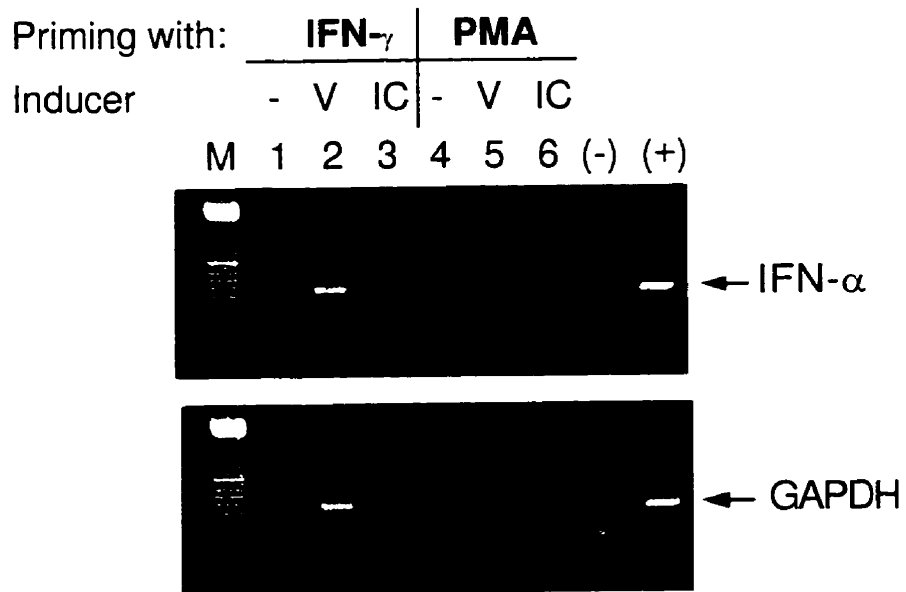**Fig. 2b.**

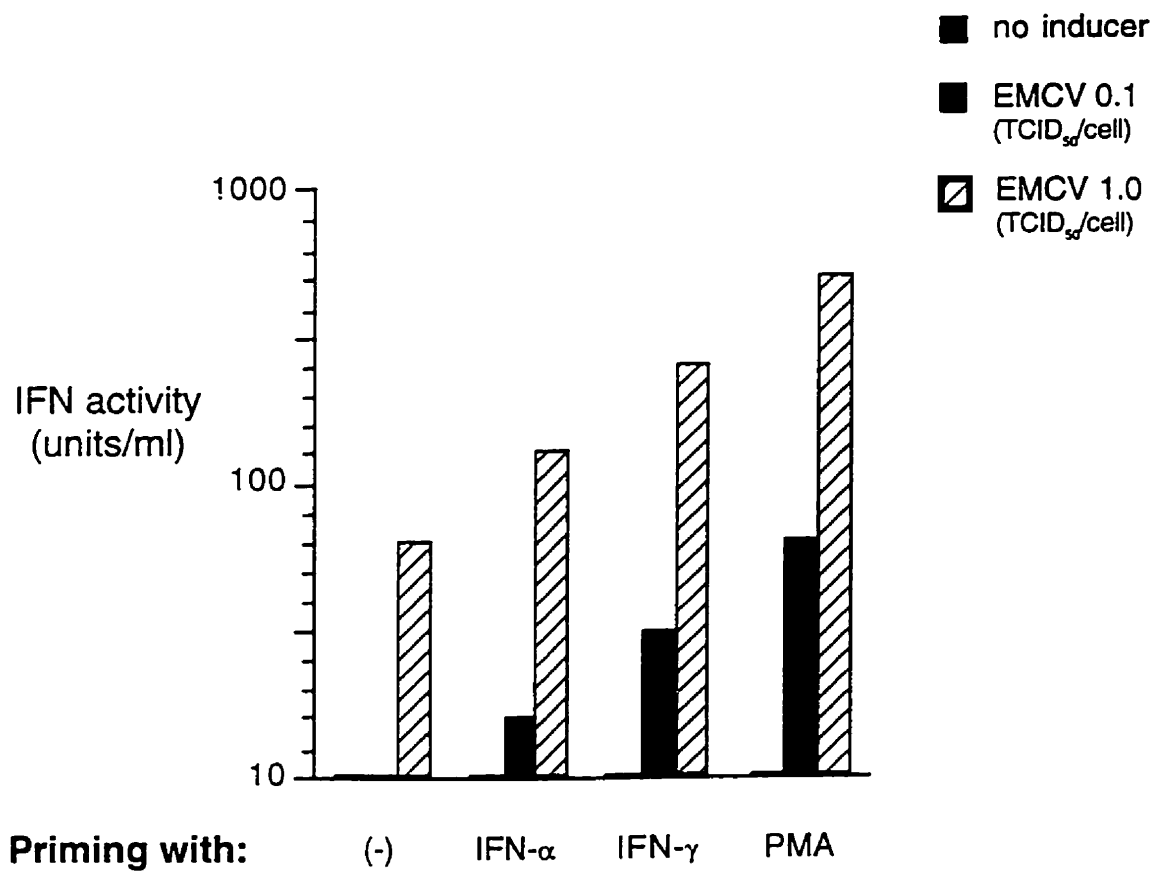**Fig. 3. Priming enhances IFN production in response to EMCV or poly [I]·poly [C].** U937 cells were primed as described previously, washed with PBS and replated with fresh medium. Following stimulation with EMCV (A) or poly [I]·poly [C] (B), at the indicated doses, supernatants were harvested and IFN activity was measured using a biological antiviral assay.

**A**



Fig. 3a.

**B**
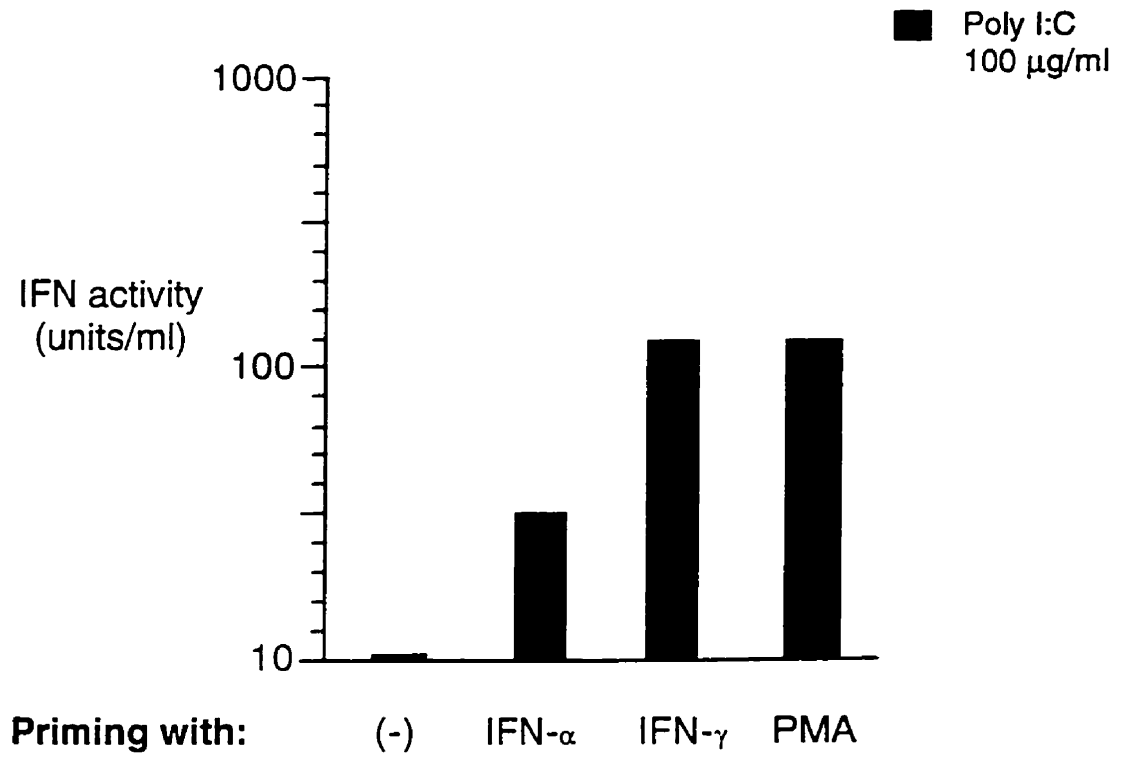


Fig. 3b.

Fig. 4. **Priming enables IFN-α induction in response to LPS or PMA.** (A) U937 cells were

left untreated or primed with IFN-α or IFN-γ for 18 hours. Following stimulation with LPS (50

ng/ml) or PMA (50 nM) for 4 hours, the cells were harvested for RNA extraction. RT-PCR was

performed to detect IFN-α and GAPDH mRNA. (B) (C) U937 cells were primed as described

previously, washed with PBS and replated with fresh medium. Following stimulation with LPS

(B) or PMA (C) at the indicated concentrations for 24 hours, supernatants were harvested and

IFN activity was measured using a biological antiviral assay.

**A**

Priming with:   (-)   |   IFN-α   |   IFN-γ

Inducer   - LPS PMA | - LPS PMA | - LPS PMA

M   1   2   3   4   5   6   7   8   9  (-) (+)



← IFN-α

← GAPDH
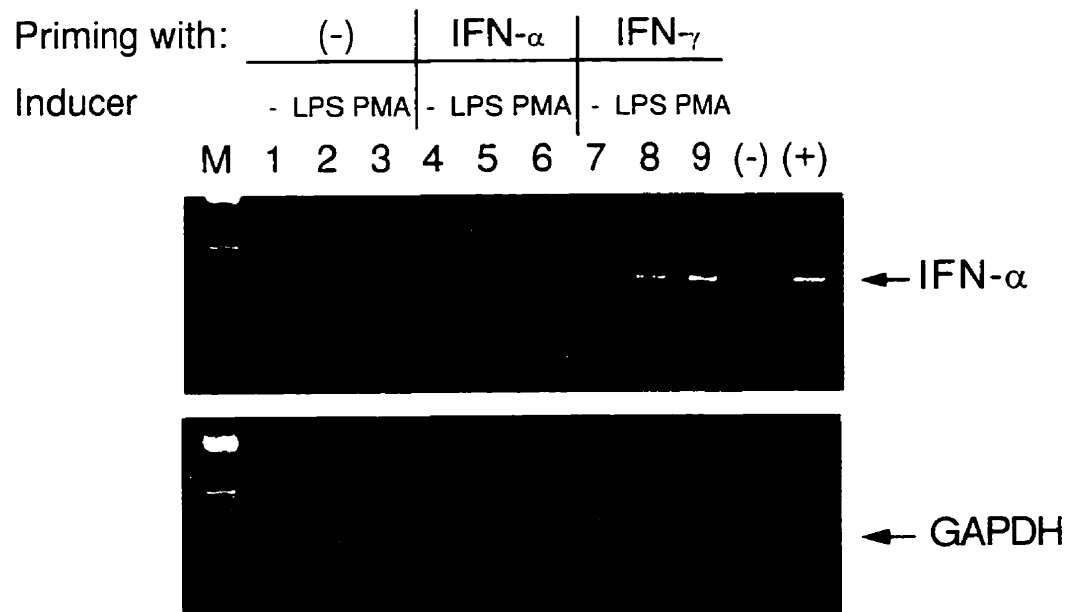
**Fig. 4a.**

**B**
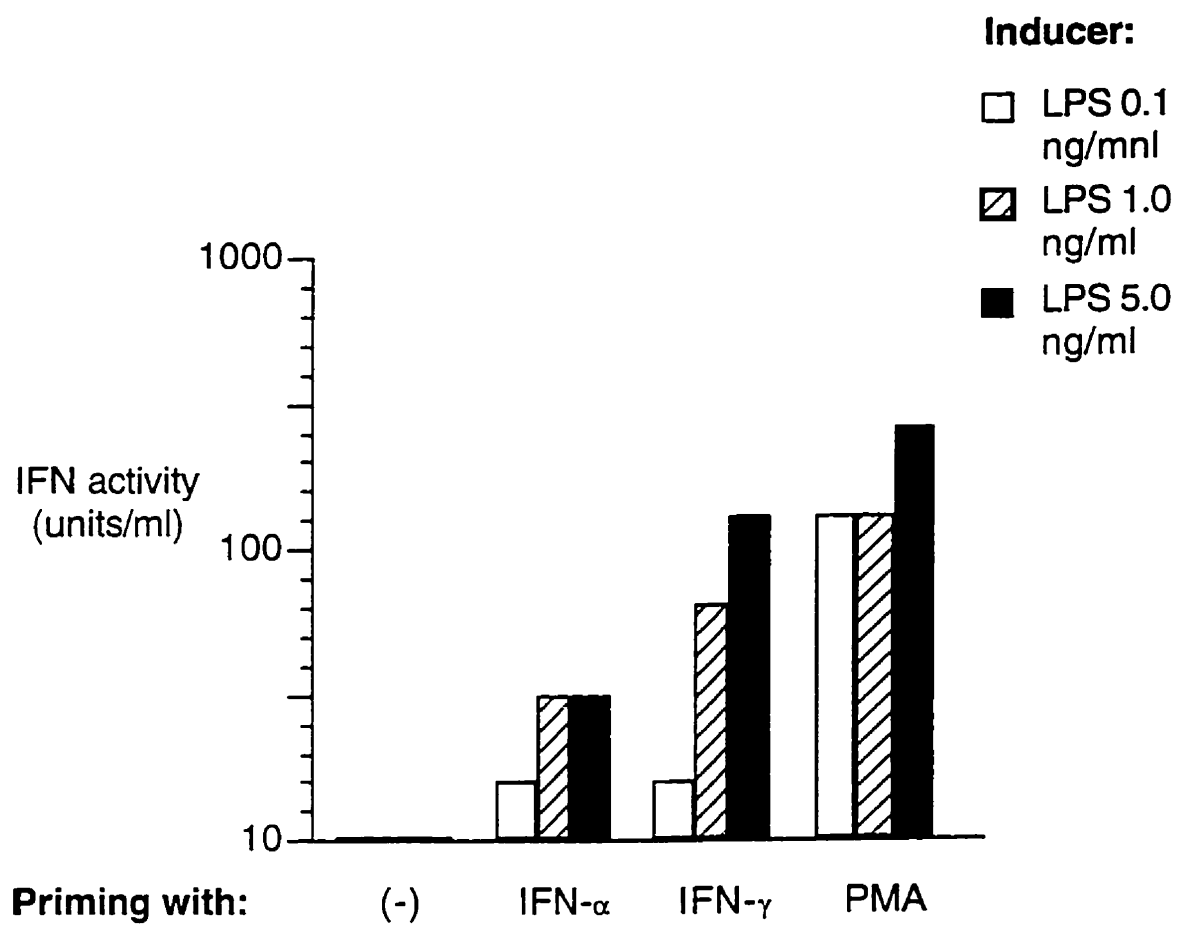


**Fig. 4b.**

C



Fig. 4c.

**Fig. 5. Differential effects on IFN production by kinase inhibitors.** (A) U937 cells were stimulated with EMCV (10 $TCID_{50}$/cells) in the absence or presence of 2-aminopurine or calphostin C, at the indicated concentrations, for 24 hours. Supernatants were collected and IFN activity was measured using a biological antiviral assay. (B) U937 cells were primed with IFN-γ (100 U/ml) for 18 hours, washed with PBS. These primed cells were then replated with fresh medium and incubated with the indicated inducers (poly [I]·poly [C] (100 ug/ml), EMCV (10 $TCID_{50}$/cells), LPS (5 ng/ml) or PMA (5 nM)) or kinase inhibitors (2-aminopurine (5mM) or calphostin C (100 nM)) for 24 hours. Supernatants were harvested and IFN activity was measured using a biological antiviral assay.

**A**



Fig. 5a.

**B**



Fig. 5b.

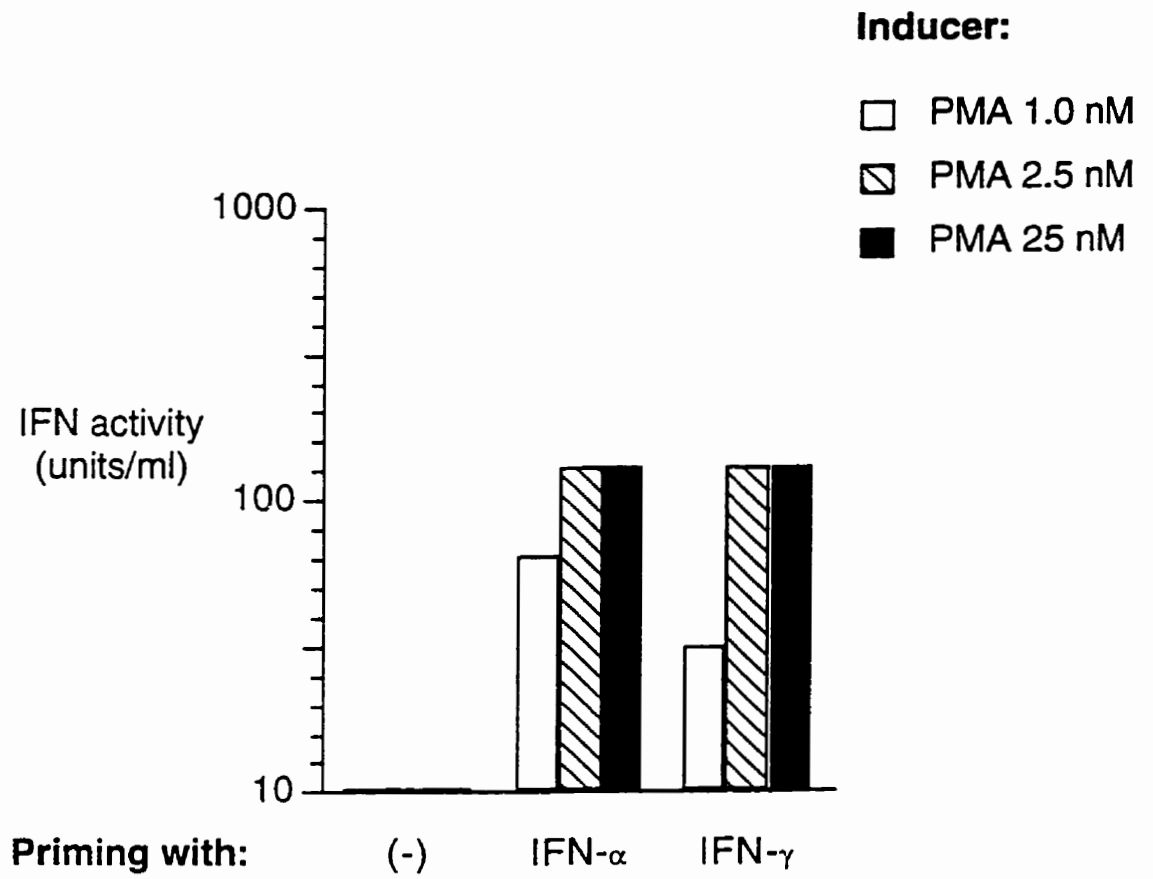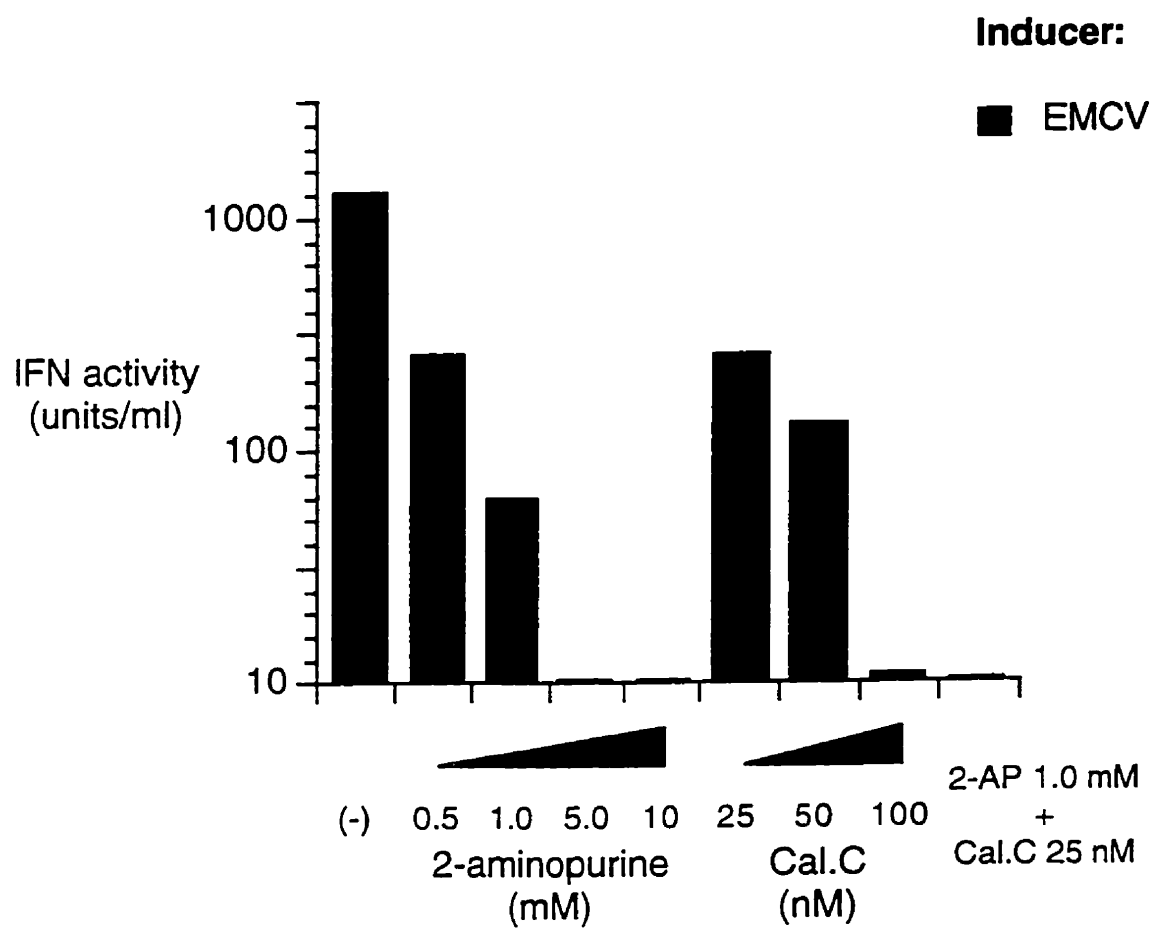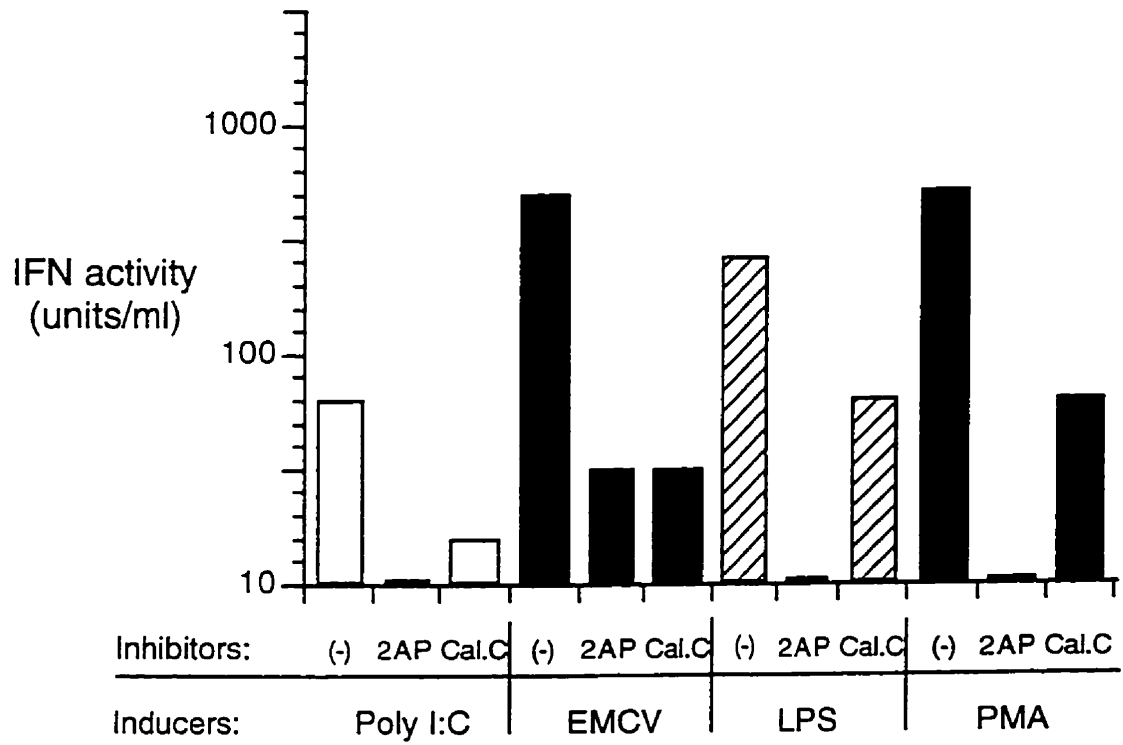# Discussion

Our preliminary experiments had revealed that the kinetics of IFN-α mRNA accumulation in U937 cells were relatively delayed in comparison, for example, with IFN-α induction in freshly isolated PBMCs (Chapter 2). In this study, we have determined that the slow kinetics of induction are not inherently intrinsic to how IFN-α genes are regulated in U937 cells, but rather, IFN-α expression can be enhanced by priming U937 cells with different factors prior to their exposure to stimuli. Our results indicate that the relatively slow kinetics of IFN-α induction by EMCV or poly [I]·poly [C] in U937 cells was not due to a lack of responsiveness to these stimuli, as evidenced by the rapid induction of IFN-β mRNA accumulation (Fig. 1). We then observed that a more rapid kinetics of IFN-α mRNA accumulation was evident in cells that had been primed with either IFNs or PMA. This is consistent, in principle, with studies on fibroblasts whereby priming results in accelerated kinetics of induced IFN-β mRNA accumulation as well as increased mRNA levels (4, 13, 21). The observation that IFN-α and IFN-γ are both proficient for priming is not surprising given that they can both stimulate many of the same biological activities, such as the induction of certain Interferon-Stimulated-Genes (e.g. IRF-1, ISG 6-16, and GBP-1)(25). In addition, IFN-α and IFN-γ are both capable of priming PBMCs for enhanced TNF-α synthesis in response to LPS (17).

Our observations that PMA primes U937 cells for enhanced IFN-α expression (Figs. 2 and 3) are seemingly contrary to the results by Sandberg *et al.* whereby PMA pretreatment of PBMCs inhibited the induction of IFN-α and IFN-β (22). Different inducers were used in our study as compared to theirs, which could account for our contrary results. Alternatively, it is possible that the differences may lie in the cell types. Since PBMCs are intrinsically highly inducible for IFN production, these cells may be more sensitive to negative regulatory signals, for which PMA may represent to PBMCs. Alternatively, U937 cells are comparatively less responsive and as such, they may also be less sensitive to inhibitory signals. At face value, our two studies are not necessarily contradictory but they simply indicate that PMA priming has

differential effects on IFN-α expression in particular cell types. In addition. it has been reported that the activation of protein kinase C activity in human fibroblasts. by priming with PMA or the synthetic diacylglycerol. OAG. enhances the levels of IFN-β protein produced following poly [I]·poly [C] stimulation (2).

The induction of IFN genes in response to LPS has been studied by other groups with varied results. LPS stimulation of mouse peritoneal macrophages induces IFN-β but not IFN-α (9). In human blood monocytes. LPS efficiently induces TNF-α and IL-1β production. but not IFN. However. IFN-α is inducible by LPS stimulation after the monocytes have been primed with IFN-γ or granulocyte-macrophage colony stimulating factor (GM-CSF) (12). In U937 cells. LPS treatment alone has been shown to induce TNF-α mRNA accumulation but it has no effect on IFN-β expression. The results from this study indicate that U937 cells become competent for producing IFN-α in response to LPS. only following priming by IFN-α. IFN-γ. or PMA (Fig. 3 and 4). Collectively. these results suggest that while cells may maintain efficient LPS-mediated signaling pathways. the pathways leading to IFN-α induction are somewhat specialized in that they require additional signals provided by priming.

Previous studies have indirectly implicated the involvement protein kinase C in IFN gene regulation. Induction of IFN-β in certain human and mouse fibroblast cell lines is inhibited by K252a and H-7. both inhibitors of protein kinase C activity. However. these agents were ineffective in inhibiting virus-induced IFN-β production in the same cells (23). In human PBMCs. IFN-α and IFN-β mRNA induction in response to virus or the synthetic IFN-inducer. imiquimod. was shown to be sensitive to the protein kinase C inhibitors. staurosporine or calphostin C (20). However. to our knowledge. there has not been any reports of IFN-α induction by protein kinase C agonists. such as PMA. Our results indicate that PMA can efficiently induce IFN-α production in IFN-primed U937 cells. at concentrations as low as 1.0 nM (Fig. 4a and 4c).

Having defined several IFN-α induction conditions as described above. we used kinase inhibitors to investigate whether these inducers used common or distinct signaling pathways.

Calphostin C is regarded as a highly specific protein kinase C inhibitor, which competes for binding by diacylglycerol and phorbol ester activators such as PMA (16). EMCV-induced IFN production was reduced by calphostin C in a dose-dependent manner, but 16 U/ml of residual IFN activity remained at the highest concentration tested, 100 nM. Furthermore, calphostin C was only partially effective in inhibiting IFN production in IFN-γ-primed cells. In particular, it was somewhat surprising that calphostin C was only partially effective in reducing PMA-induced IFN production. These results suggest that protein kinase C activity likely plays a significant auxiliary role but it is not essential for IFN-α expression.

2-aminopurine is regarded as a relatively specific inhibitor of PKR, and it has been shown to inhibit IFN-α and IFN-β induction in several cell types (3, 19, 26). As a purine analogue, 2-aminopurine has been studied as a mutagen which can be metabolized by bacteria or eukaryotic cells and incorporated into DNA to produce transition mutations of A-T <-> G-C (5). However, its kinase inhibitory properties are not dependent on any further modifications since 2-aminopurine is directly effective in preventing PKR autophoshorylation in *in vitro* kinase assays (6, 14). When 2-aminopurine is added to cells, it does not grossly alter overall cellular phosphorylation patterns nor does it inhibit protein kinase A or protein kinase C activities (18, 24). While its mode of action appears to involve competing with ATP for binding to PKR (14), the basis for its selectivity in preferentially inhibiting PKR and not most other kinases is unknown. Given its effect on IFN expression in other cell types, it was not unexpected that 2-aminopurine could completely inhibit EMCV-induced IFN production from U937 cells in a dose-dependent fashion (Fig 5a). However, EMCV-induced IFN production was only partially inhibited by 2-aminopurine in IFN-γ-primed cells (Fig. 5b). The existence of 2-aminopurine-insensitive pathways which regulate IFN-α and IFN-β expression has also been described in mouse spleen cells (3). In contrast, 2-aminopurine was completely effective in inhibiting IFN production from IFN-γ-primed cells in response to poly [I]·poly [C], LPS or PMA (Fig. 5b). Taken together, these results suggest that PKR is required for mediating IFN-α expression in response to all four of the above inducers. In further support of this notion, we

have observed that IFN-α production in response to EMCV, poly [I]·poly [C], LPS and PMA, is impaired in U937 transformant cells which are deficient in PKR activity (Chapter 4).

Currently, the mechanisms involved in the priming effect are poorly understood. Previous studies have suggested that priming induces the new synthesis of an essential signaling component such as a transcription factor. Interferon Regulatory Factor-1 (IRF-1) has been proposed to function as a positive regulator of IFN-α and IFN-β expression. IFN-α and IFN-β gene induction by poly [I]·poly [C] is deficient in mice bearing homozygous deletions of the IRF-1 gene, although virus induction of these IFN genes is unimpaired. However, IFN priming restores IFN inducibility in response to poly [I]·poly [C], thus clearly indicating the IRF-1 is not required for the priming effect. IRF-1 belongs to a family of related transcription factors which includes ISGF3γ/p48, an essential component for mediating downstream Type I IFN-induced signaling. ISGF3γ/p48 had not previously been suggested as a regulator of IFN-α and IFN-β genes but recently, mice bearing homozygous deletions of ISGF3γ/p48 have been characterized and they exhibit substantial deficiencies in Type I IFN inducibility (11). The precise role of ISGF3γ/p48 in regulating IFN-α and IFN-β genes remains to be determined. This study has identified some characteristics of U937 cells, in particular, the effects of priming agents, which may be useful for further investigation of IFN-α gene regulation.

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# Chapter Four

**Involvement of the double-stranded RNA-dependent kinase, PKR, in interferon expression and interferon-mediated antiviral activity**

## Abstract

The signaling mechanisms responsible for the induced expression of interferon (IFN) genes by viral infection or double-stranded RNA (dsRNA) are not well understood. Here, we investigated the role of the interferon-induced dsRNA-dependent protein kinase, PKR, in the regulation of IFN induction. Biological activities attributed to PKR include regulating protein synthesis, mediating IFN actions, and functioning as a possible tumour suppressor gene. Since binding dsRNA is required for its activation, PKR has been considered as a candidate signal transducer for regulating IFN expression. To examine this role of PKR, loss-of-function phenotypes in stable transformants of promonocytic U937 cells were achieved by using two different strategies, overexpression of an antisense PKR transcript or a dominant negative PKR mutant gene. Both types of PKR-deficient cells were more permissive for viral replication, compared to the control U937 cells. As the result of PKR loss, they also showed impaired induction of IFN-$\alpha$ and IFN-$\beta$ genes in response to several inducers, specifically, encephalomyocarditis virus, lipopolysaccharide, and phorbol 12-myristate 13-acetate. Interestingly, while IFN-$\alpha$ induction by dsRNA was impaired in PKR-deficient cells, IFN-$\beta$ induction remained intact. Loss of PKR function also resulted in decreased antiviral activity as elicited by IFN-$\alpha$ and, to a greater extent, by IFN-$\gamma$. These results implicate a role for PKR in the regulation of several antiviral activities.

## Introduction

Type I IFNs regulate diverse biological processes including antiviral activities, cellular growth and differentiation, and modulation of immune functions (26, 28). The induced expression of Type I IFNs, which include the IFN-α and IFN-β gene families, is detected typically following viral infections. Previous studies have identified promoter elements and transcription factors involved in regulating the expression of Type I IFNs (6, 22, 31). However, it remains unclear what are the particular biochemical cues that signify viral infections to the cell and the signaling mechanisms involved. Since many forms of dsRNA are capable of inducing Type I IFNs, this led to suggestions that the common inducing molecule between different viruses was a viral replicative intermediate containing dsRNA (19). It seems reasonable to hypothesize that the regulation of IFN genes and antiviral activities involves effector proteins responsive to dsRNA.

Of the many RNA-binding proteins, the few which are capable of binding dsRNA are distinguished by a conserved 65- to 68-amino acid "dsRNA-binding domain" (29). Among these, the IFN-induced dsRNA-dependent protein kinase, PKR, is the only one with kinase function. PKR is a serine/threonine kinase whose enzymatic activation requires dsRNA binding and consequent autophosphorylation (8, 23). The best characterized substrate of PKR is the alpha subunit of eukaryotic initiation factor-2, eIF-2α, which once phosphorylated leads to inhibition of cellular and viral protein synthesis (11). This function of PKR has been suggested as one of the mechanisms responsible for mediating the antiviral and antiproliferative activities of IFNs. An additional function for PKR is its role as a signal transducer, since 2-aminopurine, a relatively specific inhibitor of PKR, can block the induction of IFN-α and IFN-β genes by virus infection or dsRNA (21, 33). In support of this, Kumar et al. demonstrated that PKR phosphorylates IκBα, resulting in the activation of NF-κB (17). Given the well-characterized NF-κB site in the IFN-β promoter and that dsRNA alone can induce NF-κB activity (32), it has been postulated that PKR mediates the induction of IFN-β transcription by dsRNA.

To investigate the role of PKR in IFN gene regulation and cellular antiviral responses, we have utilized two different strategies to achieve a loss of PKR-function phenotype. This involved overexpression of a dominant negative PKR mutant gene, [Arg$^{296}$]PKR, or an antisense PKR gene in stable transformants of a promonocytic cell line, U937. Monocytes represent a primary source of Type I IFNs *in vivo* and accordingly, we and others have found U937 cells useful for studying IFN-α and IFN-β gene expression (3). The mutant [Arg$^{296}$]PKR gene contains a single amino acid substitution of the invariant lysine in catalytic domain II at position 296 to arginine and encodes a dominant negative protein which can specifically suppress the activity of endogenous wild type PKR *in vivo* (14, 15). An alternative approach to specifically inhibit gene expression involves antisense strategies. Recently, Maran *et al.* showed that novel 2'-5'A-linked antisense oligonucleotides, specific for PKR, suppressed PKR activity and NF-κB activation by dsRNA (20). However, it is not known whether IFN production or IFN-mediated antiviral responses was affected as a result of suppressed PKR function in the above studies. Here, we report that loss of PKR activity in U937 cells results in multiple defects in IFN production and as well, in antiviral responsiveness to IFN-α and IFN-γ.

## Methods and Materials

**Plasmids and stable transformants.** The wild type human PKR gene and the dominant negative [Arg$^{296}$]PKR mutant gene were released by HindIII digestion from the plasmids pBSKS and p6M (provided by Dr. B. R. G. Williams), respectively. They were then subcloned into the eukaryotic expression vector, pRC-CMV (Invitrogen), to generate the plasmids used in this study, namely, pPKR-AS (antisense) and p[Arg$^{296}$]PKR. Stable transformants were generated by electroporation of U937 cells with 10 μg of each plasmid using a Gene Pulser apparatus (BioRad). Clonal lines were obtained by selection with 400 μg/mL geneticin (GIBCO-BRL) and limiting dilution cloning. Cells were cultured in RPMI-1640 containing 10% fetal calf serum and geneticin.

**PKR analysis.** PKR autophosphorylation assay was performed essentially as described by Maran et al. with the following modifications (20). Cell extracts (100 μg) were incubated with poly [I]·poly [C]-cellulose for 1 hour on ice, washed three times, and incubated for 30 minutes at 30°C in 50 μl of a reaction buffer (20 mM HEPES (pH 7.5), 50 mM KCl, 5 mM 2-mercaptoethanol, 1.5 mM MgOAc, 1.5 mM MnCl$_2$) containing 1 μCi of [γ-$^{32}$P]ATP. Samples were analyzed by 10% SDS-PAGE and autoradiography. For immunoblot analysis of PKR, cell extracts (100 μg) were separated by 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were incubated with anti-PKR MAb at 1:1000 in BLOTTO, with final detection provided by using a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotech) and a chemiluminesence method (Amersham ECL).

**EMCV replication and IFN assay.** For determination of EMCV replication, U937-derived transformants were cultured in complete media alone or pretreated with recombinant human IFN-α2 (Schering) or IFN-γ (Amgen) for 18 hours. Following two washings with PBS, the cells were incubated with the indicated amounts of EMCV in serum-free media for 2 hours. The cells were washed again and 10$^6$ cells per sample was resuspended in 1 ml of media containing 1% FCS. Samples were collected at the required time points and lysed by three

rounds of freeze-thaw. Four-fold serial dilutions of the samples were added onto L929 monolayers and incubated for 48 hours, followed by staining with 0.05% crystal violet to determine cytopathic effects and $TCID_{50}$. In assaying IFN production, U937-derived transformants were similarly pretreated with IFNs as described above. Then, the cells were incubated with inducers, poly [I]·poly [C] (Pharmacia), EMCV, lipopolysaccharide (LPS, Sigma) or phorbol 12-myristate 13-acetate (PMA, Sigma), for 2 hours. Cells were washed and cultured in media containing 1% FCS. Supernatants were collected after 24 hours and IFN activity was measured by a bioassay as in a previous report (18).

**Reverse-transcriptase-polymerase chain reaction (RT-PCR).** Priming and induction of U937-derived transformants was performed as above. Total RNA was extracted from cell samples using an acid guanidinium thiocyanate procedure. First strand cDNA synthesis was performed using 2 μg of each RNA sample primed with random hexamer in a 25 μl reaction volume using 200U of MMLV reverse transcriptase (GIBCO-BRL). All PCR reactions were performed using 2 μl out of each cDNA mixture in a 50 μl reaction volume containing 50 pmol of each upstream and downstream primer, 2U Taq DNA polymerase (Promega), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 10X reaction buffer. IFN-α PCR uses consensus primers, capable of amplifying all 14 known human IFN-α subtypes genes, 5'-GGAAGCTT(T/C)CTCCTG(C/T) (C/T)TGA(A/T)GGACAGA and 5'-GGGGATCCTCTGACAACCTCCCA(G/A/T/C)GCACA which generate an expected product of 372 base pairs. IFN-β PCR uses primers, 5'- GTGTCAGAAGCTCCTGTGGC and 5'-CTTCAGTTTCGGAGGTAACC, which generate an expected product of 456 base pairs. GAPDH PCR uses primers, 5'-CCATGGAGAAGGCTGGGG and 5'-CAAAGTTGTCATGGATGACC, which generate an expected product of 196 base pairs.

# Results

**Characterization of PKR-deficient stable transformants.** Stable transformant cell lines were obtained by transfecting U937 cells with the following expression plasmids. Five representative cell lines were selected for characterization: (i) "U937-neo" was the control cell line transfected with the parental vector, pRC/CMV; (ii) "U937-AS1" and "U937-AS3" were independent clones transfected with pPKR-AS; (iii) "U937-M13" and "U937-M22" were independent clones transfected with p[Arg$^{296}$]PKR. PKR kinase activity was measured with an assay that uses poly [I]·poly [C]-cellulose for binding and activation of PKR enzyme. IFN-treated HeLa and mouse L929 cells were used as positive controls, since PKR activity in these cells had been described (Fig. 1A, lanes 1 and 8)(23). Similar to the untransfected U937 parents, U937-neo cells contained basal levels of PKR activity which increased following treatment with IFN-α (Fig. 1A, lanes 2 and 3). In contrast, PKR activity was not detected in any of the four cell lines transformed with pPKR-AS or p[Arg$^{296}$]PKR (results not shown). Furthermore, PKR activity was not restored in these cells by treatment with IFN-α (Fig. 1A, lanes 4-7), or IFN-γ (results not shown). To further confirm the inhibition of PKR expression in the pPKR-AS-transformed cells, Western blot analysis was performed using a monoclonal antibody specific for human PKR. Basal levels of PKR protein were detectable in U937-neo cells (Fig. 1B, lanes 1) which increased following treatment with IFN-α or IFN-γ (Fig. 1B, lanes 2 and 3). In contrast, PKR expression was diminished in U937-AS1 and U937-AS3 cells (Fig. 1B, lanes 4 and 6) and did not increase with IFN-α treatment (Fig 1B, lanes 5 and 7).

**Enhanced EMCV replication in PKR-deficient cells.** We first investigated whether loss of PKR function would affect the rate of EMCV replication. In control U937-neo cells following challenge with EMCV at 0.1 TCID$_{50}$/cell, viral titers peaked at approximately $10^4$ TCID$_{50}$/mL after 48 hours (Fig. 2A). However, in U937-AS1 and U937-M22 cells, EMCV replication was substantially higher reaching titers of $10^4$ to $10^5$ TCID$_{50}$/mL after only 24 hours and $10^8$ TCID$_{50}$/mL by 48 hours, a 1000-fold increase over U937-neo cells. By using a lower

virus inoculum of 0.001 TCID$_{50}$/cell, more dramatic differences in EMCV susceptibility were observed. While EMCV replication in U937-neo cells did not exceed $10^2$ TCID$_{50}$/mL, high viral titers of $10^8$ TCID$_{50}$/mL were attained in both the U937-AS1 and U937-M22 cells (Fig. 2B).

**A role for PKR in IFN expression.** The commonly invoked model for IFN action proposes that an essential function for the IFNs secreted from virus-infected cells is to protect neighboring cells against subsequent rounds of infection by progeny virus (26, 28, 31). Accordingly, the higher rates of EMCV replication in the PKR-deficient cells could have resulted from impaired IFN production or defective antiviral responses to the paracrine actions of induced IFNs. Here, we first showed that loss of PKR activity resulted in impaired IFN production in both U937-AS1 and U937-M22 cells, as compared to the control U937-neo cells. With U937-neo cells, induction by EMCV alone produced substantial amounts of secreted IFN protein (512 U/mL, Fig. 3A). In a phenomenon known as IFN priming, pretreatment of the IFN-producer cells with even small amounts of IFN enhances subsequent IFN production upon stimulation with inducers (27). Consistent with this, priming U937-neo cells with either IFN-α or IFN-γ resulted in increased production of EMCV-induced IFN activity (Fig. 3A). The effects of priming were more significant for IFN induction by non-viral inducers. Stimulation of U937-neo cells with poly [I]·poly [C], LPS, or PMA alone did not induce any detectable levels of IFN unless the cells had been primed with IFN-α or IFN-γ (Fig. 3A). In contrast, IFN production was significantly impaired in both types of PKR-deficient cells under each of the above induction conditions (Fig. 3A). Compared to U937-neo cells, IFN levels from U937-AS1 and U937-M22 cells following EMCV induction were reduced nearly 50-fold (16 U/mL or less). Furthermore, this impairment was not alleviated by IFN-α or IFN-γ priming. Also, IFN induction by poly [I]·poly [C], LPS or PMA, following IFN priming, was impaired as well in both PKR-deficient cell lines (8 U/mL or less). The IFN activity produced by U937 cells was composed of both IFN-α (over 80%) and IFN-β proteins, as determined using neutralizing antibodies (results not shown).

To examine the role of PKR in regulating the differential expression of IFN-α and IFN-β genes, steady-state levels of the respective IFN mRNAs were determined using RT-PCR. Optimal induction of IFN-α mRNA in U937-neo cells by EMCV alone required stimulation for 16 hours (Fig 3B, lane 4). Peak induction of IFN-β mRNA by poly [I]·poly [C] or EMCV alone was more rapid, occurring at 3 and 6 hours, respectively (Fig. 3B, lanes 2 and 3). In contrast, the induction of IFN-α and IFN-β mRNA following viral infection was impaired in both PKR-deficient cell lines. IFN-α mRNA induction by EMCV at 16 hours was diminished in U937-AS1 and U937-M22 cells (Fig. 3 B, lane 4). Also, the early EMCV induction of IFN-β mRNA at 6 hours was absent (Fig. 3B, lanes 3). However, IFN-β mRNA remained inducible in response to poly [I]·poly [C] (Fig. 3B, lane 2) and varying levels were induced by EMCV only after 16 hours (Fig. 3B, lane 4) in the PKR-deficient cell lines.

Next, the effect of PKR loss on the induction of IFN mRNAs was examined in IFN-primed cells. We have determined that priming enhances IFN-α induction in U937 cells. Stimulation of U937-neo with poly [I]·poly [C] alone resulted in weak IFN-α mRNA induction after 16 hours (results not shown). However, following priming with either IFN·α or IFN-γ, poly [I]·poly [C] stimulation resulted in a rapid induction of IFN-α mRNA, peaking after 3 hours (Fig. 3C lanes 2 and 5 versus Fig. 3B, lane 2). Similarly, IFN-priming also enabled a rapid induction of IFN-α mRNA in response to EMCV, peaking after 6 hours (Fig. 3C, lanes 3 and 6 versus Fig. 3B, lane 3). Furthermore, the induction of IFN-α mRNA in U937-neo cells by LPS or PMA was dependent on priming with IFN-γ (Fig. 3C, lanes 7 and 8). Consistent with the patterns observed earlier for IFN protein production, IFN mRNA expression was impaired in both PKR-deficient cell lines despite priming. The early induction of IFN-α and IFN-β mRNA following EMCV challenge was absent in both U937-AS1 and U937-M22 cells, irrespective of priming with IFN-α or IFN-γ. The induction of both IFN-α and IFN-β mRNA by LPS or PMA was also impaired in the PKR-deficient cells. While the induction of IFN-α mRNA by poly [I]·poly [C] was diminished in the U937-AS1 and U937-M22 cells, IFN-β mRNA induction by poly [I]·poly [C] again appeared unaffected (Fig. 3C, lanes 2 and 5).

**Impaired IFN responsiveness in PKR-deficient cells.** Finally, we investigated whether loss of PKR activity affected IFN-induced antiviral responses. To test this, EMCV replication was measured after treatment of cells with IFN-$\alpha$ or IFN-$\gamma$. Although generally not produced by macrophages, IFN-$\gamma$ was studied for its effects on U937 cells since it possesses direct antiviral properties and has a primary role in macrophage activation (7). While treatment with IFNs reduced EMCV titers in all cell lines, viral yields were consistently higher in the PKR-deficient cells as compared with the control cells (Fig. 4A). EMCV titers were 10-fold higher in both PKR-deficient cell lines than in U937-neo cells, following IFN-$\alpha$ treatment. Interestingly, IFN-$\gamma$-mediated antiviral activity was more severely impaired as a result of PKR loss, since EMCV titers from IFN-$\gamma$-primed U937-AS1 or U937-M22 cells were $10^2$- to $10^3$-fold higher than from control cells. We considered the possibility that these experimental conditions involving a relatively low virus inoculum may have magnified the differences in IFN-mediated antiviral responses between these cell lines. However, similar results were observed when we applied more stringent conditions for comparing IFN responsiveness by increasing the EMCV inoculum 100-fold and harvesting samples earlier, at 24 hours rather than 48 hours (Fig. 4B).

**Figure 1. PKR activity and protein levels in U937-derived stable transformant cell lines.**
(A) Functional PKR activity was determined using a poly [I]-poly [C]-cellulose assay for PKR autophosphorylation. Cell extracts were prepared from the different U937 cell lines following incubation with or without recombinant human IFN-α2 (200 U/mL) as indicated, while L929 cells were similarly treated with mouse IFN-α/β. Lane 1, HeLa; lanes 2 and 3, U937-neo; lane 4, U937-AS1; lane 5, U937-AS3; lane 6, U937-M13; lane 7, U937-M22; lane 8, L929. Positions of the human (68 kDa) and mouse (65 kDa) PKR proteins, and the molecular size standards (in kDa) are indicated. (B) Cell extracts were prepared as above and PKR protein levels were determined by Western blot analysis.
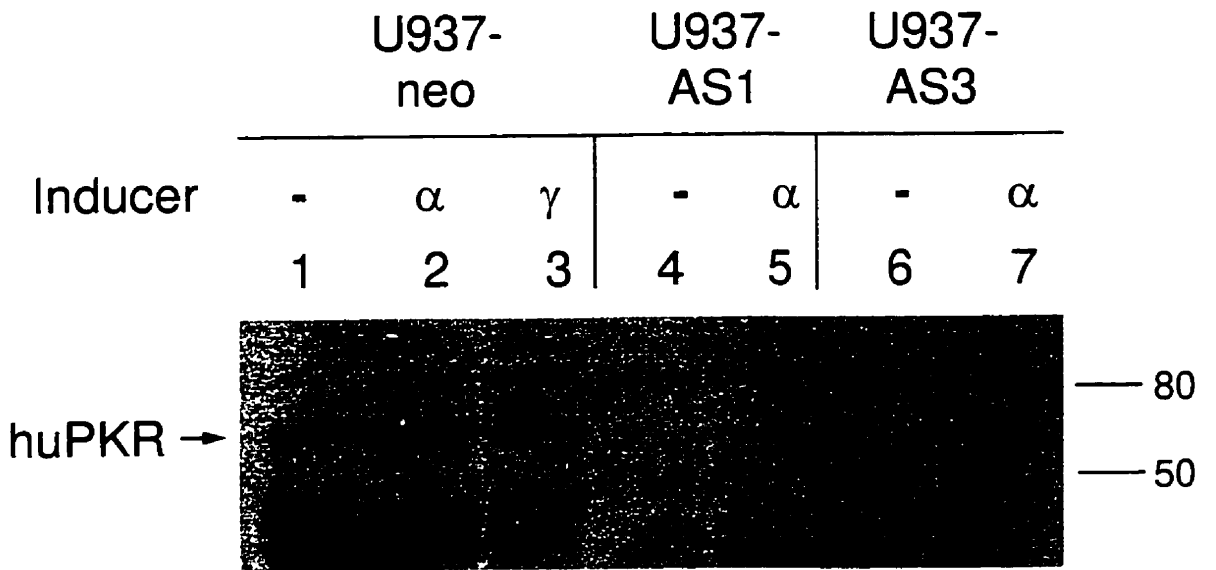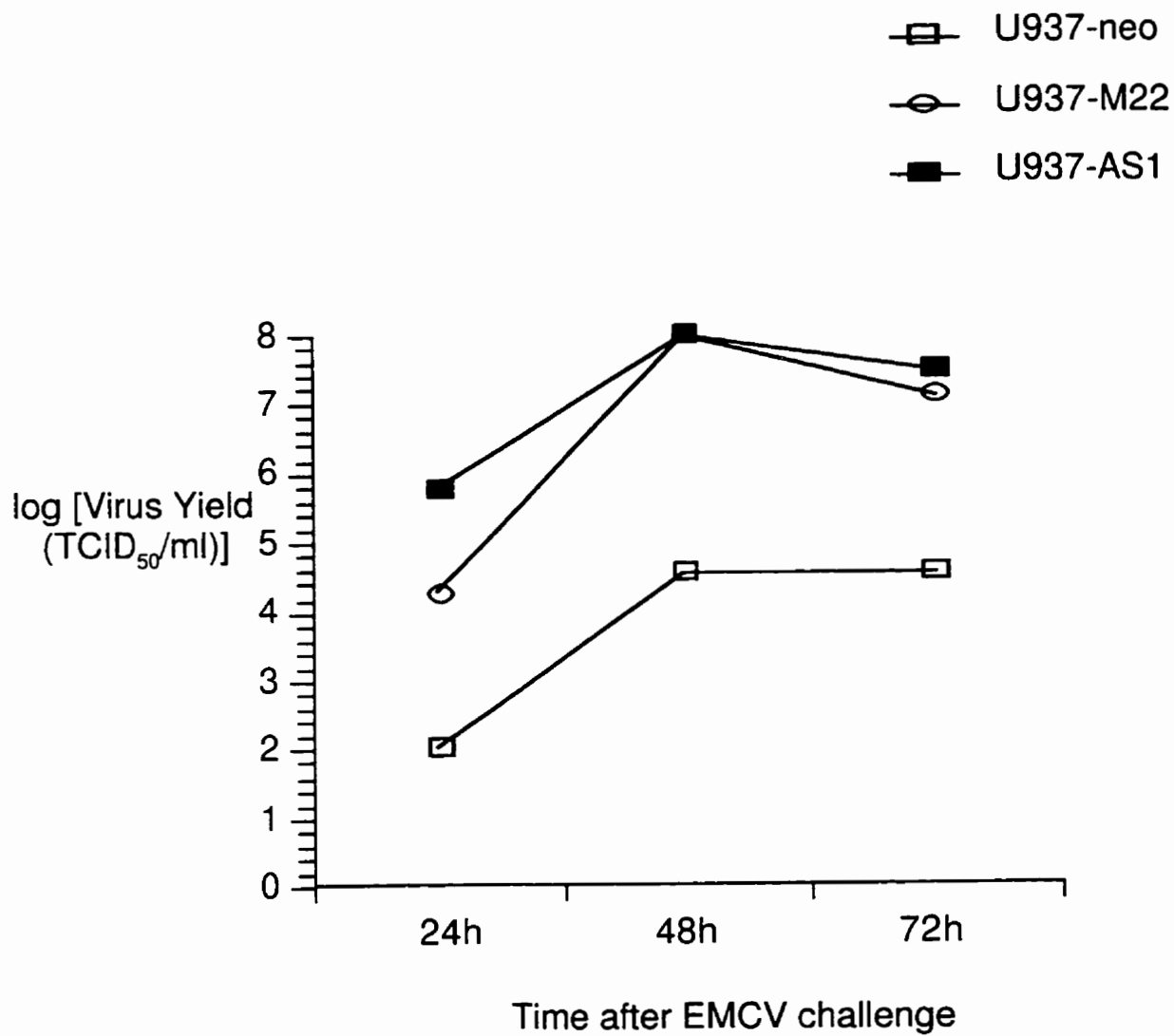
**A**
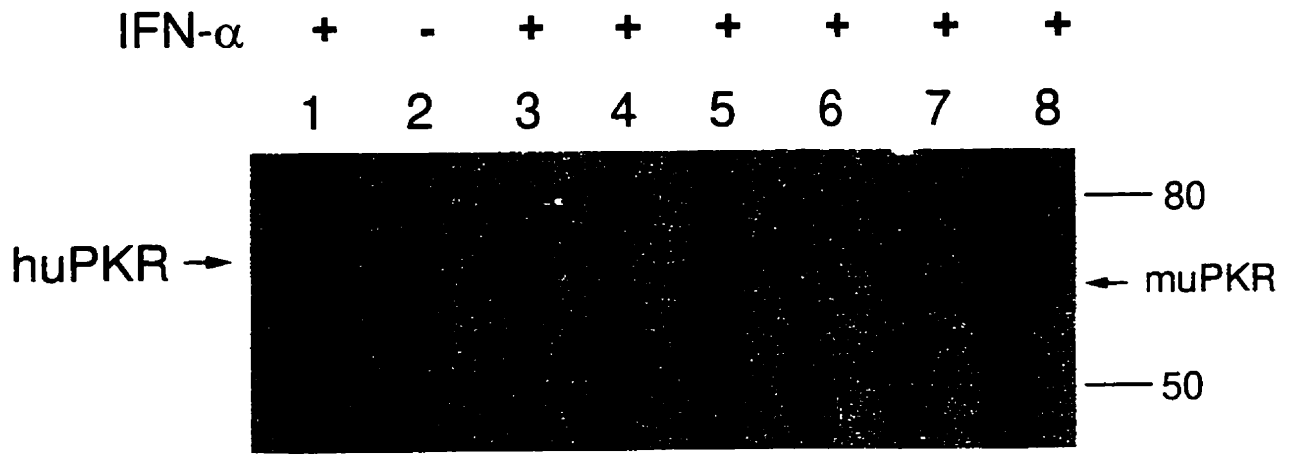


**B**



Fig. 1.

Figure 2. Kinetics of EMCV replication are enhanced in PKR-deficient cells. The different U937 cell lines were challenged with EMCV at 0.1 (A) or 0.001 (B) $TCID_{50}$/cell. Samples were harvested at the indicated times and viral yields were measured in terms of $TCID_{50}$.

**A**



Fig. 2a.

**B**
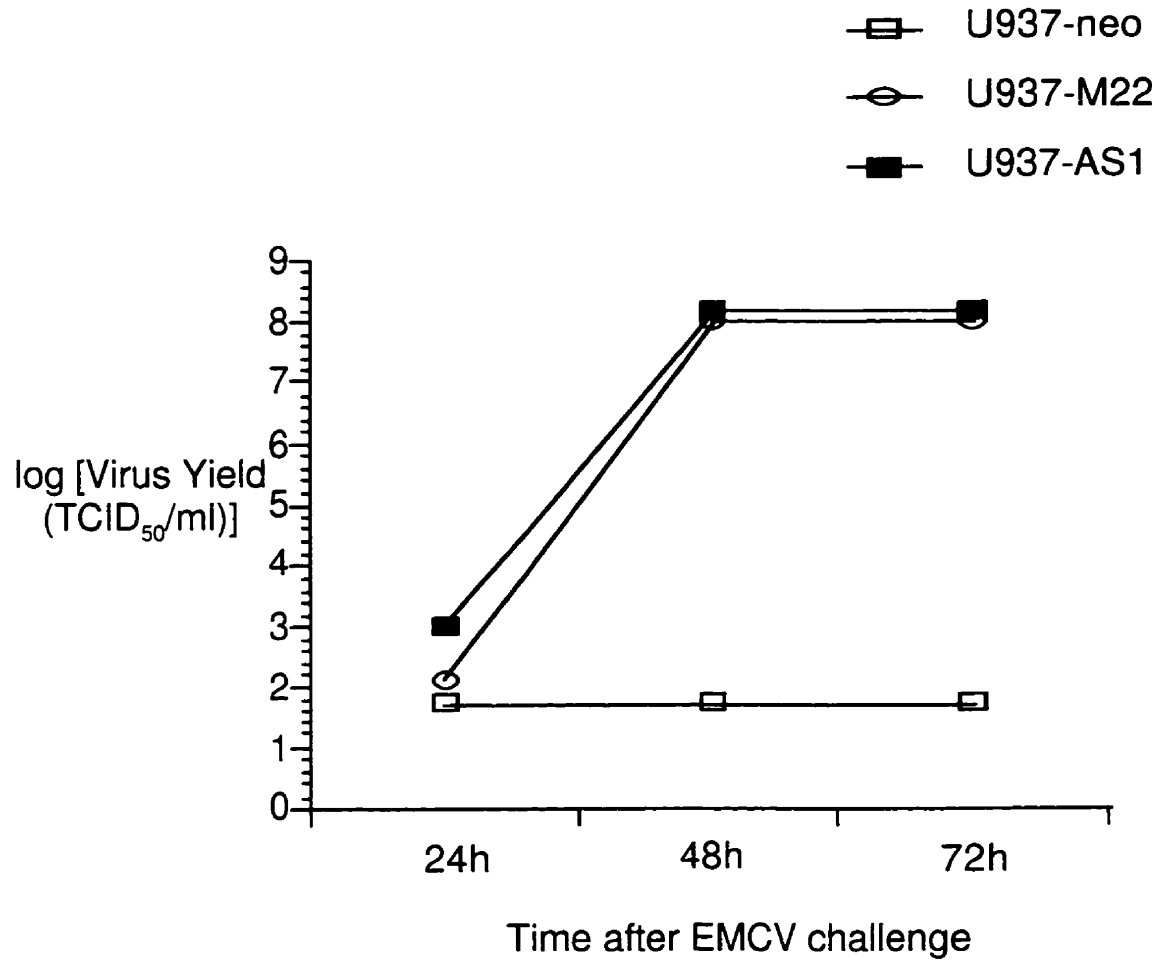


Fig. 2b.

**Figure 3. IFN expression is impaired in PKR-deficient cells.** (A) The different U937 cell lines were primed where specified with IFN-α or IFN-γ (200 U/mL). Cells were then incubated with the indicated inducers and IFN levels from each sample were determined. Following our induction conditions, U937 cells did not produce detectable IFN in response to poly [I]·poly [C] (100 μg/ml), LPS (50 ng/ml) or PMA (50 nM) alone, or after IFN priming in the absence of inducers. (B), (C) Cells were primed as described above and then incubated with inducers for the indicated times. IFN-α, IFN-β, and GAPDH mRNA were detected using RT-PCR. PCR products were visualized by ethidium bromide staining following 1.5 % agarose gel electrophoresis. Negative controls (-) represent PCR performed on RT reagents without sample RNA. Positive controls (+) represent PCR amplification of 1.0 ng of human genomic DNA. DNA markers (M) represent a ladder of increasing 100-base pairs increments.

**A**
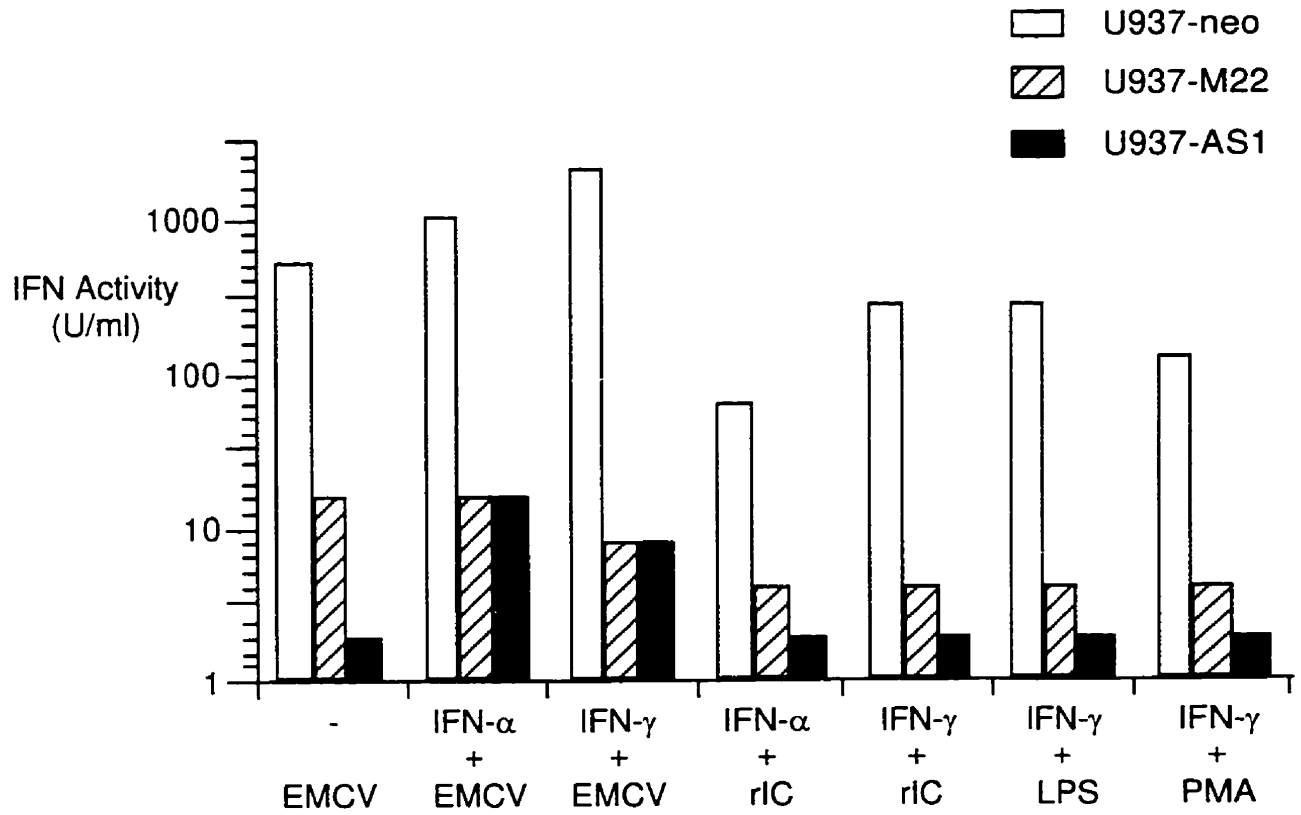


Fig. 3a.

**Fig. 3b.**
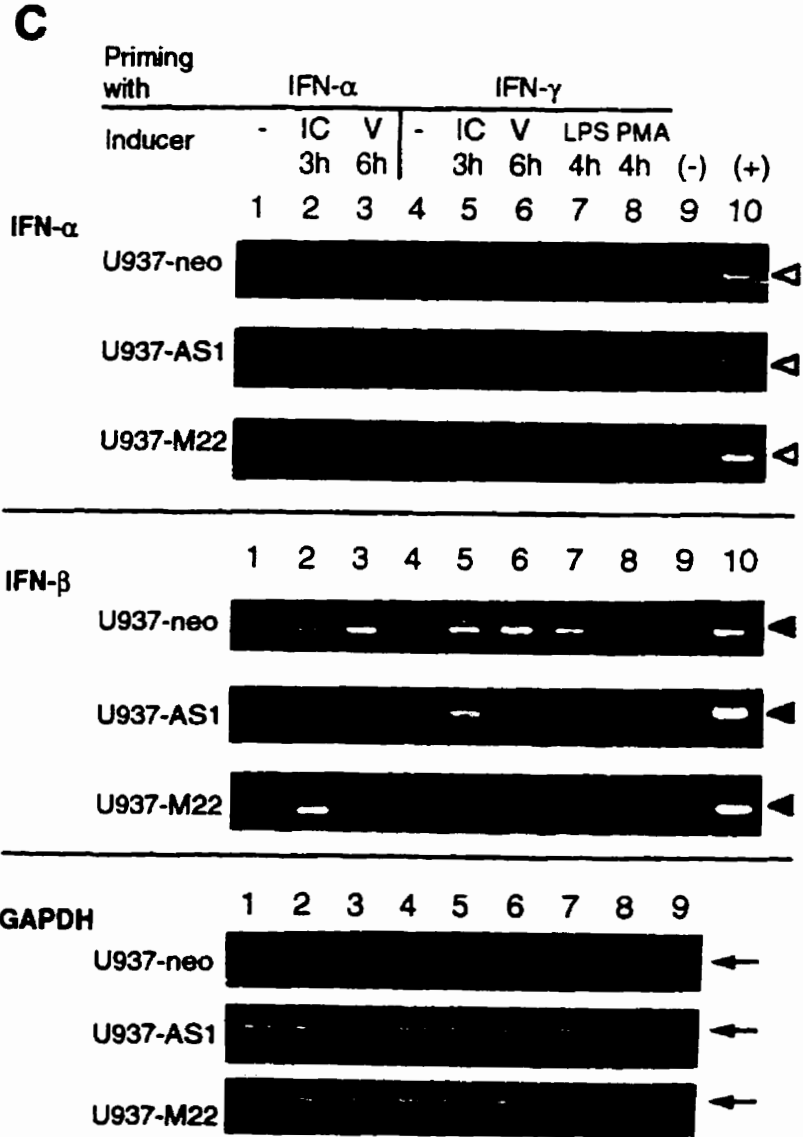
**Fig. 3c.**

**Figure 4. Inhibition of EMCV replication by IFN-α or IFN-γ is impaired in PKR-deficient cells.** (A) U937-neo, U937-AS1 and U937-M22 cells were cultured in absence or presence of the indicated concentrations of IFN-α or IFN-γ for 18 hours and challenged with EMCV at 0.1 TCID$_{50}$/cell. Samples were harvested after 48 hours for determination of virus yield. (B) Cells were similarly pretreated with or without IFNs, except they were challenged with hundred fold higher EMCV at 10 TCID$_{50}$/cell. Samples were then harvested earlier after 24 hours for determination of virus yield.

**A**
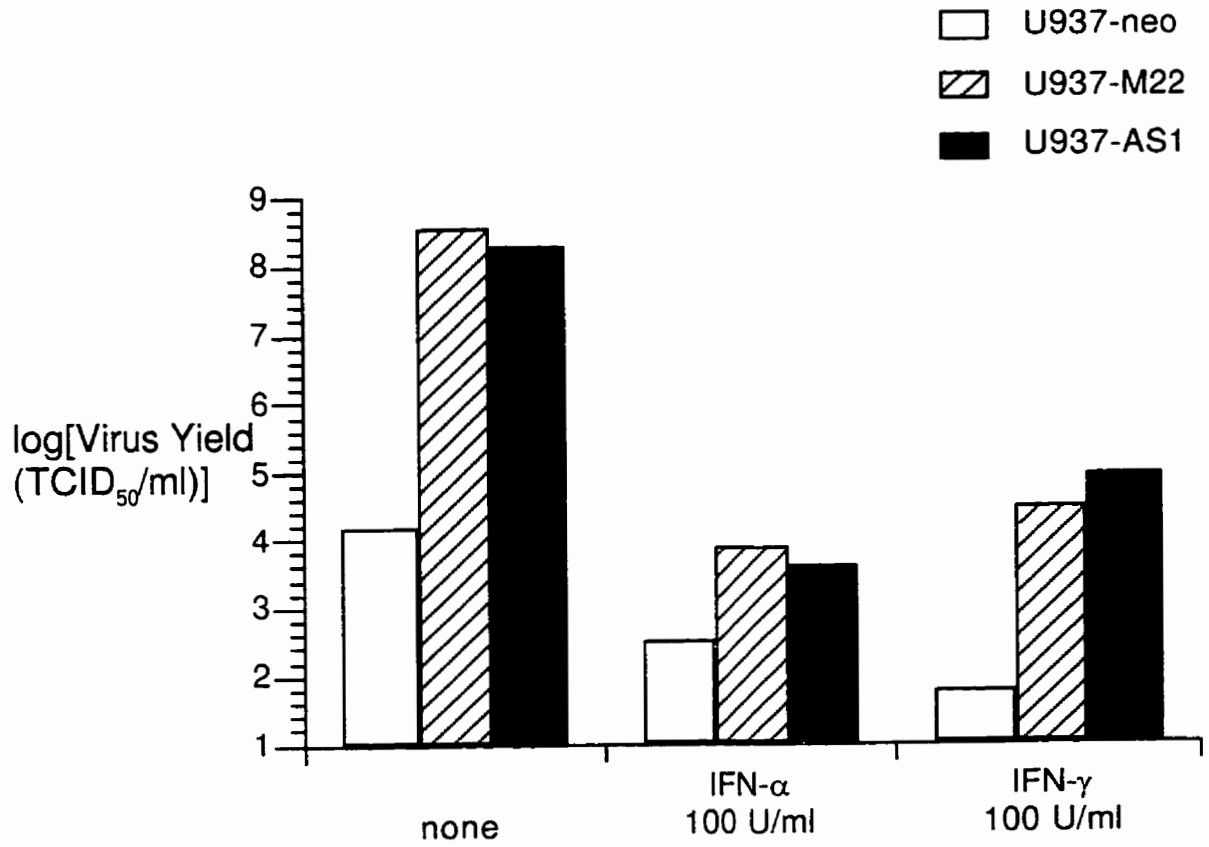


Fig. 4a.

**B**
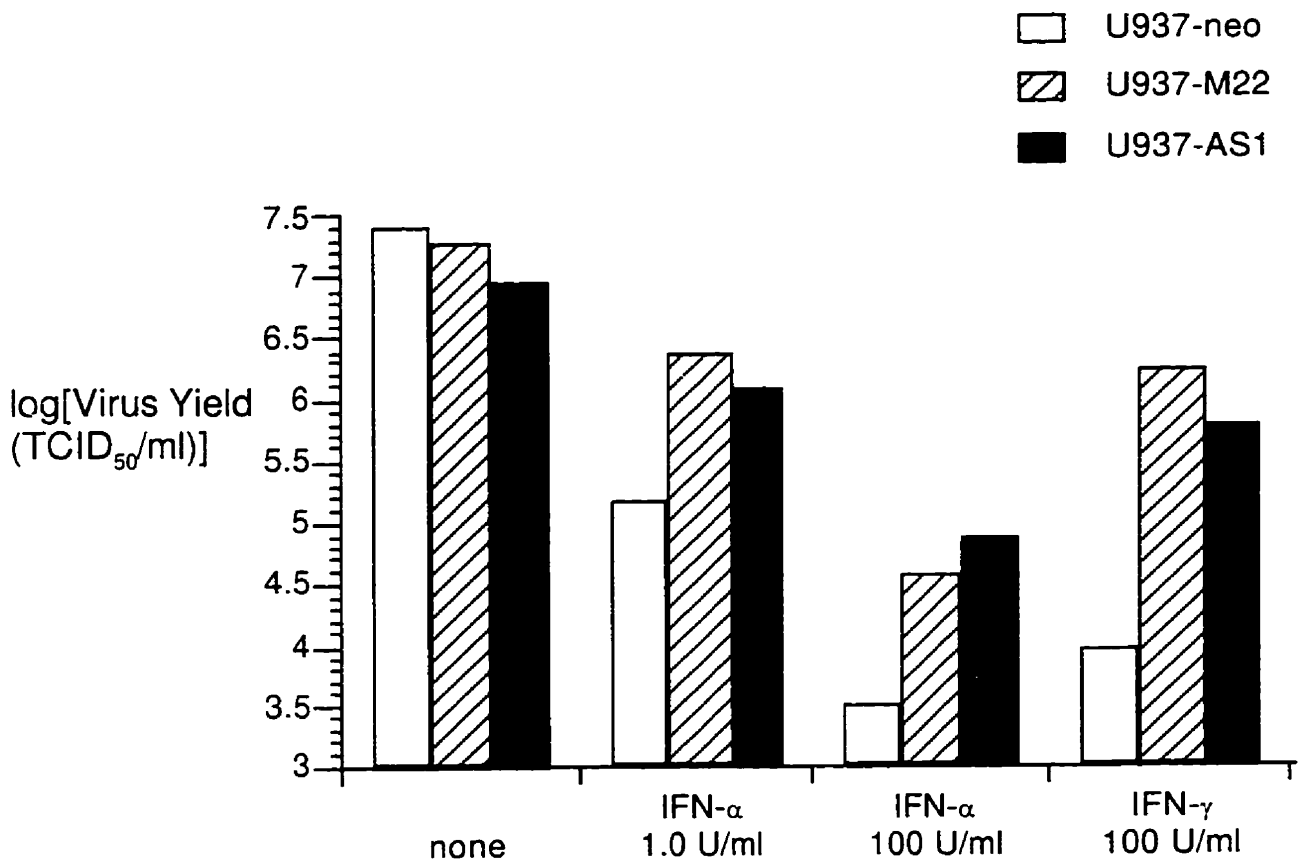


**Fig. 4b.**

# Discussion

Our data provide the first direct evidence implicating a role for PKR in the regulation of IFN-α and IFN-β genes. We have demonstrated that suppression of PKR function results in impaired IFN induction at both the protein and mRNA levels. Our data further suggest that induction of IFN-α and IFN-β genes may rely differentially on PKR-dependent and PKR-independent signaling mechanisms. The induction of both IFN-α and IFN-β mRNA by EMCV was impaired in U937-AS1 and U937-M22 cells, and yet, poly [I]·poly [C] still induced IFN-β but not IFN-α mRNA. Given this, the induction of IFN-β by poly [I]·poly [C] in these PKR-deficient cells cannot be easily explained as the result of residual PKR activity. It is possible, therefore, that alternative, PKR-independent pathways exist for dsRNA signaling. Consistent with this, tyrosine kinases have been indirectly implicated in the induction of interferon-stimulated genes by dsRNA (4). However, we cannot rule out the possibility that residual, low levels of PKR remaining in the U937-AS1 and U937-M22 cells, while insufficient for mediating IFN-α induction, are sufficient for IFN-β induction by dsRNA. Analysis of mice with homozygous deletions for PKR will be useful for the characterization of PKR-independent signaling pathways by dsRNA. Furthermore, this study suggests that activation of PKR *in vivo* can occur in response to inducers other than dsRNA, since IFN induction by the combination of IFN-γ priming and subsequent LPS or PMA stimulation required functional PKR (Fig. 3A; Fig. 3C, lanes 7 and 8). Activation of PKR without dsRNA *in vitro* has been described using heparin and other polyanionic molecules, and PKR activation *in vivo* was observed following interleukin-3 (IL-3) deprivation of a murine IL-3-dependent cell line (12, 13).

Our results also provide evidence for the participation of PKR in mediating the antiviral actions of IFN-α and IFN-γ. While PKR has not been commonly considered as a mediator of IFN-γ actions, the presence of a consensus IFN-γ responsive element, GAS, within the PKR promoter suggests that PKR may be regulated by IFN-γ (30). Consistent with this, our results here demonstrated the induction of PKR protein levels by IFN-γ (Fig. 1B). Previous studies

have linked several proteins, including the Mx, 2-5A synthetase and 2-5A-dependent RNase proteins, to IFN-α-induced antiviral activities (1, 2, 10). In particular, stable expression of the human PKR gene in mouse cells confers partial resistance to EMCV (25). Also, in embryonic fibroblasts from mice deleted for IRF-1, anti-EMCV activity by IFN-γ was even more impaired than the reduced IFN-α-mediated activity, characteristics similar to the PKR-deficient cells in this report (15). It is likely that the concerted actions of several genes including PKR contribute to the antiviral activities of IFN-α and IFN-γ. Interestingly, a tumour suppressor function for PKR has been suggested from studies showing that a malignant transformation phenotype correlates with overexpression of dominant negative PKR proteins (16, 24). Since IFNs have direct antitumour and antiproliferative activities (9), it is possible that the IFN-related deficiencies resultant from loss of PKR activity noted here may represent mechanisms which contribute to a transformation process.

PKR has been suggested to be important for controlling viral replication. However, many viruses, including adenovirus, influenza virus, vaccinia virus, and HIV, possess unique mechanisms for inactivating PKR function as means to evade the antiviral actions of the IFN system (14). We have shown that specific suppression of PKR in U937 cells resulted in a profound inability to restrict EMCV replication and that this was due to the impairment of at least two biological functions, Type I IFN expression and IFN-mediated antiviral responses. While it remains unclear which cellular proteins mediate these activities in pathways downstream from PKR, transcription factors including IRF-1, ATF-2/c-jun, and the STAT family, already implicated with regulation of Type I IFNs and IFN-stimulated genes, are possible substrates for PKR (5, 6, 22).

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# Chapter Five

## Summary and future prospects

# Discussion

## Differential expression of IFN-α subtypes

Studying the biology of the IFN-α genes is confounded by the large number of subtypes in the family and their high degree of homology. One approach to investigate their functions has been to compare the biological activities of individual recombinant IFN-α subtype proteins. A number of studies have described significant functional differences between subtypes. Elucidation of the signaling mechanisms responsible for these differences will bring a better appreciation of the biological functions of particular IFN-α subtypes. However, the relevance of such investigations needs to be supplemented by studies to determine the actual expression of IFN-α genes *in vivo* and the mechanisms by which their expression is regulated.

By using the RT-PCR approach with consensus IFN-α primers described in Chapter 2, we have observed that different steady-state levels of individual IFN-α subtype mRNA accumulate in particular cell types responding to certain inducers. However, the steady state level of mRNA transcripts in a cell is a function of several aspects of eukaryotic gene regulation that includes the rate of active gene transcription, mRNA processing, and mRNA stability. Although the relative contribution of each of these factors in controlling IFN-α expression has not been systematically analyzed, previous studies have suggested that the IFN-α genes are regulated primarily at the level of gene transcription. Firstly, the promoter regions from a number of human and mouse IFN-α genes are able to confer inducer-dependent transcriptional activity (more detailed discussion to follow) (23, 34, 35, 42). Also, a study employing nuclear transcription run-on assays indicated that *de novo* transcription of IFN-α genes occurs in response to IFN-inducers (36). Studies on the processing of IFN-α mRNA, such as polyadenylation or mRNA transport, has not been reported, and regulation at the level of gene splicing is irrelevant since IFN-α genes lack introns. Lastly, the issue of mRNA stability is important since a common characteristic of many inflammatory cytokine genes is their relatively short mRNA half-lives. This high turn-over rate is believed to be due largely to AU-

rich motifs in the 3'-untranslated region of their mRNA which serves as a recruitment signal for factors that expedite degradation of the RNA molecule (3, 4, 26). Such motifs are also present in IFN-α genes (18). One study has attempted to study the issue of IFN-α mRNA stability by using a plasmid construct consisting of a β-globin promoter linked to a fragment of the human IFN-α1 gene, that contains both the coding and 3'-untranslated regions. Stable transfection of this construct in mouse L cells resulted in a high constitutive level of IFN-α1 transgene expression, but which was not modulated in response to virus infection, suggesting that IFN-α1 mRNA stability did not represent a point of regulation (42).

For further study of the transcriptional activation and mRNA stability of IFN-α genes, it may be possible to augment the standard techniques used for measuring these parameters by incorporating our RT-PCR approach. In particular, this should be advantageous by improving on the efficiency of those portions of the assays which require detection of mRNA. The general method for measuring active gene transcription involves first isolating intact nuclei from cells and then allowing for the extension of nascent RNA transcripts to proceed *in vitro*. By including $^{32}$P-uridine during the extension reaction, only actively transcribed RNA become radioactively labeled and these species can be subsequently detected by hybridization to immobilized unlabelled probe DNA. As stated earlier, standard hybridization conditions are unable to distinguish between different subtypes. By reserving a fraction of the labeled RNA sample for analysis by RT-PCR in parallel, it would be possible to determine the precise identity of the IFN-α subtypes being transcribed. Measurements of the mRNA half-life of endogenous IFN-α transcripts has not yet been reported. Typically, mRNA half-life is studied by treating cells, which are actively transcribing the genes of interest, with the inhibitor actinomycin D in order to impose a blockage of *de novo* transcription. Determining the remaining mRNA levels at several time points following actinomycin D treatment then establishes the rate of decay and thus, permits calculation of mRNA half-life. By using the RT-PCR method with consensus IFN-α primers, it would be possible to measure the half-life of the total IFN-α mRNA pool as well as for individual subtypes. A more complete understanding of

the transcription rates and mRNA stability of endogenous IFN-α transcripts would provide a better understanding of the contributions by particular regulatory mechanisms in controlling IFN-α expression.

Although assaying for the mRNA levels for any given gene is an important part of studying its regulation, this does not substitute for measuring actual protein levels. It would be ideal to have a detection method which is effective for studying microscale samples and which can discriminate between the different IFN-α subtype proteins, but this is currently unavailable. Some monoclonal antibodies have been described which can discriminate between IFN-α2 and IFN-α4 proteins, and these were used to investigate IFN-α expression between different cell types (14). However, even if it were possible to assemble a larger panel of subtype-specific antibodies, employing such a strategy would face similar difficulties and limitations as using a panel of nucleic acid probes to distinguish between IFN-α subtype mRNAs. The binding specificity of each antibody would need to be stringently tested and controlled, and each test sample would have be to individually probed with the panel. Given the currently available methodologies, PCR detection using consensus IFN-α primers may represent the most efficient and comprehensive method for studying the expression of the IFN-α gene family.

An interesting and clinically relevant example of aberrant IFN-α expression involves the observation that elevated serum IFN-α levels are associated with the progression of AIDS in HIV-infected individuals. The cellular source of this IFN remains unknown and it does not seem to be originating from PBMCs. In fact, there appears to be a progressive decline in the IFN-α production capacity of PBMCs, in both the monocyte and NIPC compartments (12, 21), as the patients' disease status worsens (38). Recently, by performing lymph node biopsies in HIV-infected individuals, high levels of HIV replication have been observed to be occurring in the lymph nodes, with considerable association between free virions and dendritic cells. Since dendritic cells have been shown to produce IFN-α in response to HIV in cell culture experiments (11), it is reasonable to hypothesize that the lymph nodes may be the sites of production for IFN-α or other inflammatory cytokines, as well. In addition, the IFN-α proteins
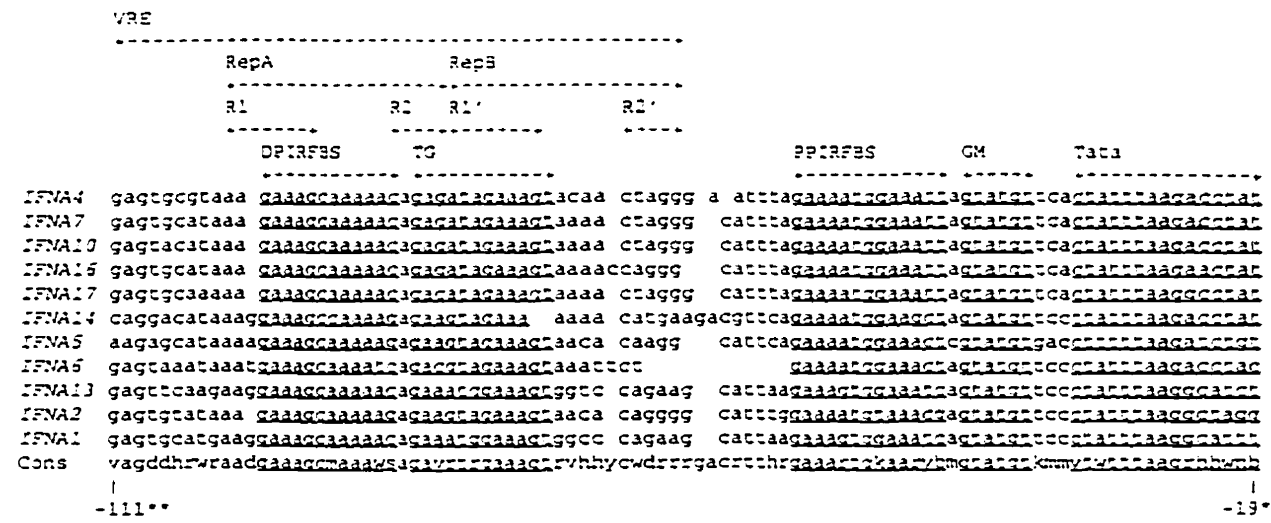
produced by PBMCs when stimulated with HIV-infected cells *in vitro*, appear to be relatively ineffective towards inhibiting HIV replication (13). While it is unknown whether this cell culture system is at all reflective of the interactions between HIV and the IFN system in infected individuals, identifying the IFN-α subtypes expressed in both situations would be helpful to better understand the role of IFN-α genes in response to HIV infection.

## Regulation of IFN-α gene transcription

It is clear that IFN-α genes are regulated at the level of transcriptional activation. However, our understanding of the regulatory mechanisms involved remains limited in two of the most important areas, the characterization of cis-acting DNA elements and the identification of unique transcription factors. Of the human IFN-α genes, only the IFNA1 promoter has been studied in detail. Deletional analyses have identified a 46-basepair region between positions -109 and -64 as a minimal virus-responsive regulatory element (VREα1) (35). It is noteworthy that the transcriptional activity of VREα1 on its own is relatively weak such that in order to reliably assay for virus-responsiveness, an exogenous enhancer element on the same reporter plasmid (e.g. SV40 enhancer) is required to boost the overall transcriptional activity (30, 35). Also, the studies on this human VREα1 element have been conducted primarily by transient transfection studies in the mouse L929 cell line. In studies by a different laboratory, the transcriptional activity of a larger IFN-α1 promoter fragment, spanning positions -131 to +25, was examined in U937 cells (19, 43). The relative induction of this IFN-α1 promoter fragment was at best, 2.5-fold, in response to priming with IFN prior to stimulation by virus infection. In comparison, a human IFN-β promoter fragment spanning from -281 to +19 exhibited up to 100-fold inducibility. At face value, these results may imply that additional sequences outside of the -131 to +25 region of the IFN-α1 promoter are required to confer high level virus-responsiveness (further discussion later). Alternatively, it is also possible that the transcriptional strength of the IFN-α1 promoter is inherently weak, as suggested by our results in Chapter 2.

A next logical set of experiments to address these issues would be to compare the IFNA1 VRE to the transcriptional strengths of other IFNA gene promoters. One recent study has compared the VREs for IFNA1, IFNA2, IFNA4 and IFNA14 (between positions -109 and -64) using reporter gene contructs (8). In transient transfection assays with Namalwa cells, it was observed that the pair of IFNA1 and IFNA2 VREs were similar in their responsiveness to virus while the IFNA4 and IFNA14 VREs were mostly unresponsive. Analyses of these sequences, however, do not readily identify any particular nucleotide positions that may be important for transcriptional activity. Over the 46 basepair length of these elements, 29 positions are conserved. Presumably, the differences in activity between the IFNA1/IFNA2 and the IFNA4/IFNA14 pairs arise from nucleotide differences among the 17 non-conserved postions, but there are none which are exclusively common to one pair over the other (8). It is likely that there are more than one functionally distinct cis-element within this 46 basepair region, as is the case for the IFN-β VRE. One approach to identify these elements would be to select two IFN-α VREs, perform base-substitution mutagenesis at the non-conserved positions, and determine which changes result in either loss or gain of activity. This type of strategy was applied in the analysis of two mouse IFN-α gene promoters, IFN-α4 and IFN-α6, which exhibit differential inducibility in L929 cells (1). A 35-basepair virus-responsive element from the highly inducible mouse IFN-α4 promoter differed in only 6 positions as compared to the corresponding region from the poorly inducible mouse IFN-α6 gene (33). By performing mutational analysis only at these 6 positions, 2 were identified as critical for high level virus-inducibility. The promoters of 11 human IFN-α genes are currently available and alignment of these promoters has permitted mapping of conserved motifs between positions -111 and -19 (Fig. 1)(20). These include two putative IRF binding sites, a TG sequence, and a highly conserved GTATGT sequence located upstream of the TATA sequence. It would be of particular interest to examine the IFNA8 gene promoter since its expression was highest among the subtypes (Chaper 2), but the IFNA8 promoter region has not yet been identified.

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**Fig. 1. Alignment of IFN-α gene promoters.** This figure was taken from Table 2 of the study by Houle and Santoro (20). Genomic DNA sequences for 11 IFN-α genes were retrieved from GenBank sequence database and the regions corresponding to positions -111 and -19 were compared and analyzed. Gaps were introduced to optimize the best alignment of the sequences. Conserved motifs are indicated by dashed lines. Studies by Weissmann and colleagues had identified the motifs designated VRE, RepA, RepB, R1, R1', R2 andn R2' (35). Houle and Santoro have further defined the motifs designated DPIRFBS, PPIRFBS, GM and TG.

```
VRE
*-------------------------------------------*
         RepA                    RepB
         *-------------------------------------*
         R1             R2   R1'           R2'
         *--------+     *-------------*     *----*
              DPIRFBS    TG                      PPIRFBS    GM      Tata
              *------------* *-----------*       *-----------* *------* *---------------*
IFNA4  gagtgcgtaaa gaaaccaaaaacagagatagaaagtacaa ctaggg a atttagaaaatggaaattagtatgtcagtattttaagacctat
IFNA7  gagtgcataaa gaaaccaaaaacagagatagaaagtaaaa ctaggg catttagaaaatggaaattagtatgttcagtattttaagacctat
IFNA10 gagtacataaa gaaaccaaaaacagagatagaaagtaaaa ctaggg catttagaaaatggaaattagtatgttcagtattttaagacctat
IFNA16 gagtgcataaa gaaaccaaaaacagagatacaaaagtaaaaccaggg catttagaaaatggaaattagtatgttcagtattttaagaaacttat
IFNA17 gagtgcaaaaa gaaaccaaaaacagagatagaaagtaaaa ctaggg catttagaaaatggaaattagtatgttcagtattttaaggcctat
IFNA14 caggacataaaggaaaccaaaagcagtagtagtaaa aaaa catgaagacgttcagaaaatggaagctagtatgttccttattttaagacctat
IFNA5  aagagcataaaagaaaccaaaaagcagtagtagtaagtaaca caagg catttagaaaatggaaactcgtatgtgacgttttttaagatccgt
IFNA6  gagtaaatcaaatgaaaccaaaattagcacgtagtaaagtaaattct      gaaaattgaaactagtatgtcccgtattttaagacccac
IFNA13 gagttcaagaaggaaaccaaaaacagtaattagtaaagtggtc cagaag cattaagaaagttgtaattagtatgttccctattttaaggcaatct
IFNA2  gagtgtataaa gaaaccaaaaacagtaatagtaaagtaaca caggggg catttgtaaaatgttttactagtatgttccctattttaaggcctacg
IFNA1  gagtgcatgaaggaaaccaaaaacagtaattgtaaagtggcc cagaag cattaagaaagttgtaaattagtatgttccctattttaaggccattt
Cons   vagddhrwraadgaaacrmaaawsagtavttttaaactrvhhycwdrrrgacttthrgaaactgkaarvhmgtattgkmmytwtttaagtnhwnh
       |                                                                                           |
       -111**                                                                                      -19*
```

*IFNA genes are listed in physical order within chromosomal band 9p21.[4,9]

**3' position IFNA2–IFNA16

***5' position IFNA1

DPIRFBS   distal putative interferon regulatory factor
          binding site
GM        GTATGT motif
PPIRFBS   proximal putative interferon regulatory factor
          binding site
RepA      repeat A
RepB      repeat B
R1        repeat A 5' octamer
R2        repeat A 3' pentamer
R1'       repeat B 5' octamer
R2'       repeat B 3' pentamer
Tata      tata box
TG        TG sequence
VRE       virus-response element

**Fig. 1.**

**Fig. 2. Genomic map of the Type I IFN gene cluster.** This figure was taken from Fig. 4 of the study by Diaz et al. (9). The relative locations of different IFN genes (solid bars) and pseudogenes (open bars) are indicated and restriction sites are designated by vertical lines. Arrowheads above or below the bars indicate the direction of transcription for individual IFN genes. The locations of the two potential LCRs discussed in this chapter, between IFNP12 and IFNP11, and IFNAP22 and IFNA14, are marked by the large vertical arrows.
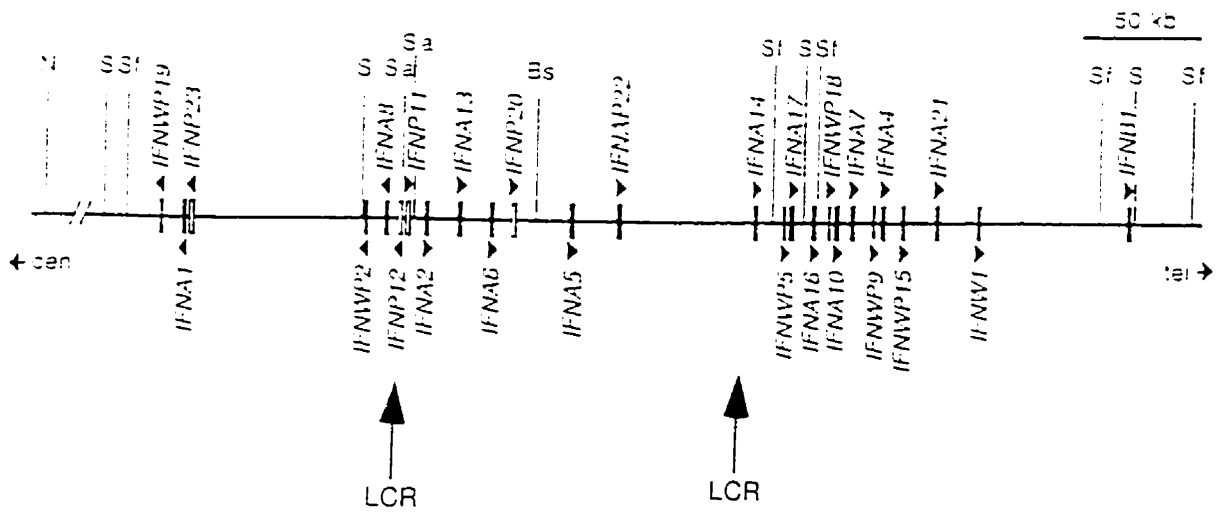
Fig. 2.

The genomic map of the Type I IFN gene cluster on the short arm of human chromosome 9 (9p) has been determined (Fig. 2)(9). IFNW genes are interspersed between the IFNA genes while IFNB is located at the most distal portion of 9p. The majority of IFN genes are arranged in tandem with the direction of transcription towards the telomere. The remaining IFN genes at the proximal side of this cluster are also arranged in tandem but their transcription direction is towards the centromere. The authors suggest that this segment likely arose as an inverted duplication during evolution. Several examples of gene families arranged in clusters have been studied including the β-globin genes, the myogenic regulatory factor genes, the Hox genes. and the granzyme genes (31). With regards to transcriptional regulation, there is an emerging generalization that these gene clusters typically contain a locus control region (LCR) which affects the expression of all the genes in the cluster. The only LCR that has been well-defined belongs the β-globin locus. Evidence for the existence of similar LCRs in the other gene loci mentioned above is indirect and arises from studies involving the creation of gene-knockout mouse models. Gene disruption is usually performed by replacing a portion of the target gene with a PGK-neo cassette which, at the same time, serves as a selectable marker for the homologous recombination event in embryonic stem cells. There are now several examples involving targeted disruptions of individual genes in these clusters which unexpectedly results in reduced expression of the remaining non-targeted genes in the cluster (22, 31, 32). In these cases, it has been speculated that transcription from the inserted PGK-neo gene somehow perturbs the activity of an LCR that is reflected by the suppression of the entire gene cluster.

There is no evidence yet supporting the existence of an LCR in the Type I IFN gene cluster but the relatively weak transcriptional activity of IFN-α VREs does suggest that additional regulatory elements may be involved. Given that the Type I IFN cluster has been isolated on a YAC contig, it may be possible to perform a screen across this region for a putative LCR or additional cis-elements using an enhancer trap strategy. This cluster encompasses 400 kilobases, however, and such a screen could be highly laborious. From studies of the β-globin LCR and putative LCRs in other gene clusters, these control regions tend

to be located upstream of the gene cluster. Using this as a guide, there are two regions in the Type I IFN cluster which may represent good candidates to contain regulatory elements. Firstly, by assuming that an inverted duplication event did occur to create the block of IFN genes at the proximal end of the cluster, this would locate the original most upstream region between IFNAP11 and IFNAP12 (Fig. 2). If an LCR or enhancer element is located here, the nearest functional IFN-α genes are IFNA8, IFNA2, IFNA13 and IFNA1. Therefore, it can be postulated that the relatively strong transcription of these genes is attributed to their physical proximity to a potential LCR/enhancer element. Previously, on the basis of sequence similarities, the IFN-α genes were categorized into one of two groups and it was hypothesized that each group may have evolved separately from the products of the first duplication of a primordial IFN-α gene (18). The structure of the IFN cluster does support this hypothesis since each group is organized within opposite halves of the cluster. Specifically, the genes encompassed between IFNA14 and IFNA21 are believed to represent one group. Therefore, it is possible that a second LCR/enhancer, a duplication of the original upstream region, may reside immediately upstream of IFNA14 (Fig. 2). Both of these candidate regions can be screened using enhancer trap strategies but initially, it may be informative to simply determine whether there are any conserved sequences between these two regions.

The identification of a well-defined transcriptional element from an IFN-α gene is critical for the characterization of required IFN-α-transcription factors. Such an element could be used in a number of different approaches to isolate such transcription factors and as well, it would serve as the basis for creating reporter plasmids to actually assay for transcriptional activity. Candidate transcription factors are usually first identified as proteins which can bind with high affinity to the transcriptional element of interest. For example, only extracts from virus-induced cells contain activated transcription factors, NF-κB and ATF-2, which can bind to the PRDII and PRDIV elements of the IFN-β gene, respectively (10). These factors are identifiable on the basis of their ability to form stable protein-DNA complexes that can be detected using *in vitro* binding assays such as an electrophoretic mobility shift assay or a

Southwestern filter binding assay. It has been reported that a number of constitutive factors bind to the VREα1 element but novel virus-inducible complexes have not yet been characterized. These results include analysis of extracts made from mouse L929 cells, as well as human Namalwa and U937 cells (19, 25). If the transcriptional activity of the IFN-α1 VRE is indeed intrinsically weak, it may not be unexpected that inducible factor binding was not detected since mobility shift assays are generally less sensitive than functional transcriptional assays. However, not all transcription factors are necessarily regulated through activation of their DNA-binding ability. Many transcription factors are intrinsically able to bind to their cognate DNA sites, but their effective DNA-binding in vivo is regulated by different mechanisms such as subcellular retention or being sequestered by inhibitory factors.

Once a strong IFN-α promoter element has been identified, a variety of biochemical and genetic approaches may be used to isolate the cognate transcription factor(s). These could include large scale column fractionation of activated cell extracts in order to purify a candidate factor that, for example, can be monitored by mobility shift assays. While this approach is the most straightforward, it is technically challenging since the levels of transcription factors in cells are usually very low. Alternatively, a genetic approach which has proved successful in a variety of situations involves expression cloning from an mammalian expression library. This library is transfected into cells along with a plasmid containing, for example, an IFN-α gene promoter or regulatory element which drives the transcription of a reporter gene. Those transfected cells in which activation of the reporter gene can be subsequently detected would presumably harbour a library plasmid clone encoding for an functionally active regulatory protein. It is usually necessary that the cells used in this type of a screen lack the presumed protein of interest in order to provide a negative background for the assay. In other words, cell lines such as HeLa or NIH3T3 cells, which do not normally express IFN-α genes, would represent a reasonable choice. Alternatively, it may also be feasible to use U937 cells for such a screen by taking advantage of the observation that IFN-α expression in response to inducers such as LPS or PMA is strictly dependent on IFN-priming. Assuming that priming induces de

*novo* synthesis of a regulatory protein which is not normally present, then unprimed U937 cells would also represent an appropriate negative background.

Before implementing any type of screen for IFN-α-specific transcription factors, it is worthwhile to consider a number of cloned genes as candidates. Earlier studies had suggested that the transcription factor, IRF-1, may be a regulator of IFN-α expression (29). Subsequent studies have ruled out its role as a primary activator since IFN-α expression can still be induced in mice homozygously deleted for the IRF-1 gene (28). These results, however, do not rule out an auxiliary role for IRF-1 in IFN-α regulation, perhaps in cooperation with other transcription factors. NF-κB has been well-studied as an important positive regulator of the IFN-β gene (41). NF-κB is a heterodimeric complex which can consist of a variety of combinations between different protein partners encoded by the Rel gene family. One of the gene members encodes a p50 subunit and mice homozygously deleted for the p50 gene exhibit increased susceptibility to infection by bacteria such as *Listeria monocytogenes* and *Streptococcus pneumoniae* (37). Surprisingly, however, p50 knockout mice are more resistant to infection by EMCV. As a possible mechanism for this resistance, the inducibility of IFN-β in p50-deficient mice is severalfold greater than in control mice, suggesting that p50 may also play an important role as a repressor in regulating IFN-β gene induction. However, it is unknown whether the expression of IFN-α genes in these mice is affected. Although NF-κB sites do not appear to reside in the IFN-α1 VRE, it is possible that NF-κB may still be involved in the regulation of other IFN-α subtype genes, especially given the dominant role of NF-κB in regulating so many inflammatory cytokine and early response genes. Finally, the most recent and intriguing candidate gene for regulating IFN-α expression is ISGF3γ/p48 since mice deleted of this gene exhibit profound deficiencies in IFN-α and IFN-β induction (17). The function of ISGF3γ/p48 has primarily been studied for its role in mediating downstream IFN-signaling (7). Its additional functions in regulating IFN gene activation will need to be investigated in greater detail.

Fig. 3. **Model of IFN-α gene regulatory pathways.** The expression of IFN-α genes are inducible by not only virus and dsRNA, but also LPS and PMA. Since IFN induction by all of these inducers are inhibited in PKR-deficient cells, PKR may function as a common signal transducer for regulating IFN-α genes. While PKR is known to be directly activated by virus and dsRNA, the mechanisms for its activation by LPS and PMA remains unclear. In addition, the transcription factor(s) which directly mediate IFN-α gene transcription have not yet been identified. Therefore, some criteria for testing future candidate IFN-α transcription factors should include activation by virus, LPS and PMA, and regulation by PKR.
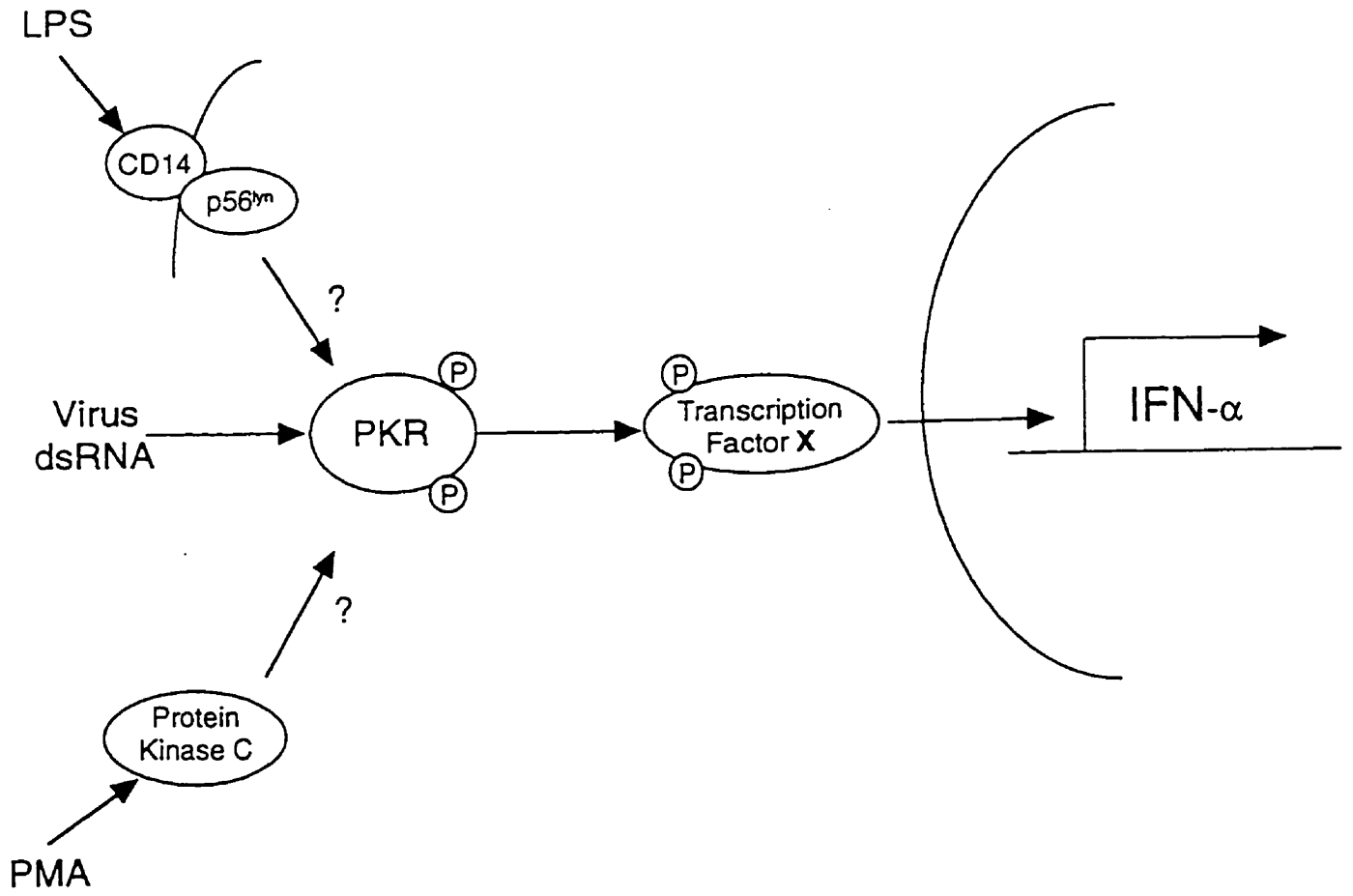
**Fig. 3.**

## Role of PKR in IFN-α gene regulation

The results in Chapter 4 demonstrate a necessary role for PKR in mediating the induction of IFN-α gene expression, at least in monocytic cells. PKR is best known for its role in protein translational control upon activation by dsRNA but these results further support a role for PKR as a signal transducer in transcriptional regulation that is responsive to virus infection or dsRNA. It remains to be determined what are the downstream substrates of PKR which lead to the initiation of IFN-α gene transcription. For IFN-β induction, PKR's role appears to involve phosphorylation of IκB, resulting in the activation and binding of NF-κB to PRDII in the IFN-β promoter (24, 27). As mentioned earlier, ISGF3γ/p48 is currently a candidate for mediating IFN-α expression. Given that ISGF3γ/p48 is known to be a phosphoprotein, it could potentially be a substrate for phosphorylation by PKR. To address this issue, the simplest experiments would involve determining whether ISGF3γ/p48 can be phosphorylated by PKR *in vitro* using either recombinant or natural proteins purified from cells. A second set of experiments would be to determine whether ISGF3γ/p48 becomes phosphorylated in vivo under conditions when PKR is known to become activated (ie. in response to dsRNA or virus). In addition, if ISGF3γ/p48 does require PKR for its activation, it would be predicted that ISGF3γ/p48 phosphorylation is deficient in either of the PKR-deficient U937 cell lines described in Chapter 4. Similar experiments could be performed to examine the phosphorylation of IκB in these mutant U937 cells. A more stringent line of experiments would rely on mapping of the PKR phosphorylation sites on either of these proteins. Mutant IκB or ISGF3γ/p48 proteins could then be engineered which contain substitutions only at the PKR phosphorylation sites. Transfection of these proteins into U937 cells, for example, would be predicted to result in a dominant negative phenotype which blocks signaling from PKR-mediated stimuli.

The requirement of PKR for IFN-α inducibility by LPS or PMA in U937 cells was unexpected. While PKR is directly activated by binding dsRNA, its activation has not previously been associated with stimulation by LPS or PMA. Recently, it was reported that LPS stimulation of mouse macrophages results in the induction of PKR mRNA and protein

levels but it is unclear whether the kinase function of PKR also became activated as a result (15). PMA is known primarily as an activator of protein kinase C while LPS stimulation is associated with the activation of CD14-associated protein tyrosine kinase $p56^{lyn}$ (39) and subsequent activation of the stress-induced kinase, p38, a MAP kinase-related member (16). It is possible that LPS-activated $p56^{lyn}$ kinase/p38 kinase or PMA-activated protein kinase C may directly phosphorylate and activate PKR in a kinase cascade (Fig. 3). It would be important to determine initially whether PKR even becomes phosphorylated and activated following LPS or PMA stimulation of cells. To determine whether PKR is a downstream substrate of these kinases, the simplest experiment would be to use either purified proteins or recombinant version of these kinases and determine whether they can phosphorylate PKR *in vitro*. To address whether $p56^{lyn}$ kinase, p38 kinase, or protein kinase C function as upstream activators of PKR in the cell, current strategies involve transfection of dominant negative/catalytically inactive mutants of these kinases into cells and then, assessing whether phosphorylation or activation of PKR is affected. Since many signaling pathways are now known to interact with each other, it is not entirely surprising that PKR also participates in this crosstalk.

PKR activity may be necessary for IFN-α gene activation, but there are additional aspects to take into account when considering whether PKR activation alone is sufficient for IFN-α induction. Firstly, the observation that calphostin C can reduce IFN production suggests that protein kinase C may play an auxiliary role in IFN-α regulation. This is reinforced by the finding that PMA, a protein kinase C agonist, is an efficient IFN inducer. Furthermore, since PMA is a well known activator of transcription factors like NF-κB and AP-1, these may also be considered as potential regulators of IFN-α induction. Secondly, there is evidence supporting alternative virus- and dsRNA-induced signaling pathways which are independent of PKR. For example, induction of IFN-α and IFN-β in mouse splenocytes is not inhibited by 2-aminopurine (5). Also, novel dsRNA- and virus-induced ISRE-binding factors have been described, some of which are similarly resistant to inhibition by 2-aminopurine (2, 6). Thirdly, all cellular phosphorylation events are opposed by the dephosphorylation activities of

phosphatases. A Type I phosphatase has been purified which reversibly dephosphorylates active PKR resulting in loss of its kinase activity (40). While it is unknown how this phosphatase is regulated, its activity during the course of a viral infection may be as important as PKR activation, for regulating downstream IFN-α-specific transcription factors.

Recently, a PKR knockout mouse model has been established (44). Embryonic fibroblasts derived from these mutant mice are deficient in IFN-α and IFN-β induction, and as well, NF-κB activation. However, following challenge of the PKR knockout mice directly with virus or dsRNA, IFN expression appeared to be normal as measured at the protein level from serum and at the mRNA level from different organs. The authors also observed that priming effectively restored IFN induction in the embryonic fibroblasts. This led them to suggest that priming signals in the mice, arising from low levels of endogenous IFNs or other cytokines, may be responsible for the apparently unaffected inducibility of IFN-α and IFN-β genes. This certainly provides the most credible evidence for alternative pathways regulating IFN expression which are PKR-independent. This mouse model also provides an ideal background with which to investigate and characterize these pathways.

My thesis work began with developing a PCR strategy which could amplify the entire IFN-α gene family. Having investigated the expression of IFN-α subtypes in a number of cell types, further study of the human monocytic cell line, U937, revealed how IFN-α expression can be significantly affected by priming. This line of experiments also revealed that LPS and PMA can act as IFN inducers but that they have strict requirements for a priming signal. Lastly, the generation of PKR-deficient cell lines provided direct evidence for the involvement of PKR in regulating Type I IFN expression. IFN-α gene regulation can serve as an important model system for studying the mechanisms required for mediating effective cellular responses to virus infections. It is also not unreasonable to expect such research to yield further insights into the pathogenesis of viral diseases and certain human illnesses associated with aberrant IFN-α expression. Ultimately, better clinical treatments for these diseases may become available with the ability to positively or negatively modulate IFN-α expression *in vivo* where it is required.

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# IMAGE EVALUATION
## TEST TARGET (QA-3)

1.0

1.1

1.25    1.4    1.6

1.0

1.1

1.25

2.5
2.8    2.2
3.2    2.0
3.6
4.0    1.8

|← 150mm →|

|← 6" →|
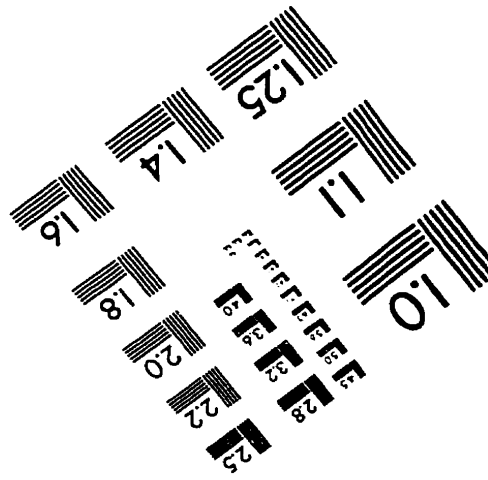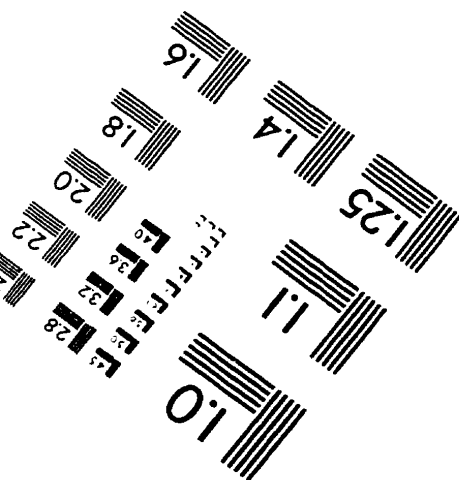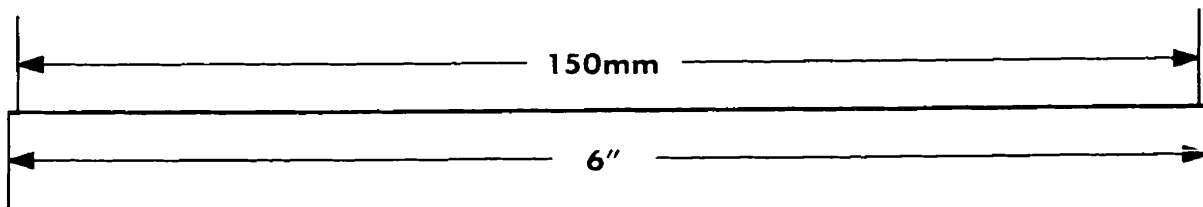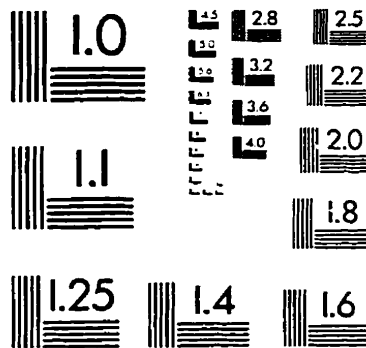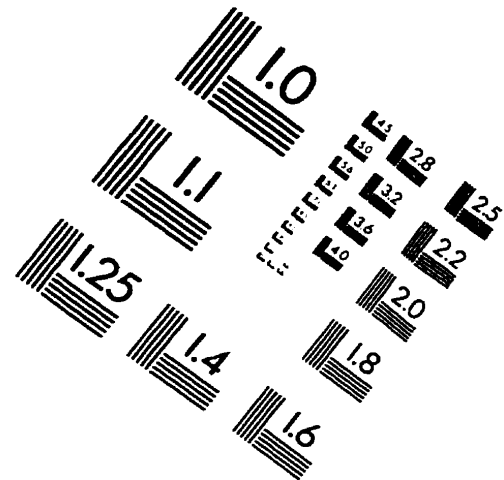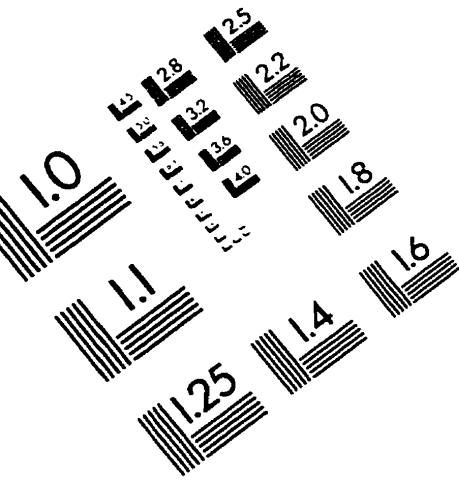
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