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Signal Transduction and Transcriptional Regulation in the Response to Type I Interferon

A thesis presented to the faculty of the Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Daniel Solomon Kessler

April 1, 1990
The Rockefeller University
New York

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For my parents, my first teachers

All musicians share
the same
obscurity.

Give up

and pick up
your horn.

(From *The Grand Concourse* by Milton Kessler, 1990)

Acknowledgements

This thesis is the result of the support, instruction, discussion and collaboration of numerous individuals, whose patience and generosity I enjoyed during my years at Rockefeller.

Within the laboratory I thank Richard Pine, Danny Lew, Thomas Decker, Xin-Yuan Fu, Chris Schindler, Nancy Reich, as well as the rest of the Darnell group whose friendship and humor have made the past four years so pleasant. I also thank my two collaborators outside of the lab, Larry Pfeffer and Susan Veals. I am indebted to David Levy for guiding my introduction to molecular biology and as a constant source of critical insight that maintained the momentum of the project. Most of all, I thank my advisor, James Darnell, for consistent support, generosity with his time and thoughts, always valuable advice, and for allowing me to freely pursue my interests.

Finally, I express my gratitude for the love and support of my friends and family. My parents, Sonia and Milton, have always provided great enthusiasm and confidence, and shared wise thoughts that guided me through difficult periods and decisions. The affection and interest of my sister Paula, brother David, and aunt Ethel were a constant source of support. And I thank Elettra, whose companionship and love have filled these years with joy.

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List of Abbreviations

2-AP	2-aminopurine
bp	base pair
BSA	bovine serum albumin
cAMP	2',5'-cyclic adenosine monophosphate
CHX	cycloheximide
CSF-1	colony stimulated growth factor-1
DAG	diacylglycerol
DME	Dulbecco's modified Eagle's medium
DMS	dimethylsulfate
DNase	deoxyribonuclease
DTT	dithiothreitol
EGF	epidermal growth factor
GAP	GTPase activating protein
GBP	guanylate binding protein
HLA	human leukocyte antigen
kD	kilodalton
IFN	interferon
IP_3	inositol 1,4,5-trisphosphate
ISG	interferon stimulated gene
ISGF	interferon stimulated gene factor
ISRE	interferon stimulated response element
MHC	major histocompatibility locus
NEM	N-ethyl maleimide
NGF	nerve growth factor
OAS	2',5'-oligoadenylate synthetase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PMSF	phenylmethylsulfonyl fluoride
pol II	RNA polymerase II
RNase	ribonuclease
SDS	sodium dodecyl sulfate

Abstract

The interaction of type I interferon (IFN) with a specific cell surface receptor elicits a number of physiological changes, including the attainment of a state in which viral replication and cellular proliferation is inhibited. The response to IFN is mediated by a group of IFN-induced proteins which are regulated at the transcriptional level. Therefore, a central event in the cellular response to IFN is the coordinate transcriptional induction of a specific set of interferon stimulated genes (ISGs). In this thesis, I have addressed the cellular strategies employed to transmit specific cell surface signals across compartmental boundaries, ultimately resulting in specific transcriptional modulation in the nucleus. The analysis of the 5' regulatory sequences of two ISGs has identified a conserved interferon stimulated response element (ISRE) present in all ISGs, which is necessary and sufficient for transcriptional responsiveness to IFN. Three nuclear DNA-binding factors were identified that specifically bind the ISRE, one which was constitutive and two whose presence was dependent of IFN treatment. The analysis of the kinetics, protein synthesis-dependence, and precise sequence requirements for ISRE-binding of these interferon stimulated gene factors (ISGFs) implicated a single IFN-inducible factor, ISGF3, as the positive regulator of ISGs. ISGF3 was induced rapidly in the absence of ongoing protein synthesis, characteristics identical to transcriptional activation of ISGs. The study of IFN-resistant variant cell lines which do not respond to IFN physiologically, but do express normal IFN receptors, supported the role of ISGF3 as the ISG activator. In these resistant cell lines the lack of ISG induction correlated with a defect in ISGF3 activation. Furthermore, it was found that ISGF3 activation occurred in the cytoplasm of stimulated cells through a series of events involving post-

translational activation of a latent cytoplasmic protein, and subsequent association with a second cytoplasmic protein forming a heteromeric complex which accumulated in the nucleus. Additional characterization of the ISGF3 complex revealed a heterotetromeric complex (48, 84, 91, and 113 kD subunits). The activation and formation of ISGF3 required IFN-dependent post-translational activation of at least one of the high molecular weight subunits (84, 91, or 113 kD), leading to facilitated nuclear translocation of this group of three proteins. Upon nuclear accumulation these proteins associated with the 48 kD ISRE-binding subunit resulting in an enhancement of DNA-binding affinity and formation of the stable, transcriptionally active ISGF3-ISRE complex. In the type I IFN system, specific transcriptional regulation in response to a cell surface ligand is mediated by activation of a latent cytoplasmic transcription factor which is a highly specific intracellular messenger.

Chapter 1

Introduction

The survival of both unicellular and multicellular organisms relies upon the ability to accurately sense the immediate environment, and to respond to external stimuli in an appropriate manner. In eukaryotes the interactions between extracellular stimuli and cellular physiology are elemental to the control of cellular growth, differentiation, and homeostasis, processes dependent on modulation of the quantity or activity of specific cellular gene products. While the control of gene activity is complex and may act at several levels, initiation of messenger RNA synthesis has emerged as a primary control point in the regulation of differential gene expression (Gurdon, 1974; Davidson, 1976; Darnell, 1982, 1985; Gehring, 1987; Maniatis et al., 1987; Almendral et al., 1988; Cochran et al., 1983; Greenberg et al., 1985; Muller et al., 1984; Derman et al., 1981). Therefore, cellular mechanisms exist that mediate specific nuclear changes in response to extracellular conditions. The existence of numerous external cues, including physical stimuli (temperature, light, etc.), polypeptides (hormones, growth factors, cytokines, etc.), and nonprotein molecules (steroids, amino acids, etc.), each provoking precise changes in cellular physiology, implies the existence of complex signal transduction mechanisms for specifying cellular response to a given stimulus.

The exact cellular strategies employed to provoke specific transcriptional changes in response to extracellular cues depends on the nature of the extracellular signal. In the case of physical stimuli, such as temperature, no specialized apparatus is required for transmission across the cell membrane and all cellular proteins are potential targets. Similarly, hydrophobic signals, including steroids, freely traverse membrane boundaries and directly influence cellular targets. In contrast, hydrophilic molecules, such as polypeptides, are dependent on specialized cell surface proteins which convey extracellular signals to the cell interior. Therefore, signal transduction in response to polypeptide signals is necessarily a more complex process involving conversion of extracellular stimuli into distinct intracellular messengers which interact with cellular machinery to alter physiology, often through specific transcriptional changes.

In an attempt to describe cellular strategies for signal transduction in response to polypeptide ligands the study of defined systems is useful. The analysis of an experimental system consisting of a characterized ligand which binds to a specific cell surface receptor, thus eliciting precise transcriptional changes, may allow a better understanding of signal transduction mechanisms. Towards this end a number of general questions must be

addressed:

1) What are the *cis*-acting transcriptional regulatory elements that are the ultimate targets for signal transduction?

2) What are the *trans*-acting nuclear targets of signal transduction that mediate transcriptional control?

3) What is the nature of the intracellular messengers that convey cell surface events to the nucleus?

4) How is specificity maintained in the response to receptor-ligand interaction?

This thesis represents an attempt to address these questions in the type I interferon system. The approach taken in studying this system can be described as "inside-out". By describing the *cis*- and *trans*-regulatory elements controlling interferon-stimulated transcriptional activation it may be possible to reveal the nuclear, cytoplasmic, and cell surface components that interact sequentially in transducing cell surface signals to the nucleus. This approach will ultimately provide a detailed description of the molecular events constituting signal transduction in the type I interferon system, perhaps revealing a general strategy for signal transduction.

As an introduction to the thesis the following sections consist of a brief description of our current understanding of transcriptional regulation and signal transduction, and a general overview of the interferon system.

Transcriptional regulation

An abundance of evidence has implicated the initiation of mRNA synthesis as a principal regulatory point in gene expression. The transcriptional activity of an individual gene is determined by the interaction of two sets of regulatory components, *cis*-acting DNA elements and *trans*-acting protein factors. The combinatorial action of *cis* and *trans* elements accounts for precisely refined regulation of specific gene transcription in a variety of cell types or physiological conditions (McKnight and Tjian, 1986; Mitchell and Tjian, 1989; Johnson and McKnight, 1989; Struhl, 1989). Among these regulatory elements are those generally required for transcriptional activity and those involved in the regulation of specific gene subsets through interaction with the general transcription apparatus (reviewed in Saltzman and Weinmann, 1989).

Cis-regulatory elements

Mutational analyses and expression studies *in vitro* and *in vivo* have shown that initiation of mRNA transcription by RNA polymerase II is controlled by *cis* regulatory elements positioned within several hundred base pairs of the transcription initiation site, as well as by additional elements acting from thousands of base pairs. The proximal or promoter elements,

such as the TATA, GC, and CCAAT boxes, act in a distance and orientation dependent manner, and are present in many pol II transcription units (Khoury and Gruss, 1983; Maniatis et al., 1987; Muller et al., 1988; Mitchell and Tjian, 1989). The TATA box, present in the promoter regions of most, but not all pol II regulated genes, is of central importance in the formation of transcriptionally active promoter complexes (Corden et al., 1980; Wasylyk and Chambon, 1981).

Distal elements, known as enhancers, are distance and orientation independent regulators of transcriptional initiation (Banerji et al., 1981, 1983; Queen and Baltimore, 1983; Gillies et al., 1983; Herr and Clarke, 1986; Ptashne, 1986a; Ondek et al., 1988; Schaffner et al., 1988). This diverse class of regulators is responsible for cell-type specific and signal-dependent transcriptional control in a large number of cases. Specific enhancers, having either a positive or negative regulatory effect, are often present in multiple copies, or in combination with distinct enhancers. Therefore, unique configurations of enhancer elements effect precise transcriptional programs on particular genes.

Trans-acting factors

The regulatory functions of *cis* elements are mediated by sequence-specific DNA-binding proteins (reviewed in Ptashne, 1986a, 1986b,

1988, 1989; Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Struhl, 1989). Despite being low abundance, technological developments now facilitate their study through DNA-affinity purification and analysis in a variety of *in vitro* systems (Roeder, 1974; Galas and Schmitz, 1978; Weil et al., 1979; Fried and Crothers, 1981; Garner and Revzin, 1981; McKay, 1981; Samuels et al., 1982; Engelke et al., 1983; Dignam et al., 1983a, 1983b; Sawadogo and Roeder, 1985; Kadonaga and Tjian, 1986; Rosenfeld and Kelly, 1986). In addition, structure-function analysis of cloned transcription factors has shed light on conserved protein structures responsible for DNA-binding and transcriptional activation. Recent work has identified a number of important DNA-binding structures, including the homeodomain (a member of the helix-turn-helix class), POU domain, zinc finger, leucine zipper-basic domain, and helix-loop-helix (Ginsberg et al., 1984; Miller et al., 1985b; Laughon and Scott, 1984; Scott et al., 1989; Herr et al., 1988; Landschulz et al., 1988; Murre et al., 1989). In addition, regions composed of a predominant amino acid type (glutamine, proline, or acidic) play an important role in transcriptional activation (Brent and Ptashne, 1985; Ma and Ptashne, 1987a; Ptashne, 1988; Courey and Tjian, 1988; Mermod et al., 1989). Identified transcription factors exist, however, for which DNA-binding and transcriptional activity do not

correspond to any recognized protein structure (Williams et al., 1988; Norman et al., 1988).

A factor of central importance in transcriptional regulation is the TATA box-binding protein TFIID (Matsui et al., 1980; Segall et al., 1980; Reinberg et al., 1987; Nakajima et al., 1988; Van Dyke et al., 1989). The interaction of TFIID with the TATA element is an essential, early event in the formation of a transcriptionally competent pol II complex. *In vitro* studies of transcriptional initiation have resulted in the following model of this process: TFIID bound at the TATA box initiates a cascade of assembly of general transcription factors TFIIA, -B, -E, -F, and pol II, forming a stable preinitiation complex (Hawley and Roeder, 1985; Fire et al., 1984; Sawadogo and Roeder, 1985; Reinberg et al., 1987; Reinberg and Roeder, 1987; Van Dyke et al., 1988, 1989; Buratowski et al., 1989). The importance of TFIID action in transcriptional initiation is supported by several observations. The assembly of nucleosomes onto template DNA resulted in inhibition of transcription *in vitro*. However, preincubation of template with TFIID prior to nucleosome formation was sufficient to overcome repression. Furthermore, addition of the enhancer-binding factor USF and TFIID at the time of nucleosome formation resulted in much less repression (Workman and Roeder, 1987).

This suggests that the transcriptional regulatory action of enhancer-binding factors is mediated through TFIID, either through modulating its affinity for the TATA box or altering its interactions with other components of the transcriptional apparatus (Fig 1.1). This proposition is supported by *in vitro* analyses of TFIID interaction with its binding site, and the alteration of this interaction by transcriptional activating proteins. The mammalian activator, ATF, and TFIID interact cooperatively when simultaneously bound to a promoter, resulting in a qualitative alteration of TFIID-DNA interaction, as shown by DNAse I footprinting (Horikoshi et al., 1988b). These interactions facilitate formation of a transcriptionally competent preinitiation complex (Hai et al., 1988). In addition, a yeast activator, GAL4, had similar effects on mammalian TFIID binding (Horikoshi et al., 1988a).

The validity of this description of transcriptional regulation is supported by the observation that yeast transcription factors function in mammalian cells, as well as the converse. This fact has been demonstrated for a number of activators (Kakidani and Ptashne, 1988; Webster et al., 1988; Fischer et al., 1988; Struhl, 1988; Schena and Yamamoto, 1988), and more recently for the general factors TFIID and TFIIA (Buratowski et al., 1988; Horikoshi et al., 1989; Hahn et al., 1989).

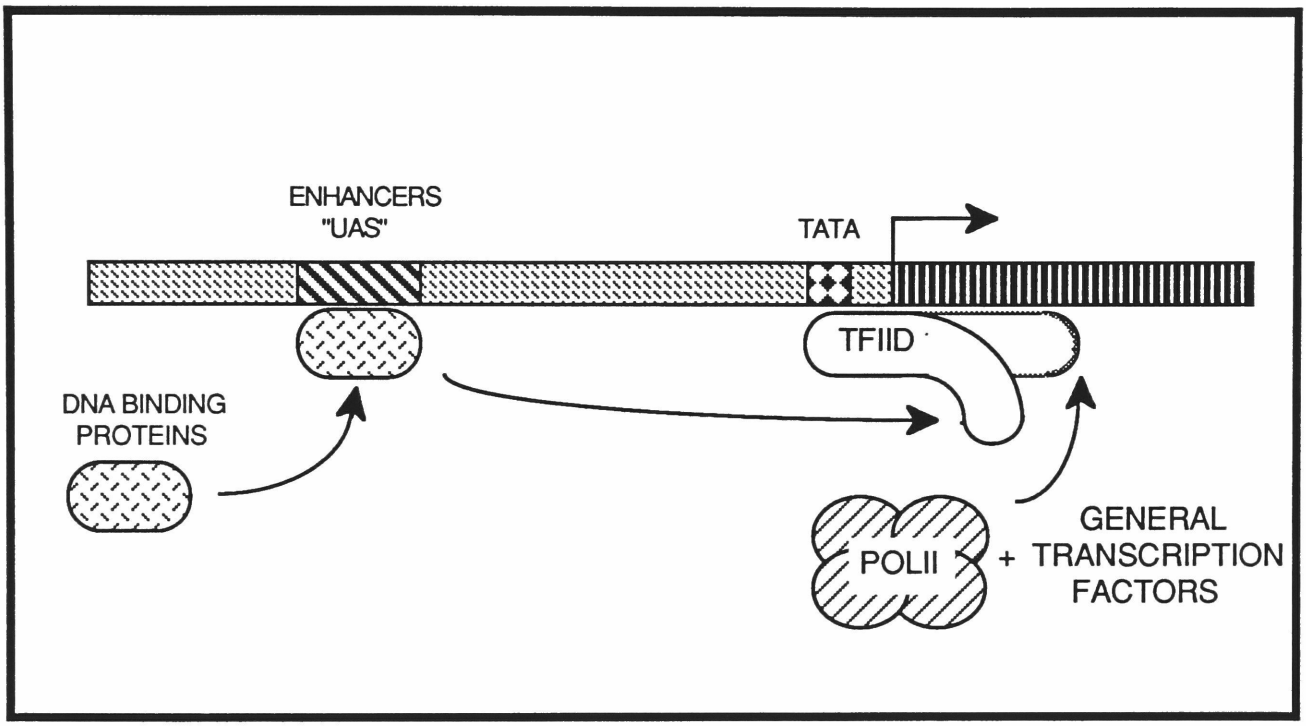


Figure 1.1 A schematic representation of eukaryotic mRNA transcriptional regulation. A regulatory protein specifically binds to a distal DNA element (enhancer) and interacts with TFIID at the proximal TATA element. The action of the upstream factor induces an alteration of TFIID-TATA box interaction which facilitates assembly of a transcriptional complex, including a number of general transcription factors and RNA polymerase II. Once assembled, the preinitiation complex is competent for transcriptional initiation.

Posttranslational regulation of transcription factors

In the case of "primary" transcriptional response to extracellular signals, pre-existing cellular components are targets for signal-dependent modification, ultimately resulting in the activation of latent transcription factors. While this scenario is assumed to account for many cases of gene induction by extracellular stimuli, in only a very few number of cases has any mechanistic detail become available. It is now widely believed that cell surface receptors mediate intracellular responses, including transcriptional changes, through the generation of second messenger molecules, including cAMP and Ca^{2+}, which subsequently activate cellular protein kinases and phosphatases (for reviews: Pastan, 1972; Rozengurt, 1986, 1989; Rozengurt et al., 1988; Hunter et al., 1988; Storms and Bose, 1989; Waterfield, 1989; Williams, 1989). However, the general role of these messengers in regulating transcription by way of posttranslational modulation of transcription factors remains an open question.

The regulatory role of changes in transcription factor phosphorylation has been most extensively explored for the activator CREB. This regulator activates transcription of the somatostatin gene, as well as others, in response to an intracellular rise in cAMP concentration (Montminy and Bilezikjian,

1987). The activation of CREB is thought to be mediated by cAMP-dependent protein kinases, and indeed this factor is a substrate for this kinase *in vitro*. This proposition is supported by *in vitro* studies in which CREB DNA-binding and transcriptional activity is dependent upon its phosphorylation state (Yamamoto et al., 1988a; Hoeffler et al., 1988; Gonzalez et al., 1989). In addition, *in vivo* experiments have shown that stimulation of a reporter construct by CREB is dependent on co-transfection with the catalytic subunit of cAMP-dependent protein kinase (Riabowol et al., 1988a, 1988b). An additional case of phosphorylation increases correlating with transcription factor activity is the hyperthermia responsive activator HSTF (Parker and Topol, 1984; Zimarino and Wu, 1987; Wu et al., 1987; Sorger et al., 1987; Sorger and Pelham, 1988; Rougvie and Lis, 1988).

Recently, a role for dephosphorylation in factor activation has been proposed for the activator AP-1, a heterodimer of c-Jun and c-Fos. The proposed model suggests that stimulation of cells results in activation of a cellular phosphatase which dephosphorylates c-Jun resulting in increased AP1 DNA-binding and transcriptional activity (Berk and Schmidt, 1990; Karin and Hunter, unpublished).

Heterotypic interactions and transcriptional regulation

Signal-dependent and developmental control of transcriptional activity often relies on the association or dissociation of distinct DNA-binding proteins, or non-DNA-binding proteins in some cases. The formation of heteromeric complexes has been shown to enhance or reduce DNA-binding affinity, to generate novel DNA-binding specificity, or to modulate transcriptional activity of DNA bound proteins.

A model for glucocorticoid receptor activation has been developed involving relocalization of the liganded receptor to the nucleus (for reviews: Evans, 1988; Beato, 1989). Unliganded receptor, in association with hsp90, is present in the cytoplasm, perhaps anchored to the cytoskeleton (Catelli et al., 1985; Sanchez et al., 1987, 1988). Ligand binding induces hsp90 dissociation, revealing nuclear translocation signals, and results in subsequent binding of the receptor to specific nuclear regulatory sites and transcriptional activation or repression of target genes (Pratt et al., 1989; Picard and Yamamoto, 1987; Picard et al., 1988; Yamamoto et al., 1988b).

The transcription factor NF-kB is active in nuclei of activated lymphocytes and can be post-translationally activated by a number of stimuli in other cell types (Sen and Baltimore, 1986a, 1986b; Lenardo et al., 1987;

Visvanathan and Goodbourn, 1989). Induction of NF-kB involves disassociation of an inhibitory cytoplasmic protein I-κB, allowing nuclear translocation of the DNA-binding activity (Baeuerle and Baltimore, 1988a, 1988b, 1989; Baeuerle et al., 1988; for review: Lenardo and Baltimore, 1989; Hunt, 1989). This inhibitory protein appears to be a substrate for protein kinases, consistent with the nature of the stimuli that induce activation of NF-kB (Shirakawa and Mizel, 1989). Therefore, phosphorylation of its cytoplasmic anchor probably facilitates relocalization of this transcriptional activator to the nucleus.

Determination of dorsoventral polarity in drosophila embryogenesis provides a striking example of regulated nuclear localization in the control of gene activity. *Dorsal* is a putative transcriptional regulator, highly similar to mammalian *c-rel*. While homogeneously distributed in the early embryo, 90 min after fertilization *dorsal* protein is selectively transported to nuclei in ventral, but not dorsal regions, producing a dorsoventral gradient of nuclear *dorsal* activity. The functional significance of this pattern is supported by analysis of mutations which alter dorsoventral polarity. Analysis of a group of 12 genes involved in establishing dorsoventral polarity suggests that 11 genes are requires to transmit positional information to the *dorsal*

morphogen, while a single gene, *cactus*, is probably the cytoplasmic anchor of *dorsal* protein (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Hunt, 1989). In this system, as opposed to the NF-kB system, it appears that the transcription factor, rather than the cytoplasmic anchor, is the target for modification.

The Fos and Jun proto-oncogene products form a stable heterodimeric complex with DNA-binding characteristics distinct from the separate proteins (Rauscher et al., 1988b; Bohmann et al., 1987). Jun homodimers bind to a defined site recognized by a group of related proteins (AP1 family and GCN4), while Fos does not form stable homodimers, nor does it exhibit detectable affinity for DNA, despite containing domains highly similar to those in Jun responsible for dimerization and DNA-binding (Landschulz et al., 1988; Kouzarides and Ziff, 1988; Turner and Tjian, 1989; Rauscher et al., 1988a). In contrast to the isolated proteins, formation of stable Fos:Jun heterodimers significantly increased the affinity of Jun for DNA (Rauscher et al., 1988a; Nakabeppu et al., 1988; Halazonetis et al., 1988), leading to specific changes in gene expression (Chiu et al., 1988). The existence of multiple *c-fos* and *c-jun* related genes and the inducibility of a subset of these genes by a variety of stimuli points to the complex regulatory potential of heteromeric complexes

derived from these gene families (Curran and Franza, 1988; Cohen et al., 1989; Chiu et al., 1989; Schutte et al., 1989; Lamph et al., 1988).

Control of mating type in yeast relies upon multiple heteromeric complexes in which common proteins exhibit distinct DNA-binding affinity and specificity (for review: Herskowitz, 1989). In this system four DNA-binding proteins act in different combinations to regulate three classes of genes. In **a** cells isolated MATα1 and PRTF have very low affinity for their respective binding sites, but through heterotypic interactions bind with high affinity to adjacent sites, thus acting in concert to positively regulate α-specific genes (Bender and Sprague, 1987). In diploid yeast the DNA-binding specificity of MATα2 is altered by collaboration with MAT**a**1 resulting in repression of haploid-specific genes (Goutte and Johnson, 1988; Miller et al., 1985a). In α cells the cooperative interaction of MATα2 with PRTF forming a third heteromeric regulatory complex, resulting in repression of **a**-specific genes (Keleher et al., 1988; Sauer et al., 1988).

An additional example of heteromeric regulators of gene activity is the *HAP2/3/4* complex. This stable ternary complex activates transcription of the yeast *CYC1* gene in response to nonfermentable carbon sources. The target for the heterotrimeric complex is the upstream site UAS2 which contains a

CCAAT box sequence (Forsburg and Guarente, 1989). Interestingly, certain mammalian CCAAT box-binding factors appear to be heteromeric, and one, CP1, consists of heterologous subunits that are functionally homologous to HAP2 and HAP3 (Chodosh et al., 1988a, 1988b). The existence of a mammalian homolog of HAP4 remains to be shown.

In certain cases viral modulation of transcription is mediated by viral proteins which activate or repress gene activity despite having no intrinsic DNA-binding activity (Nevins, 1987). These viral activators, such as the herpes simplex virus protein VP16, directly interact with cellular transcription factors, forming heteromeric complexes with novel DNA-binding and/or transcriptional activity (Kovesdi et al., 1987; Simon et al., 1988; Hoeffler and Roeder, 1985; Lin and Green, 1988; Hardy et al., 1989; Sadowski et al., 1988; Triezenberg et al., 1988). For example, VP16 forms a complex with the ubiquitous factor OCT1 which, by virtue of the acidic activating domain of VP16, becomes a potent activator of transcription. Interestingly, OCT2, a lymphoid cell-specific activator, binds the same site as OCT1, but neither activates the same set of genes nor interacts with VP16 (Gerster and Roeder, 1988), pointing to the importance of heteromeric interactions in regulation by a site bound by multiple factors.

Signal-dependent or developmental modification of transcription factors enhances cellular regulatory possibilities. The combinatorial action of multiple DNA-binding proteins provides an economical means for precise regulation of specific groups of target genes. Therefore, differing specificities of DNA sequence recognition and protein:protein interactions allow formation of multiple heteromers with distinct regulatory functions.

Signal transduction in response to cell surface ligands

The following section addresses current ideas about cellular mechanisms for signal transduction resulting in transcriptional modulation. In particular, the ability of a limited group of well-studied intracellular messengers to transmit specific ligand-transmembrane receptor interactions to the nucleus, resulting in activation of a precise set of genes, is considered.

Polypeptide ligands bind to cell surface receptors with high specificity, and in general there is no crosstalk between cell surface signals at the level of ligand binding. This cell surface event is transmitted to the cell interior and elicits precise physiological responses, often involving transcriptional changes, which rely on sequence-specific DNA-binding proteins (Murdoch et al., 1982; Murdoch et al., 1983; Greenberg et al., 1985; Greenberg and Ziff, 1984;

Siegfried and Ziff, 1989; Larner et al., 1984, 1986; Friedman et al., 1984; Almendral et al., 1988; Cochran et al., 1983; Muller et al., 1984; Clayton et al., 1985). Therefore, specificity in stimulating nuclear events is maintained by the components of the signal transduction apparatus which link external events to trancriptional control. Since different ligand-receptor pairs elicit transcriptional changes for distinct sets of genes, often in the same cell type, the signalling apparatus for a given receptor must be distinct as well. While signalling systems may share common strategies, or even common components, unique specificity conferring elements must exist in a signalling pathway, thereby maintaining appropriate cellular response.

Transmembrane receptors

As the primary source of intracellular signals, the structural and enzymatic characteristics of transmembrane receptors may shed light on the nature of intracellular messengers (for review: Waterfield, 1989). A number of the known cell surface receptors can be categorized on the basis of sequence similarity. The most extensively studied group of receptors are those having intrinsic protein tyrosine kinase activity. This class of receptor includes the insulin, NGF, EGF, PDGF, and CSF-1 receptors as well as others (Ullrich et al., 1985; Levi et al., 1988; Hempstead et al., 1989; Chen et al., 1987; Todderud and

Carpenter, 1989; Williams, 1989; Heldin and Westermark, 1989; Rettenmier et al., 1988). Clearly, addition of phosphate to tyrosine is an important component of intracellular signalling by these receptors (Cohen, 1982). This idea is supported by the growth stimulatory action of oncogenic forms of these receptors which have high, constitutive tyrosine kinase activity. Two proto-oncogenes, *c-erbB* and *c-fms*, encode growth factor receptors. The EGF receptor activity of the *v-erbB* oncogene product is constitutively activated without the need for growth factor due to a truncation at the amino terminus, deleting the EGF binding domain. The *c-fms* proto-oncogene encodes the CSF-1 receptor. Mutations in the *c-fms* gene can constitutively activate the kinase to provide growth-stimulatory signals in the absence of the ligand. Furthermore, oncogenic forms of transmembrane receptor ligands have been identified. The *v-sis* protein, which is nearly identical to PDGF, appears to cause cell transformation through its interactions with the PDGF receptor, thus activating the intrinsic tyrosine kinase activity (for reviews: Deuel, 1987; Storms and Bose, 1989; Druker et al., 1989; Sinkovics, 1988). An important question is yet to be answered for this class of transmembrane receptors: What are the physiologically important substrates of these growth factor receptor tyrosine kinases which mediate cellular response?

Consistent with the importance of phosphorylation state in signalling, recently a transmembrane receptor having intrinsic protein tyrosine phosphatase activity has been identified (Charbonneau et al., 1989; Cool et al., 1989). The CD45 receptor is present on T-cells and is a ligand-stimulated tyrosine phosphatase.

A second class of transmembrane receptors stimulate intracellular signalling through coupled G-proteins. This diverse group of receptors includes the beta- and alpha-adrenergic and muscarinic receptors, the retinal photoreceptor rhodopsin, as well as the mating factor receptors of yeast (Deuel, 1987; Weiss et al., 1988; Benovic et al., 1989; Herskowitz and Marsh, 1987; Stryer, 1988). G-proteins are a highly conserved family of membrane-associated proteins composed of alpha, beta, and gamma subunits. The alpha subunit, which is unique for each G-protein, binds GDP or GTP. Interaction with ligand-bound receptor stimulates the exchange of GDP for GTP binding to the alpha subunit of a specific G-protein, resulting in dissociation from the beta-gamma subunits. G-alpha-GTP regulates appropriate effector enzymes such as adenylyl cyclase, cyclic GMP phosphodiesterase, or phospholipases (O'Dowd et al., 1989; Bourne, 1989; Gilman, 1984; Casey et al., 1988; Bockaert et al., 1987; Burch, 1989; Firtel et al.,

1989; Neer and Clapham, 1988).

A well described G-protein-coupled receptor system is that responsible for cell type differentiation and growth arrest in yeast. The peptide ligand α-factor binds to the STE2 gene product, a member of the beta-adrenergic receptor family, resulting in dissociation of the alpha subunit from a G-protein complex, subsequently triggering cellular responses by way of unknown intracellular messengers. Protein kinases are apparently involved in α-factor signalling since two kinase-related genes, STE7 and STE11, are required for the response, and the DNA-binding protein STE12 is a probable nuclear target mediating transcriptional activation. Activation of STE12 by phosphorylation is suspected, but not yet proven, and therefore the precise nature of intracellular messengers in this system is yet to be defined (Herskowitz, 1989; McLeod, 1989).

The proposed role of G-proteins in intracellular signalling is supported by the existence of a related oncogenic GTP-binding protein, *p21ras* (for reviews: Gautam et al., 1989; Gibbs and Marshall, 1989; Marshall, 1988, 1989). The active form of *p21ras* is the GTP bound state and oncogenic mutations result in the protein being constitutively in the GTP bound active state. To transduce a signal for proliferation and transformation the active GTP form

of *p21ras* must interact with one or more cellular targets. Genetic experiments suggest that one potential effector molecule is the GTPase activating protein, GAP (McCormick, 1989). However, the mechanism by which *p21ras* interaction with GAP results in proliferation and transformation remains to be elucidated.

A growing group of transmembrane receptors fail to fall into the described classes. These receptors, such as the IL-1, IL-2, IL-6, IFNγ, and IFNα receptors, do not contain canonical structures, and therefore no insight as to their mechanism of signalling is apparent. Interestingly, it appears that the functional receptors for these ligands are composed of at least two distinct polypeptides, perhaps suggesting the importance of protein-protein interactions, rather than enzymatic action, as a primary signal (Dinarello et al., 1989; Sims et al., 1988; Kroggel et al., 1988; Waldmann, 1989; Smith, 1989; Taga et al., 1989; Aguet et al., 1988; Hemmi et al., 1989; Uze et al., 1990).

Intracellular messengers

The transmission of signals generated at the plasma membrane to cytoplasmic and nuclear targets requires the production of soluble intracellular messengers. Studies directed at identifying such messengers have implicated a group of small molecule second messengers whose levels

fluctuate in response to extracellular signals. Modulation of the intracellular concentration of these second messengers, such as cAMP, intracellular Ca^{2+}, DAG, and IP_3, has been proposed to account for the stimulation of a wide variety of physiological changes (for reviews: Pastan, 1972; Berridge, 1985; Berridge and Irvine, 1989; Rozengurt, 1989; Pilkis et al., 1988; Krebs, 1989).

It has been observed that growth factors induce transient changes in the intracellular Ca^{2+} concentration. Ca^{2+} flux is not dependent on extracellular Ca^{2+} and therefore represents a release of intracellular stores. The ligand-induced mobilization of calcium appears to result from a cascade of reactions that involve a transmembrane receptor, a G-protein and a phospholipase C which releases inositol 1,4,5-trisphosphate (IP_3) from the plasma membrane; the IP_3 acts to release calcium from smooth endoplasmic reticulum (Hughes and Putney, 1988; Joseph and Williamson, 1989; Exton, 1990). For example, binding of EGF to its receptor activates a number of immediate biochemical processes, such as increased kinase activity and alterations of intracellular free calcium (Todderud and Carpenter, 1989; Pandiella et al., 1989; Gill et al., 1987). Similarly, in PDGF-stimulated cells, ligand binding results in the increased turnover of phosphatidylinositols and the subsequent release of IP_3 (Habenicht et al., 1981; Putney et al., 1989). This

also coincides with activation of a phosphotidylinositol kinase activity (Coughlin et al., 1989). Likewise, the receptor for CSF-1 induces a guanine nucleotide-dependent activation of phospholipase C (Rettenmier et al., 1987; Jackowski et al., 1986). An additional effect of increased Ca^{2+} is the formation of Ca^{2+}-calmodulin which activates cAMP phosphodiesterase, thus impinging on a second intracellular messenger (Millar and Rozengurt, 1988).

Concomitant with receptor-mediated hydrolysis of inositol phospholipids is the production of DAG, an activator of the serine/threonine protein kinase C, which may play an important role in relaying information across the cell membrane to regulate many intracellular processes (Bell, 1986; Rozengurt, 1989). Since this enzyme also serves as a major receptor for phorbol esters, a class of tumor promoters, it may represents a major modulator of cell physiology (Nishizuka, 1989; Kikkawa and Nishizuka, 1986).

A proposed second strategy for intracellular signalling relies on cyclic nucleotides. As described above, occupied receptors are coupled to modulators of cyclic nucleotide levels by G-proteins. The G-alpha subunit activates adenylate cyclase in response to certain ligands resulting in higher intracellular cAMP levels (Pilkis et al., 1988). Among the cellular effectors of

cAMP is cAMP-dependent protein kinase which relays signals in the form of serine/threonine phosphorylation (Krebs, 1989). In particular, cAMP-dependent protein kinase is implicated in the induction of specific gene transcription, including the somatostatin, growth hormone, and c-fos genes, probably through the activation of the transcriptional activator CREB, as described above (Roesler et al., 1988; Waterman et al., 1985; Karin, 1989; Mellon et al., 1989). On the other hand, induction of prolactin gene expression through cAMP probably requires calcium-dependent signalling (Waterman et al., 1985). Therefore, it appears that the cAMP- and Ca^{2+}-dependent signalling pathways have the capacity to interact and modulate their respective effects.

Specificity in ligand-dependent transcriptional regulation

Do current ideas about intracellular signalling account for the high degree of selectivity in ligand-dependent transcriptional regulation? While a small number of genes appear to be responsive to a wide variety of stimuli (i.e., c-fos gene), the ability of a ligand to elicit a specific cellular response demands the regulation of a distinct set of genes. As mentioned above, the initial and terminal components of signalling to the nucleus involve highly specific ligand-receptor and protein-DNA interactions, and therefore intermediate steps must maintain specificity. An important question is: Can

global changes in cAMP, Ca^{2+}, IP_3, or DAG levels account for the high degree of specificity in gene regulation by numerous ligands?

In the case of cAMP stimulation of CREB there is a compelling correlation between the intracellular concentration of cAMP and activation of a set of genes by CREB (Yamamoto et al., 1988a). On the other hand, an interesting example of the noncorrelation between ligand-stimulated transcription and second messenger modulation is apparent in studies of EGF induction of c-fos transcription. Receptor bound EGF induces Ca^{2+} mobilization, enhanced kinase activity, and transcriptional induction (Pandiella et al., 1989; Todderud and Carpenter, 1989). In A431 cells, EGF stimulates rapid increases in the level of c-fos transcription and a concomitant increase in a specific enhancer-binding factor. Significantly, transcription of c-fos is markedly induced by the tumor promoter TPA and the calcium ionophore A23187, yet neither induced an increased level of the enhancer-binding activity. Therefore, these agents appear to activate c-fos transcription via a mechanism distinct from that used by EGF (Prywes and Roeder, 1986). These observations suggest that artificial perturbation of the described second messengers can result in transcriptional modulation, but these messengers may not, in certain cases, represent the relevant

intracellular messengers for transcriptional regulation by polypeptide ligands.

Intracellular signalling through the "classical" second messenger pathways involves activation of potent cellular activities, including a variety of kinases with a broad spectrum of substrates (at least *in vitro*), and therefore the maintenance of specificity in physiological responses may be difficult. In fact, artificial stimulation of these classical messenger pathways brings about dramatic changes in a variety of physiological states in many cell types, under various conditions. Therefore, in order to preserve a precise integration of cell surface events and nuclear responses, as yet undefined mechanisms must exist which bestow specificity on global changes in cAMP, Ca^{2+}, IP_3, and DAG levels. Alternatively, novel signalling pathways may exist independent of cAMP, Ca^{2+}, IP_3, and DAG which are distinct for particular ligands.

In an attempt to define novel strategies for signal transduction it is useful to identify a ligand stimulated system which is highly specific for one, or a limited number of stimuli, thus facilitating a description of the precise signalling apparatus. Our group has chosen the interferon system to address questions of signal transduction in the regulation of transcription.

The type I interferon system: an overview

Type I interferons

The interferons (IFNs) are classically defined as cellular products, secreted in response to viral infection, capable of inhibiting viral infection in a species-specific manner, and have been described in all vertebrates except amphibians (Lindenmann et al., 1957; Isaacs, 1961; Isaacs and Lindenmann, 1957; for review: De Maeyer and De Maeyer-Guignard, 1988). IFN induces an antiviral state in target cells which have not been exposed to virus, and therefore this agent directly alters cell physiology. The type I IFNs are a large family of closely related 166 amino acid proteins (after removal of an N-terminal signal sequence), which consists of two types. In humans, IFNα is represented by at least 24 distinct forms, with ~90% amino acid similarity among them, while IFNβ is represented by a single form which has ~30% similarity with the IFNα polypeptides (Weissmann and Weber, 1986). Gene conversion is thought to account for the high degree of conservation observed for the IFNα types (Todokoro et al., 1984).

Interferon induction

IFNs are synthesized, and secreted in response to viral infection of all nucleated cells (De Maeyer and De Maeyer-Guignard, 1988). The viral

induction of IFN is largely regulated at the transcriptional level, and it appears that formation of intracellular double-stranded RNA during viral infection initiates the response since treatment of cells with synthetic double-stranded RNA stimulates type I IFN production. Studies have revealed no significant qualitative differences in the inducibility of the different type I IFNs and all cell types studied are capable of inducing all type I IFNs. Although the classical inducers are animal viruses, additional agents stimulate IFN production. Among these are a variety of microorganisms, including bacteria, protozoa and bacterially-derived endotoxin, as well as certain growth factors and cytokines, such as CSF-1 and PDGF (Moore et al., 1984; Zullo et al., 1985). While important *cis*-regulatory elements of the IFN genes and a number of *trans*-acting factors which bind them have been identified, as yet, the mechanism by which virus or dsRNA stimulates IFN gene transcription is not fully understood (for review: Taniguchi, 1988).

Interferon receptor

IFNs elicit cellular response by specific interaction with a transmembrane IFN receptor (Aguet, 1980). All IFNα types, as well as IFNβ bind to a single class of type I IFN receptor, which is expressed on all cell types (Merlin et al., 1985). The receptor-ligand interaction is of high affinity (K_d

~10^{-10}M) and cells generally express relatively low numbers (1,000-5,000 per cell) (De Maeyer and De Maeyer-Guignard, 1988). The ligand-receptor complex is internalized and apparently degraded, but it seems that this process is not fundamental to physiological response for the following reasons. First, the rate of receptor internalization is constant for occupied and unoccupied receptor (Zoon et al., 1986b). Second, IFN covalently coupling to Sepharose beads, thus blocking ligand-receptor internalization, is capable of inducing the antiviral state in cells (Ankel et al., 1973). In addition, the interaction of IFN with its cognate receptor is highly species-specific, and cross-species induction results in minimal response (Rehberg et al., 1982). Recently, a cDNA for the human type I IFN receptor was isolated by expression in mouse cells (Uze et al., 1990). The cDNA encodes a protein with a single membrane spanning domain and no similarity to known proteins. However, when expressed in mouse cells the protein bound human IFN with 100-fold lower affinity than receptor present on human cells. In addition, mouse transfectants exhibit a poor sensitivity to human IFN, with only a 10-fold increase in specific activity as compared to nontransfected parental lines. Therefore, it seems likely that the isolated cDNA encodes a polypeptide which interacts with additional species-specific proteins which mediate complete responsiveness to type I

IFNs. A multisubunit structure for functional transmembrane receptors has been found for other cell surface receptors (i.e., IL-6 and NGF receptors: Taga et al., 1989; Hempstead et al., 1989).

Antiviral response

The antiviral activity of IFN operates at a number of levels, including inhibition of viral attachment, penetration, uncoating, and assembly, as well as inhibition of the accumulation of viral products by inhibiting transcription and translation. Translational control has emerged as a central regulatory point in the antiviral activity of IFN. Two described cellular systems appear to play an important role IFN-dependent translational control, the 2',5'-oligoandenylate synthetase (OAS) pathway and the dsRNA-dependent protein kinase. IFN induces a 10-100 fold increase in the intracellular concentration OAS protein resulting in the accumulation of 2'-5' linked adenylate oligomers which, in turn, reversibly activate a latent cellular endoribonuclease (RNase L). Once active, RNase L cleaves viral, cellular, and ribosomal RNAs, thus blocking production of viral gene products necessary for continued infection. IFN-dependent induction of the dsRNA-dependent protein kinase is due to de novo synthesis of the enzyme and subsequent dsRNA-dependent activation through autophosphorylation. In vitro this

serine/threonine kinase phosphorylates the α subunit of eIF2, a eukaryotic translational initiation factor. In the phosphorylated state, eIF2 is irreversibly bound to a GTP recycling factor and therefore translation is inhibited (De Maeyer and De Maeyer-Guignard, 1988).

An additional IFN-induced cellular activity which mediates the antiviral response to the mouse Mx protein. This protein renders mice specifically resistant to the influenza virus, while mice carrying the Mx- allele do not induce the Mx protein in response to IFN and are therefore sensitive to influenza infection (Horisberger et al., 1983).

Antiproliferative response

In addition to the antiviral effects, a second major response to IFN treatment is the inhibition of cellular replication in many cell types. Treatment of human diploid fibroblasts with IFN results in prolongation of all phases of the cell cycle and can ultimately lead to a 300% increase in doubling time. This effect is not dependent on significant decreases in the rate of DNA and protein synthesis, and therefore the treated cells enlarge (Pfeffer et al., 1979). The antiproliferative activity of IFN may, in part, act through antagonism of endogenously produced, or externally added growth factors, such as PDGF, EGF, and FGF (Sreevalsan et al., 1979). While the mechanisms

by which IFN blocks cellular replication is, in general, unknown, IFN-induced downregulation of EGF and CSF-1 receptors has been observed (Zoon et al., 1986a; Chen, 1986).

Transcriptional response to type I IFN

The cellular response to IFN relies in large part on the de novo synthesis of a large group of IFN-induced proteins, including those mentioned above (i.e., OAS, dsRNA-dependent protein kinase, and Mx). Study of the regulation of genes encoding the IFN-induced proteins has identified a growing set of genes transcriptionally activated by type I IFN. Among this group of interferon stimulated genes (ISGs) are several which have been analyzed in depth: ISG54 and ISG15 (so designated due to their coding capacity in kD), Mx (or IFI78 in human), OAS, 6-16, as well as the genes of the major histocompatibility group (Larner et al., 1984, 1986; Friedman et al., 1984; Levy et al., 1986; Staeheli et al., 1986; Israel et al., 1986; Reich et al., 1987; Benech et al., 1987; Sugita et al., 1987; Cohen et al., 1988; Porter et al., 1988; Abei et al., 1989). The characterization of the transcriptional response to IFNα has revealed certain distinctive features of ISG induction: 1) induction is very rapid, being detectable within 5 min of treatment, and therefore the response appears to be limited by the rate of receptor occupancy; 2) induction

is of large magnitude, with 50-100 fold increases above very low basal levels for most ISGs (i.e., ISG54 and ISG15); 3) induction is transient and transcription rate declines to basal levels within 8-15 hr of treatment for most cell types; 4) transcriptional activation occurs normally in the presence of protein synthesis inhibitors; 5) the downregulation, or return to basal level expression, requires ongoing protein synthesis, and the transcription rate will remain high in the presence of protein synthesis inhibitors (Fig 1.2); 6) cells which complete the induction cycle become refractory to further induction, a state which requires ongoing protein synthesis; 7) induction requires the continuous presence of IFN and ligand withdrawal leads to an immediate return to basal level expression (Larner et al., 1984, 1986; Friedman et al., 1984; Hannigan and Williams, 1986; Lew et al., 1989).

These characteristics of ISG induction reveal several features of transcription regulation by IFNα. First, the rapidity and protein synthesis-independence of ISG activation demands the existence of latent cellular proteins which are primary targets for IFNα-receptor initiated intracellular signalling. The latent cellular factors are post-translationally modified, and subsequently mediate transcriptional induction of ISGs. Second, the expression of the ISGs is tightly regulated and within hours of

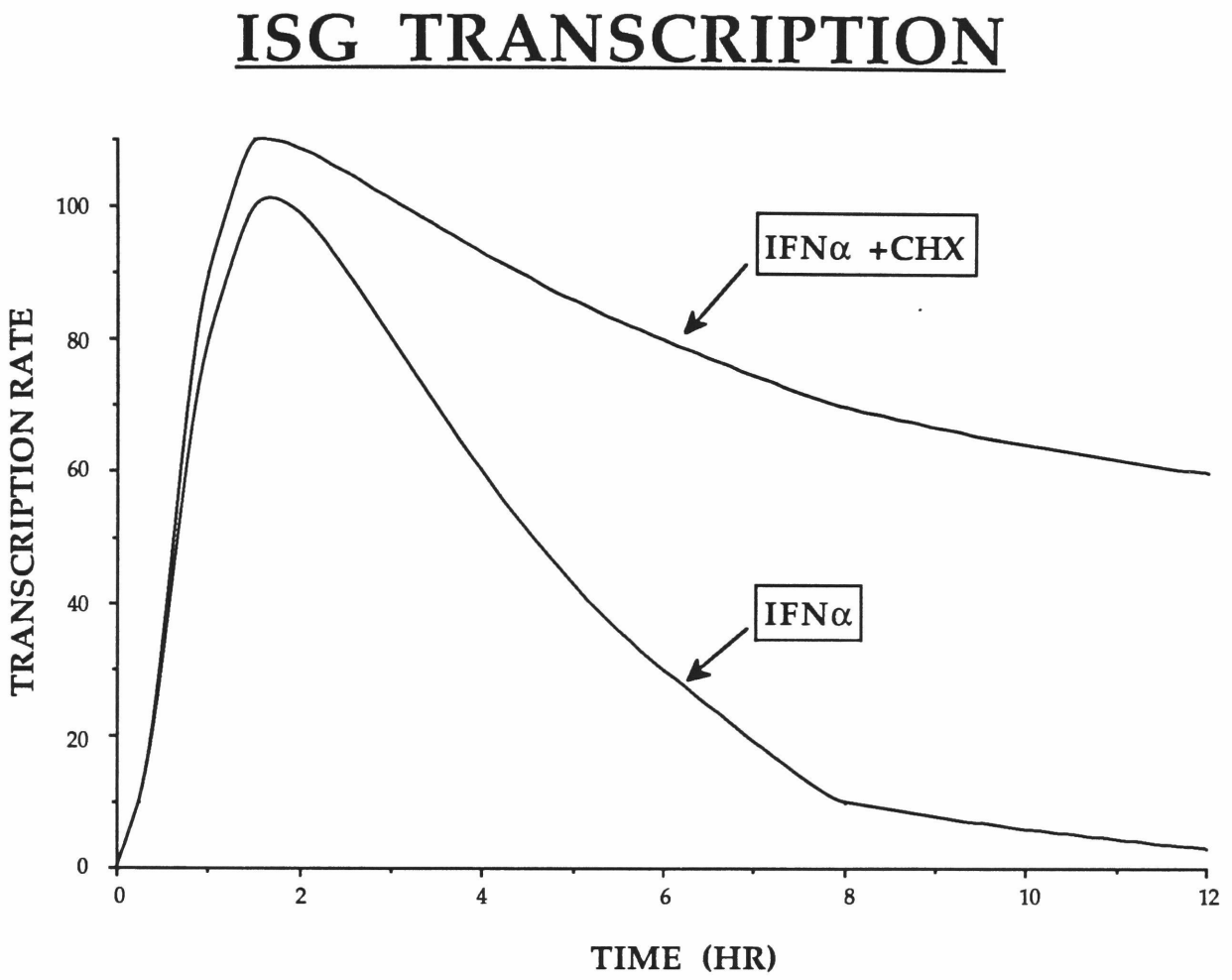


Figure 1.2 Time course of ISG induction by IFNα in human cell lines. Transcriptional induction in rapid and transient, first detectable within 5 min, peaks before 2 hr, and returns to basal levels within 8-15 hours in most cell types. Induction in the presence of a protein synthesis inhibitor (cycloheximide) has no significant effect on the activation phase of induction, but significantly delays the return to basal level transcription. These features of ISG induction imply the existence of latent cellular proteins which are post-translationally activated in response to IFNα treatment and mediate transcriptional activation. The down regulation, on the other hand, must rely on the synthesis of new cellular proteins. These characteristics of ISG induction are maintained in the majority of cell types analyzed.

induction new cellular proteins are produced which facilitate transcriptional downregulation, and maintain the ISGs in an inactive refractory state. Third, the intracellular signal produced in response to IFNα is unstable and therefore maintenance of transcriptional response relies upon continual signal generation through ligand-receptor interactions. These features of ISG induction are maintained in most cell types analyzed.

Signal transduction in the type I interferon system

The nature of intracellular signalling in response to IFNα-receptor interaction remains elusive. As mentioned above, the recent isolation of an IFNα receptor cDNA encoding a noncanonical transmembrane protein did not shed light on the character of intracellular messengers which initiate cellular response (Uze et al., 1990). The possible role of the "classical" second messengers in the response to IFN, including cAMP, DAG, and Ca^{2+}, has been explored in a number of cases and the observed changes in the concentration of these messengers are certainly involved in the cellular response to IFN (Tamm et al., 1987; Nagata et al., 1984; Schneck et al., 1982; Yap et al., 1986a, 1986b). ISG induction, however, is apparently independent of fluctuations in these second messengers and therefore a distinct signalling pathway must exist that mediates IFN-dependent transcriptional regulation (Larner et al.,

1984; Lew et al., 1989; Yan et al., 1989). In these studies, the ability of a number of pharmacological agents, which perturb the intracellular concentration of second messengers, to modulate ISG induction was explored. None of the tested agents was capable of blocking ISG induction by IFN, nor were these able to induce ISGs in the absence of IFN (Table 1.1). The obvious conclusion from these experiments is that changes in the intracellular level of cAMP, DAG, and Ca^{2+}, or in the activity of a group of signalling proteins (kinases and G-proteins) is not sufficient to mediate ISG induction. Furthermore, no additional growth factor, cytokine, or hormone has been identified which directly induces ISG transcription. Therefore, ligand binding to the type I IFN receptor generates a highly specific intracellular messenger resulting in a very large transcriptional response of a distinct set of genes. The IFN system is therefore an attractive experimental system for addressing questions of signal transduction in the regulation of specific gene transcription.

Guide to the thesis

As mentioned above, the experimental approach adopted in this study is essentially "inside-out", first addressing nuclear events and proceeding to cytoplasmic components of the response pathway. Chapter 2 describes the

Perturbation of classical second messenger pathways does not modulate IFNα-stimulated gene transcription

Agent	Concentration	Target
EGTA	5 mM	
A23187	10 μM	Ca^{2+}
Ionomycin	100 nM	
DiC_8	150 μM	Protein kinase C
PMA	150 nM	
8-Br-cAMP	1 mM	
Dibutyryl-cAMP	1 mM	Protein kinase A
Forskolin	50 μM	
Cholera toxin	1 μg/ml	G-proteins
Pertusis toxin	0.1 μg/ml	
H7	50 μM	
H8	50 μM	Kinases
HA1004	50 μM	
2-aminopurine	10 mM	

Table 1.1 Agents which perturb the "classical" second messengers do not inhibit or mimic induction of ISGs by IFNα. These listed agents were assayed for the ability to block transcriptional induction of ISGs by IFNα, or for the ability to induce ISGs in the absence of IFNα treatment. In no case did these agents cause any significant perturbation of ISG induction at the transcriptional level. These results are compiled from the following: Lew et al., 1989; Larner et al., 1984, 1986; Yan et al., 1989.

identification of the interferon stimulated response element (ISRE), which is present in all type I IFN-inducible genes, and the preliminary characterization of three nuclear factors (one constitutive and two IFN-inducible) that specifically bind the ISRE. Chapter 3 describes the analysis of IFN-resistant variant cell lines, which provides support for the positive regulatory role of a single IFN-induced ISRE-binding factor (ISGF3) as proposed in Chapter 2. Chapter 4 concerns an extensive mutagenesis analysis of the ISRE and subsequent functional studies of the mutants in vitro and in vivo, as well as additional characterization of the IFN-induced ISRE-binding factors. Chapter 5 describes a series of studies that demonstrate the cytoplasmic activation of ISGF3 (the positive regulator of ISG induction), an event which involves the association of latent cytoplasmic proteins to form active heteromeric ISGF3. Chapter 6 describes additional characterization of the ISGF3 complex, indicating that possibly four distinct polypeptides assemble in an IFN-dependent manner that involves regulated nuclear translocation and allosteric modulation of DNA-binding affinity through protein-protein interactions. In Chapter 7 the thesis is summarized and future prospects for this study are presented.

Chapter 2

Identification of the Interferon Stimulated Response Element and interferon-induced factors which bind it specifically

Introduction

It is now well established that individual peptides bound to specific cell-surface receptors cause prompt changes in the transcription of specific sets of genes. Type I (Friedman et al., 1984; Larner et al., 1984; Kelly et al., 1985; Decker, 1989) and type II interferons (Luster et al., 1985; Decker, 1989), platelet-derived growth factor (PDGF; Greenberg and Ziff, 1984; Treisman, 1985; Lau and Nathans, 1987) and epidermal growth factor (EGF; Murdoch et al., 1982) have all been demonstrated by appropriate nuclear assays to increase transcription of groups of genes. In contrast to steroid hormones, which bind directly to transcription factor receptors, the basis by which polypeptide-generated signals from the cell surface reach the nucleus and cause transcriptional changes is not understood. The signal from the cell surface might either modify pre-existing transcription factors or cause the synthesis of new proteins that modulate transcription or are transcription factors themselves. Therefore, it is important to describe nuclear factors involved in transcriptional response to cell surface signals, and in this way initiate an attempt to understand the integration of cell surface signals and transcriptional regulation.

We have previously identified several genes whose transcription in

human cells is dependent upon treatment with type I (α- or β-) interferons (IFNs) (Larner et al., 1984, 1986). This group of genes is referred to as interferon-stimulated genes (ISGs). The genomic 5'-flanking regions of two such genes, the ISG54 and ISG15 genes (so named because they encode proteins of approximately 54 and 15 kD, respectively) have been identified and shown in recombinant plasmid and adenovirus vectors to be capable of directing IFN-stimulated transcription in transfected cells (Levy et al., 1986; Reich et al., 1987). The response of ISGs to IFN is not merely a positive one because the immediate transcriptional increase brought on by IFN treatment is soon followed by a regulated decrease in transcription (Larner et al., 1984, 1986). The positive phase of this cycle does not required protein synthesis, but the return to and the maintenance of a low transcription rate of ISGs does require ongoing protein synthesis. These observations suggest that transcriptional activation by IFN is effected through pre-existing cellular components but is limited by proteins newly formed during the IFN response.

In this chapter, we identify a sequence in the 5'-flanking region of the ISG54 gene that can direct IFN-stimulated transcription. This sequence contains an element, the interferon stimulated response element (ISRE), that

was found to match a sequence present in many ISG promoters. Mutations within the ISRE of the ISG54 gene eliminated transcriptional response to IFN.

In the nucleus of untreated cells, there is at least one factor that specifically recognized the ISRE. However, in IFN-treated cells, two other factors appear. The factor that is first induced is not affected by inhibition of protein synthesis and is a candidate for a positive-acting transcription factor that is modified in response to IFN. The later appearing factor does not appear in cycloheximide-treated cells and is, perhaps, a candidate for a negative-acting IFN-induced transcription factor. By competition experiments and through the use of the mutant ISRE sequence in DNA-binding assays, we conclude that the same oligonucleotide region is bound by both IFN-induced factors.

Results

ISG54 minimal inducible promoter

Recombinant plasmids containing either 3 kb or 800 bp of 5'-flanking sequence plus the first 288 bp of ISG54 fused to a reporter template (adenovirus E1B intron and 3' exon) were shown previously to be equally capable of directing IFN-stimulated transcription (Levy et al., 1986). To more

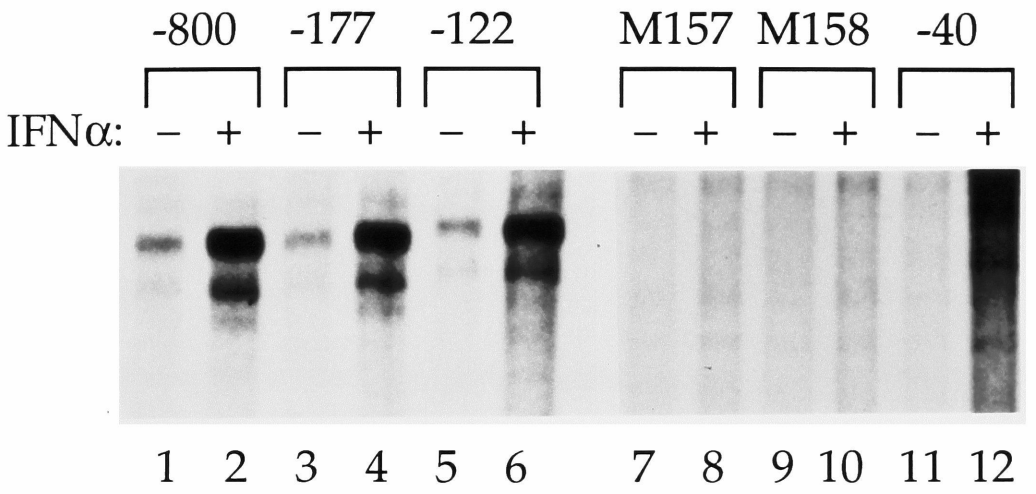
accurately localize the sequences necessary for IFN-stimulated transcriptional induction of ISG54, additional 5'-deletion mutants were prepared and used in transient transfection assays. The constructs used in Figure 2.1A (5' boundaries diagrammed in panel B) contained either 800, 177, 122, or 40 nucleotides of 5'-flanking sequence, only 55 nucleotides of the first exon, and none of the cellular intron. Expression of the E1B reporter sequence was scored by protection against nuclease digestion of a labeled antisense RNA (Fig 2.1A, lanes 1-6 and 11-12). These plasmids containing 122 nucleotides or more of 5'-flanking region gave equivalent levels of uninduced, basal expression and exhibited an equal increase in expression in response to IFN treatment (lanes 1-6). However, the construct containing only 40 nucleotides of 5'-flanking sequenced did not give a transcription signal (lanes 11-12); therefore we concentrated on sequences between -40 and -122 as important for ISG expression. This region contains a TATA box at -30 and a CCAAT box at -110.

Protein-binding site identified by DNAse I footprinting

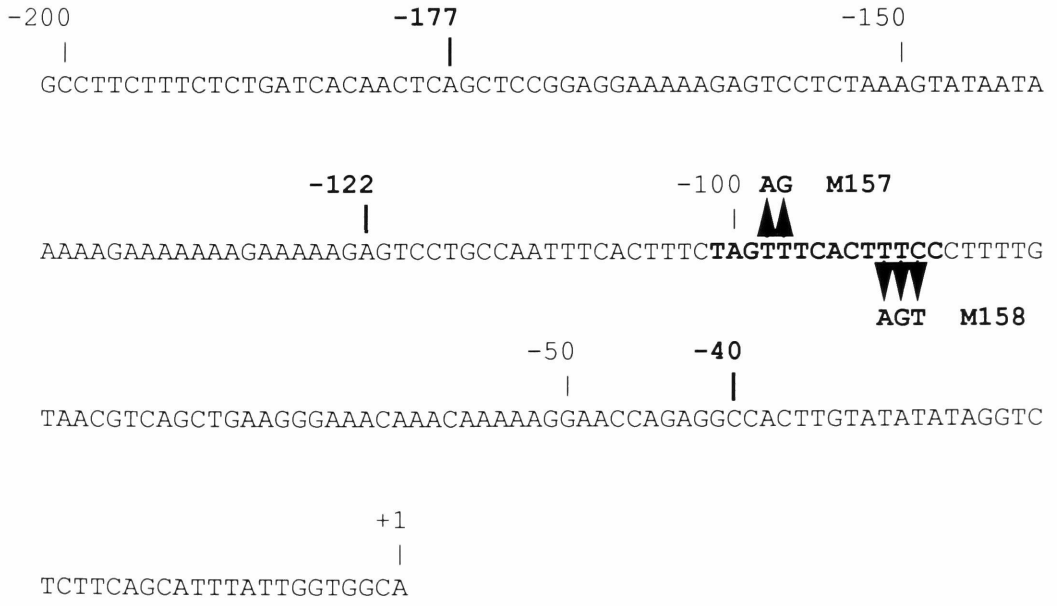
Because the region from -122 to +55 conferred IFN sensitivity to recombinant plasmids, we assumed that there might be a regulatory element specific for IFN-induced nuclear factors within this region. We performed

Figure 2.1 ISG54 Minimal inducible promoter. (**A**) Promoter mutations abrogate expression in transfected cells. HeLa cells were transfected with fusion constructs containing 800 bp (lanes 1 and 2), 177 bp (lanes 3 and 4), 122 bp (lanes 5 and 6), or 40 bp (lanes 11 and 12) of wild-type ISG54 promoter sequence or with mutated -122 bp constructs containing 2 nucleotide changes (lanes 7 and 8), or 3 nucleotide changes (lanes 9 and 10) from the wild-type sequence, as diagrammed in panel (**B**). RNA from transfected cells untreated (odd-numbered lanes) of treated with IFN (even-numbered lanes) was assayed for expression of the transfected plasmid. Endogenous ISG54 and cotransfected pRSVcat were assayed for expression as controls (data not shown).

A


-800
-177
-122
M157
M158
-40
IFNα: – + – + – + – + – + – +
1 2 3 4 5 6 7 8 9 10 11 12

B


-200
-177
-150
GCCTTCTTTCTCTGATCACAACTCAGCTCCGGAGGAAAAAGAGTCCTCTAAAGTATAATA
-122
-100
AG
M157
AAAAGAAAAAAAGAAAAAGAGTCCTGCCAATTTCACTTTCTAGTTTCACTTTCCCTTTTG
AGT
M158
-50
-40
TAACGTCAGCTGAAGGGAAACAAACAAAAAGGAACCAGAGGCCACTTGTATATATAGGTC
+1
TCTTCAGCATTTATTGGTGGCA

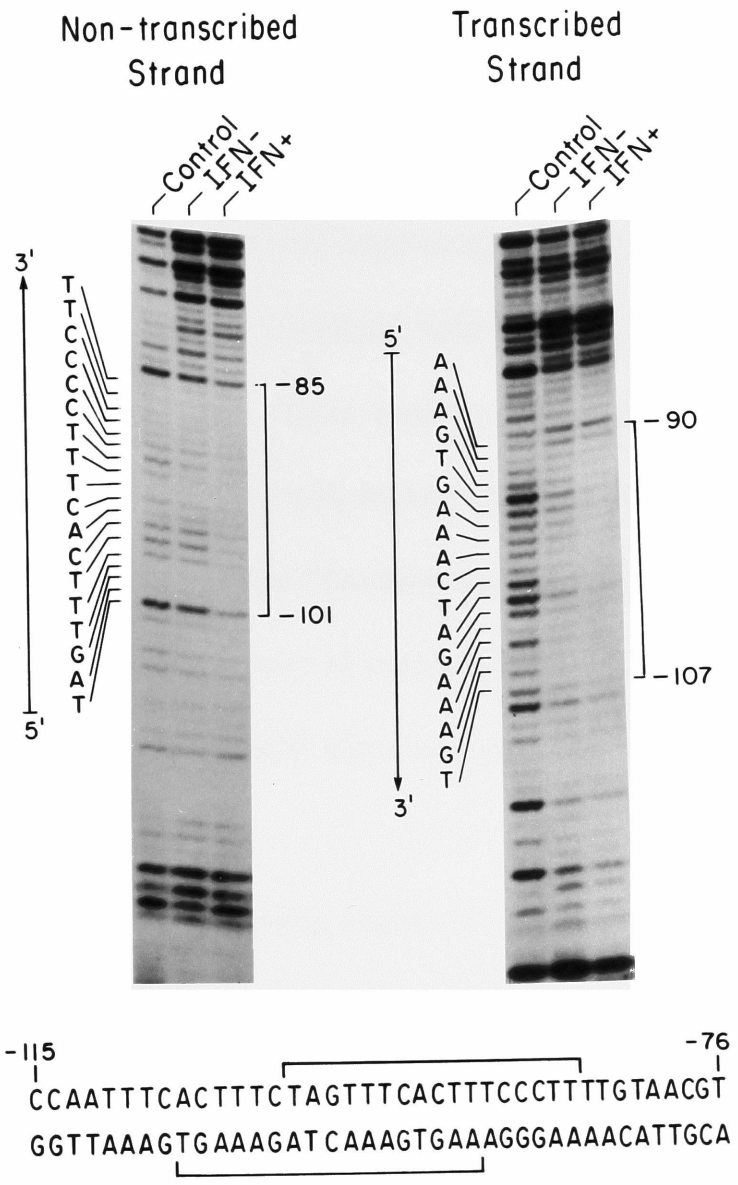
DNAse I footprint experiments (Galas and Schmitz, 1978), using crude nuclear extracts from normal and IFN-treated HeLa cells (Fig 2.2). Binding reactions contained end-labeled DNA fragments from the promoter region (-122 to +55, asymmetrically labeled on either of the two strands) and 100 μg of nuclear proteins. After incubation, the samples were digested with DNAse I, and the deproteinizied DNA was subjected to denaturing acrylamide gel electrophoresis.

Protection against DNAse digestion was detected on both strands of the DNA (Fig 2.2). The most prominent protection was in the region between -85 and -105 on both strands. Specifically, no protection nearer to the RNA start site could be detected. Using equal amounts of protein there was greater protection with extracts of IFN-induced cells. The region of greatest protection was 5-10 bases downstream of the CCAAT box motif. These initial experiments thus directed our attention to the -80 to -100 region as an important region in regulation of ISG54.

Mutations define functional importance of the ISRE, a conserved DNA element

To test the functional importance of the individual nucleotides within this DNA region, mutations were introduced into a plasmid construction that

Figure 2.2 DNase I footprint of protein binding to the ISG54 promoter. DNA fragments from -122 to +55 of ISG54 were uniquely labeled at the 5' end for the nontranscribed strand (left) or at the 3' end for the transcribed strand (right). Footprinting reactions were performed as described in Materials and methods. Control reactions contained no cell protein, whereas the other reactions contained 60 μg of protein from whole cell extracts of untreated (IFN-) of IFN-stimulated (IFN+) HeLa cells. The sequences of the protected region are indicated with numbering relative to the cap site, and are diagrammed below.


Non-transcribed Strand
Transcribed Strand
Control
IFN-
IFN+
Control
IFN-
IFN+
3'
5'
TTCCCCTTTTCACTTTGAT
-85
-101
5'
3'
AAAGTGAAACTAGAAAGT
-90
-107
-115
-76
CCAATTTCACTTTCTAGTTTCACTTTCCCTTTTGTAACGT
GGTTAAAGTGAAAGATCAAAGTGAAAGGGAAAACATTGCA

contained the -122 to +55 sequence of ISG54 fused to the E1B reporter gene. Mutations were directed to nucleotides protected from DNase digestion in the footprinting experiment. In mutant M157, the thymidines at position -98 and -97 were changed to adenine and guanine residues, respectively. In mutant M158, the sequence T-T-C at position -91 to -89 was changed to A-G-T (shown in Fig 2.1B).

Each of these mutant plasmids was used in transient transfection assays in comparison with a construction containing wild-type sequence. Little or no mRNA was transcribed from either mutant plasmid, regardless of whether transfected cells were treated with interferon or not (Fig 2.1A, lanes 7-10), indicating that the same sequence functions in both basal and induced transcription. Upon prolonged exposure of autoradiograms, a weak, properly initiated RNA was detectable for M157 (data not shown). M158, however, gave no detectable signal.

The definition in the 5'-flanking region of crucial nucleotides for expression of the ISG54 gene led us to compare the sequence of this gene with other genes induced by IFNα. A strong homology was noted with ISG15 (Reich et al., 1987). The homologous sequence in ISG15 was found within the 115 5' flanking nucleotides that were shown to be necessary for transcriptional

activation. This similar sequence was in the opposite orientation, consistent with the enhancer-like capacity of the ISG15 5'-flanking sequence (Reich et al., 1987). Other genes known to be strongly induced by IFN, such as 6-16 (Porter et al., 1988), C202 (Samanta et al., 1986), and H-2 (Israel et al., 1986; Vogel et al., 1986), also contain a strong homology limited to about 14 nucleotides. This region of homology is much smaller, but it is found within a sequence proposed by Friedman and Stark (1985) to be important for response to IFN. Their hypothesis was based on sequence comparison among IFN-induced genes whose sequences were known (metallothionine and histocompatibility genes) but whose IFN-responsive regions had not been identified. By comparison of the ISG54 and ISG15 homology with a collection of 5'-flanking sequences from genes known to respond to IFN, we were able to derive a consensus motif, YAGTTTC(A/T)YTTTYCC (Table 2.1). However, the functionally important regions of some of these genes have yet to be described.

Several distinct nuclear factors bind the ISRE

If a specific sequence within the -122 to -40 region of ISG54 is responsible for the regulated cycle of increase and subsequent decrease in ISG transcription following IFN treatment, protein extracts from IFN-treated cells

Interferon Stimulated Response Element

Sequence	Gene
Human:	
T AG T T T C A C T T T C C C	ISG54
C AG T T T C G G T T T C C C	ISG15
T AG T T T C A C T T T C C C	ISG56
T AC T T T C AG T T T C AT	GBP
G AG T T T C A T T T T C C C	6-16a
C AG T T T C A T T T T C C C	6-16b
TGG T T T C G T T T C C T C	OAS
C AG T T T C T T T T C T C C	HLA-A
C AG T T T C T G T T T C C T	Factor B
AGG T T T C A C T T T C C A	IP-10
Mouse:	
C AG T T T C A C T T C T G C	H-2K
C AG T T T C A C T T C T G C	H-2D
C AG T T T C C C T T T C AG	H-2L
T AG T T T C A C T T T T T G	Tla
C AG T T T C T C A T T T AC	202
G AG T T T C G T T T C T G A	Mx
Consensus:	
Y AG T T T CA/TY T T T Y C C	

Table 2.1 Interferon stimulated response element: conserved element of IFNα-stimulated genes. Listed are sequences present in the promoters of IFN-responsive human and mouse genes which are highly similar to the sequence in ISG54 that is necessary for transcriptional response to IFN. ISG15 (Reich et al., 1987); GBP (Decker and Lew, unpublished); 6-16 (Porter et al., 1988); OAS (Benech et al., 1987); HLA-A (Koller and Orr, 1985); Factor B (Wu et al., 1987); IP-10 (Luster and Ravetch, 1987); mouse class I histocompatibility (Moore et al., 1982; Kimura et al., 1986; Korber et al., 1985); Tla (Fisher et al., 1987); 202 (Samanta et al., 1986); Mx (Staheli et al., 1986).

might be expected to contain multiple proteins that bind within this region. The gel-shift or gel-retardation assay first described by Fried and Crothers (1981) and Garner and Revzin (1981) was used to search for such proteins.

A labeled DNA fragment (-122 to +55 of ISG54) was mixed with whole cell extracts prepared in 1.2 M KCl buffer from uninduced cells and from cells induced with IFN for 2 hr. The protein-DNA mixture was subjected to nondenaturing gel electrophoresis and autoradiography (Fig 2.3). Two cell types, a transformed human fibroblast (clone 75.1, ATCC) and HeLa cells, were used for extracts shown in Figure 2.3A. With the HeLa cell extracts (lanes 3-14), two prominent DNA-protein complexes were noted with extracts from uninduced cells (lanes 3-7); only one of these (denoted B1 or interferon stimulated gene factor-1) was completely competed by a 35-fold molar excess of unlabeled homologous DNA (lane 8), a characteristic of sequence-specific interactions. However, extracts from IFN-treated HeLa cells (lanes 9-14) produced a prominent new band (denoted B2 or ISGF2) that was not found in uninduced cells and was also subject to homologous competition (lane 14). The same bands seen in the B1/ISGF1 region using uninduced extracts were also found using extracts of induced cells. As a control for extraction efficiency of the treated and untreated cells, both extracts were found to contain the

same amount of NF-1 (Nagata et al., 1983; Pruijn et al.. 1986), a nuclear factor that binds to sequences in the terminus of adenovirus (data not shown).

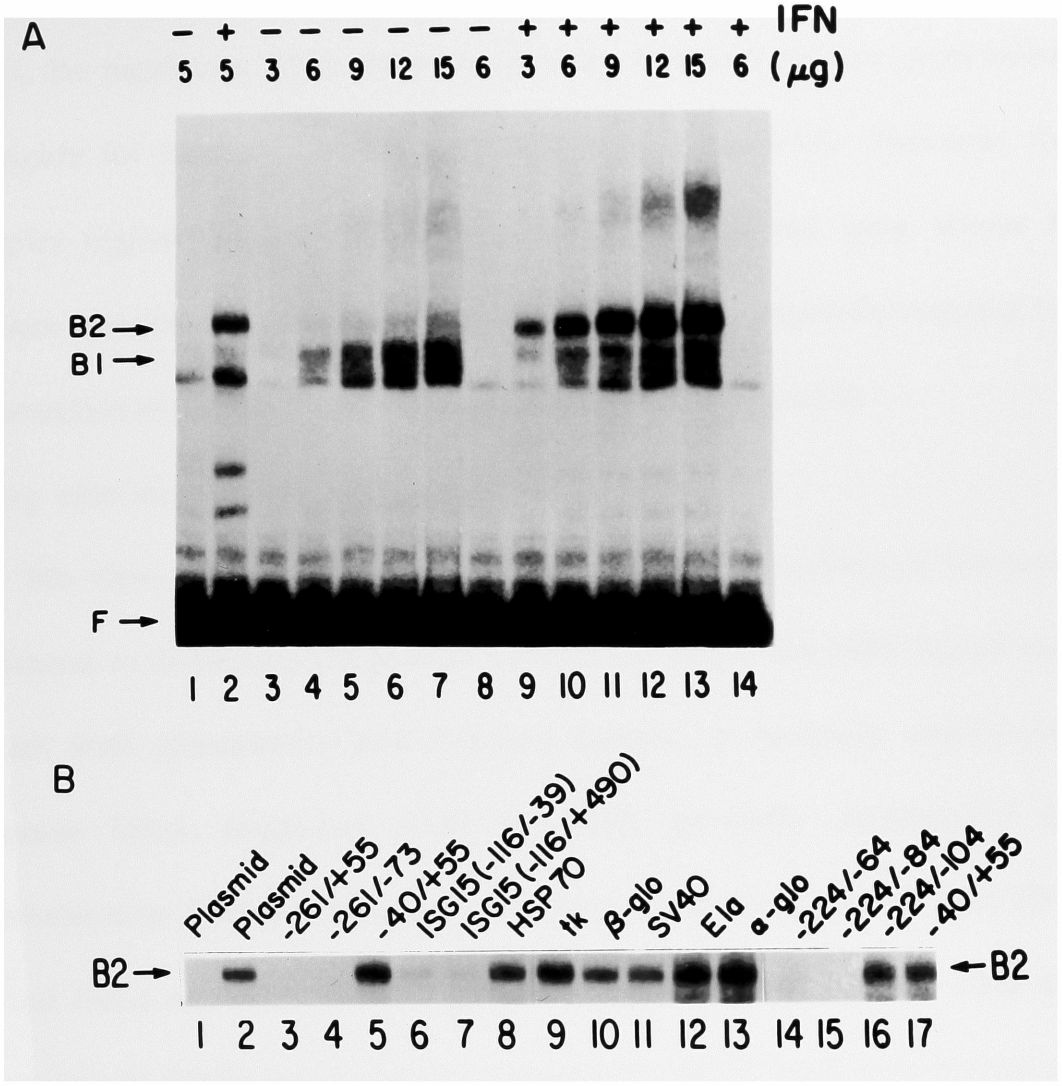
In addition to showing the induced B2/ISGF2 band, the experiment in Figure 2.3A shows the effect of adding increasing amounts of the two cell extracts to the binding reactions. Even at the highest concentration of protein from uninduced cells, only a faint signal was detected in the B2/ISGF2 region of the gel, much less distinct than the B2/ISGF2 band produced by fivefold less protein from induced cells (cf. lanes 7 and 9). This B2/ISGF2-like signal from uninduced extracts may not be due to the same protein producing IFN-induced B2/ISGF2 because with high amounts of protein, complexes that migrated slower than B1/ISGF1 and B2/ISGF2 were seen with extracts of induced and, to a much lesser extent, uninduced cells. These slower moving bands could be aggregates due to high protein concentrations exceeding the resolving power of the gel-shift assays, or alternatively, may represent concentration-dependent higher order complexes of B1/ISGF1 and/or B2/ISGF2.

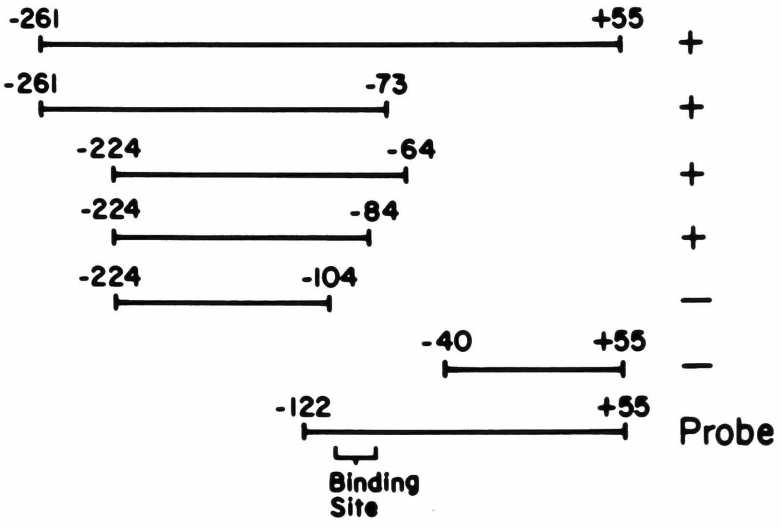
Extracts from a fibroblast cell line (Fig 2.3A, lanes 1 and 2) also showed an IFN-induced DNA-binding protein that formed a complex migrating similarly to B2/ISGF2 (lane 2). A complex having similar mobility to

B1/ISGF1 was present at a much lower level in extracts of uninduced cells, as compared to induced HeLa cell (lanes 1 and 2, migrating just above a major nonspecific complex). In addition to the IFN-induced B2/ISGF2 complex, faster migrating IFN-specific gel-shift bands (near the bottom of lane 2) were generally seen in extracts from IFN-treated fibroblast lines but were rarely found using HeLa extracts. These complexes could represent proteolytic degradation of the major IFN-induced binding activity in extracts of those cell lines. In addition to extracts of HeLa cells and fibroblasts, IFN-induced bands like B2/ISGF2 were routinely observed with extracts of IFN-treated human primary fibroblasts and lymphoblastoid (Daudi) cells but not with extracts from untreated cells (data not shown).

To determine which sequence within the -122 to +55 region were involved in forming the B2/ISGF2 complex, a series of competition experiments were performed. In these experiments (Fig 2.3B), ~35-fold molar excess of various unlabeled DNA segments was incubated with the labeled -122 to +55 DNA probe during the protein-binding reaction. Several significant results came from these experiments (Fig 2.3B). First, only DNA segments containing sequences between -84 and -104 were found to compete for protein binding. Thus, the site responsible for the B2/ISGF2 gel-shift band

Figure 2.3 Gel-shift protein-DNA binding assays with whole cell extracts. **(A)** Gel shifts were performed by mixing the indicated amounts of whole cell extracts from untreated or IFN-treated WI38 fibroblasts (lanes 1 and 2) or HeLa cells (lanes 3-14) with a -122/+55-bp end-labeled ISG54 probe. Lanes 8 and 14 represent reactions in which unlabeled homologous competitor DNA was present in a 35-fold molar excess. The positions of the unbound probe (F) and the B1/ISGF1 and B2/ISGF2 retarded complexes are indicated. **(B)** Gel-shift assays were performed using whole cell extracts from untreated (lane 1) or IFN-treated cells (lanes 2-17). (Lanes 1-13) Results using WI38 extracts; (lanes 14-17) results from HeLa cell experiments. Nonspecific and specific competitor DNA (0.5 μg) included in each reaction is indicated above each lane, as described in the text. Only the band corresponding to the B2/ISGF2 complex is shown. A summary of the competition results is diagrammed below.


A
IFN
– + – – – – – – + + + + + +
5 5 3 6 9 12 15 6 3 6 9 12 15 6 (μg)
B2
B1
F
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
Plasmid
Plasmid
-261/+55
-261/-73
-40/+55
ISG15(-116/-39)
ISG15(-116/+490)
HSP 70
tk
β-glo
SV40
E1a
α-glo
-224/-64
-224/-84
-224/-104
-40/+55
B2
B2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

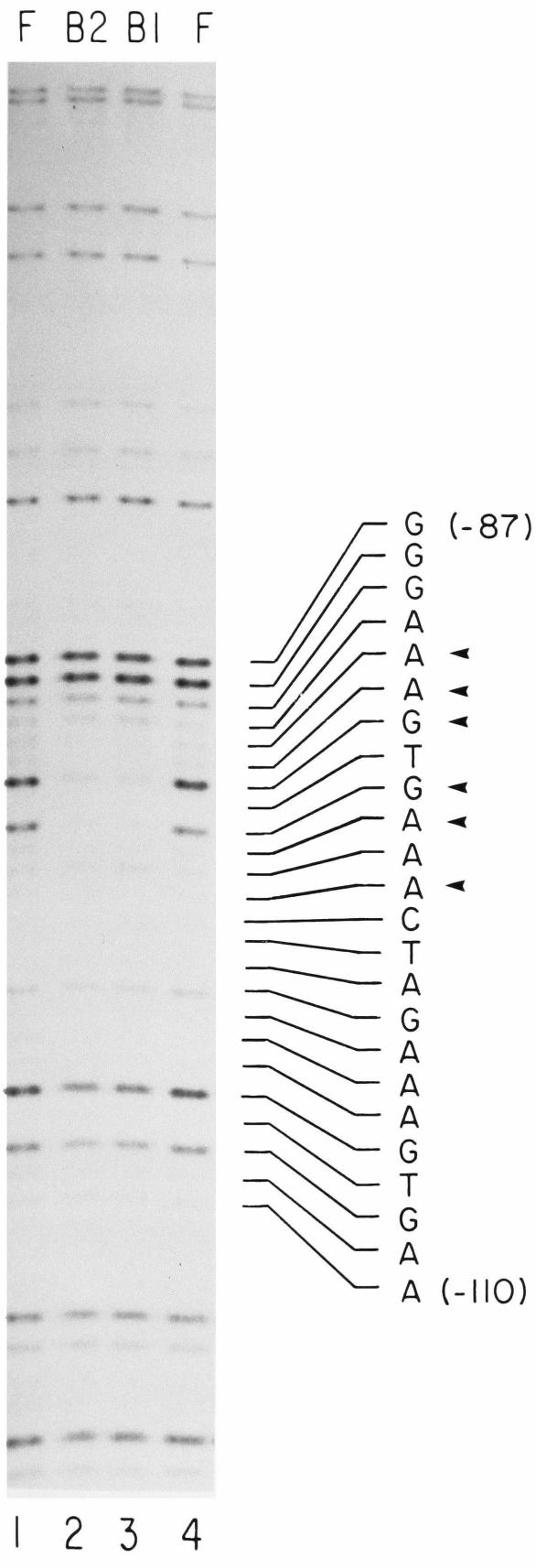

-261
+55
+
-261
-73
+
-224
-64
+
-224
-84
+
-224
-104
–
-40
+55
–
-122
+55
Probe
Binding Site

is in the same region as the major footprint region described in Figure 2.2. Second, the regulatory DNA elements of many viral and cellular genes failed to compete for formation of the B2/ISGF2 band (lanes 8-13). However, the promoter region for the ISG15 gene, an IFN-stimulated gene whose 5' sequence contains the ISRE homology (Table 2.1), did completely compete for the formation of the B2/ISGF2 complex (Fig 2.3B, lanes 6 and 7).

Binding sites detected by methylation interference

We then turned to the use of a methylation-interference footprint experiment to determine the precise nucleotides within the ISRE region that interact with constitutive and induced factors. A uniquely end-labeled antisense DNA fragment (-122 to +55), partially methylated by dimethylsulfate (DMS) (Maxam and Gilbert, 1980), was mixed with IFN-induced HeLa cell extract and a gel-shift experiment performed. Both the constitutive B1/ISGF1 band and IFN-induced B2/ISGF2 band were located by autoradiography, and the labeled DNA was recovered. The recovered DNA was then cleaved at modified bases by piperidine treatment and resolved by gel electrophoresis. If methylation of a particular G or A prevents protein-DNA complex formation, such a methylated guanine or adenine will not appear in the sequence ladder (Fig 2.4). This experiment showed that the G

Figure 2.4 Methylation interference of protein-DNA complex formation identifies a single binding site. Preparative binding reactions were prepared using partially methylated -122/+55 ISG54 probes 3'-end labeled on the transcribed strand and whole cell extracts from IFN-treated HeLa cells. Free probe (lanes 1 and 4) and retarded complexes B2/ISGF2 (lane 2) and B1/ISGF1 (lane 3) were resolved by nondenaturing gel electrophoresis, the DNA was recovered, and the cleavage products following piperidine treatment were separated on an 8% acrylamide-42% urea gel.


F B2 B1 F
G (-87)
G
G
A
A
A
G
T
G
A
A
A
C
T
A
G
A
A
A
G
T
G
A
A (-110)
1 2 3 4

residues at -95 and -93 on the antisense strand were crucial for DNA-protein binding for both B1/ISGF1 and B2/ISGF2. The single guanine residue within this region on the opposite strand (position -99) was also affected, though less dramatically, in a similar experiment performed with label in the sense strand (data not shown). The adenine residues at -96, -92, and -91 on the antisense strand are probably also necessary for interaction with binding proteins because these steps of the sequence ladder were depressed somewhat in both recovered complexes. The proteins responsible for forming the B1/ISGF1 and B2/ISGF2 complexes contact the same nucleotides within the ISRE. This finding explains our earlier observation that the ISRE was protected by both induced and uninduced HeLa extracts in DNase footprinting analysis (Fig 2.2).

The nature of the proteins responsible for the B2/ISGF2 complex

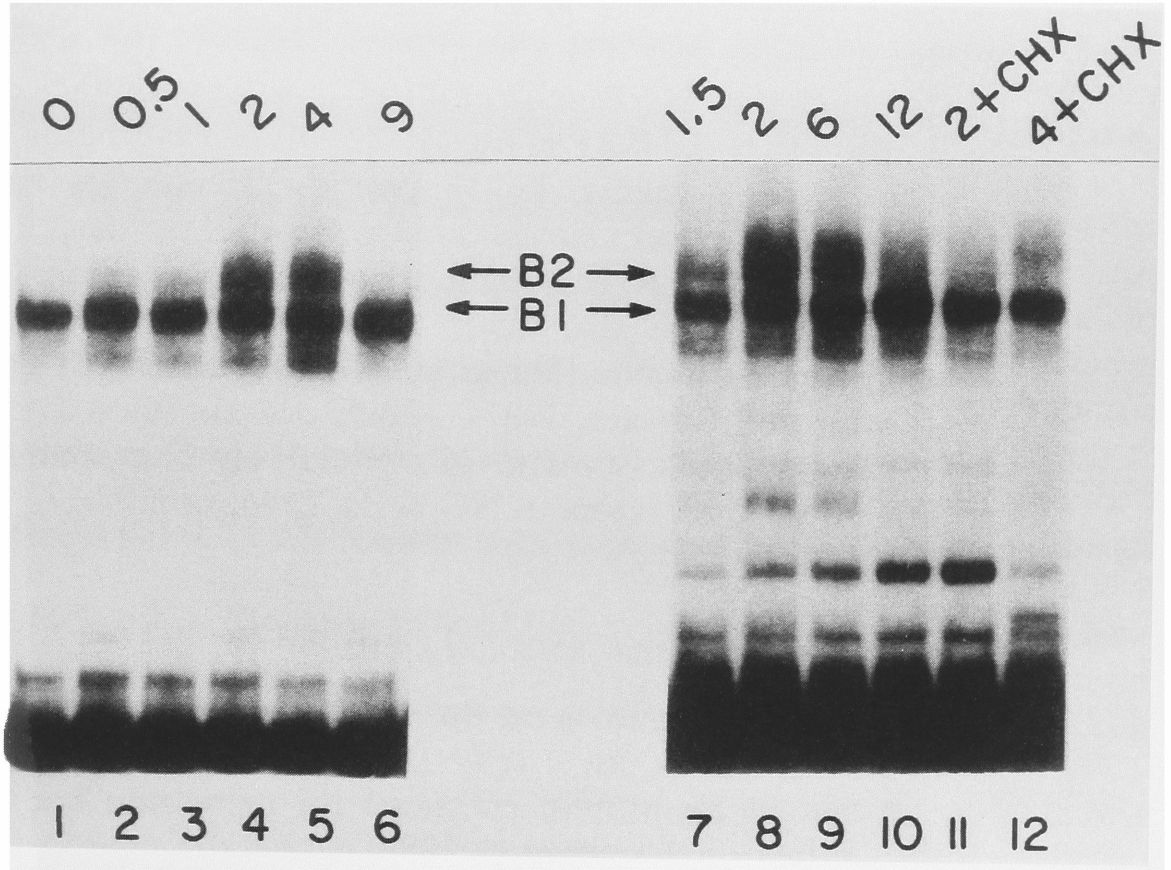
Transcriptional activation of ISG54 (and other ISGs) by IFN is very prompt (Larner et al., 1984; Reich et al., 1987) and does not require protein synthesis (Larner et al., 1986; Levy et al., 1986). We therefore tested the kinetics of IFN-stimulated B2/ISGF2 induction in the presence or absence of cycloheximide. In many experiments, two of which are shown in Figure 2.5, the clear appearance of B2/ISGF2 required at least 1.5 hr of IFN treatment and

reached maximal levels only after 2-4 hr. The B2/ISGF2 gel-shift activity than declined at ~10 hr. There was a dramatic effect of cycloheximide in blocking the induction of the B2/ISGF2 activity (Fig 2.5, lane 11). In addition, protein synthesis was required to maintain a high level of B2/ISGF2. Cells treated with IFN for 2 hr to induce B2/ISGF2 and for an additional 2 hr with cycloheximide plus IFN had much less B2/ISGF2 than cells treated for 4 hr with IFN alone (lane 12). Thus, the B2/ISGF2 activity did not correlate with the expected properties of a positive-acting factor: There was a time lag before its appearance, its appearance was blocked by cycloheximide, and it disappeared after being induced when cells were treated with cycloheximide. All of these characteristics, however, correlate with the decline of IFN-induced transcription and suggested that B2/ISGF2 may be involved in downregulation, rather than the induction, of the ISG transcription.

IFN-induced factors in low salt nuclear extracts

In the DNA-protein binding assays described thus far, extracts of whole cells prepared with 1.2 M KCl were employed. Such extraction conditions were chosen initially in an attempt to ensure release of all DNA-binding proteins. To concentrate nuclear proteins more specifically, isolated nuclei were prepared and proteins extracted in small volumes of buffer containing

Figure 2.5 Kinetics of B2/ISGF2 factor induction. Gel-shift assays are shown using whole cell extracts from HeLa cells treated with IFN for the indicated times. Experiments show in lane 11 used extracts from cells treated with IFN and cycloheximide (35 μg/ml) for 2 hr, and in lane 12 cells were treated with IFN for 2 hr followed by IFN plus cycloheximide for an additional 2 hr.


0
0.5
1
2
4
9
1.5
2
6
12
2+CHX
4+CHX
B2
B1
1
2
3
4
5
6
7
8
9
10
11
12

various salt concentrations (Dignam et al., 1983a). The previously detected IFN-induced factor responsible for B1/ISGF1 and B2/ISGF2 appeared to be quantitatively recovered by a 0.4 M salt extraction of nuclei.

In addition, extracts of nuclei prepared by this protocol using 0.2-0.4 M NaCl also contained proteins that produced additional complexes from treated cells that were not seen in whole cell extract cells (Fig 2.6A, lanes 4 and 5). Most importantly, one of these bands, denoted B3/ISGF3, has been regularly observed to be maximally induced within 1 hr of treatment. Although the appearance of B3/ISGF3 is a characteristic of all low salt nuclear extracts of IFN-treated cells, its intensity is only approximately one-tenth as great as that of B2/ISGF2, under these assay conditions (see below). Therefore, exposure times that make B3/ISGF3 easily visible cause overexposure of the B1/ISGF1 and B2/ISGF2 region of the gel-shift pattern.

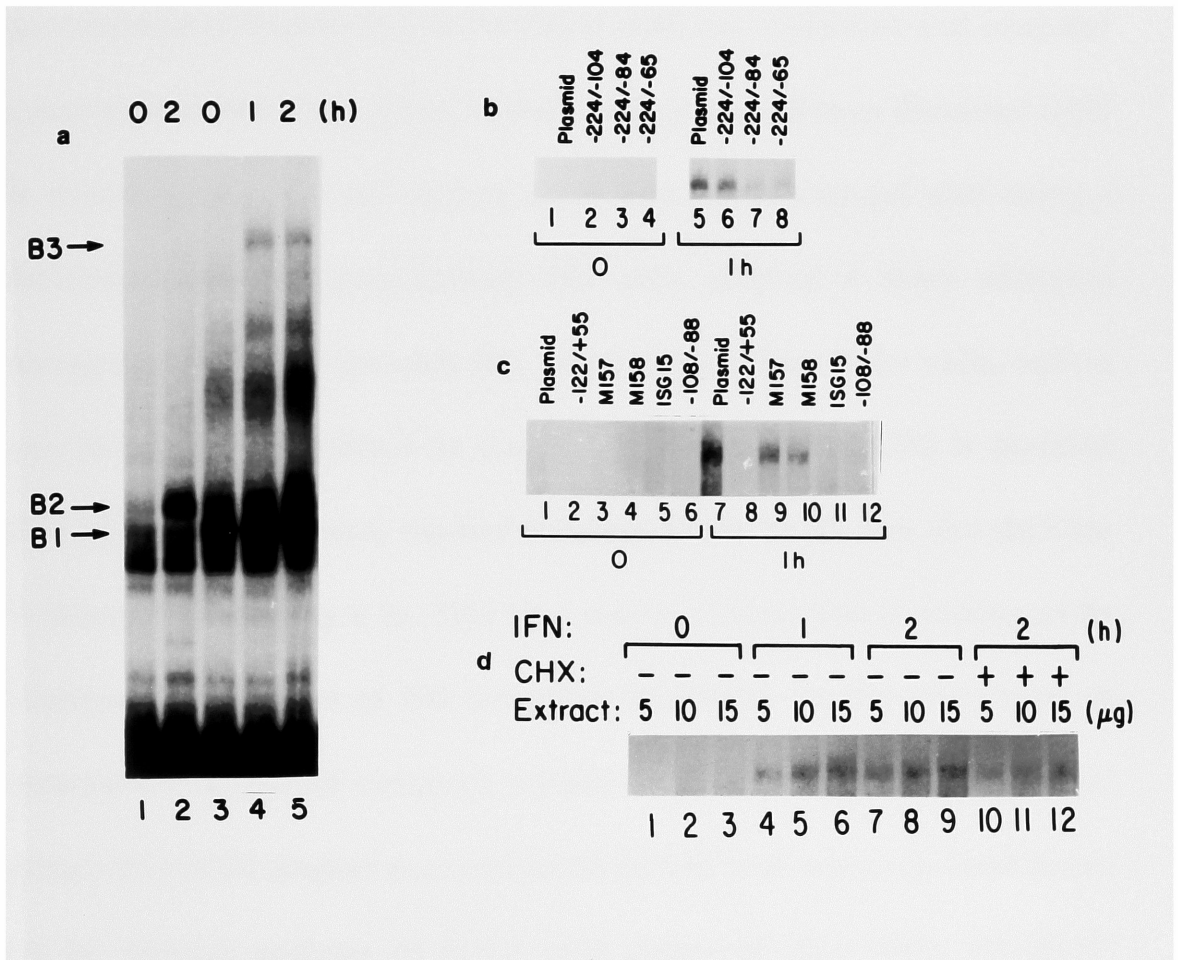
In addition to B3/ISGF3, in all nuclear extracts, there were proteins that produced gel-shift bands between B2/ISGF2 and B3/ISGF3, but the amounts of these bands varied considerably in different extracts. It is possible that the protein(s) responsible for these bands is related to B2/ISGF2. In subsequent experiments where the factor(s) responsible for B2/ISGF2 has been partially purified by heparin-agarose and oligonucleotide affinity columsn (R. Pine et

al., 1990), the broad gel band that migrates between B2/ISGF2 and the position of B3/ISGF3 becomes more prominent as more protein is used in a gel-shift reaction and therefore probably represents a concentration-dependent higher-order complex. (This is also observed with total cell extracts, see Fig 2.3, lane 13).

Upon identification of B3/ISGF3 as a gel-shift activity induced prior to B2/ISGF2, several additional experiments were carried out to investigate the nature of the B3/ISGF3 complex. The results of these experiments are shown in Figure 2.6B-D. First, competition experiments with various segments of the ISG54 promoter showed that the same sequences between -88 and -104 that competed for B2/ISGF2 formation also competed for B3/ISGF3 (Fig 2.6B). The ISG15 promoter also competed both bands, and an oligonucleotide with the sequence -84 to -108 was also effective in competition. However, DNA segments containing the mutant oligonucleotides M157 and M158 competed only very poorly (Fig 2.6C). Thus, the sequences to which B2/ISGF2 and B3/ISGF3 bind overlap considerably and may be identical.

The sensitivity of B3/ISGF3 induction to protein synthesis was tested by treating cells with both IFN and cycloheximide. In contrast to the results shown in Figure 2.5, that B2/ISGF2 induction is inhibited by cycloheximide

Figure 2.6 Gel shifts with low salt extracts detect an additional IFN-induced factor that correlates with transcriptional stimulation. (**A**) Gel-shift experiments with the -122/+55-bp ISG54 probe using whole cell extracts (lanes 1 and 2) or low salt nuclear extracts (lanes 3-5) made from HeLa cells treated with IFN for the indicated durations. (**B**) Competition gel-shift experiments with low salt extracts using a 35-fold molar excess of competitor DNA containing the indicated sequences and extracts from untreated HeLa cells (lanes 1-4) or from 1 hr (lanes 5-8) IFN treatments of HeLa cells. (**C**) Competition experiments using plasmids containing wild-type -122/+55 sequence, point mutations M157 and M158, the ISG15 promoter (-116/-39), or a double-stranded synthetic oligonucleotide containing ISG54 sequences from -108 to -88, as indicated. Low salt nuclear extracts were prepared from untreated (lanes 1-6) or 1 hr (lanes 7-12) IFN-treated HeLa cells. (**D**) Gel-shift experiments using the indicated amounts of low salt nuclear extracts made from HeLa cells treated with IFN for the indicated periods of time. For the results shown in lanes 10-12, cycloheximide was present for 2.25 hr, beginning 15 min prior to the 2 hr IFN treatment. For the experiment in **B-D**, only the band corresponding to B3/ISGF3 complex is shown.

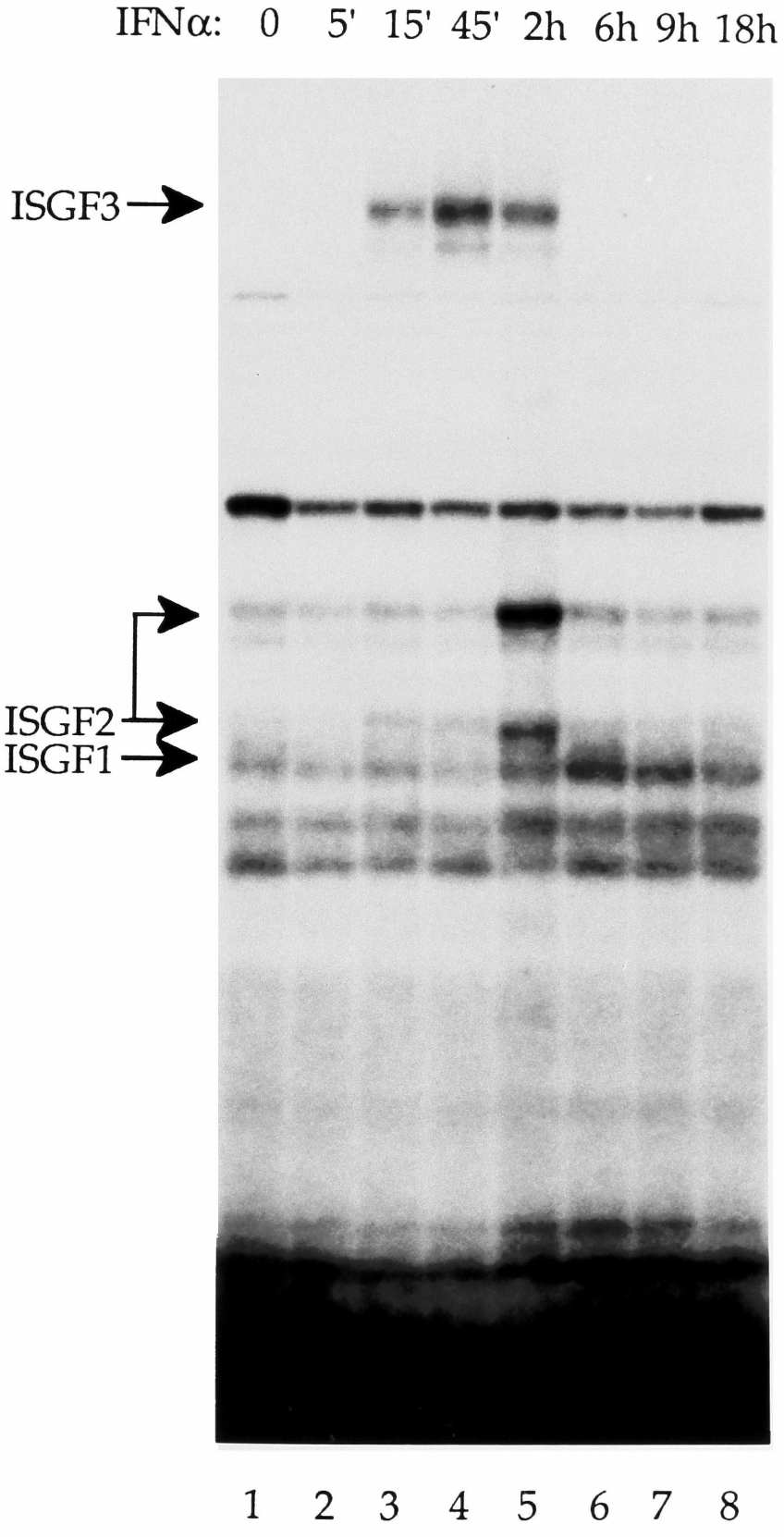

a
0 2 0 1 2 (h)
B3
B2
B1
1 2 3 4 5
b
Plasmid
-224/-104
-224/-84
-224/-65
Plasmid
-224/-104
-224/-84
-224/-65
1 2 3 4 5 6 7 8
0
1h
c
Plasmid
-122/+55
M157
M158
ISG15
-108/-88
Plasmid
-122/+55
M157
M158
ISG15
-108/-88
1 2 3 4 5 6 7 8 9 10 11 12
0
1h
d
IFN: 0 1 2 2 (h)
CHX: - - - - - - - - - + + +
Extract: 5 10 15 5 10 15 5 10 15 5 10 15 (μg)
1 2 3 4 5 6 7 8 9 10 11 12

treatment, the DNA-binding activity required for forming the B3/ISGF3 complex was induced without ongoing protein synthesis (Fig 2.6D).

Kinetics of B3/ISGF3 induction

The development of an optimized gel shift protocol using an oligonucleotide corresponding to the ISG15 ISRE has confirmed and extended the observations described above. Extracts were prepared from fibroblast (FS2) cells treated with IFNα for varying times and assayed by gel shift using a radiolabeled ISRE oligonucleotide. FS2 cells display a more stringent downregulation of IFN-induced ISG transcription than HeLa cells, with a return to basal level within 6 hr (Larner et al., 1986). B3/ISGF3 is detected within 15 min of treatment, reached maximal levels by 45 min and declines to undetectable levels by 6 hr. This time course of induction is striking in its similarity to the kinetics of ISG induction. B2/ISGF2, on the other hand, is undetectable at 45 min of treatment, a point at which ISG transcription is near maximal. B2/ISGF2 reaches maximal levels at 2 hr and returns to basal levels by 9 hr. Similar analysis of HeLa cells indicated that while B3/ISGF3 induction is detectable within 15 min, as in FS2 cells, the factor does not return to basal levels until 8-12 hr of treatment, consistent with ISG induction in HeLa cells (data not shown). The results obtained with an optimized

Figure 2.7 Induction of B3/ISGF3 correlates with transcriptional activation of ISGs. Gel-shift experiments were carried out using an end-labeled oligonucleotide corresponding to the ISG15 ISRE and low salt nuclear extracts of FS2 cells treated with IFN for the indicated durations. B3/ISGF3 induction is detected within 15 min of treatment, peaks by 45 min, and returns to undetectable basal levels by 6 hr. B2/ISGF2 (forming a concentration-dependent higher order complex on the probe used) levels are maximal at 2 hr of treatment and returns to basal levels by 9 hr of treatment. The indicated DNA-protein complexes are the only specific complexes formed, as shown by competition experiments (data not shown).


IFNα: 0 5' 15' 45' 2h 6h 9h 18h
ISGF3
ISGF2
ISGF1
1 2 3 4 5 6 7 8

protocol support the proposition that B3/ISGF3 is a positive regulator of ISG transcription.

Discussion

We have shown previously that one of the primary responses of cells to IFN treatment is a two-phase transcriptional cycle (Larner et al., 1984, 1986). Initially, a set of normally quiescent genes is activated to high rates of transcription. This activation occurs within minutes of IFN binding to high affinity cell-surface receptors and shows no requirement for ongoing protein synthesis. Subsequently, transcription falls to near pretreatment levels, controlled by an active process dependent upon continued protein synthesis, suggesting the need for renewal of some protein component that is turning over rapidly (Larner et al., 1986). For ISG54, both phases of the IFN-stimulated transcription cycle can be mediated, at least in part, by DNA sequences at the 5' end of the gene (Levy et al., 1986; Reich et al., 1987).

The results presented in this paper lend support to the idea that transcriptional regulation of ISG54, in particular, and coordinate expression of ISGs, in general, involve a specific 5'-flanking sequence, the ISRE, to which several protein factors were demonstrated to bind. There is one constitutive

factor (B1/ISGF1 complex) and at least two different factors that are activated by IFN treatment (B2/ISGF2 and B3/ISGF3). The latter two factors possess appropriate characteristics to serve as a positive activator (B3/ISGF3 complex) and as a negatively acting transcriptional factor (B2/ISGF2 complex).

The existence of an ISRE in the ISG54 promoter has been established by a number of different experiments. Deletion analysis delineated the minimal 5'-flanking sequence necessary for IFN induction, and IFN-induced factors were found to bind within this region. Plasmids carrying point mutations in the proposed ISRE no longer directed the IFN-stimulated transcriptional response and no longer bound nuclear factors. Although 800 bp of 5'-flanking sequence are sufficient for both positive and negative transcriptional regulation (Levy et al., 1986), we do not yet have direct evidence that the ISRE alone receives the negative signals or directs the subsequent repression of ISG transcription.

Although a portion of the ISRE showed varying degrees of identity with viral and cellular enhancers, the E1A, SV40 early, and HSP70 and other promoter/enhancer segments failed to compete for binding of any factors with the ISG54 promoter fragment. This result distinguishes the factors described in this paper from those reported by Israel et al. (1987) and Baldwin

and Sharp (1987), which were based on binding of proteins to mouse H-2 and β2-microglobulin promoters. These previously described binding activities were not detectably modulated by IFN; in addition, they were effectively competed by SV40 early enhancer sequences and mapped to regions of the H-2 promoter outside the ISRE homology.

One heterologous DNA did compete for binding and did so for all three factors. This was a promoter fragment from ISG15. The only extended sequence identity between these two IFN-stimulated promoters lies within the ISRE (Table 2.1; Reich et al., 1987), reinforcing the conclusion that the ISRE is the site for all factor binding. In further experiments, ISG15 promoter fragments have been found to form similar complexes to those described here (Reich and Darnell, 1989). We have also found that 5'-flanking segments from the 6-16 gene (Porter et al., 1988) and IP-10 gene (Luster and Ravetch, 1987), which contain homologous ISRE sequences (Table 2.1), effectively competed for the binding of factors to ISG54 (data not shown).

A model for transcriptional control

The previous studies on the rise and subsequent fall of transcription of ISGs after IFN treatment and the requirement for protein synthesis for repression but not for induction (Larner et al., 1986) guided the experiments

designed to test the possible functions of the IFN-induced factors that gave rise to B2/ISGF2 and B3/ISGF3 complexes. The B3/ISGF3 factor was maximally induced within 60 min and then declined and did not require protein synthesis for induction-characteristics that suggest the B3/ISGF3 complex could be responsible for ISG activation.

The B2/ISGF2 factor, although strongly induced by IFN, did not appear until about 2 hr after treatment and then only in cells that continued to synthesize new proteins during IFN treatment. Moreover, protein synthesis was also required to maintain the B2/ISGF2 factor at high levels. These are attributes characteristic of the negative phase of the ISG transcription cycle when gene expression declines and is maintained at low levels by a process involving newly synthesized proteins (Larner et al., 1986). Therefore, the B2/ISGF2 complex represents an attractive candidate repression. However, recent work has identified B2/ISGF2 as a protein identical to IRF-1 (Miyamoto et al., 1988), and a variety of observations contradict the negative regulatory role of B2/ISGF2 (Pine et al., 1990). Therefore, the role, if any, of B2/ISGF2 in IFN-induced transcriptional regulation of ISGs remains to be demonstrated.

Repression could involve interaction of the activator (B3/ISGF3) with a second protein, as suggested for GAL80 repression of the yeast galactose

regulon (Johnston et al., 1987; Ma and Ptashne, 1987b). Thus, a single DNA-binding protein that is modified sequentially could account for all the characteristics of ISG induction. In addition, it is possible that the constitutive B1/ISGF1 activity might supply the single sequence specificity observed. Further understanding of the mechanisms underlying the different phases of the IFN-induced transcriptional cycle will require purification of the proteins responsible for this regulation.

Materials and methods

Cell culture

Cultured cells were maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum and antibiotics and subcultured by trypsinization. HeLa cells (clone S3), SV-40 transformed WI38 human lung fibroblasts (CCL 75.1), and FS2 human primary diploid fibroblasts were obtained from ATCC, Rockville, Maryland. For IFN treatment, recombinant bacteria-derived IFNα, a kind gift of Dr. S. Pestka (Hoffman-La Roche, Nutley, New Jersey), was added to the culture media at 500U/ml. Cycloheximide (Sigma) was used at 35 μg/ml.

Transfections

HeLa cells were transiently transfected by DNA complexed with DEAE-dextran (Sompayrac and Danna, 1981; Lopata et al., 1984). Plasmid DNA containing ISG54 sequences (1 μg) and a control plasmid pRSVcat (Gorman et al., 1982) (0.8 μg) were mixed in 500 μl of DME and adjusted to 1 mg/ml DEAE-dextran (Pharmacia). This mixture was overlaid on HeLa cell monolayers in 100-mm culture dishes and incubated at 37°C for 4 hr. Following removal of the DNA mixture, cells were rinsed with 15% glycerol in HEPES-buffered saline and then incubated in medium for 6-12 hr. Subsequently, each transfected culture was trypsinized and divided into two duplicate cultures. After an additional 24 hr incubation period, one dish was treated with IFN, and the second served as a control. This procedure minimized fluctuations due to variations in transfection efficiency. Following a 4 hr IFN treatment, cells were harvested and cytoplasmic RNA obtained by NP-40 lysis and phenol extraction (Wilson and Darnell, 1981). To assay endogenous and transfected gene expression, ^{32}P-labeled cRNA synthesized *in vitro* (Melton et al., 1984) was hybridized with 10 μg of HeLa cell RNA, duplexes were digested with T2-RNase, protected fragments resolved on denaturing acrylamide-urea

gels and detected by autoradiography.

Cell extracts

Nuclear and whole cell extracts were prepared by a modification of the procedure of Dignam et al. (1983a, 1983b). Monolayer cells were harvested by scraping, washed with phosphate-buffered saline (PBS) by centrifugation, and swelled and homogenized in Buffer A [20 mM HEPES (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. For nuclear extracts, nuclei were collected by centrifugation, washed with 0.14 M KCl in Buffer A, and resuspended at 1 x 10^8 nuclei per milliliter in Buffer A. For high salt extracts, nuclei or homogenized cells (for whole cell extracts) were extracted in 1.2 M KCl, 10% glycerol, and 0.1 mM EDTA in Buffer A. Insoluble material was removed by centrifugation at 200,000*g*, and the clarified supernatants were dialyzed against 100 mM KCl, 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, and 10% glycerol. Dialysates were clarified by centrifugation at 10,000*g*. Low salt extracts were prepared by resuspending nuclei directly in 0.21 mM KCl, as described by Dignam et al. (1983a, 1983b).

Protein-DNA binding assays

Gel-shift assays were performed essentially as described by Fried and Crothers (1981). End-labeled DNA fragments (7 fmoles; 0.25 ng) were incubated with 10-15 μg of protein in 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 320 μg/ml poly(dIdC):poly(dIdC), and 4% Ficoll in a final volume of 12.5 μl for 20 min at room temperature. Protein-DNA complexes were resolved from free probe on 4% acrylamide gels run in 25 mM Tris-borate and 0.25 mM EDTA. Gel-shift assays using an ISRE oligonucleotide was essentially identical except protein-DNA complexes were resolved on 6% polyacrylamide gels. The oligonucleotide used was a 33mer containing the ISG15 ISRE region.

For DNase I footprinting, DNA-protein binding reactions (as described above) were scaled up to 30 μl in the same buffer lacking Ficoll and containing 100 μg/ml poly(dIdC):poly(dIdC). Following a 20 min incubation at room temperature, DNase I was added at 16 μg/ml and incubated for 4 min at 30°C. DNase digestion was terminated by the addition of 10 mM EDTA, 0.2% SDS, and 100 μg/ml proteinase K. Following 15 min at 60°C, DNA was recovered by phenol-chloroform extraction and ethanol precipitation and was fractionated on denaturing acrylamide-urea gels. Methylation interference

experiments were performed as described by Sen and Baltimore (1986b).

In vitro mutagenesis

Point mutants were introduced into the ISRE by oligonucleotide-directed DNA repair of gapped heteroduplexes, as described by Morinaga et al. (1984). Heteroduplexes were formed between a plasmid containing an ISG54-E1B fusion gene and a second plasmid containing E1B sequences and no promoter. Two oligonucleotides were designed to produce 2 nucleotide changes (M157) of three nucleotide changes (M158) within the ISRE and simultaneously create restriction enzyme recognition sites. Their sequences were 5'-CTTTCTAGAGTCACTTTCCC-3' (M157) and 5'-CTAGTTTCACTAGTCCTTTTGTAACG-3' (M158).

Oligonucleotides were annealed with the gapped heteroduplexes, which were subsequently repaired using DNA polymerase I (Klenow fragment) and nucleotides. Bacterial colonies were screened by hybridization (Grunstein and Hogness, 1975), using the mutagenic oligonucleotides as labeled probes. Filters were hybridized at 37°C (M157) or 47°C (M158) and washed at 42°C (M157) or 57°C (M158) in 6X SSC, and mutated plasmids were isolated and confirmed by restriction analysis and DNA sequencing. Mutants were observed at a frequency of ~5%.

Chapter 3

Cells resistant to interferon are defective in activation of a promoter-binding factor

Introduction

Binding of type I interferon (IFNα/β) to its cell surface receptor elicits several physiological responses in cultured cells, including inhibition of cell proliferation and inhibition of viral replication (Lengyel, 1982; Tamm et al., 1987). These ultimate effects of IFN, detectable after 24 hours, are preceded by a transient transcriptional activation of a group of interferon-stimulated genes (ISGs) whose protein products seem likely to be involved in physiological responses to IFN (Larner et al., 1984, 1986; Friedman et al., 1984; Kelly et al., 1984; Levy et al., 1986; Kusari and Sen, 1987; Reich et al., 1987). Transcriptional activation of ISGs is associated with the rapid induction of DNA-binding proteins (Levy et al., 1988a; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988) that bind to the interferon stimulation response element (ISRE), an ISG regulatory element that is both necessary (Levy et al., 1986, 1988a; Reich et al., 1987; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988) and sufficient (Cohen et al., 1988; Reich and Darnell, 1989) for interferon stimulation. One IFN-induced DNA-binding activity, designated interferon stimulated gene factor-3 (Levy et al., 1988a; ISGF3 was previously designated B3), is implicated in transcriptional activation because its accumulation directly parallels transcriptional activation. As is the case for transcriptional

activation of ISGs, the production of this activity in response to IFN treatment is rapid, does not require new protein synthesis, and likely results from an IFN-induced post-translational modification of an inactive precursor (Levy et al., 1988a). In this report we show that three human cell lines (Daudi, Raji, and HeLa lines) are resistant to the physiological effects of IFN even though they have normal numbers of high affinity IFNα receptors. IFN-induced transcriptional activation of three ISGs (ISG15, ISG54, and ISG56) was found to be greatly reduced in the IFN-resistant lines as compared with sensitive lines. In addition, the IFN induction of the ISGF3 DNA-binding activity was very low or undetectable in all IFN-resistant lines studied. These results indicate that the IFN-resistant lines are defective in the activation of a promoter-binding factor, an early event in the IFN response, and thus fail to transcribe a set of genes necessary for the physiological response.

Results

Interferon-resistant cell lines have normal interferon receptors

The IFN-sensitivity of human tissue culture cells [two HeLa cell lines, two subcloned Daudi cell lines (Dron and Tovey, 1983), and the Raji cell line] was determined by quantitation of the antiproliferative effect of IFNα on

these lines as well as by determination of the resulting antiviral state. IFNα treatment significantly inhibited proliferation of sensitive Daudi cells and one HeLa cell line, while proliferation of the Raji and variant Daudi cell lines was unaffected by IFNα treatment, and the proliferation of a second HeLa line was only slightly impaired (Table 3.1). In addition, vesicular stomatitis virus (VSV) infection of sensitive HeLa or Daudi cells was largely blocked by IFNα treatment, while treatment of the variant HeLa or Daudi line was ineffective (Table 3.1).

A trivial explanation of the IFN-resistance of these cell lines would be the possible absence of IFN receptors. The presence of type I IFN receptors was determined by the binding of ^{125}I-IFNα to IFN-sensitive and resistant cell lines. Scatchard analysis of IFN binding data revealed that the resistant and sensitive lines expressed similar numbers (1000-4000 receptors/cell) of high affinity ($K_d \approx 50$-100 pM) cell surface receptors for IFNα (Table 3.1). Some differences between lymphoblastoid and HeLa cell lines were observed, but these are not relevant to differences between IFN-sensitive and -resistant cells of each type.

Table 3.1 Characterization of IFN-sensitive and -resistant cell lines: IFN-receptors and physiological responses

Cell line	Number of IFNα receptors/cell	Affinity of IFNα binding (Kd), pM	Doubling time (hrs.) +IFN	Doubling time (hrs.) Control	Log reduction in virus replication
HeLa-sen	1,200	100	29	18	3.5
HeLa-res	1,100	120	19	18	1.1
Daudi-sen	3,700	50	45	24	4.0
Daudi-res	3,000	45	24	23	0.3
Raji	1,900	60	23	23	0.4

sen=IFN-sensitive, res=IFN-resistant

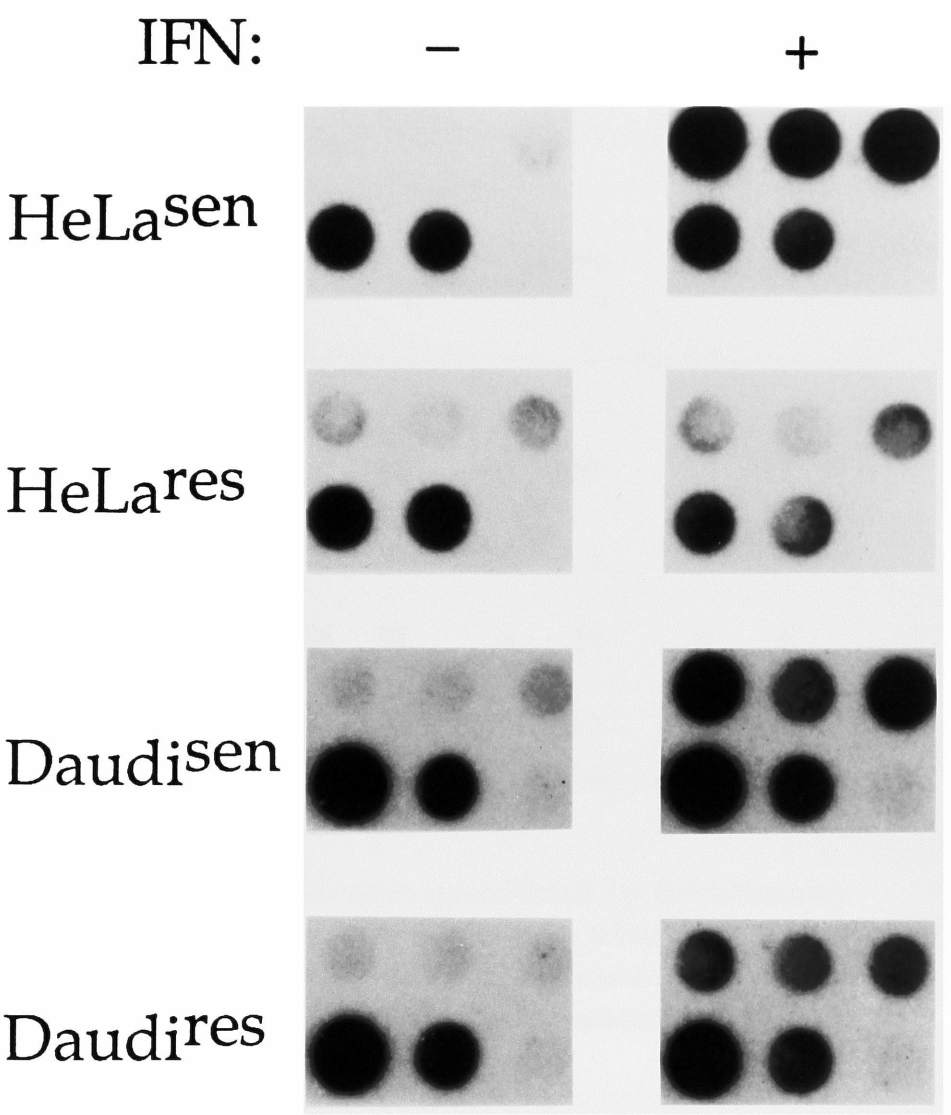
Transcription of ISGs in IFN-resistant cells

Subcloned IFN-resistant Daudi cell lines have been studied by several laboratories (Dron and Tovey, 1983; Silverman et al., 1982; Hannigan et al., 1984; Dron et al., 1985; McMahon et al., 1986). Neither the increase in mRNA encoded by a group of IFN-induced genes [561=ISG56 (Tiwari et al., 1987), 1-8, 6-16, and 9-27 (McMahon et al., 1986)], at least three of which (561, 6-16, and 1-8) are known to be transcriptionally induced, nor the IFN-induced post-transcriptional decrease of c-myc (Jonak et al., 1984; Knight et al., 1985; Dron et al., 1986) and μ-chain (Meurs et al., 1988) mRNAs occurs in resistant Daudi cells treated with IFNα. Some mRNAs that are known to increase after IFN treatment of sensitive cells (thymosin β4, metallothionein II, HLA2A, and 2'-5' oligo-A synthetase) also increased in resistant cells (McMahon et al., 1986). In no case was the level of regulation for any of these mRNAs determined in the IFN-resistant cell lines.

We therefore measured the IFN-induced transcription of ISGs in IFN-sensitive and resistant Daudi and HeLa cells in response to IFN by *in vitro* nuclear run-on transcription assays (Fig 3.1 and Table 3.2). A two hour IFNα treatment resulted in strong transcriptional signals for ISG15, ISG54, and ISG56 in sensitive Daudi and HeLa cells compared to a very low basal

transcription rate. In contrast, resistant lines showed a very weak transcriptional response of these genes. There was no detectable increase in ISG54 or ISG56 transcription and at most two-fold induction of ISG15 transcription in resistant HeLa cells. The basal levels of transcription for the three genes were somewhat higher in the resistant HeLa cells than in the sensitive HeLa cells. In resistant Daudi cells IFN did induce transcription of these genes, but only about 20% as well as in sensitive Daudi cells. As shown previously for fibroblasts (Larner et al., 1986), transcription of ISG15, ISG54, and ISG56 in the sensitive HeLa line had declined six hours after IFN addition, with a more rapid decline for ISG54 and ISG56 than for ISG15 (Reich et al., 1988, and data not shown). The transcription rate of the 2'-5' oligo-A synthetase gene (Rutherford et al., 1988) was also tested in several experiments. For this gene the transcription signals after interferon treatment were considerably weaker than for the other genes. However, the IFN sensitive HeLa cells were at least five-fold more responsive than the resistant lines. The Daudi sensitive cells were only about two times more responsive and again the transcription signals were low (data not shown). The reports that this gene is normally expressed in the IFN-resistant Daudi line (McMahon et al., 1986; Dron et al., 1986), based on mRNA accumulation,

Figure 3.1 Transcriptional analysis of ISGs in IFN-sensitive and -resistant HeLa and Daudi cell lines. *In vitro* nuclear run-on transcription assays were performed with nuclei from untreated cells (left panels) or cells treated with IFNα for two hours (right panels). Radiolabeled nascent RNA was hybridized to samples of ISG54, ISG56, ISG15, β-actin, tubulin, and pGEM1 DNAs affixed to nitrocellulose. The pattern of DNA dots on each filter is diagrammed below. sen=IFN-sensitive and res=IFN-resistant.



ISG54	ISG56	ISG15
actin	tubulin	pGem1

Table 3.2 Quantitation of IFNα-induced transcription[a]

Gene:	ISG15		ISG54		ISG56		OAS	
IFN:	-	+	-	+	-	+	-	+
Cell line:								
HeLa-sen	0	135	0	322	0	125	0	20
HeLa-res	6	47	2	13	2	4	0	4
Daudi-sen	2	51	0	57	0	15	0	6
Daudi-res	0	20	0	20	0	13	0	3

sen= IFN-sensitive, res= IFN-resistant

[a]Run-on transcription assays were quantitated by densitometry of preflashed X-ray film. Several exposures were obtained to insure that signals were linear. Results are normalized to the signal from actin for each time point and presented as arbitrary units.

might suggest a difference in cell lines or possibly that post-transcriptional control of this mRNA is also important in Daudi cells.

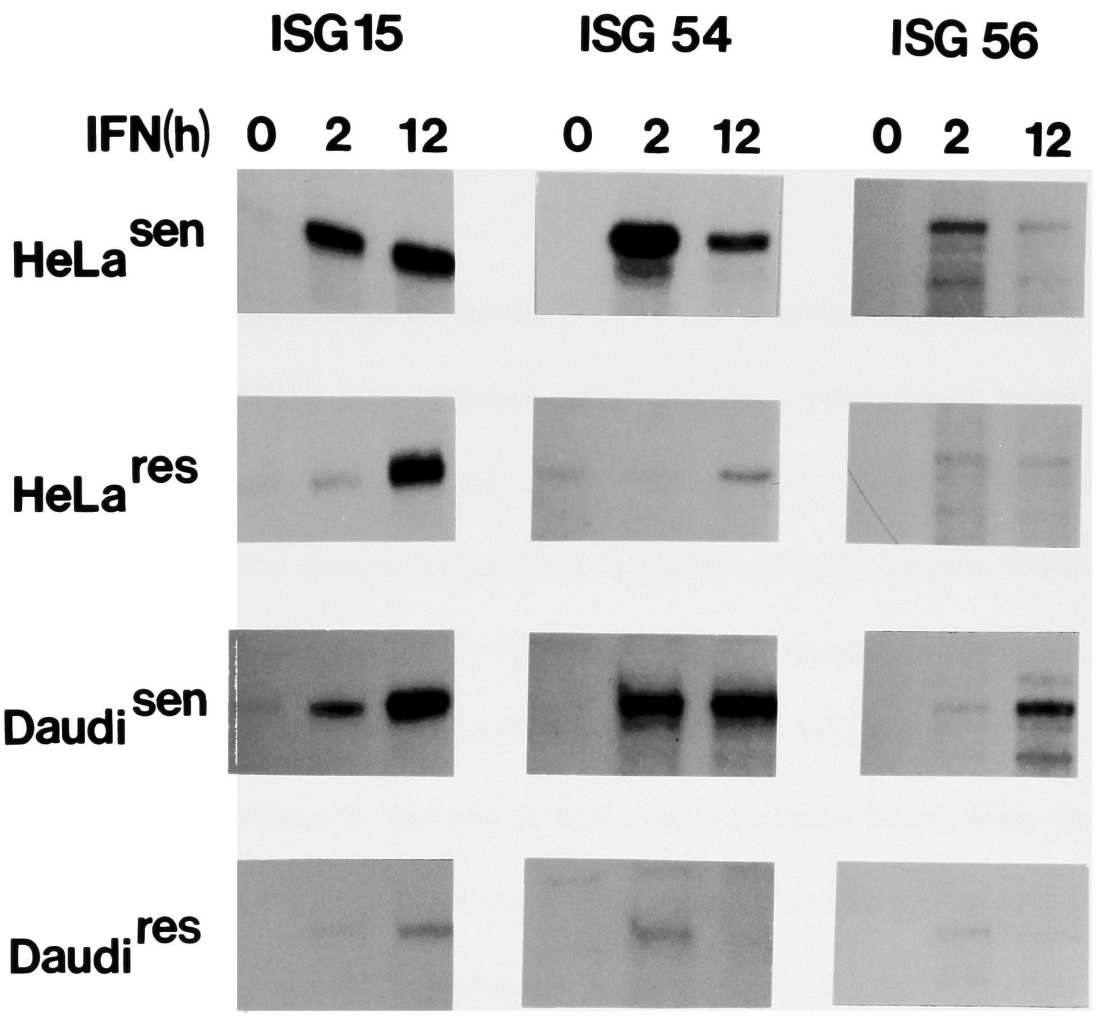
mRNA accumulation by IFN-sensitive and resistant cells

The accumulation of ISG mRNA for ISG15, ISG54, and ISG56 generally paralleled the transcription rate in all cell lines (Fig 3.2). In addition, very little ISG mRNA accumulated in IFN-treated Raji cells (data not shown). The presence of high levels of ISG mRNAs at 12 hours in sensitive Daudi cells reflects the maintenance of high transcription rates, even 20 hours after IFN addition (Pine and Darnell, 1989), which appears to be a peculiarity of this cell line (Tiwari et al., 1987). An exception is the maintenance of a high level of ISG15 mRNA even after 12 hours of IFN treatment in both sensitive and resistant HeLa lines. Given the low transcription rate of ISG15 after 12 hours of IFN treatment (data not shown), these results imply that ISG15 mRNA is more stable in HeLa cells than the other ISG mRNAs.

ISRE-binding proteins in IFN-sensitive and resistant cells

We have recently described DNA-binding activities in human cells (HeLa and fibroblasts) that bind to the ISRE (Levy et al., 1988a). Nuclear extracts from treated or untreated cells contain one activity, ISGF1 (previously designated B1). IFN-treated cells contain two additional DNA-binding

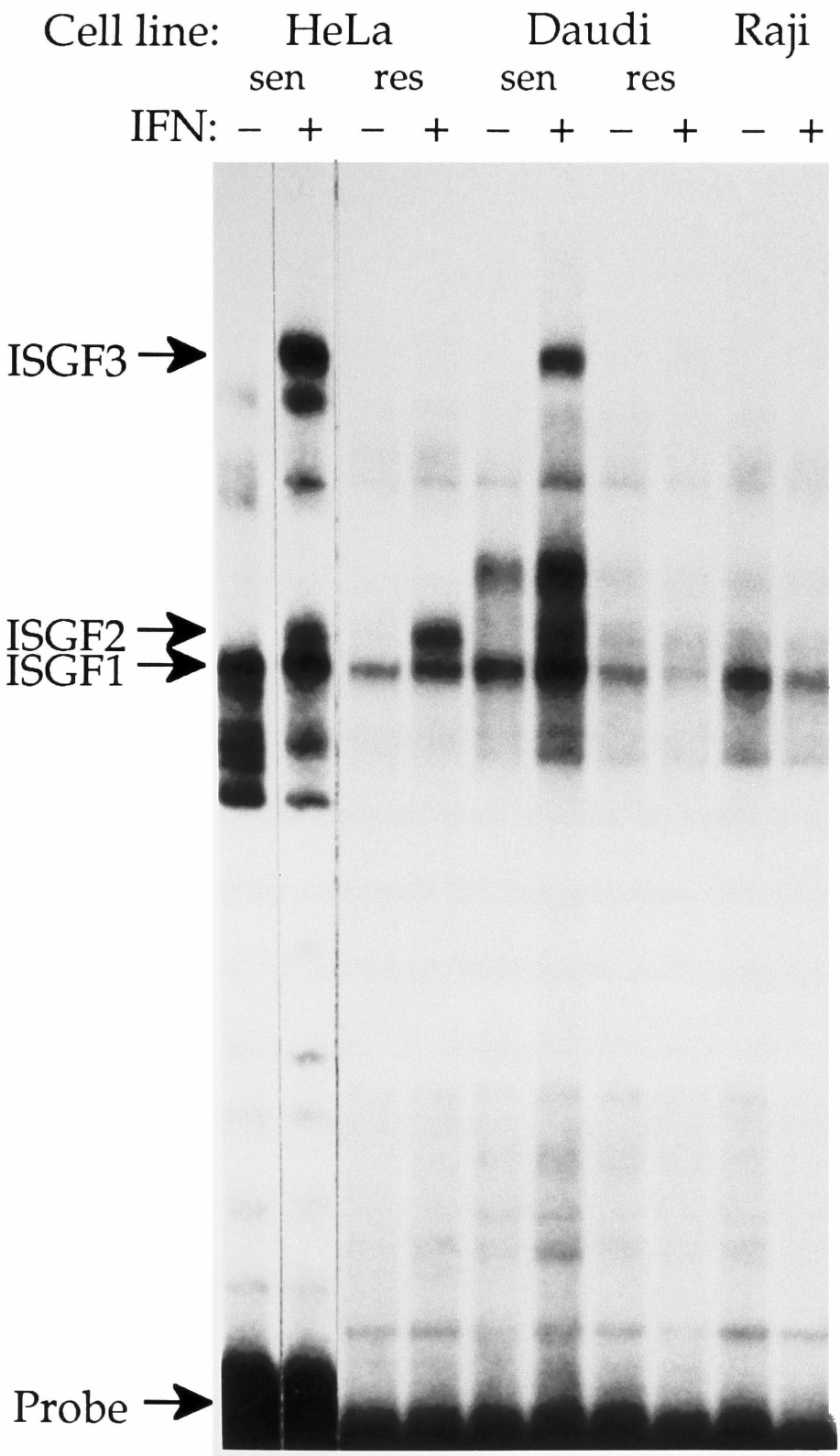
Figure 3.2 ISG mRNA accumulation in IFN-sensitive and -resistant cell lines. Total cellular RNA was isolated from untreated cells or cells treated for 2 or 12 hours with IFNα. The RNA from each sample was assayed by nuclease protection with cRNA probes for ISG15, ISG54, and ISG56. A probe for γ-actin (Enoch et al., 1986) was used to demonstrate that equivalent amounts of RNA for each sample were assayed (data not shown).


ISG15
ISG 54
ISG 56
IFN(h)
0 2 12
0 2 12
0 2 12
HeLa sen
HeLa res
Daudi sen
Daudi res

activities, ISGF2 and ISGF3 (previously designated B2 and B3). The level of ISGF3 activity increases rapidly in IFN-treated cells, even in the presence of cycloheximide, as does ISG transcription. Therefore, ISGF3 is a candidate for a positive activator of transcription. ISGF2 is produced only after 90 minutes of treatment and requires protein synthesis. While ISGF2 induction parallels ISG downregulation this activity appears not to be directly involved in ISG regulation (Pine et al., 1990).

To detect any differences in the production of these DNA-binding activities in IFN-sensitive and -resistant cells, extracts prepared two hours after IFN treatment were incubated with a radiolabeled DNA fragment containing the ISRE and assayed by gel retardation (Fried and Crothers, 1981) (Fig 3.3). While IFN-treated sensitive HeLa (lane 2) and Daudi lines (lane 6) showed clear induction of ISGF2 and ISGF3, IFN-treated resistant HeLa cells produced only ISGF2 (lane 4). Extracts of IFN-treated resistant Daudi (lane 8) and Raji (lane 10) cells contained very low induced ISGF3 levels (only detectable by over-exposure of the autoradiograph shown in Fig 3.3), although a constant low level of ISGF2 was present with or without IFN treatment. We emphasize that the level of ISGF3 activity was very low or undetectable in extracts of all IFN-resistant lines studied.

Figure 3.3 Gel retardation analysis of IFN-induced nuclear DNA-binding activities in IFN-sensitive and -resistant cell lines. Nuclear extracts of untreated cells (lanes 1, 3, 5, 7, 9) or cells treated for two hours with IFNα (lanes 2, 4, 6, 8, 10) were incubated with the -115/-39 ISG15 probe to form specific complexes. Extracts of IFN-sensitive HeLa (lanes 1 and 2) and Daudi (lanes 5 and 6) show IFN-dependent production of the ISGF2 and ISGF3 complexes. IFN-resistant HeLa cells (lanes 3 and 4) produce only the ISGF2 complex. IFN-resistant Daudi (lanes 7 and 8) and Raji (lanes 9 and 10) lines produce a low level of the ISGF2 complex regardless of treatment and overexposure of this experiment reveals a very low but detectable induced level of the ISGF3 complex. The ISGF1 complex forms with extracts of both treated and untreated cells. The faster migrating bands present in lanes 1 and 2 are due to partial degradation of the ISGF1 complex and is variable between extracts. The complex migrating between the ISGF3 and ISGF2 complexes in lanes 5-10 is a slower mobility form of the ISGF1 complex present in extracts of the lymphoblastoid lines. The specificity of the three indicated complexes for the ISG15 fragment was demonstrated by the addition of 60-fold molar excess of unlabeled ISG15 sequences which abolished the formation of detectable complexes from an IFN-sensitive HeLa extract (data not shown).


Cell line:
HeLa
Daudi
Raji
sen
res
sen
res
IFN: − + − + − + − + − +
ISGF3
ISGF2
ISGF1
Probe

Discussion

The experiments presented here suggest that the critical defect in the IFN-resistant cell lines studied is the failure to activate an ISRE-binding factor in response to IFN treatment and that this factor, ISGF3, is required for IFN-induced activation of ISG transcription. Furthermore, the action of the proteins whose synthesis is stimulated by the early burst of IFN-induced transcription must be responsible for the antiviral state and for causing cessation of cell growth.

As to the nature of the defect in the resistant lines it seems clear it is not an absence of IFN receptors; these were present in normal numbers and had a normal affinity for IFNα. Moreover, IFNα did induce ISGF2 in the resistant HeLa cells. In all resistant lines studied an event leading to the activation of ISGF3 and the associated ISG transcriptional induction appeared to be defective. Since the precursor to ISGF3 exists in IFN-sensitive cells (i.e. no protein synthesis is required to produce ISGF3 activity) two possible explanations of the IFN-resistant phenotype are suggested: 1) Resistant HeLa cells have no precursor to ISGF3 while resistant Daudi and Raji cells have some (a 20% transcriptional response was seen in these resistant lines), but not enough to elicit the late effects of IFN (antiproliferation and the antiviral

state); or 2) The ISGF3 precursor is present in resistant cells but the IFN-induced intracellular signal that activates this precursor is reduced or nonexistent in these cells. This second possibility implies that the IFN-dependent signal responsible for inducing ISGF3 is distinct from that required for ISGF2 induction since high levels of ISGF2 are produced in the resistant HeLa cell line, in the absence of ISGF3 induction.

As more is learned about the nature of proteins involved in IFN-induced transcriptional activation, an event that appears to involve post-translational modification of the precursor of ISGF3, the availability of cell lines that fail to perform this activation should be of considerable use.

Materials and methods

Cells and interferon

Interferon responsive HeLa S3 cells, obtained from ATCC, and non-responsive variant HeLa cells, a gift from Dr. E. Knight (DuPont), were maintained in monolayer cultures in DME supplemented with 10% fetal bovine serum. IFN-responsive and -nonresponsive Daudi cells were obtained from Dr. A. Hovanessian et al. (1986) and were cultured as static suspensions in RPMI-1640 supplemented with 10% fetal bovine serum.

Homogeneous preparations of recombinant IFN-αA were generously provided by Dr. P. Sorter (Hoffman-LaRoche) and added to media for HeLa cells at a concentration of 15 pM (500 U/ml) and for Daudi and Raji cells at 0.75 pM (25 U/ml).

Assay of IFN binding

Aliquots of cells were incubated at 3 X 10^6 cells/ml at 14°C with ^{125}I-IFNα-con1 (Alton et al., 1983) at varying concentrations (3-100 pM) for 100 min. Cell-associated radioactivity was determined after centrifugation through a mixture of phthalate oils (Pfeffer et al., 1987). Specific binding was determined as the difference between ^{125}I-IFNα bound in the absence and presence of unlabeled IFNα (5 nM). The data were plotted according to the method of Scatchard (1949) and analyzed by a non-reiterative least squares curve fitting program. The slope of plotted data yielded affinity of binding (K_d) and the y-intercept (bound ligand) was used to determine number of IFNα receptors/cell.

Antiproliferation assay

HeLa cells were plated at 1 X 10^5 cells per 25 cm^2 flask, treated for three days with IFNα at 15 pM, and counted. Lymphoblastoid cell lines were grown in stationary suspension culture at a concentration of 2x10^5 cells/ml of

medium and treated for three days with IFNα at 0.75 pM and counted. The ratio of cell number on day 3 to that on day 0 was used to calculate mean doubling times.

Antiviral assay

Cultured HeLa cells in 60 mm Petri dishes were incubated overnight with 15 pM IFNα, washed twice with PBS, and incubated with serial 10-fold dilutions of vesicular stomatitis virus (VSV-Indiana strain) for 1 h at 37°C. Cell monolayers were washed with PBS, overlayed with 0.9% agar, and virus plaques were counted 24-30 h post-infection. Viral titer on control HeLa cells was 1-3 X 10^6 plaque forming units/60 mm dish. Lymphoblastoid cells were incubated overnight with 0.75 pM IFNα and then infected with VSV at m.o.i. of 0.1 PFU/cell. Twenty-four hours later virus yield was assayed in L cells. Viral titer in infected control lymphoblastoid cells varied from 0.6 to 2.0 x 10^6 PFU/ml of medium.

RNA transcription and accumulation assays

Nuclear run-on transcription was performed as described (Larner et al., 1984). 3-5 X 10^7 nuclei were used in each reaction and 5-20 X 10^6 cpm of labeled nuclear RNA was hybridized to excess plasmid DNA bound to nitrocellulose. RNA accumulation was measured with radiolabeled antisense

RNA probes hybridized with 10 μg of total cellular RNA by RNase T_2 resistance (Levy et al., 1986). The ISG15 probe was a 180 bp Taq I second exon fragment; the ISG54 probe a 250 bp Eco RI-Taq I second exon fragment; and the ISG56 probe a 400 bp Eco RI-Bgl II cDNA fragment (Levy et al., 1988a; Reich et al., 1987).

DNA-protein binding assay

Nuclear extracts were prepared essentially as described by Dignam et al. (1983a). Gel retardation analysis was performed essentially as described by Fried and Crothers (1981). End-labeled ISG15 fragment (-115 to -39) was incubated with 5-10 μg of nuclear protein in 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 320 μg/ml poly(dIdC):poly(dIdC), 40 μg/ml of a mutated ISG54 ISRE as non-specific competitor (M158 in Levy et al., 1988a), and 4% Ficoll in a final volume of 12.5 μl for 20 min. Protein-DNA complexes were separated from free probe on 4.8% polyacrylamide gels as described (Levy et al., 1988a).

Chapter 4

Two interferon induced nuclear factors bind a single promoter element in interferon stimulated genes

Introduction

Transcriptional activation of gene expression underlies the primary response of cultured human cells to interferon (IFN) treatment, resulting in accumulation of a number of new proteins (Larner et al., 1984, 1986; Friedman and Stark, 1985) and subsequent long term changes in cellular physiology. In a study of mechanisms responsible for this immediate transcriptional response elicited by IFN binding to specific cell surface receptors, we identified cDNA clones representing IFN induced mRNA species (Larner et al., 1984) and have isolated and characterized genomic DNA sequences for two such interferon stimulated genes, termed ISG54 (Levy et al., 1986, 1988a) and ISG15 (Reich et al., 1987) to indicate that polypeptides of 54 and 15 kD, respectively, are encoded by these genes. Analysis of progressive deletions of 5' flanking DNA sequences indicated that approximately 120 nucleotides upstream of each mRNA start site was necessary to direct the IFN transcriptional response (Levy et al., 1986, 1988a; Reich et al., 1987). A 77 bp fragment from this region of ISG15 has been shown to function as a position and orientation independent IFN stimulated enhancer (Reich et al., 1987). Sequence comparison of these functional regions revealed a 12 out of 15 bp sequence similarity in opposite orientations in the ISG54 and ISG15 promoters (Levy et

al., 1988a; Reich et al., 1987). Similar sequences have been found in promoter regions of all other IFN-α/β stimulated genes described so far (Levy et al., 1988a, 1988b; Reich et al., 1987; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988). This conserved sequence, which constitutes an IFN-stimulated response element (ISRE), was protected in DNase I footprint experiments using crude extracts from IFN-responsive cells. Introduction of two different point mutations into the ISG54 ISRE abolished transcriptional activity of the DNA in transfection experiments (Levy et al., 1988a).

Nuclear proteins which specifically recognize the ISRE have been characterized further by gel retardation assays and by methylation interference footprints (Levy et al., 1988a). At least three protein-DNA complexes, which were distinguished by differential mobilities in gel retardation assays, can be formed between factors from IFN treated cells and DNA fragments containing the ISRE (Levy et al., 1988a; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988). One complex (termed interferon stimulated gene factor-1 or ISGF1) is formed by proteins found in untreated or IFN treated cells which do not vary in abundance or binding ability during the IFN response. Two additional complexes (termed ISGF2 and ISGF3) are formed by factors present only in

IFN-treated cells. All three of these DNA-protein complexes involve recognition of functional ISRE sequences (Levy et al., 1988a).

The IFN induced factors giving rise to the ISGF2 and ISGF3 complexes can be distinguished by differential kinetics of accumulation (Levy et al., 1988a) and have been separated by biochemical fractionation (Pine et al., 1990; X-Y. Fu et al., submitted). ISGF3, appears rapidly following IFN treatment even in the absence of new protein synthesis, paralleling the kinetics and characteristics of IFN stimulated transcription (Larner et al., 1986; Levy et al., 1986, 1988a). In contrast, ISGF2 requires ongoing protein synthesis for its induction and is not detectable until approximately 90 min following IFN treatment (Levy et al., 1988a). These characteristics suggested that ISGF3 may be directly involved in ISG transcriptional induction whereas ISGF2 cannot be required for gene activation.

In order to delineate the specific DNA recognition requirements of ISGF2 and ISGF3, and in hope of separating genetically the activities of these two DNA-binding proteins, we have carried out extensive mutagenesis of the ISG54 ISRE, site directed mutagenesis of the ISG15 ISRE, as well as DNase footprinting experiments for both promoters. From the analysis of 20 mutations in ISG54 and four specific changes within ISG15, we find that the ISGF2

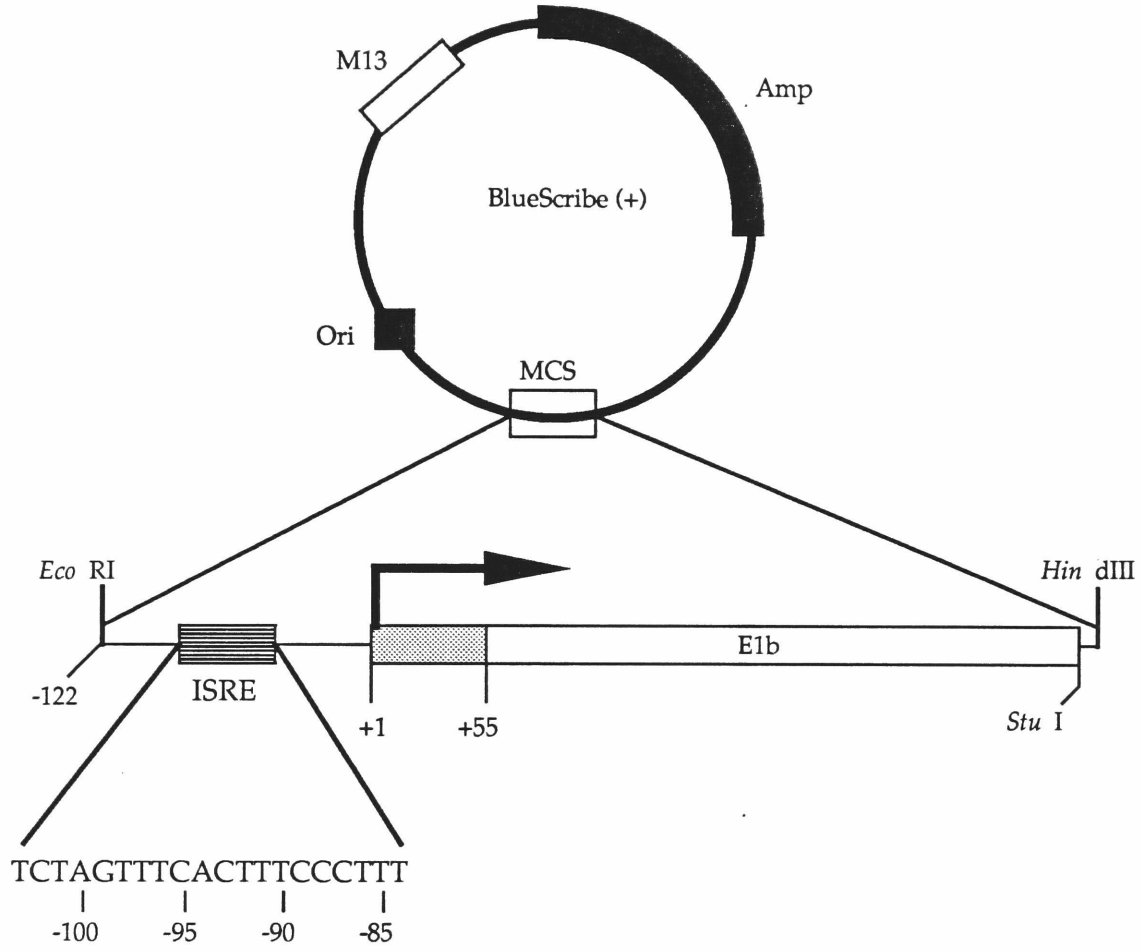
binding site is entirely encompassed within a slightly larger binding site for ISGF3, consistent with DNase protection results. Furthermore, the critical contacts for ISGF3 binding *in vitro* correlate with sequences necessary for maximal transcriptional activity *in vivo*. In addition, photoaffinity crosslinking revealed DNA-binding proteins of distinct molecular weight for ISGF2 and ISGF3.

Results

ISG54 mutagenesis

The position and sequence of the ISRE in a recombinant ISG54-adenovirus fusion plasmid (Levy et al., 1986) is diagrammed in Fig 4.1. By modification of the technique of Kunkel (1985), mutations were introduced into this plasmid using a mixture of synthetic oligonucleotides complementary to the ISRE that were synthesized to contain approximately 1 to 2 errors in every 20 nucleotides. The oligonucleotide mixtures were hybridized to single-stranded DNA containing the ISRE sequence and enzymatically converted to double strands; mutants were selected by transformation into appropriate bacterial hosts. Identification of each mutation was achieved by DNA sequence analysis. Over thirty individual

A



B

Functional ISREs

TAGTTTCACTTTCCC	ISG54
CAGTTTCGGTTTCCC	ISG15
GAGTTTCATTTTCCC	6-16a
CAGTTTCATTTTCCC	6-16b
TGGTTTCGTTTCCTC	OAS

Figure 4.1 Recombinant ISG54 expression plasmid. **A.** The diagram shows the organization of the ISG54 expression plasmid used for mutagenesis, factor binding studies, and in vivo expression experiments. The 20 nt region of the ISRE which was mutated by random oligonucleotide mutagenesis (see Materials and Methods) is indicated, as well as the position of the portion of the ISG54 first exon (+1 to +55) fused to the adenovirus 5 E1b gene. **B.** ISRE sequences are shown which have been documented by direct transcriptional analysis and transfection studies to mediate rapid induction by IFN in human cells (Levy et al., 1986, 1988a; Reich et al., 1987; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988).

mutations were studied by both transfection analysis to establish functional effects of mutant sequences and by gel retardation assays to test alterations in DNA-protein interactions. At least one mutation was recovered for each nucleotide between -102 and -86 except for nucleotides at -96 and -93. For gel retardation experiments, we used nuclear proteins that had been partially purified by column chromatography to separate ISGF2 and ISGF3. Factors forming the IFN induced ISGF2 and ISGF3 complexes with ISRE DNA fragments were the only sequence specific DNA binding activities in these protein fractions (data not shown).

Protein interactions with wild type ISG54 and ISG15 DNA sequences

ISRE sequences are not absolutely conserved (Levy et al., 1988a, 1988b; Reich et al., 1987), and ISG54 and ISG15 are no exception (see Fig 4.1B). Their ISREs differ at residues -101, -94, and -93 of the ISG54 sequence. [We have adopted a numbering system based on residue numbers upstream of the ISG54 cap site as shown in Table 4.1.] The ISG15 ISRE in the ISG15 promoter is inverted relative to the ISG54 orientation; its actual position is from nucleotides -94 to -108 upstream. This natural variation in the consensus sequence of these two genes is compatible with the normal cycle of IFN induced transcriptional increase and decrease since both wild type genes

respond in such a manner to IFN treatment (Reich et al., 1988). Likewise, there are differences in ISRE sequences from other IFN stimulated genes. As shown in Fig 4.1B, ISREs from 6-16 (Porter et al., 1988) and 2'-5' oligo (A) synthetase (OAS; Rutherford et al., 1988; Cohen et al., 1988), two other human genes in which ISREs have been demonstrated to be functional by expression *in vivo*, the central 2 nucleotides show greatest variability. It is interesting to note that the OAS ISRE differs most from ISG54. We have found that this gene is transcriptionally induced to a much lesser extent than either ISG54 or ISG15 (data not shown) which may be a direct consequence of lower affinity binding of factors to this divergent ISRE.

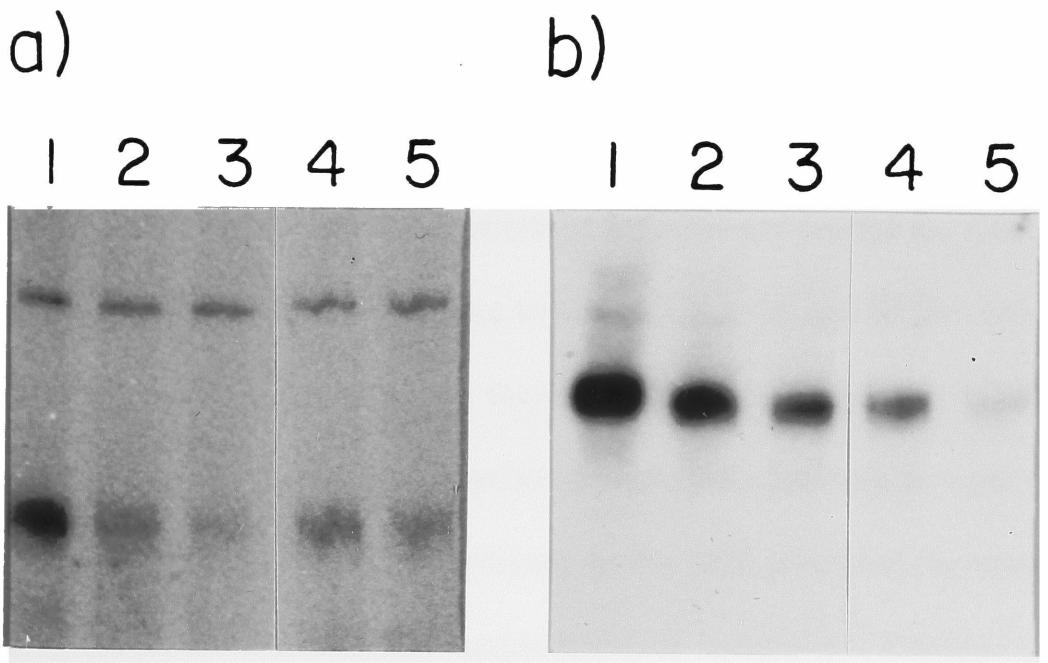
We have noted consistently that oligonucleotide probes derived from ISG54 gave much stronger ISGF2 than ISGF3 gel shift pattern while ISG15 probes gave gel shift complexes that were approximately equal in intensity for both ISGF2 and ISGF3. This difference in binding properties is illustrated in Fig 4.2 where a labeled ISG15 probe was mixed with partially purified preparations of either ISGF2 or ISGF3. To compare relative binding affinities of ISG15 and ISG54 for these two different proteins, plasmids carrying ISRE sequences from each gene were used as unlabeled competitors in gel retardation assays. Titration of increasing amounts of unlabeled competitor

DNA against constant amounts of labeled probe, protein, and total DNA showed that the ISG54 version of the ISRE preferentially competed ISGF2 complex formation (Fig 4.2A, lane 3 versus lane 5) while the ISG15 sequence competed better for ISGF3 (Fig 4.2B, lane 5 versus lane 3). These results illustrate the stronger interaction of proteins forming the ISGF3 complex with ISG15 whereas ISGF2 interacts more strongly with ISG54. Since we were particularly interested in how mutations would affect binding of ISGF3 (i.e., the putative positive acting factor), the gel retardation experiments testing mutant sequences shown in Fig 4.3 employed labeled ISG15 probes so that a strong ISGF3 as well as strong ISGF2 band could be clearly discerned in non-competed samples. However, under these conditions wild type ISG54 does not compete completely with ISG15 for ISGF3 complex formation. Therefore, subtle effects of mutations on ISGF3 binding may not be readily apparent.

Protein interactions with mutant ISG54 ISREs

Figure 4.3 shows a representative sample of gel shift experiments in which a labeled ISG15 DNA fragment (-115 to -39) was incubated with partially purified ISGF3 (panel A) or ISGF-2 (panel B) preparations in the presence of competitor DNA plasmids. Plasmids containing vector alone, wild type ISG54, or each of a series of different plasmids containing mutant

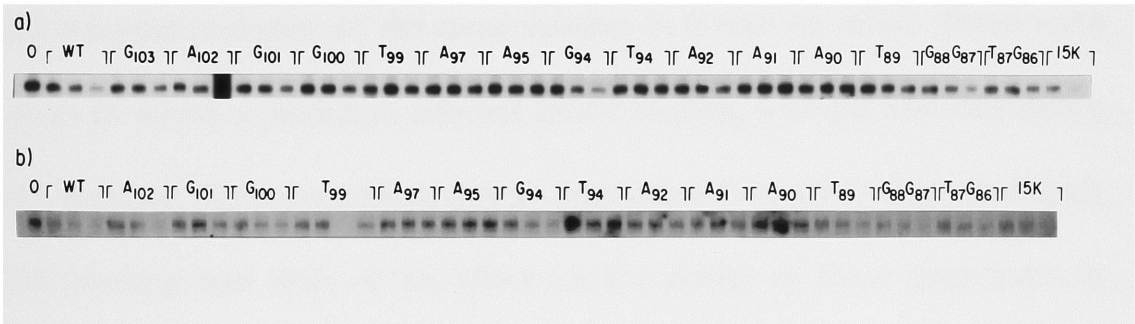
Figure 4.2 ISGF2 and ISGF3 bind to the ISRE sequences of ISG54 and ISG15 with different affinities. Radiolabeled ISG15 ISRE probe (-115 to -39) was used in gel retardation competition experiments with partially purified preparations of ISGF2 (**A**) and ISGF3 (**B**) as described in Materials and Methods. Lanes 1 show competition with vector and non-specific DNA only; binding reactions for lanes 2 contained 25 fold molar excess of the ISG54 ISRE sequence while lanes 3 represent a 75 fold molar excess. Lanes 4 show competition with a 25 fold molar excess of the ISG15 ISRE, and lanes 5 represent competition with a 75 fold excess of ISG15 sequences. In **A** the top band is nonspecific as indicated by its lack of competition by wild type DNA sequences. The ISGF2 specific complex is the bottom band. In **B,** the only gel band shown is the ISGF3 specific complex.


a)
1 2 3 4 5
b)
1 2 3 4 5

ISG54 sequences were tested at three concentrations of competing DNA equivalent to 25, 75, and 225 fold molar excess. Wild type competitor (lanes marked WT) reduced binding to the probe by approximately 95% at the highest concentration of competitor. In contrast, several mutant DNAs, e.g., M99 through 95 and M92 through 90, showed little or no ability to compete for formation of the ISGF3 complex. This and other similar results in four additional such competition experiments established that sequences flanking and including the two sets of three thymidine residues are crucial for ISGF3 binding to the ISRE. Note that mutations in residues 102, 89, and 87 also have significantly less competitive ability than wild type DNA for the ISGF3 complex indicating that ISGF3 contact points include these residues as well.

Not all changes at a particular position in the ISRE produced the same effect. One particularly dramatic example involves residue 94. Guanine is present at this site in the ISG15 sequence, whereas ISG54 has an adenine at this position. Transition of A to G in ISG54 did not affect the ability of this mutant ISG54 DNA to compete. However, a transversion of A to T at this site greatly reduced the competitive ability of the mutant sequence. Thus, a T at 94 abolished ISGF3 binding to the ISG54 ISRE while a G at the same position

Figure 4.3 Protein binding abilities of ISG54 point mutants. Gel retardation competition assays were performed with a radiolabeled ISG15 ISRE probe and different ISRE sequences. Mutated sequences are indicated above lanes by nucleotide and position of the substituted base. Lanes marked 0 represent competition with non-specific DNA only; lanes marked WT and 15K represent competition with wild type ISG54 and ISG15 ISREs, respectively. Competitor DNA was used in 25, 75, and 225 fold molar excess. **A**. Partially purified ISGF3. **B**. Partially purified ISGF2.


a)
O WT G103 A102 G101 G100 T99 A97 A95 G94 T94 A92 A91 A90 T89 G88G87T87G86 15K
b)
O WT A102 G101 G100 T99 A97 A95 G94 T94 A92 A91 A90 T89 G88G87T87G86 15K

allows normal interaction with ISGF3. The C at 101 also showed greater effect of transversions than transitions, similar to the finding at position 94.

The series of lanes in Fig 4.3B shows mutant DNA competition for formation of the ISGF2 complex. All mutations that impair ISGF2 complex formation (nucleotides 101-95 and residues 92-90) are included among mutations that also affected competition for the ISGF3 complex. Likewise, mutation of residue 94 to T abolished competition for ISGF2 as it did for ISGF3 whereas mutation of the same residue to G had no effect. There were no cases in which a mutation affected ISGF2 binding without also disrupting ISGF3 binding. However, mutations at residues 102 or 89-87 that do impair ISGF3 binding had little or no effect on the ability of these sequences to compete for ISGF2 binding. Thus it appears that the region of the ISRE important for ISGF2 recognition is contained within, but less extensive than, the ISGF3 binding site (nucleotides 102-87), but that the sequence requirements of most importance (101-90) are exactly the same for the two factors. The effect of mutations on the ability to compete for either ISGF2 or ISGF3 is summarized in Table 4.1.

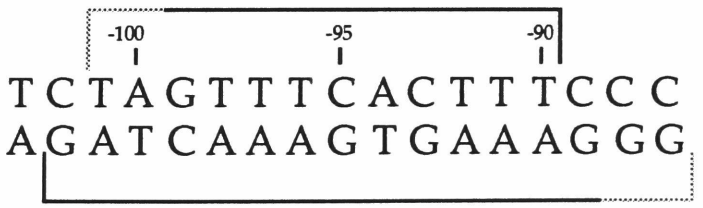
Protein interactions with mutant ISG15 sequences

Table 4.1 Wild type and mutant ISRE sequences. The numbered positions indicate nucleotides upstream from the ISG54 RNA initiation site and these numbered are used throughout. The ISG15 ISRE is inverted in its natural setting relative to this representation and corresponds to -111 to -94 from the ISG15 initiation site. Mutations are indicated by the nucleotides which differ from wild type with a subscript denoting the mutated position. Columns headed ISGF2 and ISGF3 indicate extent of binding of each sequence by partially purified preparations of the IFN induced nuclear factors. Column headed IND indicates the ability of each sequence to direct IFNα-dependent transcription *in vivo*. Minus (-) indicates less than 25% wild type activity for binding or expression; plus/minus (+/-) indicates 25-75% activity; and plus (+) indicates equal or nearly equal activity to that of the wild type sequence; nd=analysis not performed (see Figs 4.2 and 4.3). The lower diagram summarizes the nucleotide requirements for ISGF2 and ISGF3 binding to the ISRE. The heavy line indicates absolute requirement for binding while the light line indicates partial requirement.

SUMMARY OF ISRE MUTAGENESIS: BINDING AND FUNCTIONAL ANALYSIS

	100 95 90 85	ISGF2	ISGF3	IND
ISG54	T C T A G T T T C A C T T T C C C T T T	+	+	+
G_{103}	G	+	+	+
A_{102}	A	+	+/-	+/-
C_{101}	C	+	+/-	+/-
G_{101}	G	-	-	-
G_{100}	G	+/-	-	-
T_{99}	T	+/-	-	-
$A_{98}G_{97}$	A G	-	-	-
A_{97}	A	-	-	-
A_{95}	A	-	-	-
G_{94}	G	+	+	+
T_{94}	T	-	-	-
A_{92}	A	-	-	-
A_{91}	A	-	-	-
A_{90}	A	-	-	-
T_{89}	T	+	+/-	+/-
$G_{88}G_{87}$	G G	+	+	+
$T_{87}G_{86}$	T G	+	+/-	+/-
ISG15	C A G T T T C G G T T T C C C T T T	+	+	+
C_{99}	C	+	+	nd
G_{95}	G	-	-	nd
G_{89}	G	+	-	nd
G_{88}	G	+	-	nd

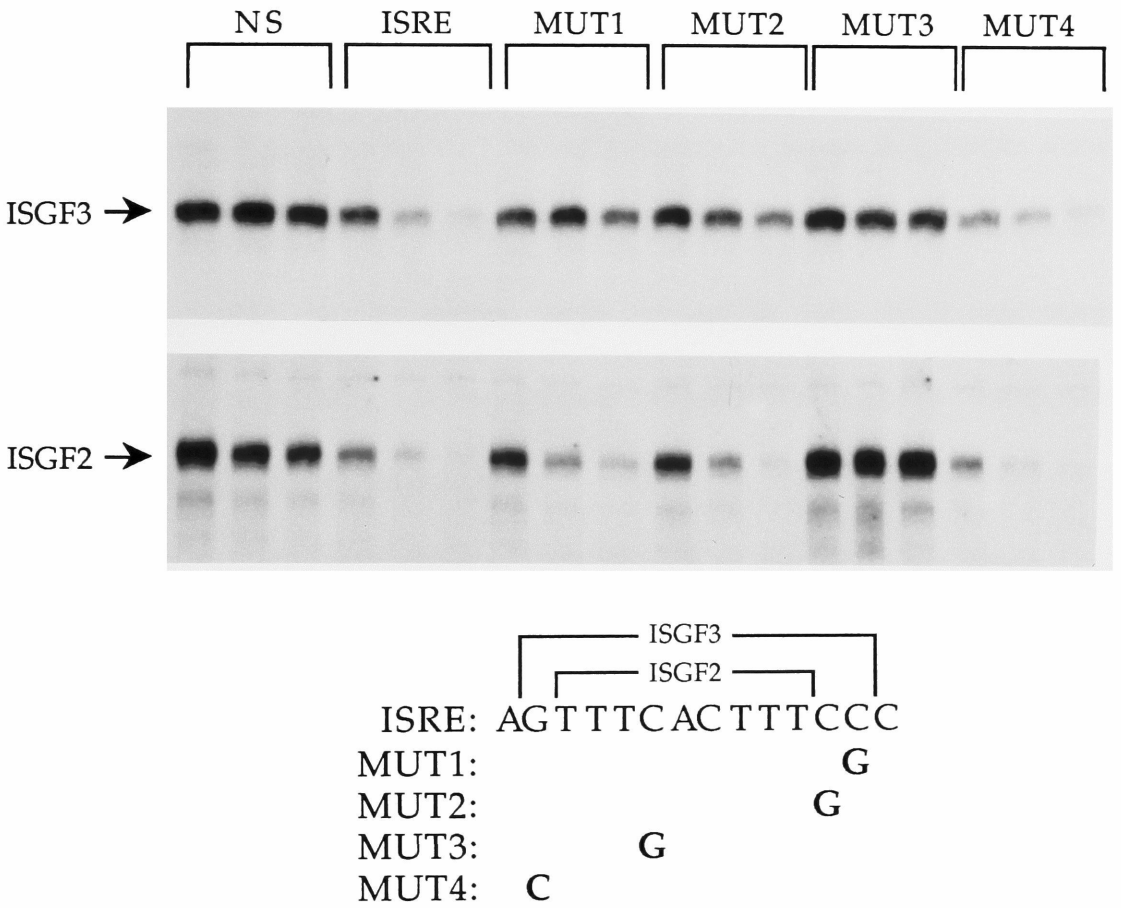
ISGF-2



ISGF-3

Since ISGF3 binding to ISG54 contacts nucleotides between -102 and -86, mutations were introduced into an ISG15 ISRE oligonucleotide at four sites within the equivalent region (actually -111 to -94 in the ISG15 sequence) as shown in Table 4.1. The effects of these mutations on ability to compete for ISGF3 and ISGF2 complex formation are shown in Fig 4.4, upper and lower panels, respectively. A significant loss of competition for ISGF3 was evident for mutations at positions 88, 89 and 95 (by the ISG54 numbering system); only the mutation at 99 retained near complete competitive ability. In contrast, the pattern of ISGF2 competition for these mutants was quite different. Not only was the mutation at 99 essentially wild type for ISGF2 competition, but also mutations at 88 and 89 were almost equal to wild type oligonucleotide in competitive ability. Only the mutation at 95 (in the middle of the ISRE) abolished competition for both ISGF2 and ISGF3 binding to the ISG15 oligonucleotide. These results strongly support the binding data with ISG54 mutants: central nucleotides from 90 to 100 are crucial for both ISGF2 and ISGF3 binding. However, residues flanking the core ISRE at 88, 89, and 102 play no role in ISGF2 binding while still being involved in ISGF3 recognition. Again, the broader ISGF3 binding site encompasses the ISGF2 binding domain.

Figure 4.4 Protein interactions with mutant ISG15 ISRE oligonucleotides. Radiolabeled ISG15 ISRE probe (-115 to -39) was used in competition gel retardation assays with partially purified ISGF3 (upper panel) or ISGF2 (lower panel) as described in Fig 4.3. The lower diagram summarizes the nucleotide requirements for the binding of ISGF2 and ISGF3 to the ISRE as well as the mutant sites used for competition.

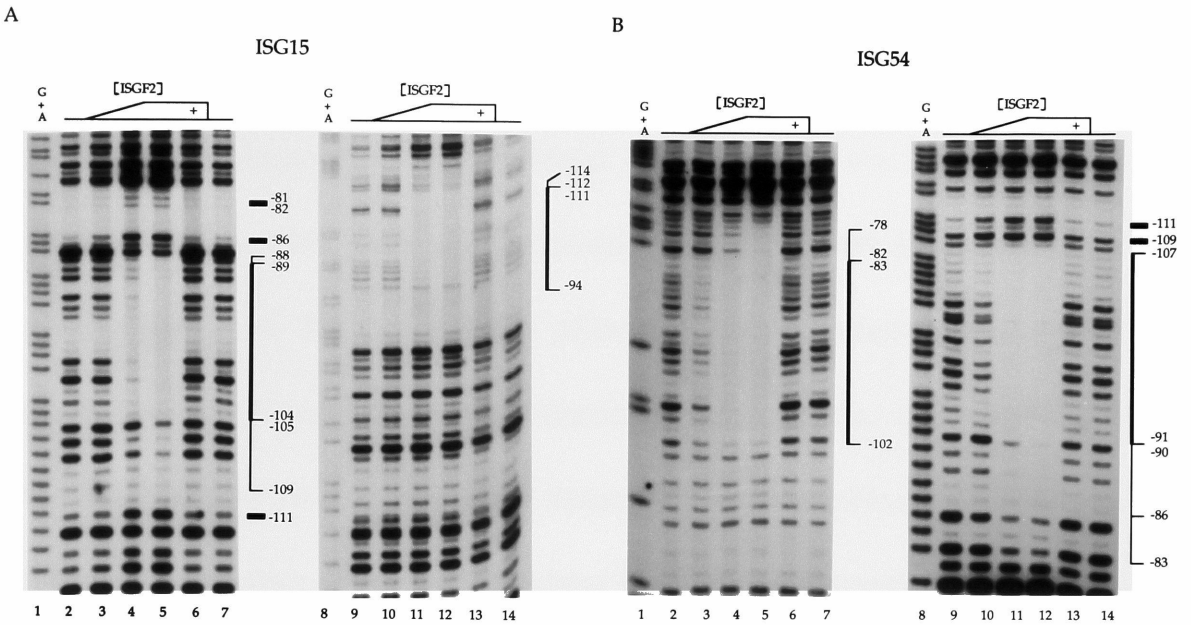

NS
ISRE
MUT1
MUT2
MUT3
MUT4
ISGF3
ISGF2
ISGF3
ISGF2
ISRE: AGTTTCACTTTCCC
MUT1: G
MUT2: G
MUT3: G
MUT4: C

DNAse I footprinting of ISGF2 and ISGF3

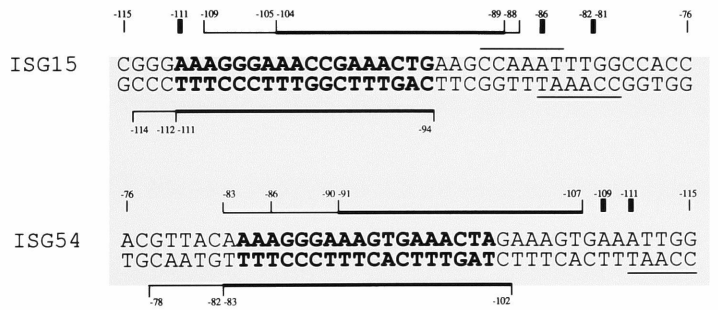
DNase I protection experiments (Fig 4.5) performed on both strands of fragments that contained the essential regulatory and promoter elements of either ISG15 or ISG54 (Reich et al., 1987; Levy et al., 1988a) showed that highly purified ISGF2 specifically protected only a small area of each strand, which encompassed the ISRE (compare lanes 4 and 5 to lane 6, or lanes 11 and 12 to lane 13 in panel A and panel B). No other sites in these fragments were bound by the purified preparation of ISGF2 (data not shown). Figure 4.5C shows schematically that protection with respect to each ISRE was very similar, although the ISREs are in opposite orientations in these two genes relative to the direction of transcription.

Similarly, specific protection of both strands of the ISG54 promoter fragment by ISGF3 was detected only at the ISRE (Fig 4.6). The nature of the protection by ISGF3 was distinct from that for ISGF2. ISGF3 binding did not result in strong hypersensitivity at neighboring sites, as was the case for ISGF2. In addition, the area of protection by ISGF3 was somewhat more extensive on the upper strand, as compared with ISGF2 protection, indicating that ISGF3 may contact additional nucleotides at the ISRE, consistent with the mutagenesis results.

Figure 4.5. Purified ISGF2 binds only to the ISRE region in ISG15 and ISG54 promoter fragments. (**A**) A 130 bp fragment including from -115 to -37 of the ISG15 promoter was 5' end-labeled either at the upstream end of one strand (left panel) or the downstream end of the other strand (right panel). Samples without ISGF2 had BSA instead, and samples without specific oligonucleotide had a nonspecific oligonucleotide. Lanes 1 and 8, a G plus A ladder was generated from the respective end-labeled fragments; lanes 2, 7, 9, and 14, no ISGF2; lanes 3 and 10, 0.8 ng of ISGF2; lanes 4 and 11, 1.6 ng of ISGF2; lanes 5 and 12, 3.0 ng of ISGF2; lanes 6 and 13, 3.0 ng of ISGF2 plus a 500-fold molar excess of specific competitor DNA. The position of changes in the DNase cleavage pattern are indicated to the right of each panel. Thick bars indicate DNase hypersensitivity, thin and thick lines indicate partial or complete protection against DNase digestion. (**B**) A 176 bp fragment of the ISG54, from -122 to +54, was labeled and treated as described for panel A. (**C**) The DNase footprint of ISGF2 around the ISRE in ISG15 or ISG54 is depicted schematically, as described for panel A, in relation to the nucleotide sequence of each strand. The ISRE is shown in bold type, and CCAAT boxes are marked by an adjacent line.


A
ISG15
G + A
[ISGF2]
+
-81
-82
-86
-88
-89
-104
-105
-109
-111
-114
-112
-111
-94
1 2 3 4 5 6 7
8 9 10 11 12 13 14
B
ISG54
-78
-82
-83
-102
-111
-109
-107
-91
-90
-86
-83

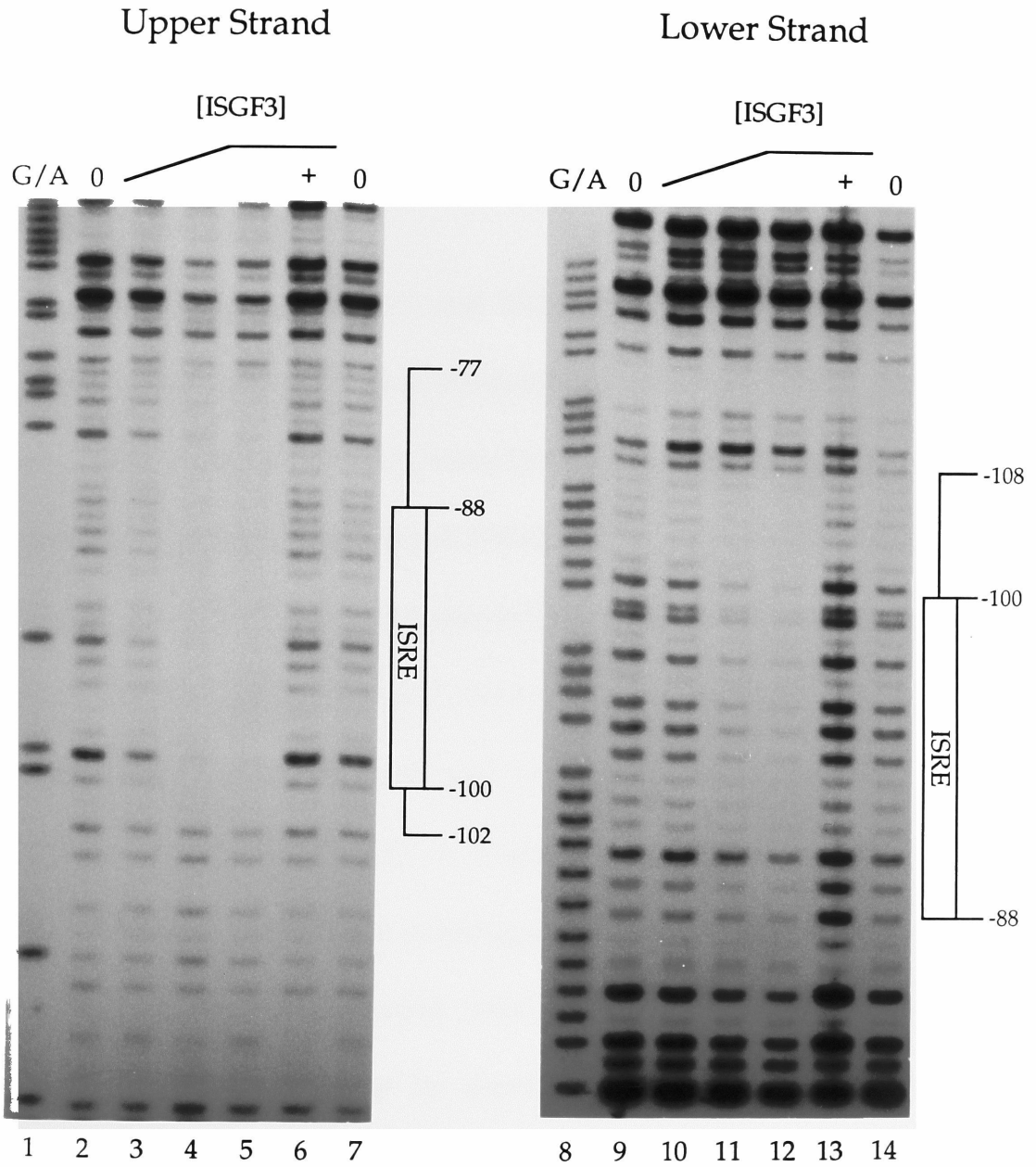
C


-115
-111
-109
-105 -104
-89 -88
-86
-82 -81
-76
ISG15
CGGGAAAGGGAAACCGAAACTGAAGCCAAATTTGGCCACC
GCCCTTTCCCTTTGGCTTTGACTTCGGTTTAAACCGGTGG
-114 -112 -111
-94
-76
-83
-86
-90 -91
-107
-109
-111
-115
ISG54
ACGTTACAAAAGGGAAAGTGAAACTAGAAAGTGAAATTGG
TGCAATGTTTTCCCTTTCACTTTGATCTTTCACTTTAACC
-78
-82 -83
-102

Functional effects ISG54 mutations

The proposition that ISGF3 functions in cells as a positive acting transcription factor responsive to IFN treatment suggests that protein binding to mutated ISRE sequences *in vitro* would correlate with the ability of these sequences to direct IFN dependent transcription *in vivo*. We have at present no functional test for mutations affecting the regulated decline in transcription occurring late in the response, although we do know from transcriptional analysis of promoter constructs in recombinant adenovirus vectors that repression of the IFN response is mediated through 5' flanking sequence (Levy et al., 1986). Therefore, in the present set of experiments we have used transient transfection assays to measure the ability of different ISRE sequences to activate transcription following IFN treatment. The mutations that had the greatest effect on fully induced levels of steady state mRNA corresponded to the same mutations that had the greatest effect on *in vitro* binding of ISGF3. Figure 4.7 shows representative examples of these assays. A number of mutants at nucleotides 100-90 reduced mRNA accumulation to background levels whether or not IFN was added to the transfected cells. The change at residue 94 to G (the ISG15 nucleotide at this position) did not affect function, but the transversion to T at this site eliminated transcriptional

Figure 4.6 ISGF3 binds specifically to the ISG54 ISRE. Conditions were identical to those described in Figure 4.5. Note that in contrast to ISGF2, ISGF3 protection of the upper strand (left panel) extends to the 3' end and causes no hypersensitivity.

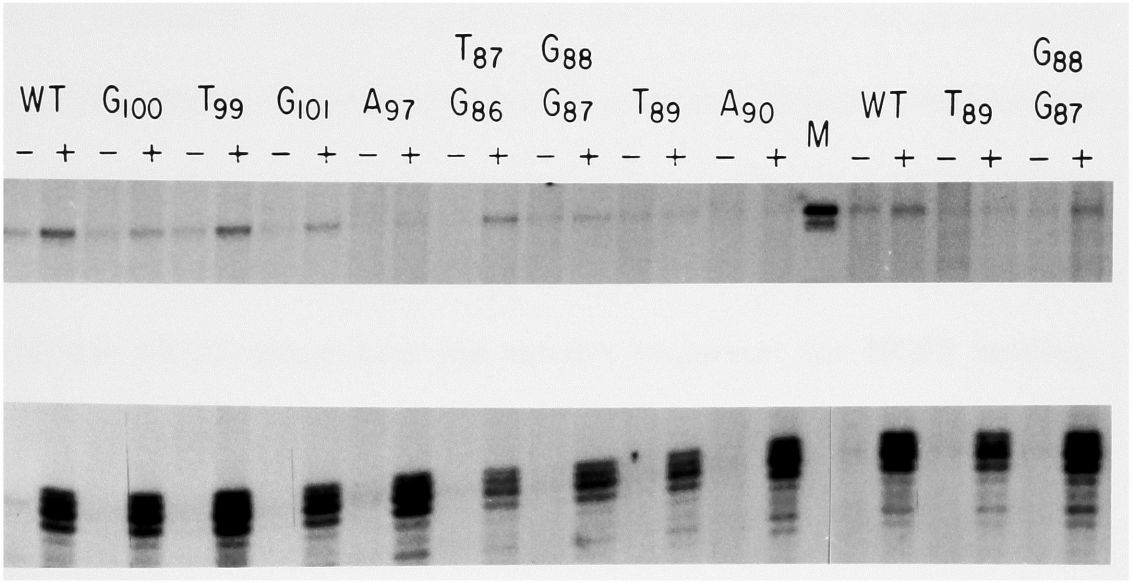

Upper Strand
[ISGF3]
G/A 0 + 0
-77
-88
ISRE
-100
-102
1 2 3 4 5 6 7
Lower Strand
[ISGF3]
G/A 0 + 0
-108
-100
ISRE
-88
8 9 10 11 12 13 14

activity as it did protein binding. The requirements for full inducibility implicate ISGF3 as the critical factor for transcriptional activity. Bases 89, 101, and 102 play a role in transcriptional activation because expression was decreased approximately 50% by mutations at these sites. The complex formed by ISGF3 binding to the ISRE involves the same nucleotides required for full inducibility (Table 4.1).

Photoaffinity crosslinking of ISGF2 and ISGF3

As a further step in the characterization of ISGF2 and ISGF3 interaction with the ISRE, proteins in contact with DNA were detected by photoaffinity crosslinking. High purity fractions of ISGF2 and ISGF3 were incubated with a uniformly labeled 5'-bromodeoxyuridine substituted oligonucleotide containing the ISG54 ISRE sequence. DNA-protein complexes were exposed to ultraviolet radiation in solution and covalently linked complexes were resolved by SDS-PAGE (Fig 4.8). A single 75kD complex was obtained for ISGF2, which corresponds to a polypeptide of ~65kD after adjustment for linked DNA. Complex formation was blocked by inclusion of excess unlabeled ISRE DNA, but not by unrelated DNA, thus establishing the specificity of ISRE binding (Fig 4.8A). For ISGF3, a specific 55kD complex was detected, corresponding to a polypeptide of ~45kD (Fig 4.8B). These

Figure 4.7 Transient expression of ISG54 point mutants. Plasmids containing ISG54 wild type or mutant ISRE sequences were transfected into HeLa cells. Specific RNA expression was measured by RNase protection without (-) or following (+) a 4 h treatment with IFNα. Upper portion shows the RNA signal from the expression construct; lower lanes shows expression from endogenous ISG54 used as control (see Materials and Methods). The lane marked M is a molecular size marker. The experiment shown is representative of the data, and the complete analysis involved at least two assays for each mutant construct listed in Table 4.1.

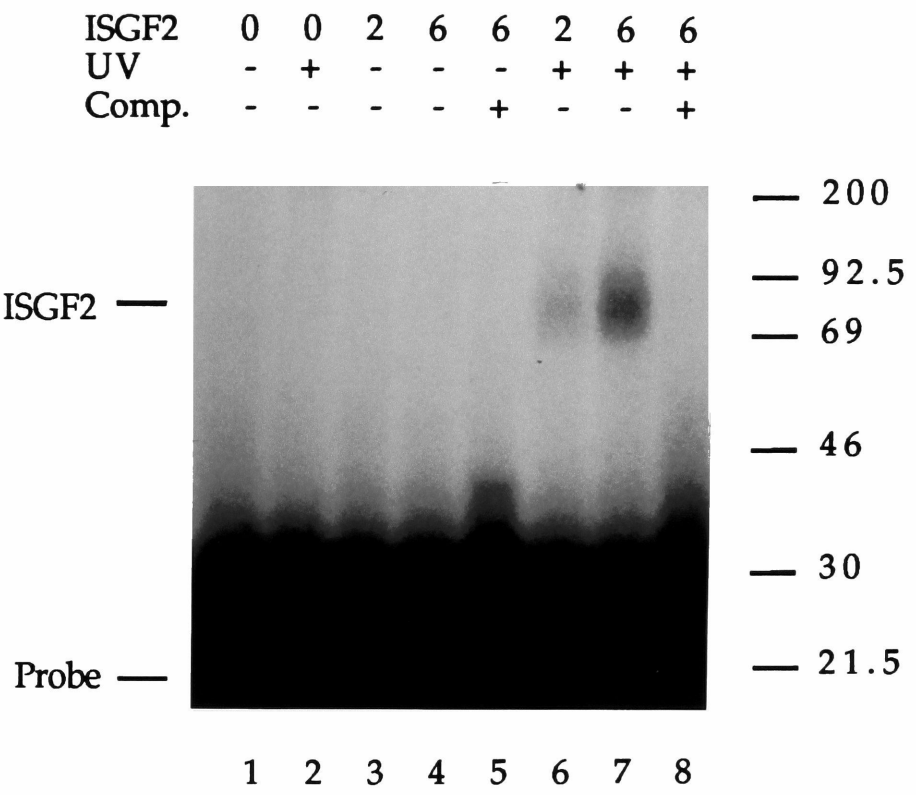

WT
G100
T99
G101
A97
T87 G86
G88 G87
T89
A90
M
WT
T89
G88 G87
– + – + – + – + – + – + – + – + – + – + – + – + – + – +

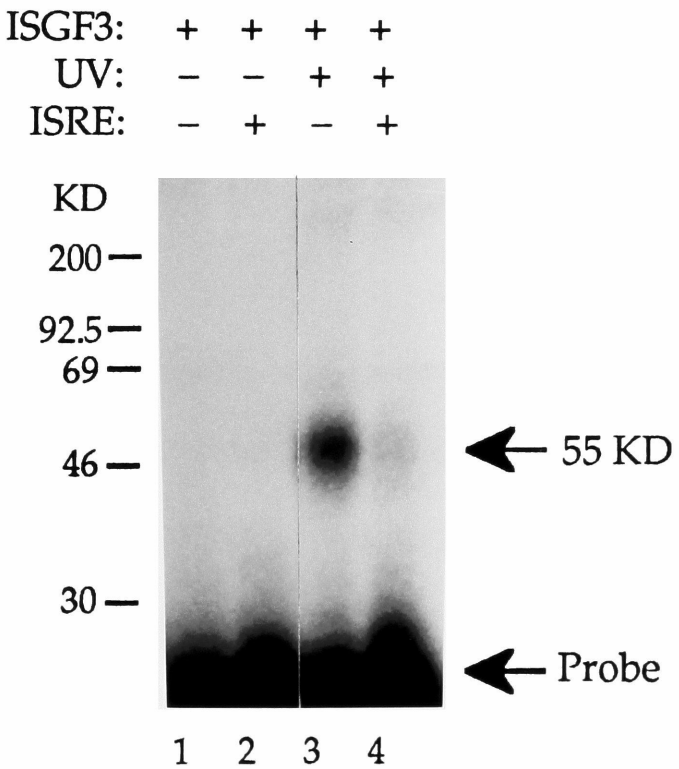
observations further support the nonidentity of ISGF2 and ISGF3 and provide an initial physical characterization of these ISRE-binding proteins.

Discussion

The experiments presented here show that, although ISGF2 and ISGF3 bind to essentially the same promoter element, subtle and presumably functionally important differences exist in the exact sequence requirements for binding of the two IFN induced nuclear factors. Thus the ISGF2 binding site is contained completely within the broader ISGF3 site, and ISRE residues critical for ISGF2 recognition are equally important for ISGF3 binding. However, these two factors differ in several significant characteristics: the factors form protein-DNA complexes with distinct electrophoretic mobilities; ISGF2 and ISGF3 can be separated into discrete fractions by column chromatography; and ISGF3 pre-exists in cells in an inactive form prior to IFN treatment which gains the ability to bind DNA following stimulation by IFN. ISGF2, in contrast, accumulates slowly and only when *de novo* protein synthesis is allowed following IFN addition (Levy et al., 1988a). Thus it seems likely that ISGF2 and ISGF3 are different proteins that compete *in vivo* for the same DNA binding site. Photoaffinity crosslinking confirms the

Figure 4.8 Photoaffinity crosslinking of ISGF2 and ISGF3 to the ISG54 ISRE. (Top panel) Samples from a UV crosslinking experiment (see Materials and methods) were used for SDS PAGE through a 10% separating gel, then the gel was dried and autoradiographed. The crosslinked complex between ISGF2 and the probe, and the free probe, are indicated at the left. Protein markers (Amersham), are indicated at the right. ISG54 ISRE oligonucleotide labeled with bromodeoxyuridine and [α-^{32}P]dCTP were used as the probe in a DNA-binding reaction in which addition of purified ISGF2, UV irradiation, and inclusion of a 500 fold molar excess of unlabeled ISG54 ISRE oligonucleotide were as follows: lane 1, no ISGF2, UV or competitor; lane 2, UV only, lane 3, ISGF2 only; lane 4, ISGF2 only; lane 5 ISGF2 plus competitor; lane 6, ISGF2 and UV; lane 7, ISGF2 and UV; lane 8, ISGF2 and UV, plus competitor. (Bottom panel) ISGF3 crosslinking as described for the top panel.


ISGF2 0 0 2 6 6 2 6 6
UV - + - - - + + +
Comp. - - - - + - - +
200
92.5
69
46
30
21.5
ISGF2
Probe
1 2 3 4 5 6 7 8


ISGF3: + + + +
UV: – – + +
ISRE: – + – +
KD
200
92.5
69
46
30
55 KD
Probe
1 2 3 4

proposition that these distinct binding activities represent different polypeptides (Fig 4.8).

ISGF2 binds to the nine residue central core of the ISRE. Seven of these residues are absolutely conserved in ISREs which have been demonstrated to be transcriptionally functional (Fig 4.1B and Reich et al., 1987; Levy et al., 1988a, 1988b; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988). Of the other two residues, position 93 is more variable than 94 which appears to require a purine. The ISG54 mutagenesis reported here reinforces the requirement for a purine at this position for both ISGF binding and for ISG expression, suggesting an important protein-DNA contact at this site which is not absolutely nucleotide specific. The sequence requirements for ISGF3 binding include all those for ISGF2, and in addition nucleotides that flank the core ISRE (positions 102, 101 and 89-87). These extended sequence requirements for ISGF3 binding correspond to the region necessary for maximal ISG54 expression.

DNAse protection experiments were consistent with the mutagenesis results. ISGF2 and ISGF3 both bound specifically to the ISRE, but no other sequence in the promoters of ISG15 and ISG54. However, qualitative differences in the precise pattern of protection point to important differences

in the interaction of these factors with the ISRE. ISGF2 protects the ISRE and causes strong hypersensitivity at neighboring sites (including a CCAAT sequence). On the other hand, ISGF3 protection of the ISRE region is somewhat more extensive on one strand and causes no hypersensitivity. The extent of ISRE protection by ISGF2 and ISGF3 is consistent with the differences in nucleotide requirements for binding, as defined through mutagenesis.

These findings are consistent with observations of variant cell lines that are resistant to the physiological effects of IFN (Kessler et al., 1988a). The resistant cell lines fail to arrest proliferation and cannot develop an antiviral state in response to IFN. We have found a corresponding inability of these cell lines to increase ISG transcription in response to IFN, and they do not activate the ISGF3 DNA binding factor. Taken together, these results strongly implicate ISGF3 as the IFN-stimulated gene activator acting through the ISRE DNA sequence. The role of ISGF2 in ISG regulation is, as yet, unclear (Pine et al., 1990), but the ability of this factor to bind a site contained with the ISGF3 binding site points to possible regulatory interactions between these factors. With sufficient amounts of these purified proteins, an analysis of ISGF3-ISGF2 regulatory interactions will become testable. We are developing an *in*

vitro RNA transcription system in which to test directly the activity of ISGF3. Such a system may also be required to fully understand the role of ISGF2 in the IFN response.

It is interesting to contrast ISG transcriptional induction in response to IFN with the induction of IFN genes themselves when activated by virus infection. For example, mutagenesis of the transcriptional regulatory domain of human β–IFN has suggested positive and negative control regions within this promoter as well (Goodbourn and Maniatis, 1988). In fact, there is an eight nucleotide identity between the positive acting domain of β–IFN (-74 to -67; PRD-I) and the ISRE central core (98 to 91). Indeed, we have found that the regulatory domain of the β–IFN gene will effectively compete for ISGF2 binding although it does not bind appreciably to ISGF3 (see Chapter 5). Furthermore, IFN genes and ISGs respond to distinct physiological signals and act in different arms of the cellular response to viral infection. Further purification and characterization of ISGFs as well as IFN gene factors should define any additional correspondences between these interrelated inducible gene families.

Materials and methods

ISRE mutagenesis

Mutagenesis of ISG54 was carried out in an expression construct in which promoter sequences spanning from 122 nt upstream of the RNA initiation site to 55 nt into exon 1 were fused to a reporter sequence, an adenovirus 5 E1b gene fragment (Levy et al., 1988a), and cloned into a multi-functional plasmid containing an M13 origin of replication (BlueScribe, Stratagene) as diagrammed in Fig 4.1A. Single-stranded templates containing occasional uridine residues substituted for thymidine (Kunkel, 1985) were used for oligonucleotide-primed mutagenic repair performed as described previously (Craik et al., 1985) using mixed synthesis oligonucleotides (McNeil and Smith, 1985; Hutchison et al., 1986) produced using a mixture containing 3% of each of 3 incorrect nucleotides and 91% of the wild type nucleotide at each position (obtained from MCRC, Midland, TX). Successfully repaired plasmids were selected for ability to transform wild type bacteria (Kunkel, 1985), and each mutation was identified by DNA sequencing (Sanger et al., 1977). Site-directed mutagenesis of ISG15 was carried out by complete synthesis of four sets of 18 nt complementary oligonucleotides spanning nucleotides -111

to -94 of the ISG15 promoter, each containing a single deviation from the wild type sequence as shown in Table 4.1.

Binding assays

Protein-DNA binding assays were performed by gel retardation (Levy et al., 1988a; Garner and Revzin, 1981; Fried and Crothers, 1981). Partially purified preparations of ISGF3 or ISGF2 were incubated with 0.25ng of radiolabeled ISG15 promoter fragment containing the ISRE sequence (-115 to -39) in the presence of nonspecific DNA [4 μg double stranded poly(dIdC)] and a constant amount of total plasmid DNA consisting of varying amounts of wild-type or mutant ISRE sequence. The ability of each mutant DNA sequence to compete with labeled wild type ISG15 for binding to ISGF2 and ISGF3 was determined by comparison to competitions using wild type or vector DNA. Purification of ISGF2 and ISGF3 involved chromatography on heparin agarose, phosphocellulose, DEAE cellulose, non-specific and specific oligonucleotide affinity resins, and Mono Q FPLC and has been reported elsewhere (Pine et al., 1990; X-Y. Fu et al., submitted).

DNAse I protection assay

For DNase footprinting, 5% glycerol was substituted for Ficoll in the standard gel shift binding reactions. Either 1.0 μg of BSA or varying amounts

of purified ISGF2 or ISGF3, as indicated in the legend to Figure 4.5, were mixed with 2 ng of 5'-labeled ISG15 or ISG54 promoter fragments and a 500 fold molar excess of nonspecific oligonucleotide, or where indicated a 500 fold molar excess of a homologous ISRE oligonucleotide. The samples were incubated for 30 min at 22°C, then 30 min at 4°C. Finally 0.1 volumes of DNase I, at 20 ng/μl in 10 mM $CaCl_2$, was added and incubation was continued for an additional 4 min at 4°C. The reactions were stopped with excess EDTA and proteinase K digestion, DNA was recovered and electrophoresed on 8% acrylamide/8 M urea denaturing gels and the gels were dried and autoradiographed.

Photoaffinity crosslinking

A standard gel shift binding reaction was set up with either 1 μg BSA or high purity ISGF2 or ISGF3 fractions, and a uniformly labeled, 5'-bromodeoxyuridine substituted oligonucleotide containing the ISG54 ISRE sequence (Chodosh et al., 1988a). Reactions were incubated for 30 min at 22°C and samples were placed on ice and exposed to ultraviolet irradiation for 5 min in a Stratalinker 2400 (Stratagene). Included in the reactions was a 500-fold molar excess of ISRE or unrelated oligonucleotide. Products were resolved by 10% SDS-PAGE. Gels were fixed, dried and autoradiographed.

Expression assays

ISG54 mutants were tested functionally for IFN stimulated transcriptional activity by transient transfection. Plasmid DNA (10 μg of specific DNA) was introduced into HeLa CL2.2 cells by $CaPO_4$ precipitation (Graham and van der Eb, 1973), and accumulated RNA levels were measured by RNase protection (Levy et al., 1988a; Melton et al., 1984) 72 to 96 h later, with or without a final 4 h treatment with 15 pM IFN-α (500 u/ml). This regimen has been found to circumvent artifactual stimulation of ISGs by DNA-$CaPO_4$ precipitates (Pine et al., 1988) and allows transient assays for IFN stimulated transcription (Reich et al., 1987). RNase protection assays were quantitated by densitometry and normalized for RNA recovery and for transfection efficiency by measuring endogenous γ–actin production (Enoch et al., 1986), endogenous ISG54 expression, and chloramphenicol acetyl transferase activity derived from cotransfected RSVcat (Gorman et al., 1982). HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and antibiotics. Recombinant bacterial IFN-αA was a generous gift from Dr. P. Sorter (Hoffman-La Roche, Nutley, NJ).

Chapter 5

Cytoplasmic activation of ISGF3

Introduction

Cellular responses to changes in the extracellular environment require information transfer from outside the cell to the interior. When extracellular stimuli elicit transcriptional responses, the signal transduction process reports information from the cell exterior to the nucleus. Responses to small molecules such as ions, heavy metals, and steroids, or to physical agents such as heat may be mediated by direct transmission of the inducing agent into the cell, in some cases into the nucleus (Maniatis et al., 1987; Minghetti and Norman, 1988; Furst et al., 1988; Westin and Schaffner, 1988; Greenberg et al., 1985). However, a profoundly different situation is presented by responses to polypeptide stimuli, and perhaps also by induction dependent on cell-cell or cell-matrix contact, in which internalization of the ligand is not required for transcriptional stimulation. In a growing number of cases, rapid changes in expression of limited sets of genes have been linked to occupation of specific cell surface receptors by polypeptide ligands (Larner et al., 1984; Friedman et al., 1984; Friedman and Stark, 1985; Greenberg and Ziff, 1984; Lau and Nathans, 1985; Lau and Nathans, 1987; Almendral et al., 1988; Prywes and Roeder, 1986; Greenberg et al., 1986b; Zullo et al., 1985) or to direct cell-cell

contact (Clayton and Darnell, 1983). Since many cytokines and growth factors lead to fluctuations in the levels of one or more small molecule "second messengers" in target cells, it is possible that signal transduction is mediated through the actions of specific protein kinases affected by these agents (Nishizuka, 1986; Berridge, 1987; Rozengurt, 1986; Kikkawa and Nishizuka, 1986; Yamamoto et al., 1988a). Although it has been shown that agents which artificially perturb the intracellular levels of second messengers can lead to changes in gene transcription (Riabowol et al., 1988a; Greenberg et al., 1986a; Fisch et al., 1987; Pine et al., 1988) it is not at all clear how the specificity of each individual ligand for stimulating a limited set of genes can be maintained if the signal passes through such broadly affected pathways. We have been studying the regulation of gene expression by interferon-α (IFNα) with the ultimate goal of understanding the specific molecules involved in one such signal response pathway.

Interferons α and β were among the first polypeptide ligands demonstrated to directly and rapidly regulate transcription of a limited set of specific target genes in human cells (Larner et al., 1984; Friedman et al., 1984; Friedman and Stark, 1985; Larner et al., 1986; Levy et al., 1986). Although IFNα

stimulated genes (ISGs) are characterized as primary response genes (i.e., IFNα treatment triggers transcriptional induction which is detectable within 5-10 min and is mediated by pre-existing protein components), it has not been possible to document any involvement of known second messengers, kinases, or previously identified transcription factors in this response (Larner et al., 1984; Tamm et al., 1987; Lew et al., 1989). Our previous studies have implicated an IFN-stimulated DNA binding protein (ISGF3) as the positive transcriptional activator of ISGs (Levy et al., 1988a). This factor recognizes a conserved regulatory sequence, the IFNα stimulated response element (ISRE), present in all ISGs so far characterized (Levy et al., 1988a, 1988b; Reich et al., 1987; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988; Israel et al., 1986; Israel et al., 1987; Shirayoshi et al., 1988; Wathelet et al., 1987; Vogel et al., 1986; Gribaudo et al., 1987; Reid et al., 1989). The ISRE acts as an orientation independent, IFN dependent enhancer element (Reich et al., 1987) and serves as the binding site in vitro and probably in vivo (Levy et al., 1988a; Kessler et al., 1988a; Dale et al., 1989b) for two IFNα induced protein factors as well as for at least one constitutive factor.

ISGF3 has been implicated as the ISG transcriptional activator: its

binding activity appears in cells immediately following IFNα treatment with no requirement for de novo protein synthesis (Levy et al., 1988a). Extensive point mutagenesis of the ISRE demonstrated that the binding specificity of only this protein correlated with ISG expression (Kessler et al., 1988a; Levy et al., 1988a). Furthermore, activation of ISGF3 occurs in IFNα sensitive cells to an extent commensurate with the degree of transcriptional induction of ISGs but is not detected in cells resistant to IFNα (Kessler et al., 1988b; Levy et al., 1990). Thus, the signal transduction pathway initiated by IFNα interaction with its cell surface receptor involves activation of a latent precursor of ISGF3. In this chapter, we provide evidence that latent ISGF3 resides in the cytoplasm of uninduced cells where it is activated through a series of at least three steps initiated by IFNα treatment. An undefined modification of one component of ISGF3, stimulated by IFNα-receptor interaction, leads to association with a second polypeptide in the cytoplasm, which is followed by translocation of the active ISGF3 complex to the nucleus. The time course of these events in vivo correlates with the appearance and disappearance of nuclear ISGF3 and with the transcriptional cycle of ISGs. We have reconstituted in vitro the second step, combination of two proteins to form

active ISGF3, from cytoplasmic extracts of appropriately treated cells.

Results

ISGF3 appears in the cytoplasm of IFNα treated cells.

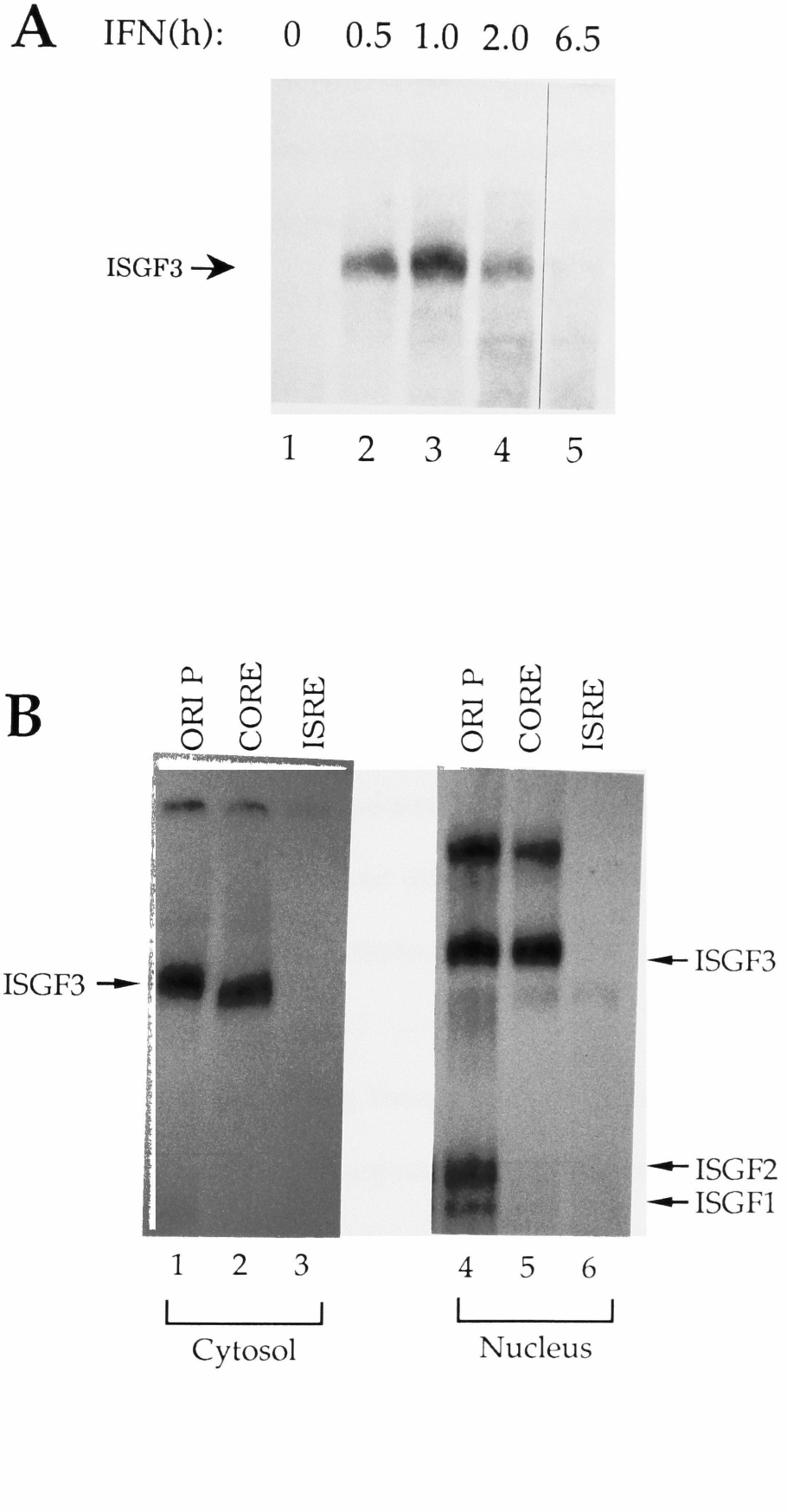
We have used gel retardation analysis (Levy et al., 1988a; Fried and Crothers, 1981; Garner and Revzin, 1981) to detect ISRE binding factors in IFNα treated cells using a labeled DNA fragment containing the ISRE from an ISG promoter. ISGF3 was detected in nuclear extracts (Levy et al., 1988a) prepared from IFNα treated cells with a time course comparable to the transient nature of ISG transcription (Fig 5.1A). Although no ISGF3 binding activity was detectable in extracts from uninduced cells (lane 1), abundant ISGF3 was observed after 30 min to 2 h of IFNα treatment (lanes 2-4), but had disappeared by 6.5 h (lane 5), a time when ISG transcription had returned to basal levels (Larner et al., 1986). Although no ISRE specific DNA binding activities were detectable in cytoplasmic fractions of untreated cells (not shown), ISGF3 was readily detected in cytoplasm after 30 min of IFNα treatment (Fig 5.1B). We have previously shown that the entire ISRE sequence is required for full ISG transcription and for ISGF3 binding. This

binding specificity differs from that of ISGF1 or ISGF2, nuclear factors whose recognition is limited to the central core sequence of the ISRE (Kessler et al., 1988a). Therefore, differential competition by core and full-length ISRE oligonucleotides provided an assay for ISGF3-specific binding. All specific DNA-protein complexes, including ISGF3, were competed by a full-length, unlabeled ISRE oligonucleotide (lanes 3 and 6). However, the binding of cytoplasmic and nuclear ISGF3 was not competed by oligonucleotides containing non-specific sequences (lanes 1 and 4) or by oligonucleotides containing only the central core of the ISRE (lanes 2 and 5). In contrast, the binding of nuclear ISGF1 and ISGF2 was effectively competed by the core sequence (lane 5). Thus, both cytoplasmic and nuclear ISGF3 displayed the same sequence specificity, matching the genetically defined requirements for ISG transactivation (Kessler et al., 1988a).

Active ISGF3 accumulates first in the cytoplasm and later in the nucleus.

The detection of active ISGF3 in the cytoplasm of IFNα treated cells suggested that the active factor might appear in the cytoplasm of treated cells prior to nuclear accumulation. Since ISG transcription is detectable within minutes after IFNα treatment, cytoplasmic and nuclear extracts were prepared

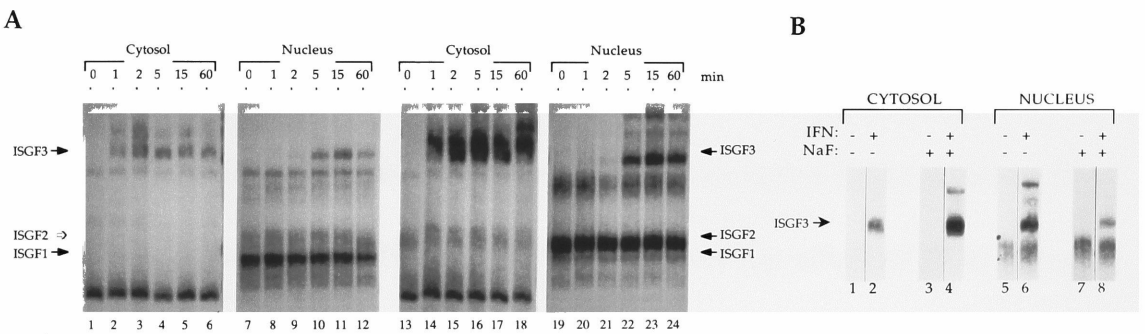
Figure 5.1 ISGF3 is induced by IFNα in both cytoplasm and nucleus. **A**. Nuclear extracts of human fibroblasts treated with IFNα for the indicated times were analyzed by gel retardation using a labeled ISRE probe. **B**. Nuclear and cytoplasmic extracts from HeLa cells primed with IFNγ for 16 h and treated with IFNα for 15 min were subjected to competition gel retardation analysis. The slower mobility complex present in lanes 4 and 5 is observed using extracts containing high amounts of ISGF3 (either nuclear or cytoplasmic, e.g., Fig 5.2B) and may result from dimer formation. The sequence of the double stranded oligonucleotide containing an ISRE core equivalent was 5'-AGGAATTTCCCACTTTCACTTCTC-3'; the ISRE oligonucleotide sequence was 5'-GGCTTCAGTTTCGGTTTCCCTTTCCCGAG-3'.


A
IFN(h):
0
0.5
1.0
2.0
6.5
ISGF3
1
2
3
4
5
B
ORI P
CORE
ISRE
ORI P
CORE
ISRE
ISGF3
ISGF3
ISGF2
ISGF1
1
2
3
4
5
6
Cytosol
Nucleus

after brief exposure of cells to IFNα (Fig 5.2A). ISGF3 was detected in cytoplasmic extracts prepared within 1 min of IFNα treatment, increased in extracts made after 5 min, and was detectable in extracts prepared after 60 min (lanes 1-6). Nuclear extracts, in contrast, displayed no ISGF3 activity until 5 min of IFNα treatment (lane 10), reaching maximal levels only after 15 min (lane 11). These extracts were prepared by mechanically shearing cells suspended in hypotonic salt solutions. An S100 fraction obtained by centrifugation of the post-nuclear supernatant contained all cytoplasmic ISGF3, suggesting that proteins with this binding activity are freely soluble in crude cytoplasm and are not associated with particles or membranes. Nuclear ISGF3, on the other hand, is not removed from nuclei by additional washes with hypotonic buffer, but rather is recovered by extraction in 0.3 M NaCl. The differential time of appearance and distinct extractability of cytoplasmic and nuclear ISGF3 imply that the cytoplasmic form is not due to artifactual leakage from the nucleus.

IFNα and IFNγ, despite acting through distinct cell-surface receptors, can act in concert to produce synergistic interactions leading to mutual reinforcement of cellular response. In HeLa cells, this synergistic response is

Figure 5.2 ISGF3 appears in the cytoplasm prior to being translocated to the nucleus. **A.** Cytoplasmic and nuclear extracts of HeLa cells were prepared after IFNα treatments for the indicated times and assayed by gel shift. Lanes 1-12 show results from cells treated only with IFNα; extracts for lanes 13-24 were derived from cells pretreated with IFNγ for 18 h. The positions of ISGF1 and ISGF2 from nuclear extracts and ISGF3 from nuclear and cytosolic extracts are indicated by arrows. ISGF2 is present only in nuclear extracts from IFNγ-treated cells because the IFNα treatments were too short to allow accumulation of this factor (Levy et al., 1988a; Pine et al., 1990). **B.** NaF partially inhibits nuclear translocation of ISGF3. HeLa cells pretreated for 18 hr with IFNγ and treated for 15 min with IFNα and 10 mM NaF as indicated were tested for the presence of cytoplasmic and nuclear ISGF3.


A
Cytosol
Nucleus
Cytosol
Nucleus
0 1 2 5 15 60
min
ISGF3
ISGF2
ISGF1
1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 23 24
B
CYTOSOL
NUCLEUS
IFN:
NaF:
ISGF3
1 2 3 4 5 6 7 8

initiated by cooperative induction of IFNα stimulated genes (ISGs). Although they were only negligibly responsive to IFNγ, combined treatment of cells with IFNγ followed by IFNα resulted in an approximately 10-fold increase in ISG transcription. Consistent with the positive regulatory role of ISGF3, IFNγ pretreatment accounted for a 10-fold higher IFNα-dependent induction of ISGF3 as compared to levels induced by IFNα alone (Levy et al., 1990). We inferred that this IFNγ "priming" effect resulted from formation of a large pool of latent ISGF3 that can be converted into the active DNA binding form after cell treatment with IFNα (Fig 5.3). We tested the effect of IFNγ priming on the kinetics of cytoplasmic and nuclear ISGF3 accumulation (lanes 13-24). Although much higher levels of ISGF3 were detected (the autoradiograph for lanes 13-24 was exposed for 1/6 as long as that for lanes 1-12), the initial cytoplasmic appearance and the time course of cytoplasmic and nuclear ISGF3 accumulation were identical to those detected in cells treated with IFNα alone.

The temporal lag between cytoplasmic and nuclear accumulation of ISGF3 suggested that cytoplasmic ISGF3 might be a precursor to the nuclear form. In other experiments directed at understanding the biochemical basis

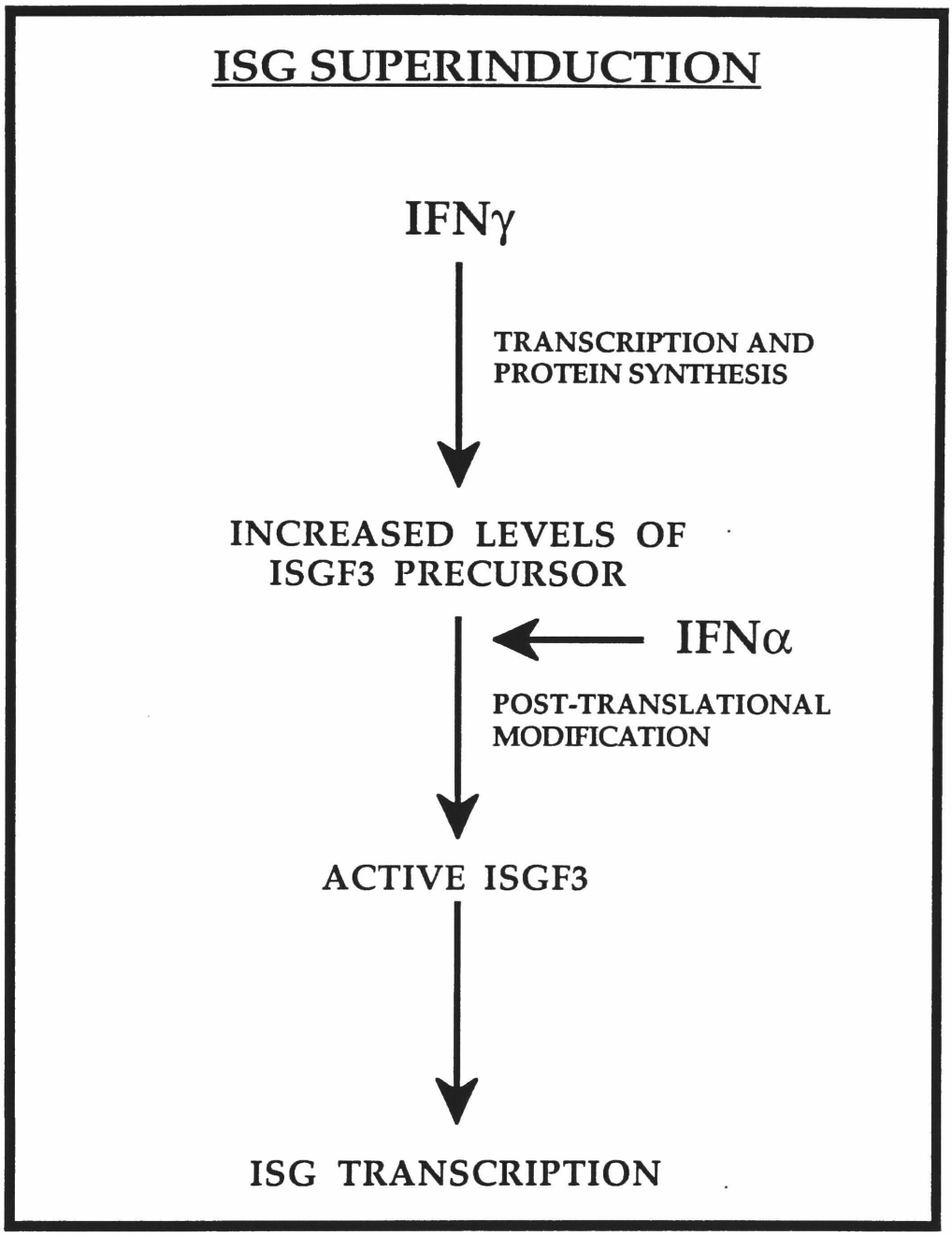


Figure 5.3 Model of IFNα and IFNγ synergy resulting in superinduction of ISGF3 levels and ISG transcription. Treatment of cells with IFNγ, by a processes requiring ongoing transcription and translation, increases the intracellular quantity of inactive ISGF3 precursor. The larger pool of precursor is post-translationally activated by subsequent IFNα treatment resulting in superinduced levels of ISGF3 which directs transcriptional activation of ISGs (Levy et al., 1990).

of the IFN-induced signal pathway, we have tested many pharmacological inhibitors for effects on the IFN response. Of many agents tested, only NaF had an inhibitory effect on ISG expression, reducing ISG54 expression to approximately 20% of the fully induced levels (data not shown). We therefore tested this agent for effects on cytoplasmic and nuclear accumulation of ISGF3. As shown in Fig 5.2B, 10 mM NaF significantly inhibited the accumulation of nuclear ISGF3 in response to IFNα (compare lane 8 with lane 6), even in cells pretreated with IFNγ in order to induce very high levels of this DNA binding activity. Normalizing to the level of the constitutive activity ISGF1, NaF reduced nuclear accumulation of ISGF3 by 5-fold. The corresponding cytoplasmic extracts, in contrast, showed 2-3 fold increased levels of ISGF3 following IFNα treatment in the presence of NaF (compare lane 4 with lane 3). Therefore, the inhibitory effect of NaF on ISG expression is not due to an absence of the initial activating signal generated by IFNα receptor occupation or to an inability to activate ISGF3, but rather results from a decrease in translocation of this factor to the nucleus.

The biochemical basis for this inhibition is not known. Fluoride is known to form AlF_4 in the presence of trace aluminum, a compound which

structurally mimics the γ-phosphoryl group of nucleotide triphosphates and exhibits properties resembling non-hydrolyzable analogues of GTP (Sternweis and Gilman, 1982). However, we have found no evidence for participation of GTP- or other nucleotide triphosphate-dependent proteins in IFNα stimulated transcription using known inhibitors and activators of G-protein, kinase, or adenylate cyclase coupled processes (Larner et al., 1984; D. Lew et al., 1989). The NaF block of ISGF3 nuclear localization is compatible with inhibition of some process requiring shuttling of phosphoryl groups. In this context, we have found that cytoplasm to nuclear transit of ISGF3 also is inhibited at low temperatures, requiring several hours instead of minutes at 15° C or less, results compatible with an energy requirement for translocation (data not shown).

Complementation of cytoplasm from IFNγ-treated cells with cytoplasm from IFNα-treated cells reconstitutes ISGF3 activation in vitro.

If ISGF3 were in fact activated in the cytoplasm from latent precursors, we hoped to find conditions for reproducing this activation in vitro. As discussed earlier, we have previously found that IFNγ priming of HeLa cells appears due to synthesis of high levels of latent ISGF3 (Levy et al., 1990). We

reasoned that extracts of IFNα treated cells might be capable of activating this latent form of ISGF3 from IFNγ treated cells (Fig 5.4)

Therefore, we mixed cytoplasmic extracts from untreated, IFNα-treated, and IFNγ-treated cells, and quantitated the resulting levels of ISGF3 by gel retardation (Fig 5.5). No cytoplasmic ISGF3 was detected in extracts from untreated (lane 1) or IFNγ-treated cells (lane 3), and, because limited amounts of extract were used, little ISGF3 was detectable in IFNα cytoplasm (lane 2). However, when cytoplasmic extracts from IFNα-treated cells were mixed with extracts from IFNγ-treated cells, high levels of ISGF3 were produced (lane 6). This level of ISGF3 was comparable to the high level detected in extracts from IFNγ-primed cells following IFNα treatment in vivo. This in vitro-produced activity displayed legitimate ISGF3 binding specificity as demonstrated by differential competition with core (lane 7) and full (lane 8) ISRE oligonucleotides. A low but detectable level of ISGF3 was also produced by mixing IFNα cytoplasm with cytoplasm from untreated cells (lane 4), indicating that, as expected, untreated cells contain only modest levels of unconverted, latent ISGF3. No ISGF3 was produced by mixing untreated cytoplasm with IFNγ-treated cytoplasm (lane 5); neither of these preparations

Figure 5.4 Schematic representation of *in vitro* activation of ISGF3. Cytoplasmic extracts (S100) prepared from cells treated either with IFNα or IFNγ are mixed *in vitro*. An IFNα-stimulated activity (present in the cytoplasmic extracts of IFNα-treated cells) activates the latent precursor to ISGF3 (present in large quantities in the cytoplasmic extracts of IFNγ-treated cells) producing ISGF3 *in vitro*.

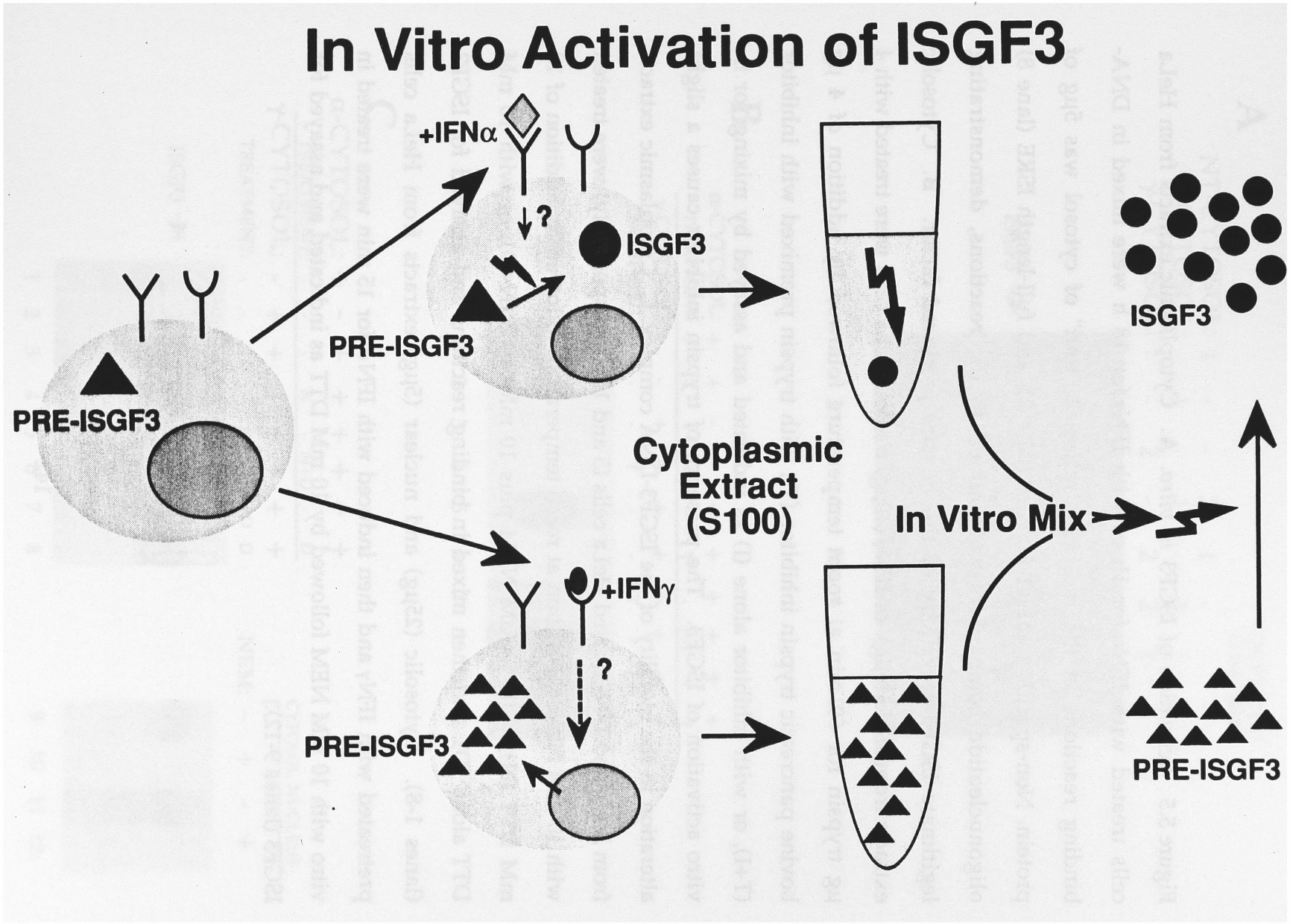
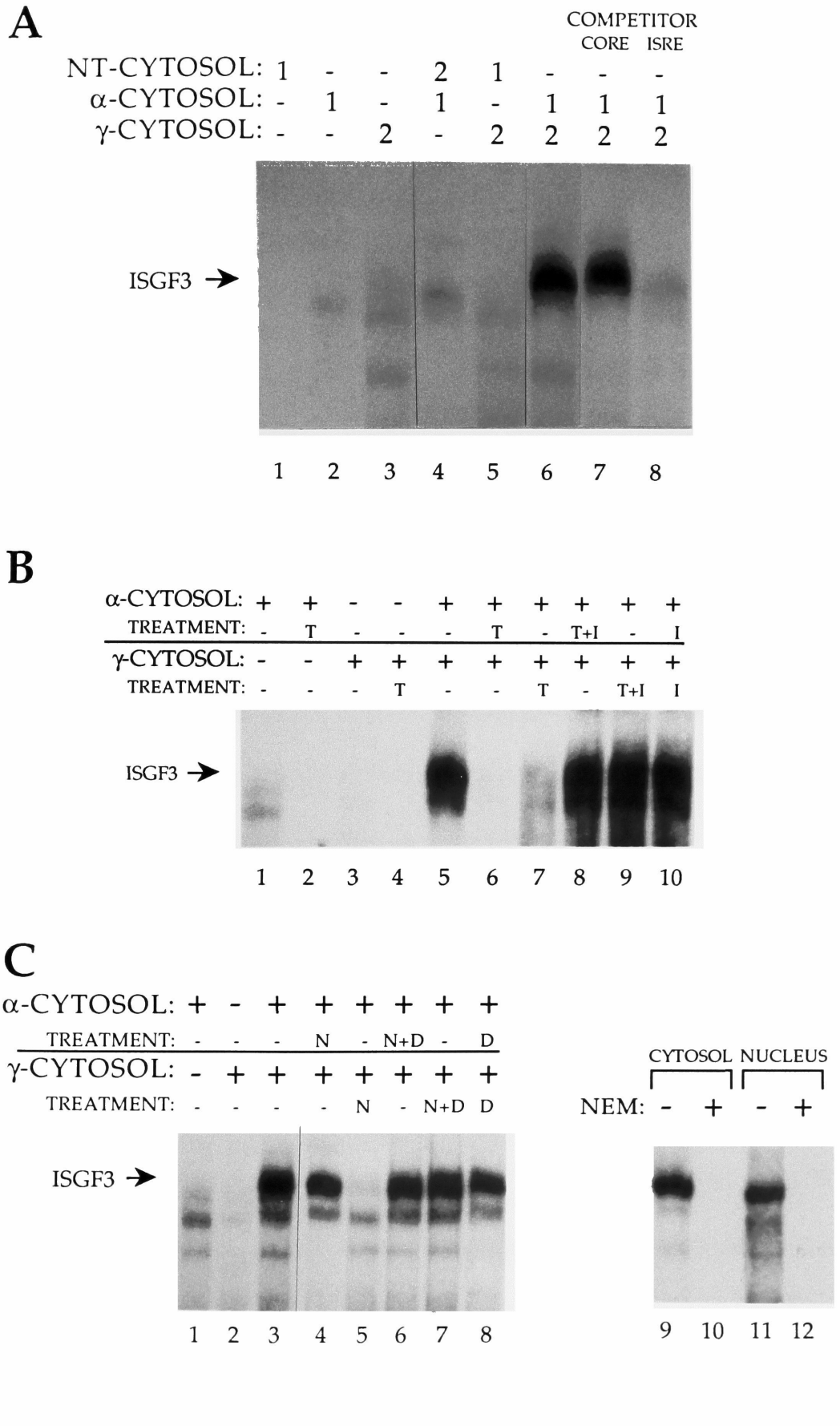

In Vitro Activation of ISGF3
+IFNα
?
ISGF3
PRE-ISGF3
PRE-ISGF3
Cytoplasmic
Extract
(S100)
ISGF3
In Vitro Mix
+IFNγ
?
PRE-ISGF3
PRE-ISGF3

Figure 5.5 Activation of ISGF3 *in vitro*. **A**. Cytoplasmic extracts from HeLa cells treated with IFNα for 1h or with IFNγ for 18 h were mixed in DNA-binding reactions in the indicated ratios. 1 "unit" of cytosol was 5μg of protein. Non-specific (lanes 1-6), core (lane 7), and full-length ISRE (lane 8) oligonucleotide competitors were added to the reactions, demonstrating legitimate ISGF3 specificity of the *in vitro* produced factor. **B**. Cytosolic extracts from IFNα (5μg) or IFNγ (10μg) treated HeLa cells were treated with 4 μg trypsin for 60 min at room temperature followed by addition of 4 μg bovine pancreatic trypsin inhibitor (T), with trypsin premixed with inhibitor (T+I), or with inhibitor alone (I) as indicated and assayed by mixing for in vitro activation of ISGF3. The presence of trypsin inhibitor causes a slight alteration in the mobility of the ISGF3-DNA complex. **C**. Cytoplasmic extracts from IFNα and IFNγ treated HeLa cells (5 and 10 μg, respectively) were treated with 10 mM NEM for 10 min at room temperature followed by addition of 10 mM DTT (N), with 10 mM NEM plus 10 mM DTT (N+D), or with 10 mM DTT alone (D), and then mixed in binding reactions and assayed for ISGF3 (lanes 1-8). Cytosolic (25μg) and nuclear (5μg) extracts from HeLa cells pretreated with IFNγ and then induced with IFNα for 15 min were treated in vitro with 10 mM NEM followed by 10 mM DTT as indicated and assayed for ISGF3 (lanes 9-12).


A
COMPETITOR
CORE ISRE
NT-CYTOSOL: 1 - - 2 1 - - -
α-CYTOSOL: - 1 - 1 - 1 1 1
γ-CYTOSOL: - - 2 - 2 2 2 2
ISGF3
1 2 3 4 5 6 7 8
B
α-CYTOSOL: + + - - + + + + + +
TREATMENT: - T - - - T - T+I - I
γ-CYTOSOL: - - + + + + + + + +
TREATMENT: - - - T - - T - T+I I
ISGF3
1 2 3 4 5 6 7 8 9 10
C
α-CYTOSOL: + - + + + + + +
TREATMENT: - - - N - N+D - D
γ-CYTOSOL: - + + + + + + +
TREATMENT: - - - - N - N+D D
ISGF3
1 2 3 4 5 6 7 8
CYTOSOL NUCLEUS
NEM: - + - +
9 10 11 12

would be expected to contain a necessary IFNα-activated signal.

Active ISGF3 is formed from distinct protein components which act stoichiometrically

We have attempted to find agents or treatments capable of selectively inhibiting or activating in vitro formation of ISGF3. NaF and decreased temperature in vivo inhibited nuclear ISGF3 accumulation and ISG expression. However, neither incubation at sub-physiological temperature nor inclusion of NaF in the in vitro reaction inhibited formation of ISGF3 using cytoplasmic extracts from IFNα and IFNγ treated cell (data not shown). Similarly, a large number of other pharmacological agents tested at concentrations known to affect kinases, phosphatases, G-proteins, proteases, as well as other cellular activities, had no effect on ISGF3 formation in vitro (Table 5.1) just as they had no effect in vivo (data not shown). Susceptibility of in vitro ISGF3 formation to protease treatment of cytoplasmic extracts indicated that protein components from both IFNα and IFNγ treated cells are necessary for ISGF3 formation in vitro (Fig 5.5B).

Protein alkylation was found to inhibit ISGF3 activity and in vitro formation (Fig 5.5C). Nuclear or cytoplasmic extracts were treated with 10 mM N-ethyl maleimide (NEM) under conditions which result in alkylation

Table 5.1 Agents which failed to affect the cell-free formation of ISGF3

TREATMENT	CONCENTRATION
H7	45 μM
H8	45 μM
HA1004	45 μM
2-Aminopurine	45 μM
EDTA	5 mM
EGTA	5 mM
NaPPi	2 mM
Sodium Vanadate	0.5 mM
NP-40	1%
Formamide	8%
AMP-PNP	5 mM
GMP-PNP	5 mM
GTPγS	5 mM
Hexokinase + glucose	0.1 mg/ml + 10 mM
PMSF	0.5 mM
Aprotinin	3 μg/ml
Leupeptin	0.5 μg/ml
Pepstatin	1.5 μg/ml
Bovine pancreatic trypsin inhibitor	0.4 mg/ml

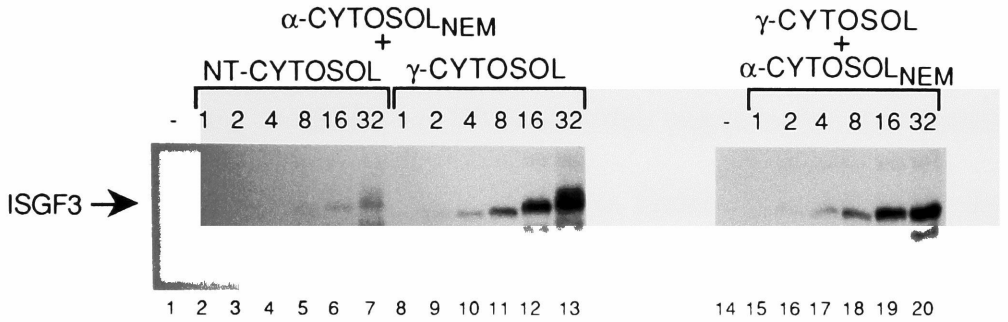
of reduced cysteine residues, and then dithiothreitol (DTT) was added to inactivate any remaining NEM. This treatment effectively eliminated the ability of ISGF3 formed in vivo to bind DNA (lanes 9-12). In addition, NEM treatment inactivated a cytoplasmic component from IFNγ cells which was necessary for ISGF3 formation in vitro (lane 5). However, it had no effect on necessary components from IFNα treated cell extracts (lane 4). These results indicated that at least two different proteins, one present in IFNα treated cells (termed "ISGF3α component") and one present in increased amounts in IFNγ treated cytoplasm (therefore termed "ISGF3γ component") were involved in ISGF3 formation. Because of its NEM sensitivity, we suspected that at least the IFNγ inducible component is part of nuclear ISGF3 and that it is the inactivation of this component by NEM that results in loss of ISGF3 binding activity.

We have exploited the NEM sensitivity of mature ISGF3 and of the ISGF3γ component to investigate the stoichiometry and kinetics of ISGF3 formation in vitro (Fig 5.6A). Cytoplasmic extracts from IFNα treated cells were treated with NEM and quenched with DTT to deplete ISGF3 activated in vivo but retain active ISGF3α. Constant amounts of this NEM treated extract

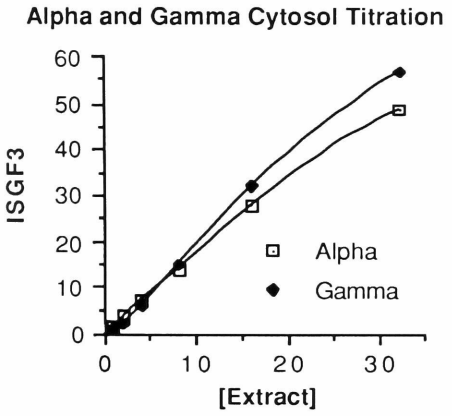
(lane 1) were mixed with increasing amounts of cytoplasmic protein from non-treated or from IFNγ treated cells. Low but linearly increasing amounts of ISGF3 were formed in response to increasing amounts of cytoplasm from untreated cells (lanes 2-7), while much higher and again linearly increasing amounts were formed after addition of increasing extract from IFNγ treated cells. Similarly, a linear increase in ISGF3 formation was observed in the converse titration when a constant amount of extract from IFNγ treated cells was mixed with increasing amounts of cytoplasm from IFNα treated cells (lanes 8-20 and quantitated in Fig 5.6B). Although we had initially hypothesized that ISGF3 formation resulted from some enzymatic action of an IFNα-activated component on a protein substrate from the IFNγ-induced cytoplasm, the direct dependence of product on starting concentration of either component suggested that both are participating stoichiometrically in product formation rather than either one functioning catalytically. The linearity and approximately equal slopes of product formation in response to addition of either component, and the lack of effect of standard inhibitors (Table 5.1), are best explained by subunit association to form an active complex.

Figure 5.6 Stoichiometry of in vitro activation of ISGF3. **A.** Cytoplasmic extracts from HeLa cells treated for 15 min with IFNα were treated in vitro with 10 mM NEM followed by 10 mM DTT to inactivate ISGF3. 4.5 μg of this extract (lane 1) were added to increasing amounts of cytoplasm (in arbitrary units equal to approximately 0.56μg protein) from non-treated (lanes 2-7) or from IFNγ-treated cells (lanes 8-13) in two-fold increments from 0.56 μg (1 unit) to 18 μg (32 units); 4.5 μg of cytoplasm from IFNγ-treated cells (lane 14) were mixed with similar amounts of NEM-treated cytoplasm from IFNα-treated cells (lanes 15-20). In vitro activation of ISGF3 was determined by gel shift assay following a 20 min incubation at room temperature. **B.** The autoradiograph shown in (**A**) was quantitated by densitometry for lanes 8-13 (filled symbols) and for lanes 15-20 (open symbols). **C.** Cytoplasmic extracts from IFNα and IFNγ treated cells were mixed and incubated at room temperature in the absence of labeled DNA for the indicated times followed by addition of probe and a 20 min binding reaction. The reactions contained 2.25 μg (lanes 1-3) or 9 μg (lanes 4-6) of each extract, as indicated by units as in **A.**

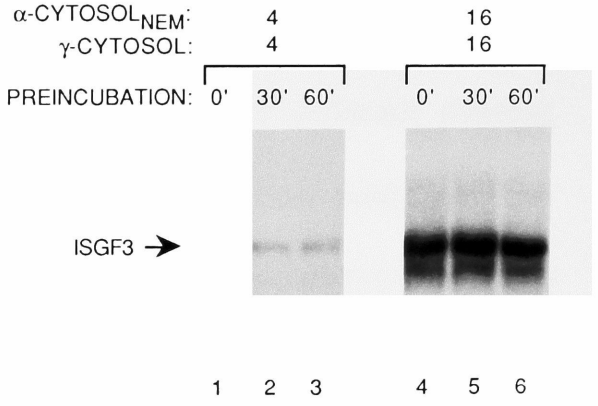
A


α-CYTOSOL$_{NEM}$
+
NT-CYTOSOL
γ-CYTOSOL
- 1 2 4 8 16 32 1 2 4 8 16 32
γ-CYTOSOL
+
α-CYTOSOL$_{NEM}$
- 1 2 4 8 16 32
ISGF3
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20

B


Alpha and Gamma Cytosol Titration
ISGF3
60
50
40
30
20
10
0
0
10
20
30
[Extract]
Alpha
Gamma

C

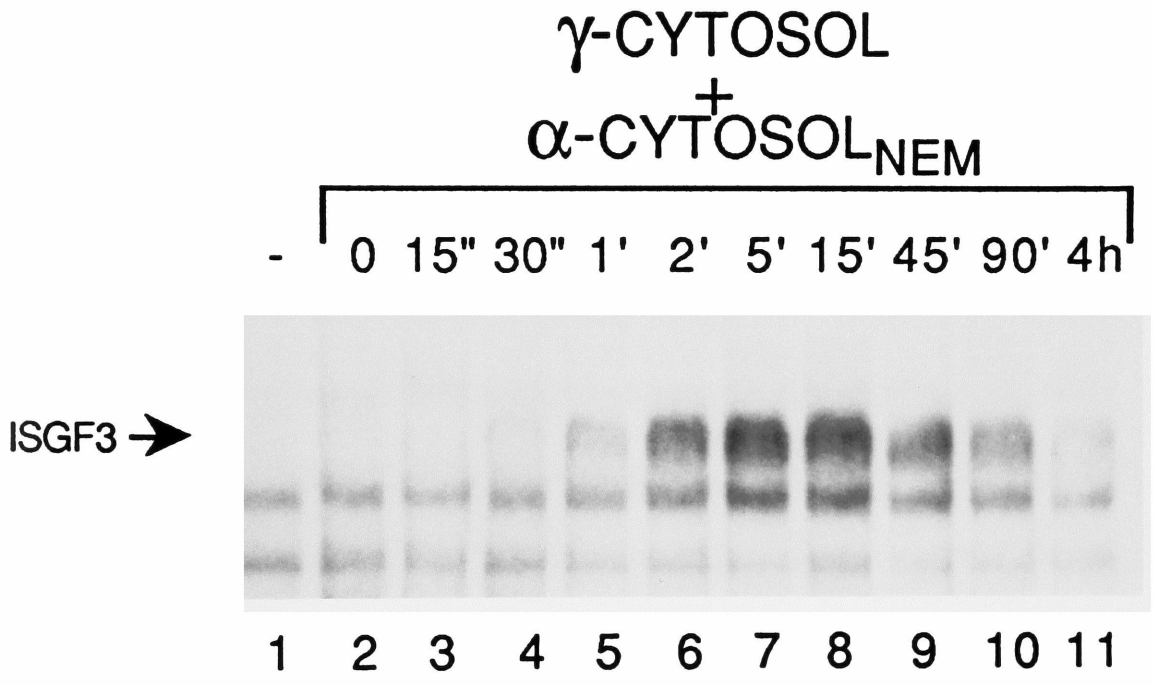

α-CYTOSOL$_{NEM}$: 4 16
γ-CYTOSOL: 4 16
PREINCUBATION: 0' 30' 60' 0' 30' 60'
ISGF3
1 2 3 4 5 6

The apparently non-enzymatic nature of ISGF3 formation in vitro prompted an examination of the temperature and kinetic parameters of the reaction. Varying reaction temperatures over the range from 4°-30°C had no effect on in vitro ISGF3 formation (data not shown). Likewise, no effect of increased time of incubation was observed. We have previously determined that optimal binding of ISGF3 to DNA in vitro requires 20 min incubations with probe (data not shown). No increase in ISGF3 formation was detected following incubation times longer than the standard 20 min (Fig 5.6C). The levels of ISGF3 formed following 30 min (lanes 2 and 5) or 60 min (lanes 3 and 6) pre-incubations were equal to those observed when no pre-incubations were allowed. This result was obtained with various different starting concentrations of extract. Thus, neither component could act catalytically on excess amounts of the other to produce increased levels of ISGF3. These results are not adequately explained by hypothesizing a rapidly decaying catalytic component because preincubation of the separate extracts prior to mixing led to no significant loss in activity (data not shown). We conclude that ISGF3 is an active DNA-binding complex formed from the stoichiometric association of ISGF3α and ISGF3γ components.

Rapid activation of ISGF3α component in vivo

The association of two precursor components to form active ISGF3 suggested that one result of IFNα action on cells is the conversion of a precursor of ISGF3α component to a form capable of associating with ISGF3γ component. Given the rapidity of IFNα action in vivo (see Fig 5.2), we tested the time course of activation of the ISGF3α component of the cell-free reaction. HeLa cells were treated with IFNα for different lengths of time, from 15 sec to 4 hr, cytoplasmic extracts were prepared, and endogenously produced ISGF3 was inactivated with NEM. The presence of active ISGF3α was determined by addition of excess cytoplasm from IFNγ treated cells followed by gel retardation analysis (Fig 5.7). Active ISGF3α component was detectable after 30 sec of IFNα treatment (lane 4) and reached maximal levels after 5-15 min (lanes 7 and 8). Increased time of IFNα treatment resulted in gradually decreasing levels of ISGF3α component capable of forming ISGF3 in vitro (lanes 9-12), although residual levels were still detectable after 4 h. These in vitro data are consistent with the time course of accumulation of cytoplasmic ISGF3 in vivo (compare with Fig 5.2) and indicate that the rate of ISGF3 formation in vivo is determined by the rate at which ISGF3α

Figure 5.7 Time course of ISGF3α component activation *in vivo*. Cytoplasmic extracts were prepared from HeLa cells treated with IFNα for the indicated times and treated with NEM to inactivate ISGF3. These extracts were mixed with cytoplasm from IFNγ-treated cells and assayed for ISGF3 production.

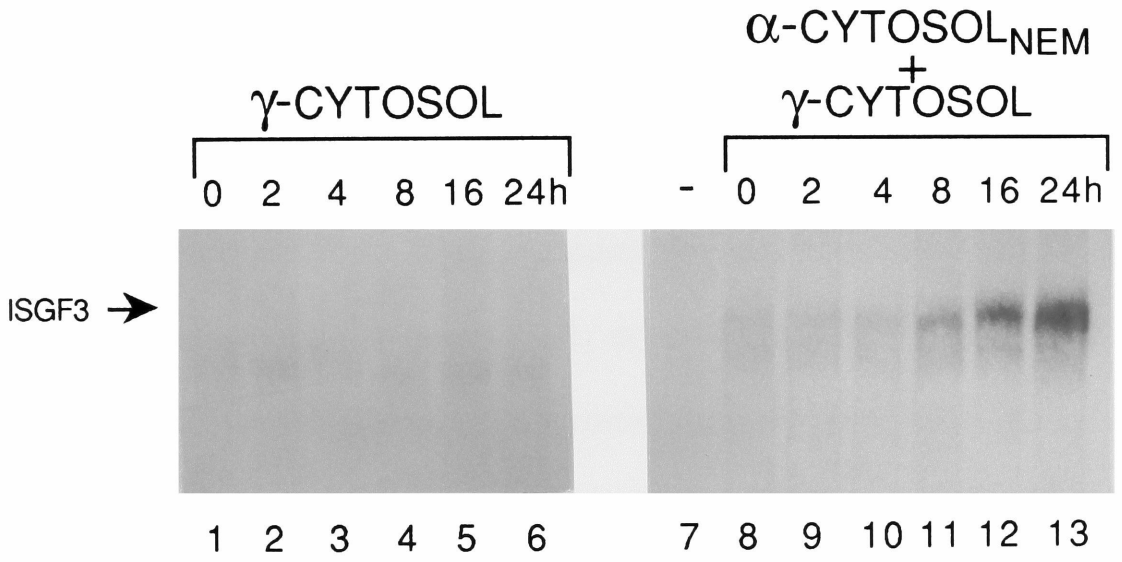

γ-CYTOSOL
+
α-CYTOSOL$_{NEM}$
- 0 15" 30" 1' 2' 5' 15' 45' 90' 4h
ISGF3
1 2 3 4 5 6 7 8 9 10 11

component is activated in response to IFNα.

IFNγ priming induces ISGF3γ component in vivo

Since ISGF3γ was detected in greater abundance in IFNγ treated HeLa S3 cells, we tested whether this factor accumulated with a time course consistent with IFNγ priming. Cytoplasmic extracts were prepared from HeLa cells treated with IFNγ for various lengths of time from 2-24 h. No ISGF3 was detected in these extracts since the cells had not been exposed to IFNα (Fig 5.8 lanes 1-6). These extracts were subsequently mixed with cytoplasmic extracts prepared from IFNα treated cells which had been treated *in vitro* with NEM to eliminate any ISGF3. Gel retardation assays performed on the mixed extracts detected *in vitro* formed ISGF3 (lanes 7-13), indicating that ISGF3γ component was induced in the cytoplasm of IFNγ treated cells over a time period consistent with the synergistic effect previously noted for transcriptional induction (Levy et al., 1990). This component, present in extracts from untreated cells, was elevated over its basal level after approximately 8 h of treatment with IFNγ, and continued to increase in abundance through 24 h of treatment. In contrast, no change in the level of ISGF3α was detected during IFNγ priming. Rather, this factor was apparently

Figure 5.8 Time course of ISGF3γ induction in HeLa S3 cells. Cytoplasmic extracts were prepared from HeLa S3 cells treated for the indicated times with 5 ng/ml IFNγ. These extracts were analyzed by gel shift for factors binding to the ISG15 ISRE probe without (lanes 1-6) or with addition of NEM-treated cytoplasmic extracts prepared from IFNα treated cells (lanes 8-13). The NEM-treated extract from IFNα-treated cells had no binding activity on its own (lane 7).


γ-CYTOSOL
0 2 4 8 16 24h
α-CYTOSOL$_{NEM}$
+
γ-CYTOSOL
- 0 2 4 8 16 24h
ISGF3
1 2 3 4 5 6
7 8 9 10 11 12 13

present in excess over ISGF3γ (see Chap 6).

Diploid fibroblasts have high constitutive levels of ISGF3γ

The diploid human fibroblast cell line FS2 responds dramatically to both IFNα and IFNγ (Larner et al., 1986; Decker et al., 1989). However, these cells showed no effect of IFNγ pretreatment on subsequent induction of ISG expression or of ISGF3 activation by IFNα. These cells showed the same induced levels of ISG transcription following IFNα induction irrespective of previous exposure to IFNγ. Nuclear extracts prepared from IFNα treated FS2 cells showed the same induced levels of ISGF3 whether or cells were pretreated with IFNγ, showing again a correlation between the abundance of ISGF3 and the level of IFNα induced transcription. ISGF3 was only detected in extracts from cells which had been treated with IFNα, and no differences in abundance of ISGF3 were found after IFNγ priming (Levy et al., 1990).

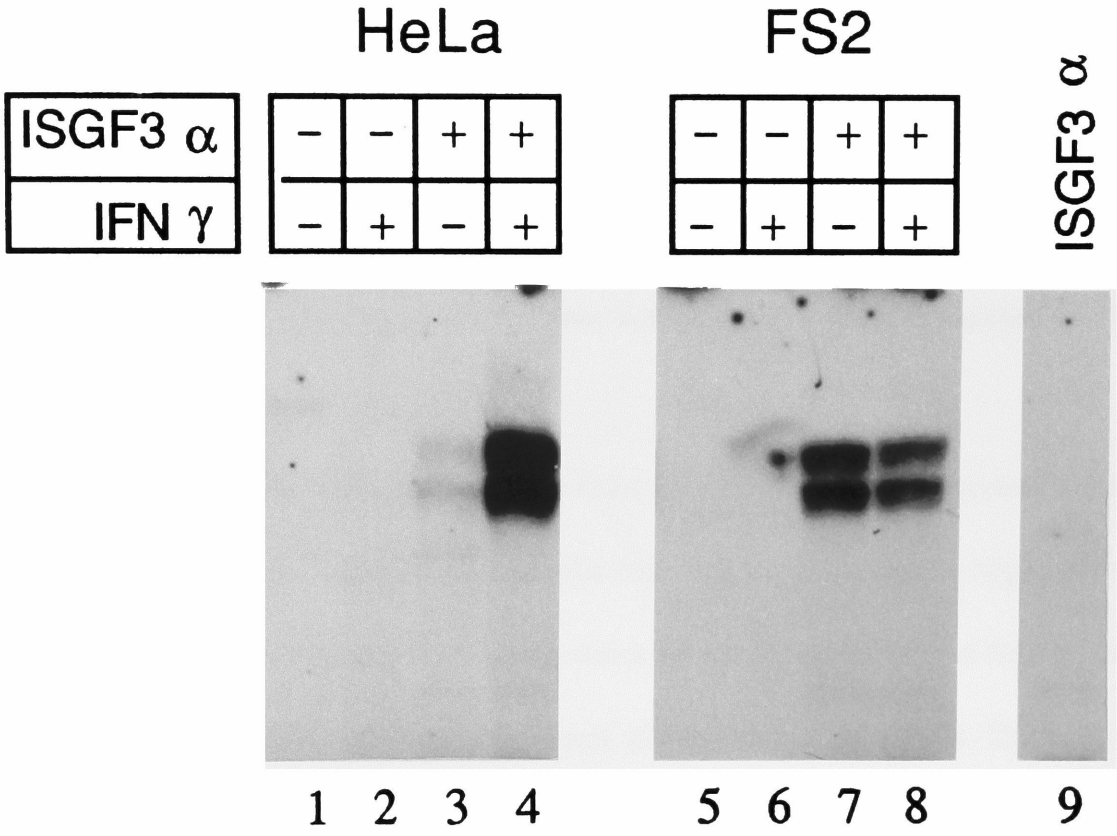
Based on comparisons of extracts made from approximately equal numbers of cells, the level of ISGF3 in FS2 cells following treatment with IFNα is comparable to that in HeLa cells following treatment with both IFNγ and IFNα. This finding suggests that FS2 cells contain a constitutively high level of the component of ISGF3 which in HeLa cells is IFNγ inducible. The

level of ISGF3γ component was assayed directly in FS2 cells and compared with that detected in HeLa cells (Fig 5.9). Cytoplasmic extracts from untreated and IFNγ treated HeLa and FS2 cells were assayed with and without addition of partially purified ISGF3α component. No ISGF3 was detected in the absence of ISGF3α addition (lanes 1-2 and 5-6) nor was any DNA binding activity present in the ISGF3α preparation (lane 9) since it contained no ISGF3γ. Addition of ISGF3α to the cytoplasmic extracts showed the presence of low levels of ISGF3γ in untreated HeLa cell extracts (lane 3) which increased to much higher levels in extracts from IFNγ treated HeLa cells (lane 4). In contrast, extracts from untreated FS2 cells (lane 7) contained constitutively high levels of ISGF3γ, comparable to the levels detected in IFNγ treated HeLa cells. These levels were not increased in extracts from IFNγ treated FS2 cells (lane 8).

Discussion

We have presented evidence that the transcriptional activator for ISGs is derived from latent components resident in the cytoplasm prior to stimulation. IFNα occupation of its cell-surface receptor initiates a series of

Figure 5.9 ISGF3γ levels in human diploid fibroblasts are unaffected by IFNγ priming. Induction of ISGF3γ component by IFNγ was compared in HeLa S3 cells (lanes 1-4) and FS2 cells (lanes 5-8) by mixing with partially purified ISGF3α component which by itself contained no ISGF3 (lane 9). In the absence of added ISGF3α, untreated (lanes 1 and 5) or 15 h IFNγ treated (lanes 2 and 6) contained no ISGF3. Addition of ISGF3α revealed a clear induction of ISGF3γ in HeLa cells in response to IFNγ (compare lanes 3 and 4) while the level of ISGF3γ in FS2 cells was unaffected by such treatment (compare lanes 7 and 8) and was approximately equal to the level detected in IFNγ treated HeLa cells.


HeLa
FS2
ISGF3 α
IFN γ
– – + +
– + – +
– – + +
– + – +
ISGF3 α
1 2 3 4
5 6 7 8
9

steps resulting in the association of two cytoplasmic polypeptides to produce a complex capable of translocating to the nucleus and binding to DNA (Fig 5.10). We have reconstituted one step in vitro by mixing cytoplasmic extracts from IFNα-treated cells with extracts from untreated or IFNγ treated cells. The stoichiometry, kinetics, and pharmacology of this reaction in vivo and in vitro are all consistent with the participation of two protein components. The two polypeptides display distinct sensitivities to alkylation by NEM. In addition, we are presently purifying these activities and have separated them chromatographically.

By our model, both proteins pre-exist in cytoplasm of untreated cells. One (ISGF3γ) is of low abundance in HeLa cells but accumulates to high levels following IFNγ induction. Cell lines vary in their constitutive levels of ISGF3γ, and in certain cell lines may limit the formation of ISGF3. The other component (ISGF3α) is directly or indirectly modified in response to IFNα treatment allowing it to associate with ISGF3γ to form active ISGF3 which translocates to the nucleus and binds the ISRE of responsive genes. Recently, we have prepared highly purified nuclear ISGF3 by oligonucleotide affinity chromatography (X-Y. Fu et al., submitted). Both ISGF3α and ISGF3γ

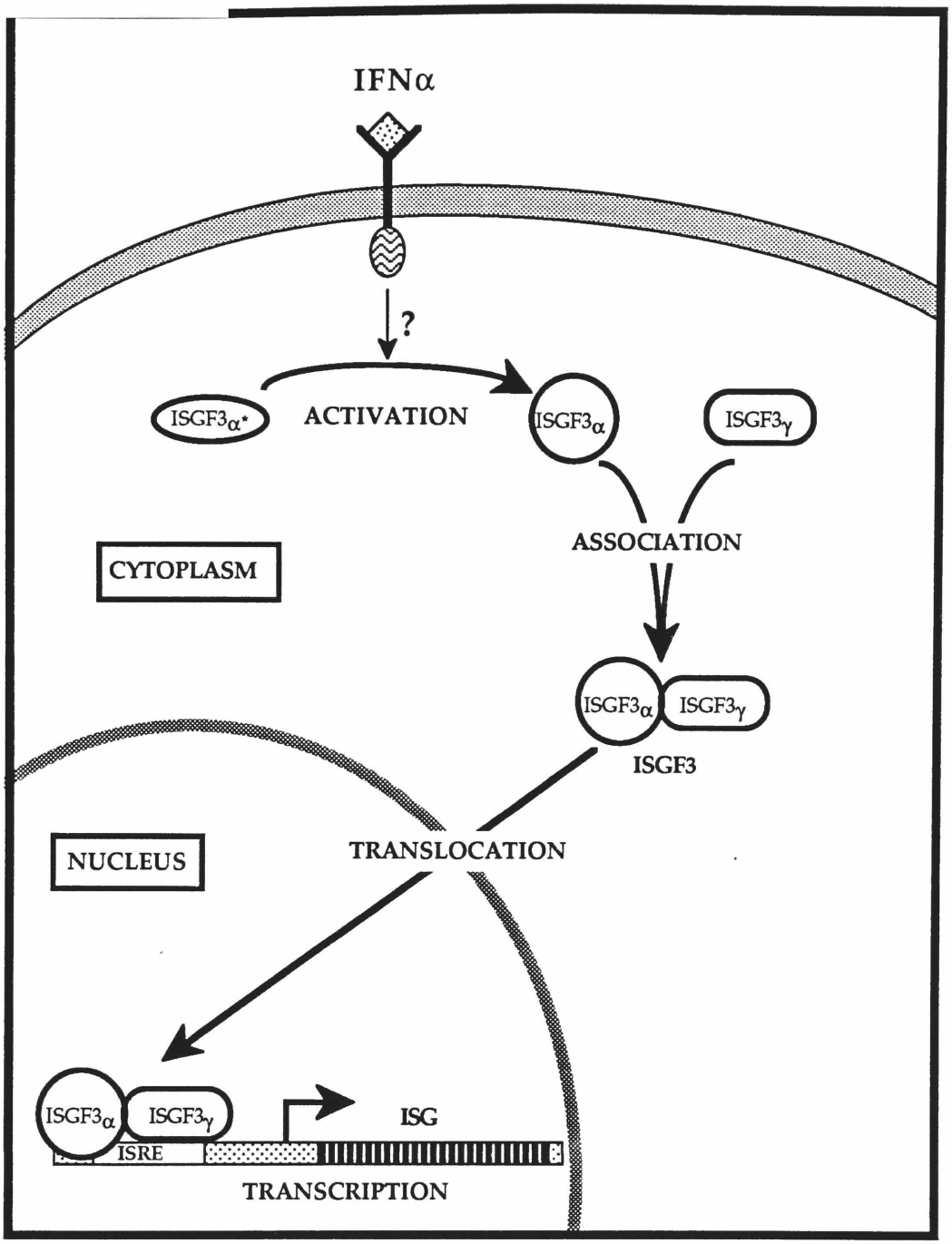


Figure 5.10 Schematic representation of ISGF3 activation. The mature, DNA binding form of ISGF3 detected in nuclei of IFNα treated cells is derived from two latent components preexisting in the cytoplasm, ISGF3α and ISGF3γ. The abundance of ISGF3γ increases in response to IFNγ; ISGF3α changes from an inactive form (ISGF3α*) to an active form through an uncharacterized modification in response to IFNα. This modified form of ISGF3α associates with ISGF3γ, and this complex translocates to the nucleus and binds ISRE sequences of IFN stimulated genes.

complementing activities were present in highly enriched fractions of the mature factor, suggesting that the nuclear factor is composed of these two components.

Although the accumulation of ISGF3γ component in response to IFNγ appears to be a peculiarity of HeLa cells (e.g., it is constitutively abundant in diploid fibroblasts), the cytoplasmic activation followed by nuclear accumulation of ISGF3 in response to IFNα has also been observed in FS2 fibroblasts and in Daudi lymphoblastoid cells (data not shown). In addition, Dale et al. (1989a) reported a soluble DNA binding activity induced by IFNα in the cytoplasm of human fibroblasts and B lymphocytes, which is very likely ISGF3. Most important, this group reported that in vivo activation of this factor occurred in enucleated cytoplasts, indicating that its presence in the cytoplasm is unlikely to result from an artifact of cell fractionation (Welshons et al., 1984).

Theme and variations in transcription factor activation

The proposed mechanism for ISGF3 activation is a simple solution to the problem of how a cell surface receptor can mediate selective transcriptional stimulation of a limited set of genes. In other cases, cell surface

signalling appears to involve traditional second messengers. It remains to be determined how perturbations of the cytoplasmic levels of small molecules such as Ca^{++}, diacylglycerol, inositol polyphosphates, or cAMP that in turn modulate activities of general protein kinases are faithfully coupled to activation of defined gene sets, retaining the fidelity of the receptor-ligand specificity that initiated the intracellular signal. On the other hand, if the transcription factor itself, in some form, is the "second messenger" then the selectivity of activation is simplified to a single recognition step between receptor and factor in the cytoplasm. It is tempting to speculate that one component of ISGF3 (most likely ISGF3α) actually interacts directly with the IFNα cell surface receptor and is modified or released in response to IFNα treatment. A general theme of the involvement of protein-protein interaction in cytoplasmic transcription factor activation becomes evident by comparing this proposed mechanism of ISGF3 formation with other signal transduction systems. For example, the glucocorticoid receptor is a silent transcription factor in the cytoplasm of uninduced cells. Steroid binding causes the hormone-receptor complex to translocate to the nucleus and bind specific target sequences in responsive genes (Yamamoto, 1985; Picard and

Yamamoto, 1987). It has recently been shown that the silent glucocorticoid receptor is associated with hsp90 in the cytoplasm (Sanchez et al., 1985) and that dissociation of this complex is triggered by steroid binding (Sanchez et al., 1987; Denis et al., 1988; Pratt et al., 1988). Thus, steroid activation of its intracellular receptor also involves protein-protein interactions, although it is release from a complex rather than complex formation which allows nuclear translocation.

Baeuerle and Baltimore (1988a) have described another example of cytoplasmic activation of a transcription factor, involving dissolution of a protein-protein complex. A silent precursor to NF-κB is complexed with a specific inhibitor in the cytoplasm of cells not expressing κ immunoglobulin light chain. Upon treatment of cells with bacterial lipopolysaccharide or phorbol esters, the cytoplasmic complex dissociates, allowing active NF-κB to migrate to the nucleus and bind DNA target sequences (Baeuerle and Baltimore, 1988b). It is interesting that NF-κB presumably can be activated through a classical second messenger pathway (i.e., protein kinase C). In distinction from the activity of ISGF3, this transcription factor has been implicated in the expression of a growing number of diverse genes in a

variety of cell types [e.g., κ light chain, interleukin-2 receptor α-chain, the human immunodeficiency virus LTR, human IFNβ, and serum amyloid protein stimulated by LPS, dsRNA, and TNF (Sen and Baltimore, 1986; Bohnlein et al., 1988; Leung and Nabel, 1988; Visvanathan and Goodbourn, 1989; Edbrooke et al., 1989; Lenardo et al., 1989).

A similar mechanism may be employed in the regulation of human hsp70 gene expression. Again, a sequence-specific DNA binding protein is activated from a silent cytoplasmic precursor, this time in response to elevated temperature (Larson et al., 1988). Although involvement of a second polypeptide as either inhibitor or activator has not been demonstrated, this activation is also a two-step process of modification or conformational change coupled with nuclear translocation.

Protein-protein associations have been shown to affect the activity of many other DNA binding proteins. For example, the proto-oncogene products c-fos and c-jun are nuclear proteins that display weak DNA binding activity. However, as heterodimers with each other (Chiu et al., 1988) or possibly with other related proteins (Nakabeppu et al., 1988) these complexes bind DNA with specificity and high affinity, correlating with efficient

transactivation of specific target genes (Rauscher et al., 1988a; Kouzarides and Ziff, 1988; Halazonetis et al., 1988). Although the association of this family of transcription factors has not been shown to be modulated in response to any extracellular signal, the activation of ISGF3 could be similar to this fundamental mechanism for creating a high affinity DNA binding protein. We suggest that for the case of specific and limited induction of transcription from cell surface receptors, the cytoplasmic activation of precursors to nuclear DNA-binding proteins may prove to be a general mechanism for assuring an accurate and rapid response.

Materials and methods

Cell culture

Cultured HeLa S3 cells obtained from ATCC, Rockville Md. and were maintained in Joklik's modified Eagle's medium supplemented with 5% calf serum and antibiotics. Human diploid fibroblasts (FS2) were obtained from D.E. Knight (E. I. duPont), and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics. For IFN treatments, human recombinant bacteria-derived IFNα, a kind gift of Dr. P.

Sorter (Hoffman-LaRoche, Nutley, NJ), was added to culture media at 15 pM (500 U/ml) unless otherwise noted; human recombinant bacteria-derived IFNγ, generously provided by Dr. D. Vapnek (Amgen, Thousand Oaks, CA), was used at 60 pM (20 U/ml).

Cell extracts

Nuclear and cytoplasmic cell extracts were prepared by a modification of the procedure of Dignam et al. (1983a, 1983b) as previously described (Levy et al., 1988a). Cells were harvested by centrifugation, washed with PBS, and lysed by homogenization in hypotonic Buffer A [20 mM HEPES (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 0.5 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. Nuclei were separated from cytoplasmic extracts by centrifugation at 200 x g, washed in Buffer A, and centrifuged at 14,000 x g prior to extraction with 0.3 M NaCl in Buffer A without detergent. Nuclear extracts were clarified by centrifugation at 14,000 x g; cytoplasmic extracts were centrifuged at 100,000 x g. For some experiments performed on small numbers of cells, cytoplasmic extracts were clarified only at 14,000 x g. No differences were noted between extracts prepared by the two procedures. For time course experiments, cells were resuspended in media at

approximately 5 x 10^7 cells/ml and treated with 1.2 nM IFNα (40,000 U/ml) in order to quickly saturate receptor binding. Cells were shaken at 37°C for the times indicated in figure legends, and then were quenched with 100 volumes of PBS at 0°C, centrifuged, and extracted as described. It was found that treated cells could remain on ice for up to 4 h with no change in the amount or distribution of cytoplasmic and nuclear ISGF3 or its subunits (data not shown).

Protein-DNA binding assays

Protein-DNA binding assays were performed by gel retardation (Garner and Revzin, 1981; Fried and Crothers, 1981) as previously described (Levy et al., 1988a; Kessler et al., 1988a). Nuclear and cytoplasmic extracts derived from equal numbers of cells were incubated with 5 fmol of radiolabeled ISG15 promoter fragment containing the ISRE sequence (-115 to -39) in the presence of nonspecific DNA [4 μg of double-stranded poly(dI-dC), 0.5 μg of plasmid DNA, and 5 mM nucleotide] in a total volume of 12.5 μl (Kessler et al., 1988a). Unless noted otherwise, binding reactions contained 5μg of nuclear or 25 μg of cytoplasmic protein. Under these conditions, greater than 90% of the labeled probe remained unbound, insuring that activity measurements were

made in probe excess. For competition assays, 100 ng of specific or non-specific double-stranded oligonucleotides were included in each binding reaction. Following 20 min incubations at room temperature, 5 µl of each binding reaction was fractionated on 4.8% polyacrylamide gels run in 20 mM tris-borate-EDTA for 3 h at 300 V.

Chapter 6

IFNα regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator

Introduction

Multicellular development, control of cell growth and differentiation, and homeostasis depends on the ability of cells to respond to extracellular signals. How cells transduce complex signals can be understood through analysis of defined signalling systems. The interferon (IFN) system provides a useful model for studying signal transduction. Type I interferons (IFNα/β) interact with a specific cell surface receptor present on nearly all human cell types. The major physiological responses to IFNs are cessation of cell growth and inhibition of viral replication, effects that depend on de novo synthesis of dozens of IFN-induced gene products (for review: De Maeyer and De Maeyer-Guignard, 1988). An initial event in the response to IFNα is transcriptional activation of a group of IFN-stimulated genes (ISGs). We have investigated the molecular events resulting in transcriptional activation of ISGs following IFNα-receptor interactions at the cell surface to define strategies used for signal transduction.

The transcriptional response of several ISGs to IFNα has been described, illustrating particular characteristics of this inducible system. Activation is rapid, detectable within 10 min of exposure to IFNα, and of large magnitude with transcriptional increases greater than 50-fold within 1 hour

of treatment (Larner et al., 1984, 1986; Friedman et al., 1984; Friedman and Stark, 1985). Activation of transcription occurs in the absence of new protein synthesis, implying a role for post-translational modification of pre-existing cellular protein(s) in transcriptional induction. However, the response is transient, returning to basal transcription levels within 8-15 hours in most cell lines. The transient aspect of the response, or down-regulation, is dependent on new protein synthesis, and the inclusion of protein synthesis inhibitors during IFNα treatment results in prolonged transcription (Larner et al., 1984). Studies with agents which perturb several identified signalling systems have failed to implicate known intracellular messengers as components of the IFNα signal transduction apparatus (Lew et al., 1989). The recent cloning of a subunit of the IFNα receptor revealed a noncanonical transmembrane protein (Uzé et al., 1990), providing no clues into the nature of IFNα signalling.

Transcriptional activation of ISGs is independent of ongoing protein synthesis, and thus the IFN stimulated signal must be transmitted through pre-existing cellular components to activate a latent transcriptional machinery. Analysis of ISG promoters has identified a cis-regulatory target, the ISRE (Porter et al., 1988; Levy et al., 1988a, 1988b; Friedman and Stark, 1985;

Wathelet et al., 1987; Israel et al., 1986; Vogel et al., 1986; Sugita et al., 1987; Reich et al., 1987), both necessary and sufficient for ISG transcriptional activation (Cohen et al., 1988; Reich and Darnell, 1989), at which this machinery must act. We and others have identified nuclear proteins which bind specifically to the ISRE (Levy et al., 1988a; Porter et al., 1988; Rutherford et al., 1988; Cohen et al., 1988; Shirayoshi et al., 1988), one of which (ISGF3) is the primary transcriptional activator of ISGs (Levy et al., 1988; Kessler et al., 1988a; Levy et al., 1989). The role of ISGF3 as the transcriptional activator has been confirmed through analysis of extensive point mutants of the ISRE (Kessler et al., 1988a), measurement of the kinetics of its activation (Levy et al., 1988a; Levy et al., 1989), absence of protein synthesis requirements (Levy et al., 1988a), and correlation of its activation with ISG transcription in a variety of responsive and resistant cell lines (Kessler et al., 1988b; Levy et al., 1990). ISGF3 is distinct from several other proteins detected binding to the ISRE. In particular, it appears to be unrelated to ISGF2 (equivalent to the mouse protein IRF-1 described by (Fujita et al., 1988), an IFN-inducible ISRE binding protein which plays no role in the primary induction of ISGs (Pine et al., 1990).

ISGF3 is initially activated in the cytoplasm of IFNα treated cells (Levy

et al., 1989; Dale et al., 1989a), pointing to a central role for ISGF3 in early steps in signal transduction as well as in subsequent transcriptional activation. Cytoplasmic ISGF3 can be detected within 1 min of stimulation, while nuclear ISGF3 is undetectable before 5 min of treatment. Furthermore, the stimulation of ISGF3 in enucleated cells confirms the cytoplasmic location of its activation. Development of an *in vitro* system for cytoplasmic activation revealed two distinct activities necessary for ISGF3 formation and suggested a multisubunit nature for ISGF3. Cytoplasmic extracts containing large quantities of an "inactive precursor" of ISGF3 were mixed with cytoplasmic extracts of IFNα-stimulated cells, yielding high levels of ISGF3 *in vitro*. One activity (termed ISGF3γ) could be detected in both untreated and IFNα treated cell cytoplasms, while the other (termed ISGF3α) was detected only following IFNα treatment. Examination of many parameters of this *in vitro* reaction failed to reveal any of the usual characteristics of enzymic processes. Rather, the data suggested that stoichiometric interactions between ISGF3α and ISGF3γ resulted in production of ISGF3. These two components had distinct characteristics: while ISGF3α was detected only following IFNα treatment (hence its name), ISGF3γ was constitutive, but found to be inducible by IFNγ treatment in certain cell lines, requiring both RNA transcription and protein

synthesis for its increase in abundance following IFNγ treatment (Levy et al., 1990). In addition, these two activities could be distinguished biochemically: ISGF3γ was sensitive to alkylation by N-ethyl maleimide while ISGF3α was not.

We have now examined the molecular composition and regulation of ISGF3. We address two aspects of signal transduction by ISGF3 in the response to IFNα: the nuclear accumulation of the individual subunits of ISGF3 and the effect of complex assembly on ISG transcriptional control. Sedimentation analysis, protein renaturation, and photoaffinity crosslinking of highly purified preparations of ISGF3 and its components, ISGF3α and ISGF3γ, revealed a heteromeric ISRE-binding complex composed of multiple, distinct polypeptides. ISGF3γ was found to be a novel ISRE-binding protein present in both the cytoplasm and nucleus of unstimulated cells. This 48 kD polypeptide bound the ISRE with high specificity but low affinity, perhaps accounting for incomplete localization to the nucleus. ISGF3α, having no DNA-binding activity, was found to be a complex probably composed of several polypeptides. Following IFNα treatment, this activity was detected in cytoplasm prior to its accumulation in nuclei. In nuclei, ISGF3α interacted with ISGF3γ and the ISRE to form mature ISGF3 displaying high affinity for

the ISRE.

Results

ISGF3γ is a novel DNA-binding activity with specificity for the ISRE

ISGF3α and ISGF3γ were defined as complementing cytoplasmic extracts which, although individually devoid of ISGF3, produced this characteristic DNA binding activity when mixed *in vitro*. To gain further insight into the composition of these individual activities, we have fractionated ISGF3α and ISGF3γ individually from separate cytoplasmic extracts and compared these activities with ISGF3 isolated from nuclei derived from stimulated cells. Cytoplasmic extracts prepared from IFNγ-treated HeLa S3 cells contained high levels of ISGF3γ activity but, since derived from cells never exposed to IFNα, no mature ISGF3 (Levy et al., 1989). Cytoplasmic extracts of IFNα-treated cells were alkylated *in vitro* with N-ethyl-maleimide (NEM) to inactivate ISGF3γ and thus contained only ISGF3α activity (Levy et al., 1989).

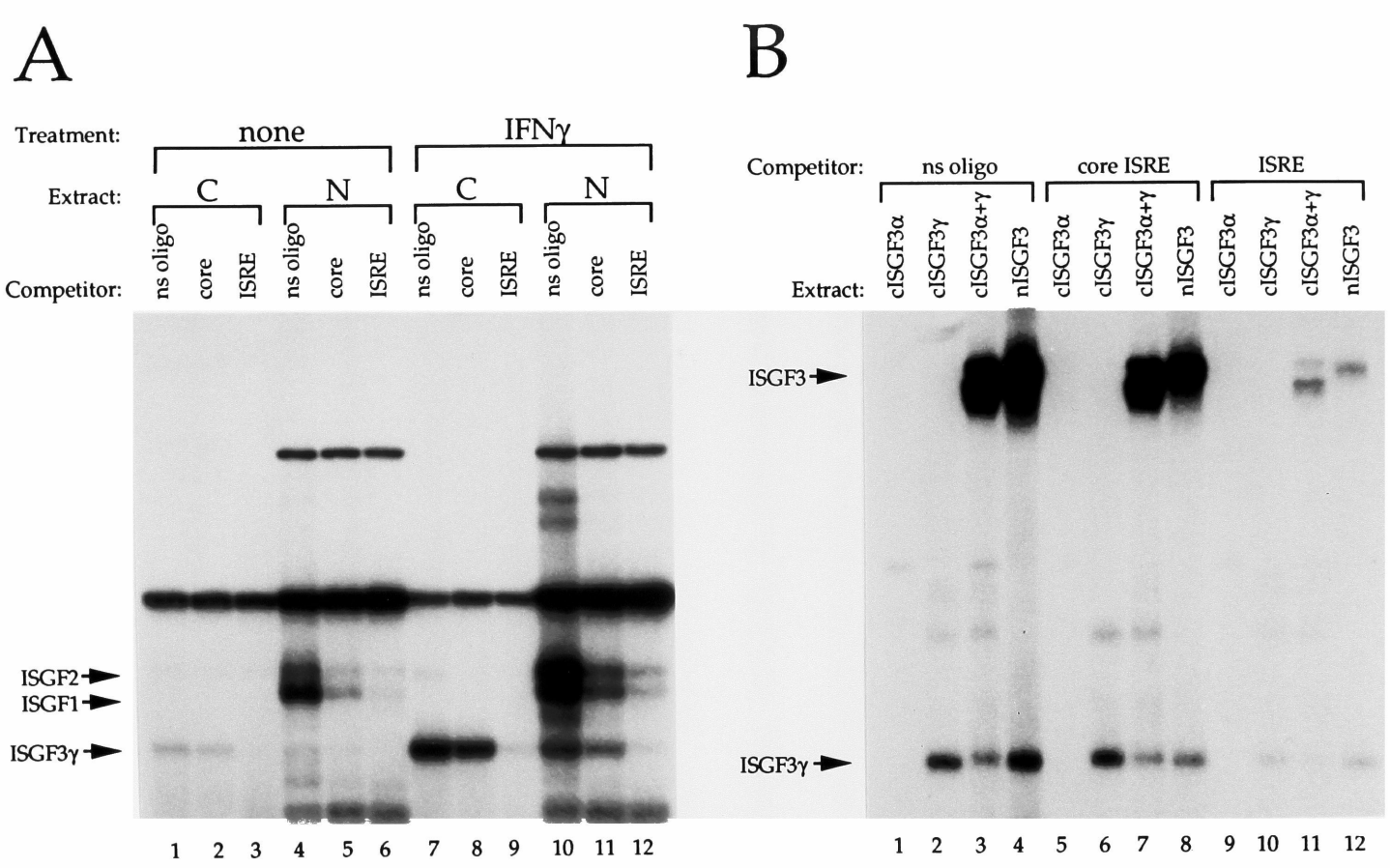
HeLa cell cytoplasmic extracts were mixed with complementing extracts in a DNA binding reaction with an oligonucleotide containing a high-affinity binding site for ISGF3 and assayed by gel electrophoresis mobility shift

analysis. Specificity of observed DNA-protein interactions was assessed by competition between a full-length ISRE oligonucleotide probe and an oligonucleotide containing only the central 9 bp core sequence of the ISRE. This core sequence is sufficient to bind other ISRE-binding proteins such as ISGF1 and ISGF2 (IRF-1) but not ISGF3 (Kessler et al., 1988a). In this assay, in addition to a gel shift band corresponding to ISGF3, we also observed a DNA binding activity with a specificity identical to ISGF3 but with much faster mobility during electrophoresis (Fig 6.1). It was detected in both cytoplasmic and nuclear extracts from untreated cells and was induced to much higher abundance by IFNγ treatment (Fig 6.1A, compare lanes 1 and 7). This activity was distinct from ISGF1 and ISGF2: it was detected in cytoplasm (lane 7) as well as nuclei (lane 10), unlike ISGF1 and ISGF2 which were found only in nuclei (lanes 4 and 10); it had ISGF3-like binding specificity; and it formed a complex with an ISRE probe having distinct electrophoretic mobility (Fig 6.1A). In addition, it was present in extracts from cells never exposed to IFNα (lane 7) and therefore distinct from ISGF3. This novel DNA binding activity, like ISGF3 was sensitive to alkylation by NEM (not shown).

The NEM sensitivity, binding specificity, and IFNγ inducibility of this DNA-binding activity prompted us to consider its relationship to previously

described ISGF3γ. ISGF3α and ISGF3γ from cytoplasmic extracts were separately purified greater than 2000-fold by biochemical fractionation (see Material and methods). Column fractions were assayed for activity by mixing with crude extracts containing the complementary activity. The resulting highly enriched fractions were sufficient to reconstitute ISGF3 when mixed with each other, suggesting that no additional components which might have been present in crude extracts were necessary for ISGF3 formation (Fig 6.1B, lane 3). The enriched preparation of ISGF3α contained no DNA binding activity on its own (lane 1). Purified ISGF3γ, on the other hand, displayed ISRE-specific gel shift equivalent to the novel IFNγ-inducible DNA binding activity observed in crude extracts (lane 2). This DNA binding activity copurified precisely with the ability to form ISGF3 upon addition of ISGF3α through every chromatographic step (data not shown). Further, addition of ISGF3α to this preparation of ISGF3γ caused a significant depletion of the faster mobility gel shift coincident with the appearance of the slower moving ISGF3 complex (compare lanes 2 and 3). Highly purified fractions of ISGF3 isolated from nuclei by ISRE-affinity chromatography (X-Y. Fu et al., submitted) also contained this DNA binding activity in addition to ISGF3 (lane 4). These results imply an identity between this fast mobility ISRE

Figure 6.1 ISGF3γ displays intrinsic DNA binding ability. **A**. Binding of cytoplasmic (noted C) or nuclear (noted N) factors from untreated (lanes 1-6) or IFNγ-treated (lanes 7-12) HeLa cells to an ISRE oligonucleotide. Competing unlabeled heterologous (ns oligo), core, or full ISRE oligonucleotide was included in the binding reactions as indicated. The mobilities of protein-DNA complexes composed of ISGF1, ISGF2, and ISGF3γ are indicated. ISGF1 and ISGF2 were observed only in nuclear extracts and binding of these proteins was competed by both core and ISRE oligonucleotides (Kessler et al., 1988b; Reich and Darnell, 1989). ISGF3γ was seen in both cytoplasm and nuclei and was highly inducible by IFNγ (Levy et al., 1990). **B**. Highly purified preparations of ISGF3α and ISGF3γ from cytoplasm (indicated c) and of ISGF3 from nuclei (indicated n) were mixed with labeled ISRE DNA in the presence of excess competitors, as indicated. ISGF3α preparations (lanes 1, 5, 9) contained no DNA binding activity prior to mixing with ISGF3γ to form ISGF3 (lanes 3, 7, 11) which displayed identical mobility and sequence specificity as nuclear ISGF3 (lanes 4, 8, 12). ISGF3γ binding to DNA (lane 2) was competed only by full-length ISRE oligonucleotides (lane 9) and was partially depleted on addition of ISGF3α coincident with formation of ISGF3 (compare lanes 2 and 3).


A
Treatment:
none
IFNγ
Extract:
C
N
C
N
Competitor:
ns oligo
core
ISRE
ns oligo
core
ISRE
ns oligo
core
ISRE
ns oligo
core
ISRE
ISGF2
ISGF1
ISGF3γ
1 2 3 4 5 6 7 8 9 10 11 12
B
Competitor:
ns oligo
core ISRE
ISRE
Extract:
cISGF3α
cISGF3γ
cISGF3α+γ
nISGF3
cISGF3α
cISGF3γ
cISGF3α+γ
nISGF3
cISGF3α
cISGF3γ
cISGF3α+γ
nISGF3
ISGF3
ISGF3γ
1 2 3 4 5 6 7 8 9 10 11 12

binding activity and ISGF3γ, suggesting that ISGF3γ is the DNA binding subunit of ISGF3.

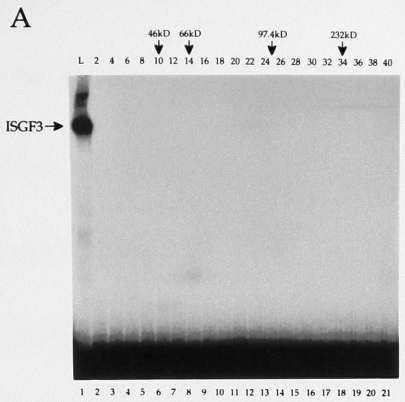
ISGF3 can be separated into its constituent components

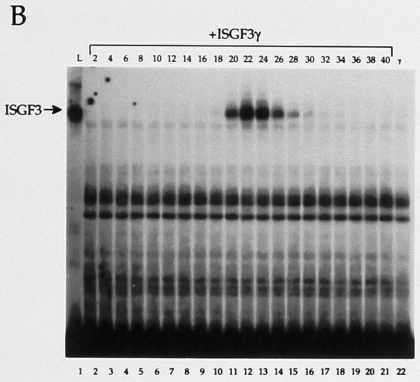
A second approach used to substantiate the model of ISGF3 composition was sedimentation analysis. A highly purified nuclear preparation of ISGF3 was fractionated on a 15-30% glycerol gradient. Although a substantial amount of ISGF3 was loaded onto the gradient (Figure 6.2A, lane 1), no ISGF3 was recovered in the gradient fractions (lanes 2-21). One explanation for this result would be that ISGF3 was an unstable complex (under these conditions) composed of distinctly fractionating subunits. To test for such separated activities, gradient fractions were mixed with ISGF3γ (Fig 6.2B) or ISGF3α (Fig 6.2C) enriched from cytoplasm. ISGF3 activity was readily reconstituted by this *in vitro* mixing. Addition of ISGF3γ to fractions 20–26 demonstrated the presence of ISGF3α-like activity (Fig 6.2B), while complementary mixes with ISGF3α revealed a distinct, non-overlapping peak of ISGF3γ activity in fractions 10–12 (Fig 6.2C). These data indicate that ISGF3α sediments with a relative molecular weight of ~100 kD, while ISGF3γ sediments as an ~50 kD protein. When these peak fractions from the gradient were mixed with each other rather than with cytoplasmic fractions, ISGF3 was

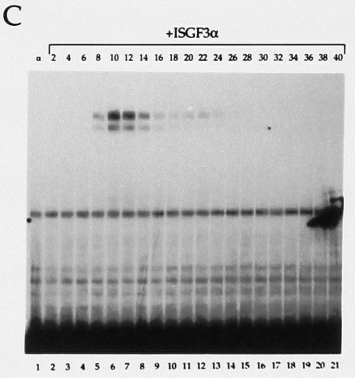
again recovered (Fig 6.2F), indicating that no additional components besides the individual ISGF3α and ISGF3γ activities are required to form ISGF3. Likewise, enriched preparations of ISGF3γ and ISGF3α derived from cytoplasmic extracts were separately fractionated by sedimentation. Each activity cosedimented with its nuclear cognate (Fig 6.2D and E) suggesting that no gross differences existed in the cytoplasmic and nuclear forms of the ISGF3 subunits.

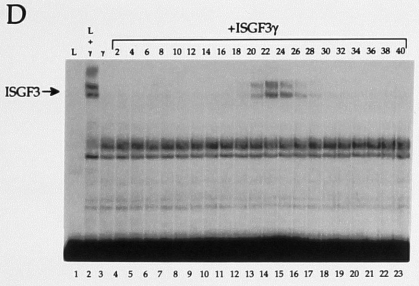
Why did ISGF3, a DNA binding complex which can be purified by affinity chromatography, separate into its constituent subunits during centrifugal sedimentation? A variation on this experiment was performed to address this apparent instability of the ISGF3 complex. Nuclear ISGF3 was incubated with a radiolabeled ISRE oligonucleotide prior to sedimentation analysis, and the sedimentation velocity of the protein-DNA complex was determined (Fig 6.2G). Under these conditions, the ISGF3-ISRE complex sedimented with an apparent molecular mass of 250–350 kD. A similar result was obtained using ISGF3 formed from mixed cytoplasmic fractions of ISGF3α and ISGF3γ (data not shown). Control experiments using an unrelated oligonucleotide probe did not stabilize the ISGF3 complex, and when fractions were assayed for ISGF3α and ISGF3γ, each was detected in fractions from

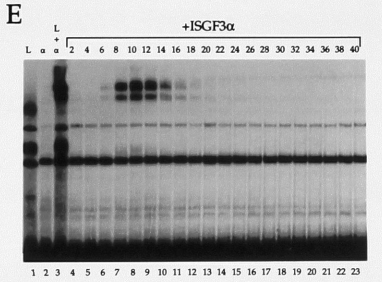
Figure 6.2 Glycerol gradient sedimentation fractionates ISGF3 into separate ISGF3α and ISGF3γ activities. ISGF3 purified from nuclei IFNα-treated HeLa cells (**A–C**) or ISGF3α (**D**) and ISGF3γ (**E**) purified from cytoplasm were loaded onto 15–30% glycerol gradients and fractionated as described in Materials and methods. Starting material (L) and each fraction were assayed for ISRE-binding activity by gel shift assay either directly (**A**) or after addition of ISGF3γ (**B** and **D**) or ISGF3α (**C** and **E**), as indicated. Sedimentation of marker proteins was determined by SDS-PAGE of fractions from a parallel gradient as indicated by their molecular weights. **F.** Pooled fractions from the gradient shown in **A** were assayed for DNA-binding activity alone (lanes 2 and 3) and following mixing *in vitro* with each other (lanes 4–6). Binding specificity of the ISGF3 formed from these fractions was tested by competition with heterologous (lane 4), core (lane 5) or ISRE oligonucleotides (lane 6). **G.** ISGF3-ISRE complexes formed *in vitro* between purified nuclear ISGF3 and labeled ISRE DNA were fractionated by glycerol gradient sedimentation. Each fraction was assayed directly for the presence of complexes by gel shift assay. Positions of ISGF3 and free ISRE are indicated as are the migrations of molecular weight standards from a parallel gradient.

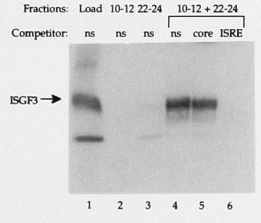

A
46kD 66kD 97.4kD 232kD
L 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
ISGF3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


B
+ISGF3γ
L 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 γ
ISGF3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22


C
+ISGF3α
α 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


D
+ISGF3γ
L
L + γ
γ 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
ISGF3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23


E
+ISGF3α
L
L + α
L α α 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23


F
Fractions: Load 10-12 22-24 10-12 + 22-24
Competitor: ns ns ns ns core ISRE
ISGF3
1 2 3 4 5 6

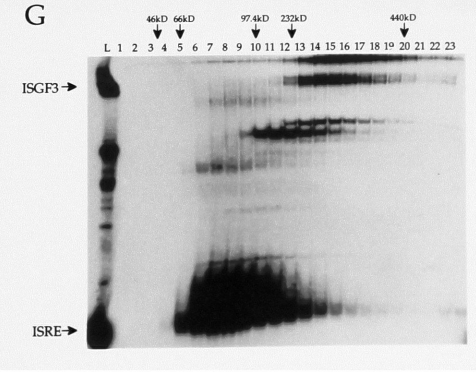
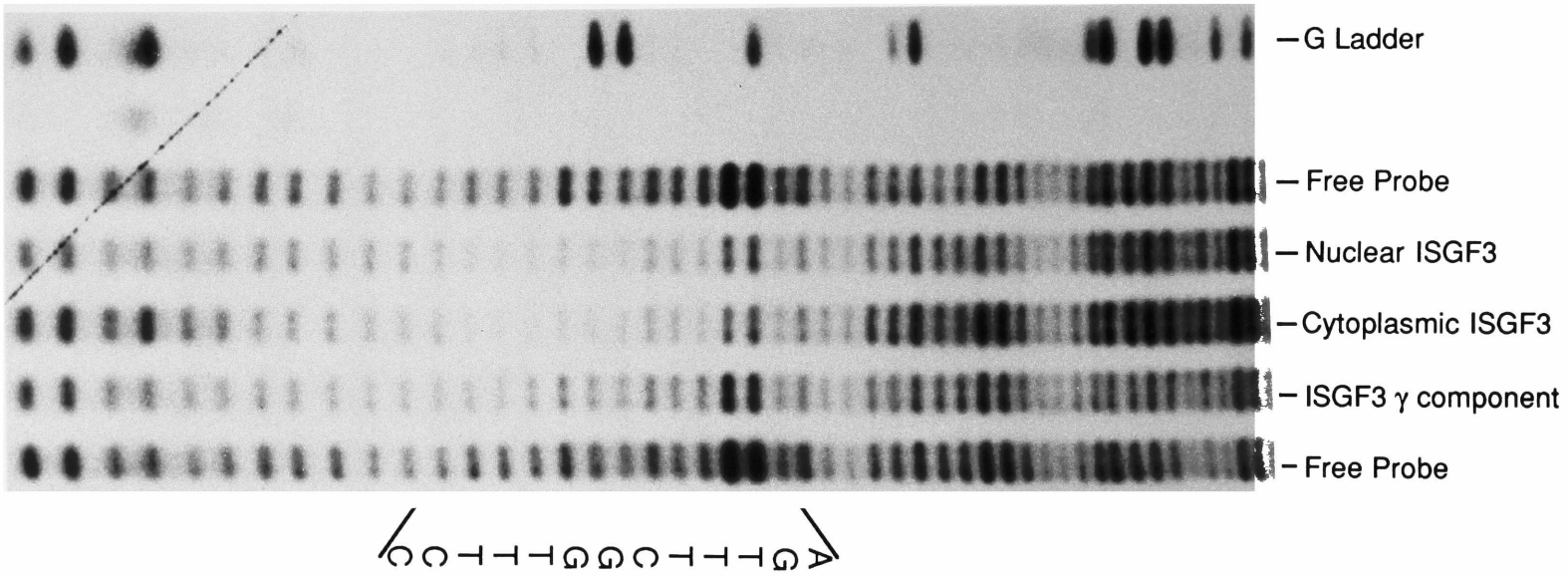

G
46kD 66kD 97.4kD 232kD 440kD
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
ISGF3
ISRE

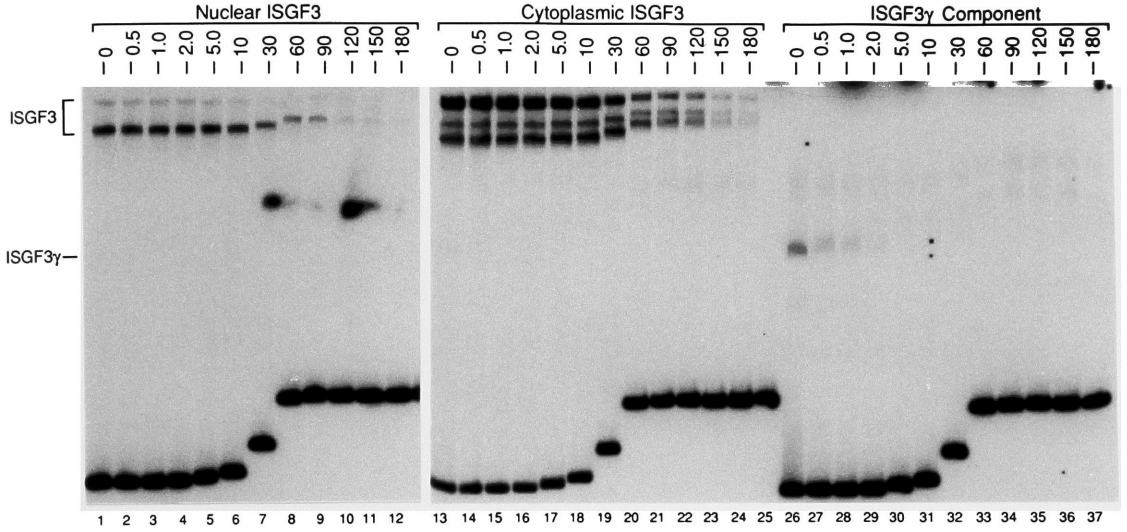
Figure 6.3 Orthophenanthroline-Cu footprinting of ISGF3 and ISGF3γ. Mobility-shift assays were performed using ISGF3 purified from nuclei or formed *in vitro* from cytoplasmic ISGF3α and ISGF3γ and using cytoplasmic ISGF3γ alone. Labeled DNA in the shift complexes as well as unbound DNA were cleaved in situ by soaking the intact gel in 1, 10-phenanthroline and copper sulfate (Kuwabara and Sigman, 1987). DNA was isolated by electroelution onto DEAE-nitrocellulose, recovered, and fractionated by denaturing gel electrophoresis, as indicated. The sequence of the ISRE is indicated to the right.


G Ladder
Free Probe
Nuclear ISGF3
Cytoplasmic ISGF3
ISGF3 γ component
Free Probe
A G T T T C G G T T T C C

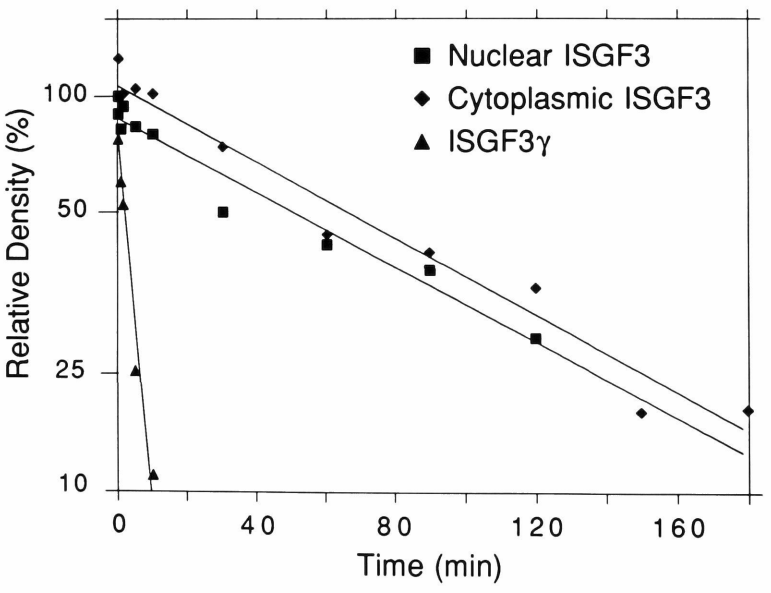
The weaker footprint by ISGF3γ suggested a less stable interaction with the ISRE. The affinities of ISGF3 and ISGF3γ for the ISRE were compared to address this possibility. The ISGF3 complex and ISGF3γ were pre-incubated with the ISRE under standard DNA-binding conditions for 20 min at room temperature, a period of time sufficient for the interaction to reach equilibrium (Levy et al., 1989). A 500-fold molar excess of unlabeled ISRE oligonucleotide was then added to the reaction and the stability of the pre-formed protein-DNA complexes was determined over time. At serial time points after addition of competitor, aliquots were removed and analyzed by gel shift (Fig 6.4A). Decay of each complex was measured over time, and half-lives were determined assuming concentration-independent dissociation kinetics (Fig 6.4B). The affinity of the ISGF3-ISRE interaction was high, having a half-life of ~53 min (the range of 4 independent measurements was 40–68 min). The ISGF3γ-ISRE interaction was strikingly less stable with a half-life of less than 2 min (the range of 3 determinations was 1.1–2.3 min). Essentially identical results were obtained for both nuclear and cytoplasmic preparations of ISGF3 and ISGF3γ when bound to a 130 bp ISG15 promoter DNA fragment containing the ISRE or when bound to a 33 bp synthetic ISRE probe. The approximately 25-fold higher affinity of ISGF3 for the ISRE

Figure 6.4 ISGF3 forms a much more stable complex with ISRE DNA than does ISGF3γ. **A**. Protein-DNA complexes were formed between nuclear ISGF3 (lanes 1–12), ISGF3 formed *in vitro* from cytoplasmic ISGF3α and ISGF3γ (lanes 13–25), or ISGF3γ alone (lanes 26–37) and a labeled ISG15 promoter fragment containing the ISRE. Stability of these complexes was determined by measuring the amount of residual labeled DNA in a specific complex at serial times following addition of unlabeled DNA oligonucleotides. Portions of each binding reaction were removed at the indicated times (min) and loaded directly onto a running mobility-shift gel. **B**. The autoradiograms shown in **A** were quantitated by laser densitometry. Log percent of the density from starting complexes was plotted against time and fitted to a simple line by least squares regression.

A


Nuclear ISGF3
Cytoplasmic ISGF3
ISGF3γ Component
0 0.5 1.0 2.0 5.0 10 30 60 90 120 150 180
ISGF3
ISGF3γ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

B


Nuclear ISGF3
Cytoplasmic ISGF3
ISGF3γ
Relative Density (%)
100
50
25
10
0
40
80
120
160
Time (min)

probably accounts for the difference observed in footprinting efficiency compared to ISGF3γ. Purified preparations of ISGF3α displayed no DNA binding activity, and yet interaction with ISGF3γ stabilized high affinity ISRE binding. These results suggest that ISGF3 is assembled from a regulatory subunit (ISGF3α) in an allosteric complex with a DNA-binding subunit (ISGF3γ).

Polypeptide composition of ISGF3

Further analysis of ISGF3 activation and function required a description of the molecular composition of ISGF3α and ISGF3γ. Four approaches to the characterization of ISGF3 polypeptides were employed: SDS gel fractionation followed by protein renaturation, photoaffinity crosslinking, sedimentation analysis, and preparative gel shift analysis. In an attempt to identify the polypeptides comprising ISGF3, fractions of cytoplasmic ISGF3γ or ISGF3α were resolved by SDS-PAGE, and protein eluted from gel slices was renatured and assayed by gel shift for the ability to form ISGF3 after mixing with complementing ISGF3α or ISGF3γ. Protein recovered from the 42–55 kD range produced ISGF3 when mixed with ISGF3α (Fig 6.5), consistent with sedimentation analysis (see Fig 6.2), indicating that ISGF3γ was a polypeptide(s) of 42-50 kD. No detectable ISGF3α activity was recovered by the

Figure 6.5 SDS-PAGE fractionation and renaturation of ISGF3γ. ISGF3γ purified from cytoplasm was fractionated on 10% SDS-PAGE. Protein recovered from individual gel slices corresponding to marker proteins of the indicated molecular weights was renatured (Briggs et al., 1986) and analyzed for ISGF3γ activity by mixing with cytoplasmic ISGF3α. ISGF3γ was recovered in a single gel slice (lane 7).

ISGF3α component
ISGF3γ component
ISGF3α + ISGF3γ
kDa
10 – 20
20 – 35
35 – 42
42 – 55
55 – 60
60 – 70
70 – 90
90 – 110
110 – 130
130 – 150
ISGF3
1 2 3 4 5 6 7 8 9 10 11 12 13

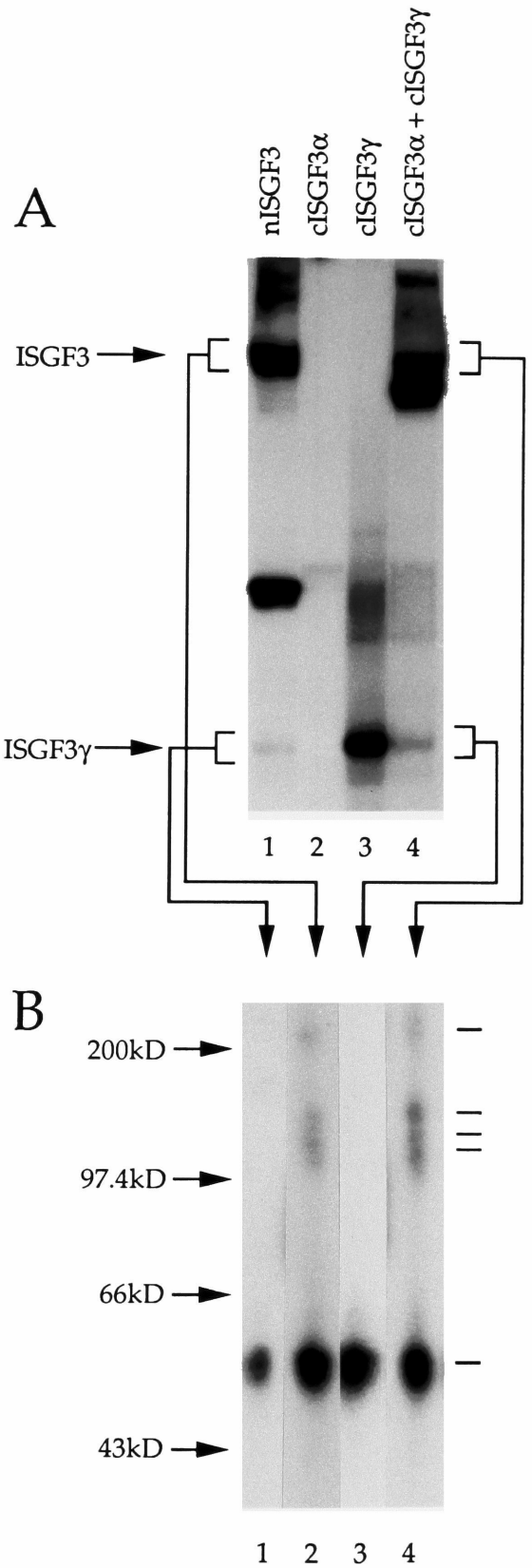
same renaturation procedure from any region of the SDS gel (data not shown). This negative result may indicate that recovery of ISGF3α activity was exceedingly low under conditions used, or, alternatively, that ISGF3α consisted of more than a single polypeptide and that the necessary subunits were not present in a single gel slice. Essentially identical results to those described here have been obtained using nuclear fractions of ISGF3 (X-Y. Fu et al., submitted).

Photoaffinity crosslinking was used to identify proteins bound to, or in close proximity with the ISRE. A uniformly labeled ISRE oligonucleotide containing 5-azido-deoxyuridine was incubated with appropriate fractions and DNA-protein complexes were resolved by gel shift. The intact gel was exposed to ultraviolet light, and ISGF3γ– and ISGF3–ISRE complexes were excised (Fig 6.6A). Cross-linked protein DNA was electroeluted directly into 8% SDS-PAGE, and radiolabeled polypeptides were identified following autoradiography. A single labeled polypeptide of ~50 kD (after adjustment for the presence of linked oligonucleotide) was detected from the ISGF3γ-ISRE gel shift complex, a size consistent with both sedimentation and renaturation analyses (Fig 6.6B, lanes 1 and 3). As expected, a polypeptide of identical mobility was detected from the ISGF3 complex, but additional higher

molecular weight polypeptides were also present (lanes 2 and 4). Three proteins in the range of 80–120 kD (after adjustment) plus a fourth very large species (>200 kD) were specifically cross-linked and therefore presumably were in close proximity with the ISRE. Once again, no gross differences between cytoplasmic and nuclear fractions were apparent (compare lane 1 with 3 and lane 2 with 4). The additional crosslinked polypeptides detected in the ISGF3 complex were unexpected since, as mentioned above, no ISRE-binding activities cofractionated with ISGF3α. It is possible that these higher molecular weight proteins represent ISGF3α and are brought into close proximity of DNA through interaction with ISGF3γ bound at the ISRE. The existence of multiple ISGF3α subunits would be consistent with the failure to renature this activity from protein of a limited size distribution following SDS-PAGE. Furthermore, covalent linkage of putative ISGF3α polypeptides by crosslinking points to ISRE interactions supplementary to ISGF3γ in the ISGF3 complex. These additional protein-DNA contacts could contribute to the greater stability of ISGF3 bound to the ISRE compared to independent ISGF3γ.

Comparison of these ISGF3 crosslinking results with those described in Chapter 4 are consistent with regard to an ~50kD protein contacting the ISRE.

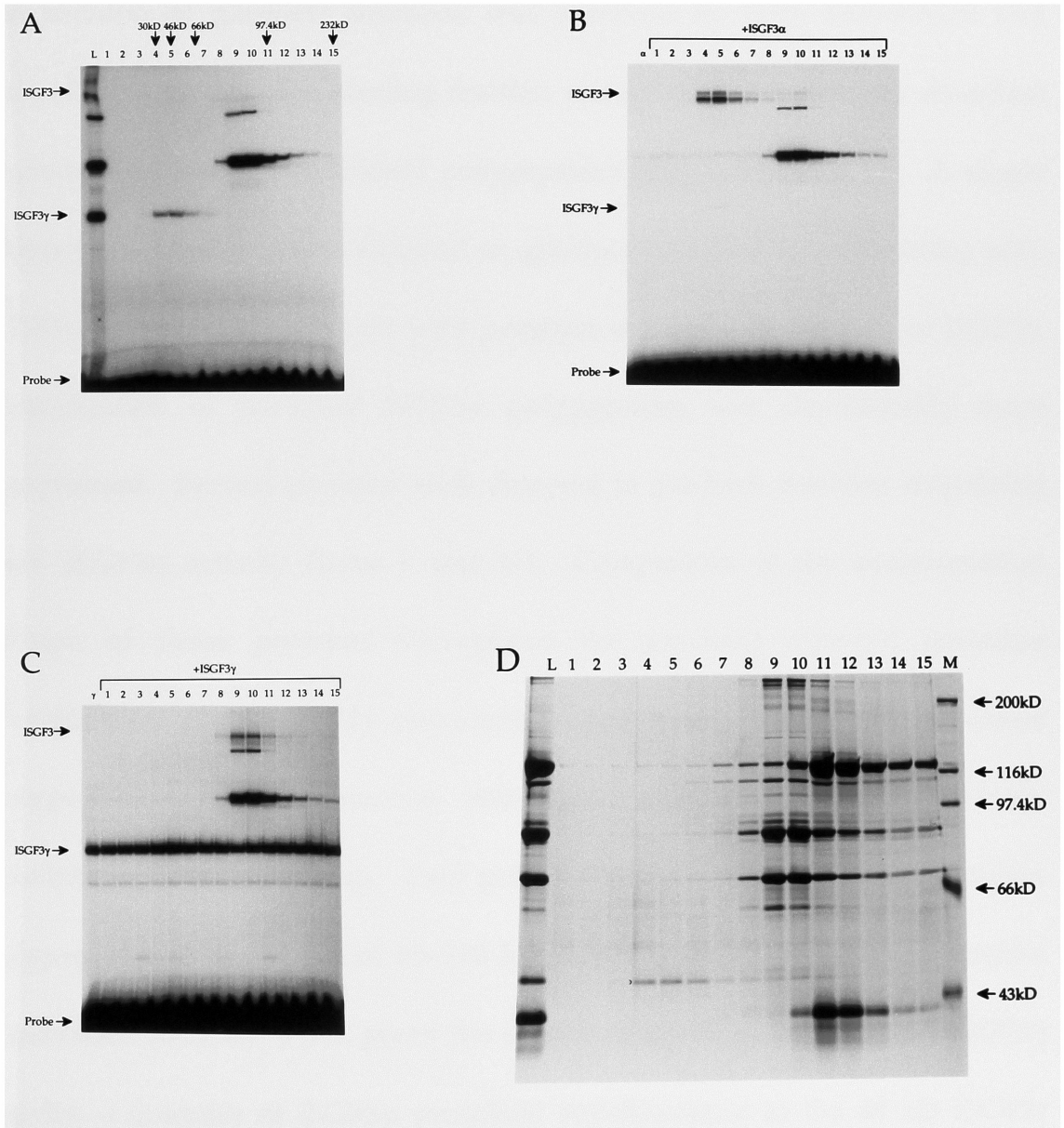
Figure 6.6 Photoaffinity crosslinking of ISGF3 polypeptides. **A**. Nuclear ISGF3 (lane 1) and cytoplasmic ISGF3α (lane 2), ISGF3γ (lane 3), and ISGF3 formed *in vitro* (lane 4) were resolved by gel shift following incubation with labeled ISRE DNA containing azido-deoxyuridine residues. The intact gel was exposed to UV light and, following autoradiography, protein DNA complexes were excised and electroeluted into 8% SDS-PAGE shown in **B**. ISGF3γ from the nuclear ISGF3 preparation was loaded in lane 1, nuclear ISGF3 in lane 2, cytoplasmic ISGF3γ in lane 3, and *in vitro* formed cytoplasmic ISGF3 in lane 4, as indicated by arrows. Specifically labeled polypeptides which were not detected when free probe was subjected to similar analysis (data not shown) are indicated by marks on the right.


A
nISGF3
cISGF3α
cISGF3γ
cISGF3α + cISGF3γ
ISGF3
ISGF3γ
1 2 3 4
B
200kD
97.4kD
66kD
43kD
1 2 3 4

However, in the earlier experiment no higher molecular weight polypeptides were observed. This result may be explained in two ways. First, the crosslinking nucleotide used in the two experiments differs. Azido-deoxyuridine is much more reactive than bromo-deoxyuridine and therefore is more efficient at crosslinking proteins that may be weakly associated with DNA. In fact, an examination of band intensity suggests that the ~50kD polypeptide is more tightly associated with the ISRE than the larger proteins. (Fig 6.6B). Second, the present crosslinking involved excision of distinct protein-DNA complexes, while the earlier experiment was carried out in solution, and therefore a mixed population of ISGF3-ISRE and ISGF3γ-ISRE complexes was sampled, thus diminishing the sensitivity of the assay.

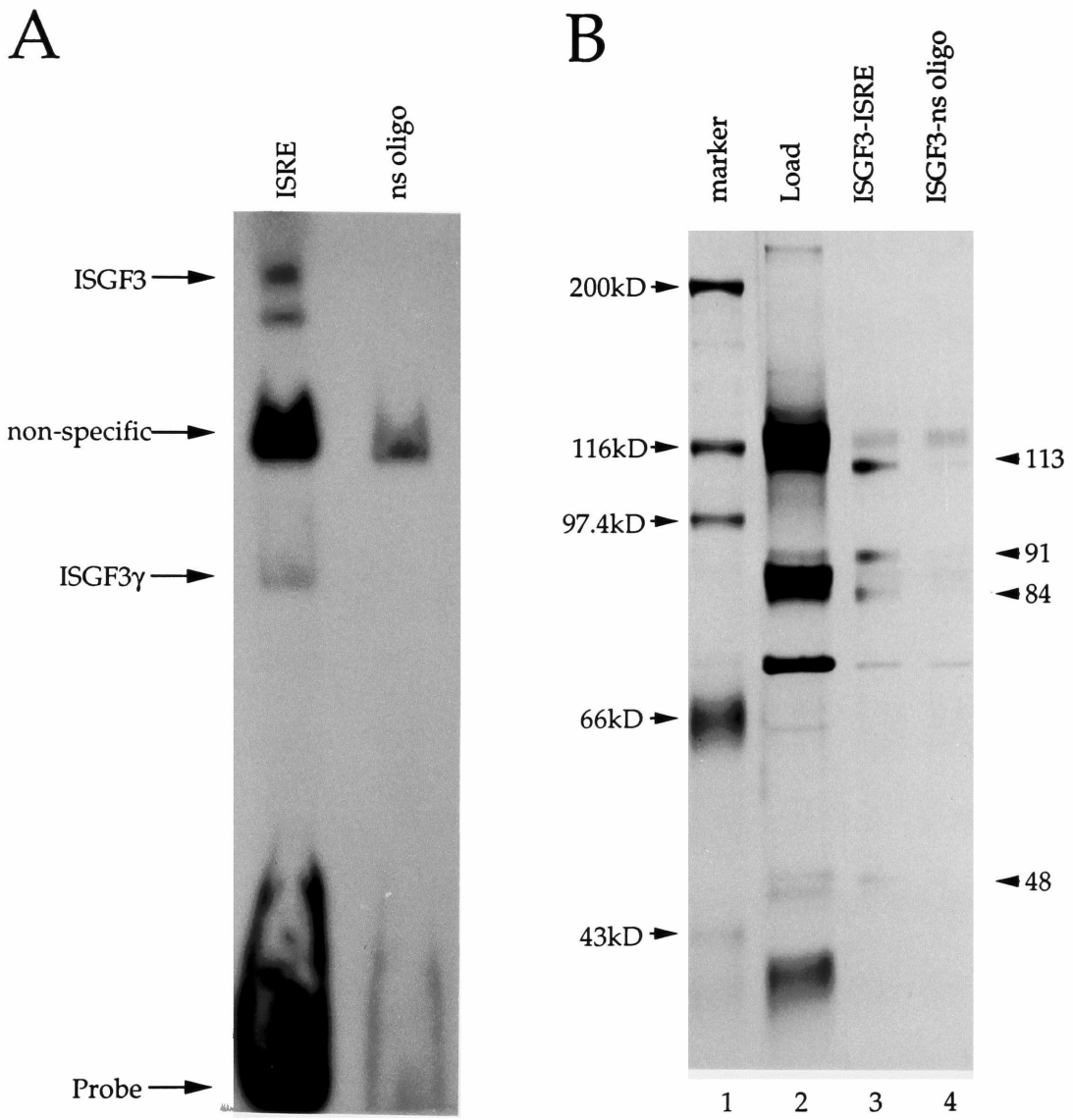
An additional sedimentation experiment using a fraction of nuclear ISGF3 consisting of only a small number of polypeptides allowed direct observation of proteins cofractionating with ISGF3α and ISGF3γ. As in the previous sedimentation analysis (Fig 6.2) the ISGF3 complex was unstable (Fig 6.7A) and mixing gradient fractions with cytoplasmic ISGF3α or ISGF3γ identified non-overlapping peaks of activity (Figure 6.7 B and C). The peak fractions for ISGF3γ activity (Fig 6.7B, lanes 4-6) also contained the faster mobility DNA binding activity identified as ISGF3γ when assayed without

Figure 6.7 Polypeptide composition of ISGF3. Nuclear ISGF3 was fractionated by glycerol gradient centrifugation, and each fraction was assayed for activity by gel shift assay either before (**A**) or after addition of cytoplasmic ISGF3α (**B**) or ISGF3γ (**C**). Note that although no ISGF3 was detected in the direct assay, ISGF3γ was detected in fractions 4 and 5. After mixing with ISGF3α, this ISGF3γ formed ISGF3 (**B**). Mixing with ISGF3γ (**C**) formed ISGF3 from material in fractions 9 and 10, signifying the presence of ISGF3α fractionating at this position in the gradient. (**D**) Portions of each fraction were analyzed by 7.5% SDS-PAGE followed by silver staining (Wray et al., 1981). Note that fractions 4 and 5 contained a single 48 kD polypeptide corresponding to ISGF3γ. Many polypeptides cofractionated with ISGF3α activity in fractions 9 and 10.


A
30kD 46kD 66kD 97.4kD 232kD
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ISGF3
ISGF3γ
Probe
B
+ISGF3α
α 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ISGF3
ISGF3γ
Probe
C
+ISGF3γ
γ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ISGF3
ISGF3γ
Probe
D
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
200kD
116kD
97.4kD
66kD
43kD

mixing (Fig 6.7A, lanes 4-6). ISGF3γ activity was quantitatively depleted with the addition of ISGF3α to form ISGF3, consistent with the participation of ISGF3γ in the ISGF3 complex (Fig 6.7B, lanes 4-6). The polypeptide composition of gradient fractions was resolved by 7.5% SDS-PAGE and stained with silver. The starting fraction consisted of three highly abundant proteins and several additional polypeptides (Fig 6.7D, lane L). A single polypeptide of 48 kD was detected in gradient fractions cosedimenting with ISGF3γ (lanes 4–6), consistent with previous estimates of the size of ISGF3γ. Identification of potential ISGF3α polypeptides was considerably more complicated. Several proteins were detected in gradient fractions containing peak ISGF3α activity (lanes 9 and 10). Comparison of the sedimentation pattern of these proteins throughout the gradient allowed tentative identification of two highly abundant polypeptides, four additional major polypeptides, and numerous minor polypeptides which precisely cosedimented with ISGF3α. Two points suggested that the less abundant polypeptides in the range of 80–120 kD rather than the two major proteins constituted ISGF3α. First, given the similar recovery of ISGF3α and ISGF3γ activity, a quantity of ISGF3α protein(s) stoichiometric to the 48 kD ISGF3γ protein might be expected. Assuming no great differences in the staining

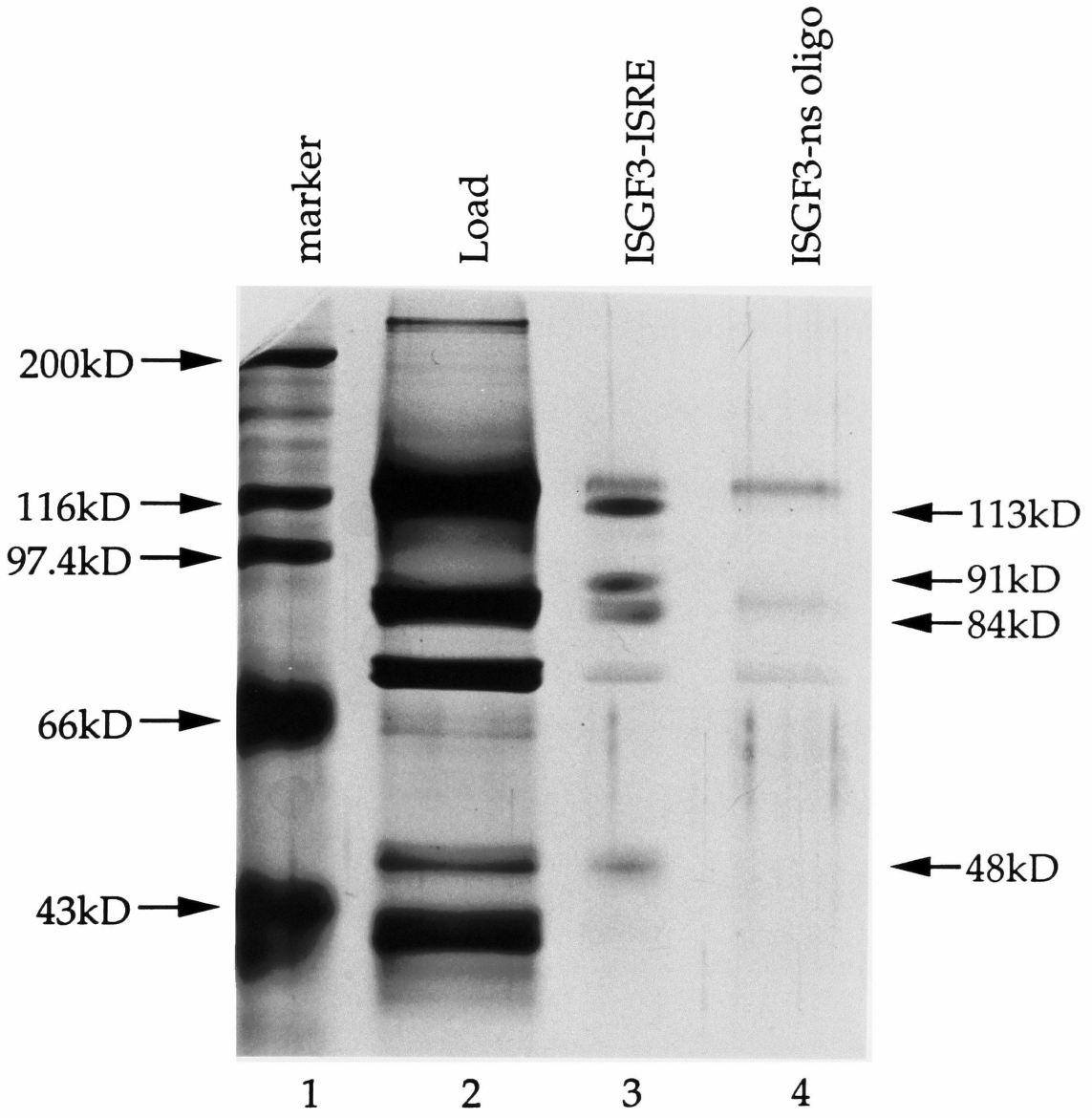
Figure 6.8 Preparative gel shift analysis of ISGF3. Nuclear ISGF3 was mixed with radiolabeled ISRE or heterologous DNA and fractionated by gel shift assay (**A**). Following autoradiography, ISGF3 and ISGF3γ protein-ISRE complexes were excised as well as the corresponding region from the heterologous DNA lane. These gel slices were electroeluted onto 8% SDS-PAGE which was subsequently stained with silver (**B**). A Mono Q fraction of nuclear ISGF3 (Load, lane 2) was used for this analysis.


A
ISRE
ns oligo
ISGF3
non-specific
ISGF3γ
Probe
B
marker
Load
ISGF3-ISRE
ISGF3-ns oligo
200kD
116kD
97.4kD
66kD
43kD
113
91
84
48
1
2
3
4

sensitivity of these proteins, the two highly abundant proteins were clearly present in large molar excess over ISGF3γ, while four proteins were present in quantities very similar to ISGF3γ. Second, the size range (80–120 kD) of a subset of the less abundant polypeptides correlated with those identified by crosslinking.

Direct identification of the polypeptide subunits of ISGF3 was possible through preparative gel shift analysis. A large scale DNA-binding reaction consisting of high purity nuclear ISGF3 incubated with an ISRE or unrelated radiolabeled oligonucleotide probe was resolved by electrophoresis (Fig 6.8A). ISGF3 and ISGF3γ complexes and parallel regions from a control lane were excised, and polypeptides were directly electroeluted into 8% SDS-PAGE and stained with silver. Seven polypeptides were recovered from the gel slice containing ISGF3 bound to the ISRE, three corresponding to high abundance proteins and four to proteins of low abundance in the starting fraction (Fig 6.8B, lane 3). A parallel region from a control reaction contained the three high abundance proteins, indicating that four low abundance proteins from the starting fraction were specifically complexed to the ISRE (lane 4). Sizes of the four specific proteins were consistent with other analyses of ISGF3α and ISGF3γ. The 48 kD protein corresponds to ISGF3γ, as shown by renaturation,

Figure 6.9 Preparative gel shift analysis of ISGF3. The experiment was carried out as described in Fig 6.8 using the same starting fraction.

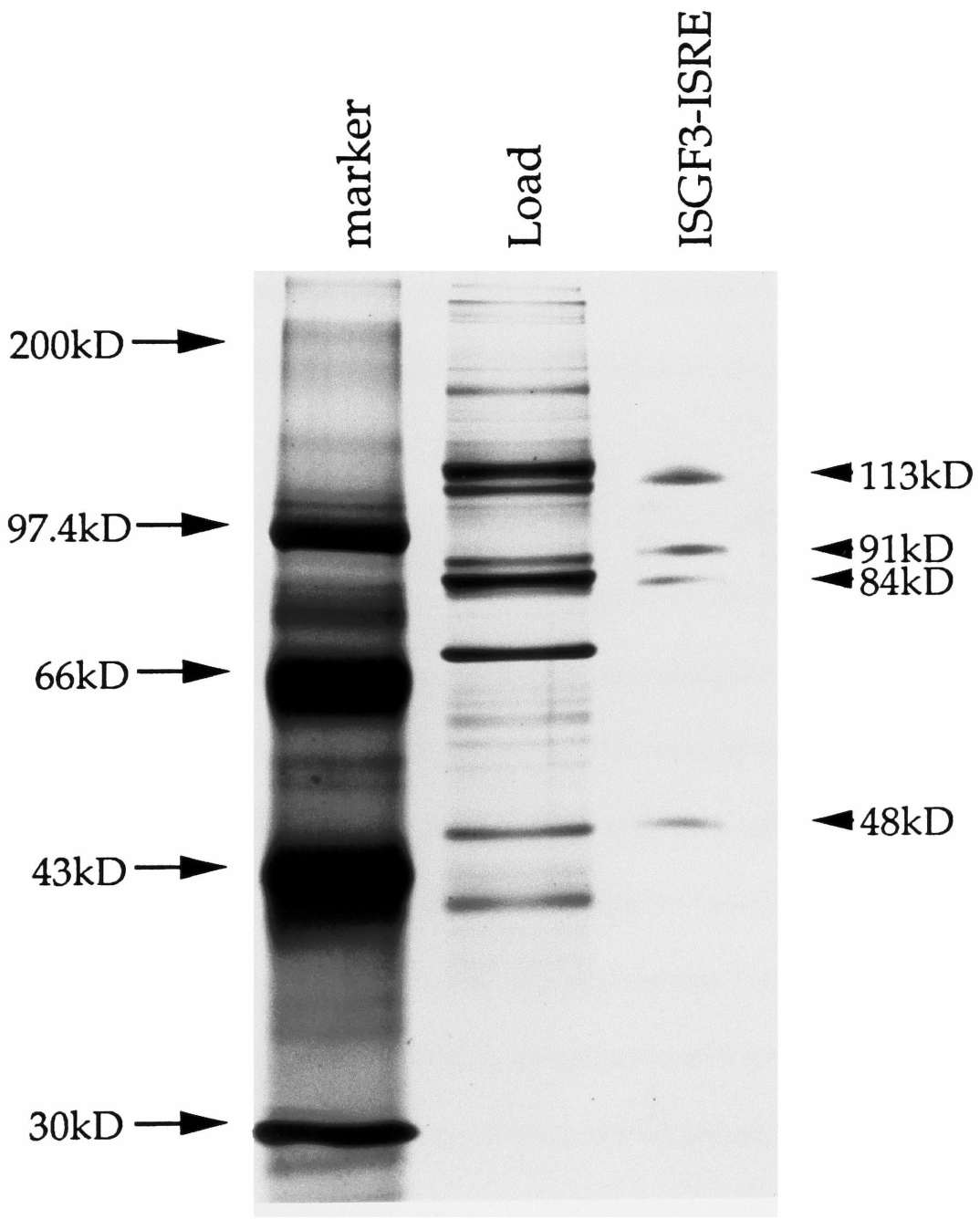

marker
Load
ISGF3-ISRE
ISGF3-ns oligo
200kD
116kD
97.4kD
66kD
43kD
113kD
91kD
84kD
48kD
1
2
3
4

crosslinking, and sedimentation, while three proteins (84 kD, 91 kD, and 113 kD) would be candidates for ISGF3α. As expected, a single 48 kD polypeptide was recovered from the ISGF3γ-ISRE complex. The three abundant, contaminating proteins present in the region of the ISGF3-ISRE complex were found throughout the one-dimensional separation, probably due to their high abundance (data not shown). This result was precisely replicated in a second, independent experiment (Fig 6.9). In a third experiment of this type, using an extract which did not contain the major nonspecific DNA-binding activity present in the previous experiments (Fig 6.8A), only the four ISRE-specific polypeptides (48, 84, 91, and 113 kD) were recovered (Fig 6.10).

Regulated nuclear translocation of ISGF3α

The activation of ISGF3 in the cytoplasm of IFNα-stimulated cells and subsequent appearance in the nucleus implied nuclear translocation of one or both of the components of ISGF3. The presence of ISGF3γ in both compartments of untreated cells suggested that active nuclear translocation of this factor may not be an essential element of IFNα signalling. ISGF3α, on the other hand, was activated in the cytoplasm within 30 sec of treatment and only appeared in the nucleus after 5 min (Levy et al., 1989). Evidence in support of a role for regulated nuclear translocation in the response to IFNα

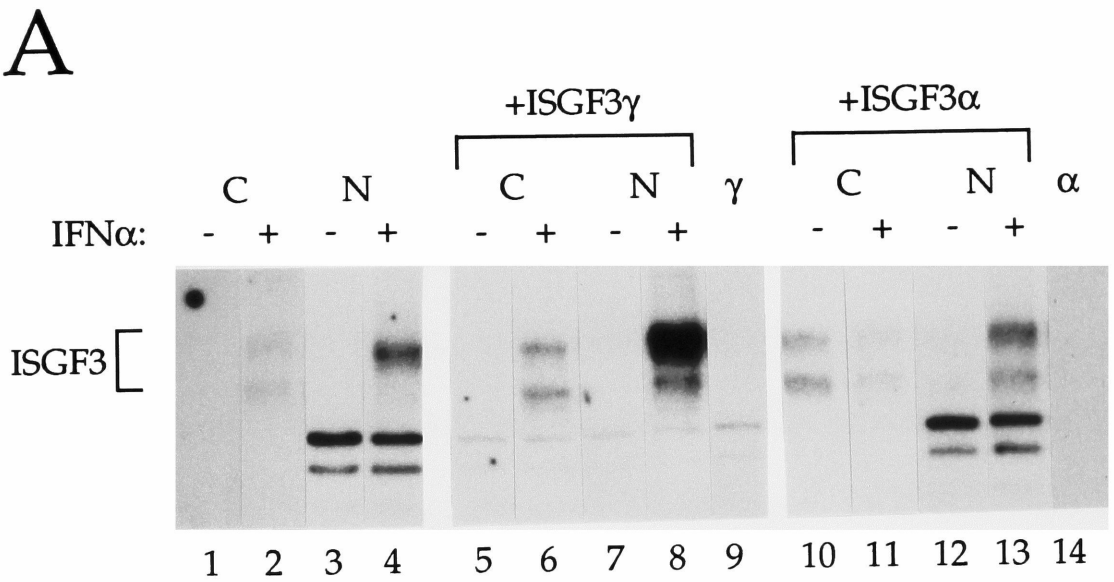
Figure 6.10 Preparative gel shift analysis of ISGF3. The experiment was carried out as described in Fig 6.8 except the fraction used did not contain major non-specific DNA-binding activities.

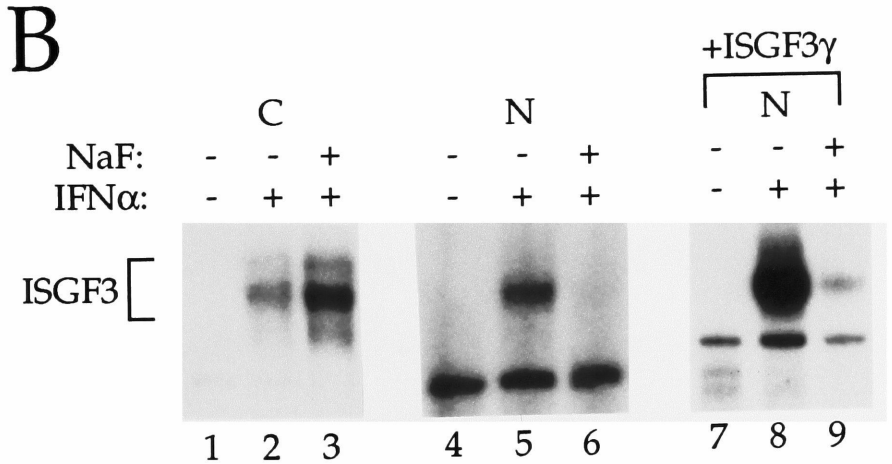

marker
Load
ISGF3-ISRE
200kD
97.4kD
66kD
43kD
30kD
113kD
91kD
84kD
48kD

was obtained through the use of NaF, an inhibitor of ISG induction. We previously reported a partial inhibition of ISG induction by IFNα acted at the level of nuclear accumulation of ISGF3 (Levy et al., 1989). In cells pretreated with NaF the total level of ISGF3 produced was normal, but a large fraction of ISGF3 was sequestered in the cytoplasm with a concomitant decrease in nuclear levels. The mechanism of inhibition by NaF is undefined but its effect points to the important role of ISGF3 nuclear translocation in the response.

A modification of the previously used NaF inhibition protocol (Chapter 5) has resulted in quantitative inhibition of nuclear accumulation of ISGF3. Control cells treated with IFNα displayed comparable levels of ISGF3 in cytoplasm and nucleus (Fig 6.11B, lanes 2 and 5). NaF pretreatment of cells for 1 hr followed by IFNα stimulation resulted in normal total ISGF3 levels, but >95% of the activity was retained in the cytoplasm (lane 3), with virtually no ISGF3 appearing in nuclei (lane 6). To explore the level at which NaF blocked nuclear accumulation of ISGF3, cytoplasmic and nuclear extracts of NaF pretreated or control IFNα-treated cells were assayed for ISGF3α and ISGF3γ. Exclusion of ISGF3 from nuclei in NaF-treated cells was reflected in extremely low levels of nuclear ISGF3α (lane 9) while ISGF3γ levels were

Figure 6.11 Regulated nuclear translocation of ISGF3. **A.** ISGF3α translocates to the nucleus in excess of the amounts of ISGF3γ. HeLa cells were treated with IFNα for 15 min as indicated. Cytoplasmic (C) or nuclear extracts (N) were analyzed for ISGF3 by gel shift assay directly (lanes 1-4) or following addition of ISGF3γ (lanes 5-8) or ISGF3α (lanes 10-13). Addition of ISGF3γ formed much greater amounts of ISGF3 than had been detected by the direct assay, revealing the presence of excess ISGF3α in nuclei from IFNα-treated cells (lane 8). Addition of ISGF3α to these samples (lane 13) did not create any more ISGF3 than had been detected directly (lane 4), indicating the lack of free ISGF3γ in these extracts. **B.** NaF blocks nuclear accumulation of ISGF3α Cytoplasmic (lanes 1-3) and nuclear extracts (lanes 4-9) were prepared from HeLa cells before (lanes 1, 4, 7) or after treatment with IFNα for 15 min (lanes 2, 5, 8) or with NaF for 1 h followed by IFNα for 15 min (lanes 3, 6, 9). Extracts were analyzed by mobility-shift using an ISRE probe either directly (lanes 1-6) or following addition of cytoplasmic ISGF3γ (lanes 7-9).


A
+ISGF3γ
+ISGF3α
C
N
C
N
γ
C
N
α
IFNα: - + - + - + - + - + - + - +
ISGF3
1 2 3 4 5 6 7 8 9 10 11 12 13 14


B
+ISGF3γ
C
N
N
NaF: - - + - - + - - +
IFNα: - + + - + + - + +
ISGF3
1 2 3 4 5 6 7 8 9

unperturbed (data not shown). Therefore, inhibition by NaF appeared to act at the level of ISGF3α nuclear translocation, an event essential for nuclear accumulation of ISGF3.

Quantitation of the cytoplasmic and nuclear levels of ISGF3 and its components provided further evidence for independent nuclear translocation of ISGF3α. Extracts prepared from untreated or stimulated cells were assayed for ISGF3 activity, and for total levels of ISGF3α and ISGF3γ (Fig 6.11A). Addition of ISGF3γ to induced extracts resulted in a marked increase in ISGF3 activity, suggesting the presence of excess ISGF3α in both the cytoplasm and nucleus of stimulated cells (compare lanes 2 and 6 and lanes 4 and 8; see also Fig 6.11B, lane 8). Addition of ISGF3α, on the other hand, while detecting constitutive cytoplasmic ISGF3γ (lane 10 and 11), did not enhance the level of nuclear ISGF3 over that obtained without mixing (compare lanes 4 and 13). These observations indicated that ISGF3α was activated in cells in excess of the level of ISGF3γ and that it accumulated preferentially in nuclei also in excess. These results argue that ISGF3α was capable of nuclear translocation independent of its association with ISGF3γ. In contrast, nuclear ISGF3γ levels corresponded to total nuclear ISGF3. Accumulation of substantial nuclear ISGF3γ was dependent on the presence

of ISGF3α, presumably stabilizing its participation in the ISGF3 complex.

Discussion

We have presented evidence that ISGF3 is a multicomponent complex, supporting our previous conclusion that it was formed from stoichiometric association of distinct subunit activities (Levy et al., 1989). Analyses of highly purified fractions of cytoplasmic ISGF3α and ISGF3γ as well as of intact ISGF3, by preparative polyacrylamide gel electrophoresis, photoaffinity labeling, and sedimentation demonstrated the distinct characteristics of these individual activities and revealed a multicomponent nature for ISGF3α. ISGF3α and ISGF3γ, either purified from cytoplasm or recovered from purified fractions of nuclear ISGF3, were sufficient to reconstitute ISGF3 *in vitro* without requiring additional cellular proteins. Interestingly, ISGF3γ was found to be a novel ISRE-binding protein present in both cytoplasm and nuclei of unstimulated cells. Its low-affinity binding to DNA was stabilized in the presence of ISGF3α, a complex of several proteins present in nuclei only following treatment with IFNα.

The molecular composition of ISGF3 appeared to be heterotetrameric, consisting of a 48 kD DNA binding polypeptide (ISGF3γ) and possibly three

regulatory proteins of 84, 91, and 113 kD comprising ISGF3α. From the present data, it is impossible to determine conclusively the exact polypeptide composition of ISGF3α. It is possible that the three polypeptides tentatively identified as ISGF3α are related to one another, either through modification or through proteolytic degradation during purification. Alternatively, there could be three distinct forms of ISGF3α, each consisting of one of these polypeptides. However, the apparent stoichiometric recovery of all three polypeptides by preparative gel shift analysis argues that they were all members of the mature ISGF3 complex.

ISGF3α appeared to be the initial target in IFNα signalling. Its activation presumably depended on as yet unidentified post-translational modifications of one or more of its polypeptide chains. ISGF3α thus fits the criteria for being the regulatory subunit of ISGF3. Activation of ISGF3α stimulated its nuclear translocation and its association with ISGF3γ and DNA where allosteric interaction increased the affinity of the mature complex for the ISRE over that of the independent DNA binding subunit (Fig 6.12).

Heteromeric transcriptional regulators

Combinatorial assembly of multicomponent transcription complexes from individual DNA-binding and transcriptional regulatory proteins

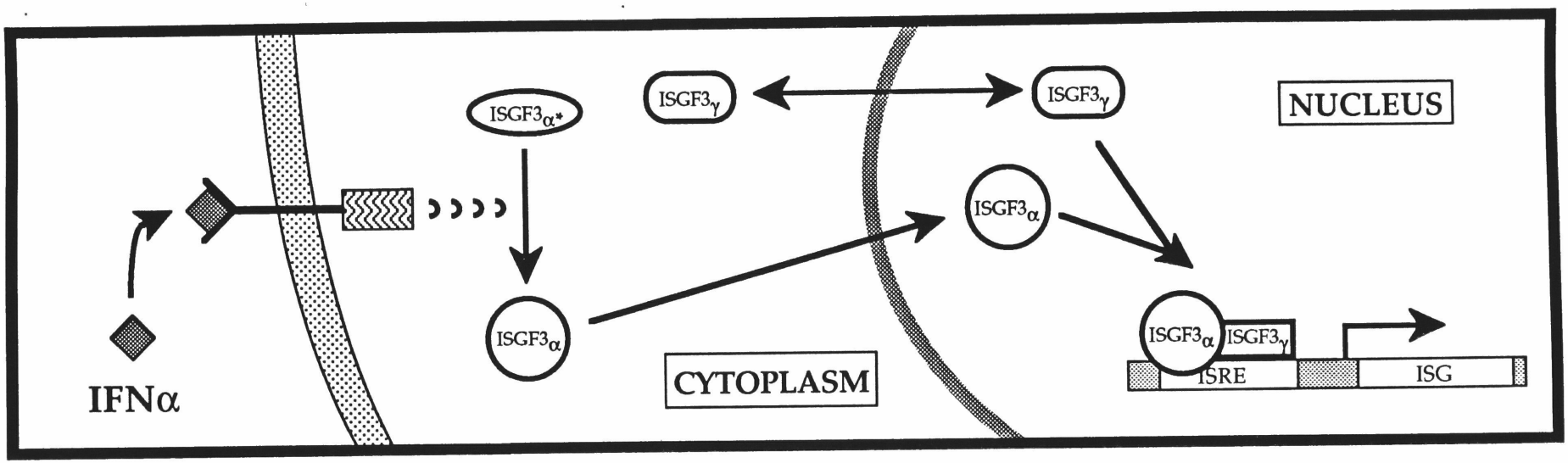


Figure 6.12 Model of signal transduction by IFNα. Ligand-dependent activation of ISGF3α in cytoplasm of IFNα-treated cells involves post-translational modification of a latent activity. Activated ISGF3α translocates to the nucleus where it can stabilize ISGF3γ binding at the ISRE through allosteric conformational changes. ISGF3γ is free to partition between cytoplasm and nuclei; association with ISGF3α and stable binding at the ISRE causes it to accumulate in nuclei following IFNα treatment. Ultimately, ISRE bound ISGF3 directly transcriptional activation of ISGs.

appears to be a common theme in eukaryotic developmental and tissue-specific transcriptional control. In many cases, this process involves interaction of multiple, sequence-specific DNA binding proteins at distinct cis-acting regulatory sites resulting in additive or synergistic effects on gene expression. Formation of heteromeric protein complexes on single DNA sites has also been found to enhance or reduce DNA-binding affinity, to generate novel DNA-binding affinities, and to modulate transcriptional activity of previously bound proteins.

Several examples have been defined recently in which interaction of distinct protein subunits modulate the affinity of the resulting complex for DNA. In some cases, these interactions appear to be involved in developmental or cell-type specific regulation of gene expression. In the yeast Saccharomyces cerevisiae, UAS2 of the CYC1 gene, a gene transcribed in response to nonfermentable carbon sources, contains a CCAAT box recognized by a trimeric transcription factor composed of the HAP2/3/4 polypeptides (Forsburg and Guarente, 1989). Mammalian cells have homologous proteins (Chodosh et al., 1988a), containing at least three distinct CCAAT box-binding factors with different sequence specificities, each of which is at least dimeric (Chodosh et al., 1988b). A second example of

modulated DNA affinity through protein-protein interaction was described for the AP-1 family of transcription factors (GCN4 in yeast and fos- and jun-related proteins in mammalian cells; see (Curran and Franza, 1988) for review). The individual subunits of these proteins display at best limited affinity for DNA, while homodimers or heterodimers, which are stable even in the absence of DNA, bind with high specificity and affinity. Differential production of individual subunits, combinatorial complex formation, and possibly differential activation are postulated to allow these proteins to participate in both positive and negative regulatory circuits (Nakabeppu et al., 1988; Cohen et al., 1989; Chiu et al., 1989; Schonthal et al., 1989; Schutte et al., 1989).

In the case of ISGF3, the ISGF3γ polypeptide binds the ISRE with low affinity while heteromeric association with the ISGF3α chains induces 25-fold greater stability for the protein-DNA complex. The low affinity interaction between ISGF3γ and DNA may explain its presence in the cytoplasmic compartment, an unexpected finding for a DNA binding protein. Since ISGF3α displays no DNA-binding activity on its own, a probable basis for high affinity ISGF3 is an allosteric effect on ISGF3γ imposed by interaction with ISGF3α. An additional possibility would be the exposure of cryptic

DNA-binding sites on one or more ISGF3α polypeptides through interaction with ISGF3γ. It should be noted that photoaffinity crosslinking of ISGF3 to the ISRE specifically labelled a set of polypeptides in the same size range as putative ISGF3α proteins which were not seen in the ISGF3γ-ISRE complex. However, if ISGF3α makes additional contacts with DNA not present in the ISGF3γ-ISRE complex, these contacts were not detected by orthophenanthroline-Cu footprinting. Inefficiency of crosslinking these larger polypeptides suggests that while they were brought into close proximity to DNA by ISGF3γ, they may not make sequence-specific contacts at the ISRE. Similar interactions between DNA-binding domains and accessory proteins which modulate the overall affinity of the resulting complex have been noted for the activation of transcription by adenovirus E1a (Reichel et al., 1989; Hardy et al., 1989) and for transactivation of chicken ovalbumin expression (Tasi et al., 1987).

Subcellular compartmentalization and nuclear translocation in gene regulation

Subcellular division of eukaryotic cells into nucleus and cytoplasm opens the possibility of differential regulation through compartmentalization since transcription factors excluded from nuclei would be inactive (Hunt,

1989). This may have particular relevance in terms of transcriptional responses to extracellular signals since their nature requires passage through the cytoplasm. The glucocorticoid receptor may be regulated through compartmentalization since, in the absence of ligand, this transcription factor appears to be anchored in the cytoplasm through interaction with hsp90 (Sanchez et al., 1985; Catelli et al., 1985) or other cellular proteins (Gasc et al., 1989; Tai et al., 1986). However, the major regulation of the transcriptional stimulatory activity of glucocorticoid receptor requires allosteric changes associated with hormone binding not involved in subcellular localization (Evans, 1988; Picard et al., 1988). Regulation of the transcription factor NF-kB during B-cell maturation, on the other hand, involves inducible changes which allow nuclear translocation as well as uncovering of DNA-binding activity (Lenardo et al., 1989). This factor is bound to a cytoplasmic inhibitory protein, I-kB, which appears to both anchor the transcription factor in the cytoplasm as well as mask its DNA-binding ability (Baeuerle and Baltimore, 1988a, 1988b). I-kB appears to be a substrate for protein kinases and becomes phosphorylated during NF-kB activation, thus releasing the DNA binding domain for nuclear translocation (Baeuerle et al., 1988b; Shirakawa and Mizel, 1989). Likewise, it has recently been shown the Drosophila *dorsal* protein (a

protein related to vertebrate c-rel and presumably involved in transcriptional control) regulates dorsal-ventral polarity during early embryogenesis by differential nuclear accumulation (Rushlow et al., 1989; Steward, 1989; Roth et al., 1989). *dorsal* is regulated through a complex pathway involving at least 12 gene products, one of which (*cactus*) appears to be a cytoplasmic anchor. It appears that the transcription factor itself, rather than its cytoplasmic anchor, is the target for modification in the regulatory cascade in the *dorsal* system.

Regulated nuclear localization is central to the activation of ISGF3. Intracellular signalling triggered by IFNα binding to its cell surface receptor targets ISGF3α for modification in the cytoplasm. Neither the nature of ISGF3α modification nor the intracellular events leading to its activation are yet known, but the consequence of these events is the ability of ISGF3α to translocate to the nucleus, associate with ISGF3γ, and assemble an active transcription complex on the ISRE. Whether release from a cytoplasmic anchor or creation or uncovering of nuclear translocation signals is the basis for this regulation is unknown and must await further characterization of these proteins. However, it would appear that ISGF3α actively translocated independent of ISGF3γ or of the formation of mature ISGF3. This translocation was blocked by pretreatment of cells with NaF. In addition,

ISGF3α was detected in nuclei from IFNα-treated cells in large excess over the level of ISGF3γ or of mature ISGF3. The DNA-binding subunit ISGF3γ, on the other hand, was detected in both cytoplasm and nuclei of unstimulated cells. This distribution probably reflects its size (<50 kD), allowing passive diffusion through nuclear pores, and its low affinity for DNA in the absence of ISGF3α, leaving it with little tendency to accumulate in nuclei independent of complete ISGF3.

Specificity in signal transduction

The precision of transcriptional responses to extracellular ligands reflects the inherent specificity of ligand-receptor interactions and therefore requires equal precision in second messenger pathways carrying the information to the nucleus. Intracellular signalling through perturbation of the levels of common second messenger molecules such as inositol phospholipids, cAMP, and calcium ions has been demonstrated for a growing number of cellular inducers (Rozengurt, 1986; Berridge, 1989; Herschman, 1989). What remains unclear from these pathways, however, is how the specificity of many different ligand-receptor pairs can be maintained through such general pathways of common components to produce distinct metabolic responses.

In the IFNα system, this problem is at least partially solved through the participation of specific DNA-binding proteins at a very early stage in the signal pathway. ISGF3γ carries specificity for the ISRE and therefore activates only ISGs and not other gene families lacking this specific sequence. However, it exerts its transcriptional activity only following IFNα stimulation since its low affinity for DNA probably precludes formation of productive transcriptional complexes in the absence of ISGF3α. The regulatory subunit ISGF3α, on the other hand, is active only in response to IFNα. Its activation in the cytoplasm allows it to be directly acted upon following IFNα-receptor interaction, and its active nuclear translocation allows it to be a specific carrier of signalling information. An as yet missing link in the biochemical characterization of this signalling pathway is the nature of the modifying signal transmitted by the IFNα receptor to initiate ISGF3α activation. This information must await isolation and characterization of the ISGF3 polypeptides.

Material and methods

Gel shift analysis

Crude extracts were prepared as previously described (Levy et al., 1989). Gel retardation analysis was performed essentially as described by Fried and Crothers (1981). End-labeled ISRE oligonucleotide (0.5-5.0 ng) derived from the ISG15 promoter, was incubated with protein fractions in 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 320 μg/ml poly(dIdC):poly(dIdC), 8 μg/ml of unrelated oligonucleotide as non-specific competitor, and 4% Ficoll in a final volume of 12.5 μl for 20 min at 22°C. For high purity fractions the quantity of nonspecific DNA in the reaction was reduced to 32 μg/ml poly(dIdC):poly(dIdC) and 8 μg/ml of unrelated oligonucleotide. Protein-DNA complexes were resolved on a 6% low ionic strength polyacrylamide gel run at 300V for 2 hr at 22°C. *In vitro* complementation was achieved by including an appropriate complementing extract in the standard DNA-binding reaction as described above.

Preparation of cytoplasmic ISGF3γ

ISGF3γ fractions were prepared from S100 extracts of HeLa S3 cells grown in suspension and treated with 1 ng/ml IFNγ for 18-24 hours as described (Levy et al., 1990). Protein precipitated by 50% saturated ammonium

sulfate was resuspended in and dialyzed against column buffer (20 mM Tris-HCl (pH 7.4) or Hepes (pH 7.6), 10% glycerol, 0.1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) containing 50 mM KCl, then chromatographed on heparin-sepharose, phenyl sepharose and FPLC Mono Q. Protein which bound to columns was eluted with linear KCl gradients or, for phenyl sepharose, column buffer containing 50% ethylene glycol. Column fractions were assayed by *in vitro* complementation assays. The specific activity of ISGF3γ was increased 1000-2000-fold over crude cytoplasm.

Preparation of cytoplasmic ISGF3α

ISGF3α fractions were prepared from S100 extracts of HeLa S3 cells as above, except that cells were treated with 10 mM NaF for 1 hour and IFNα at 10^4 U/ml for 15 minutes. Protein was alkylated with 10 mM N-ethyl maleimide (NEM) for 10 minutes, then unreacted NEM was quenched by addition of 15 mM DTT. Protein precipitated by 30% saturated ammonium sulfate was resuspended, dialyzed and chromatographed as for ISGF3γ, except DEAE-sephacel, FPLC Mono Q and FPLC Mono S columns were used. The specific activity of ISGF3α was increased 1000-2000-fold over crude cytoplasm. Nuclear ISGF3 was prepared as described (X-Y. Fu et al., submitted).

Sedimentation analysis

Gradients (15-30% glycerol, 20 mM Hepes [pH 7.9], 50 mM KCl, 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF) were prepared and fractionated with a Hakke-Buschler gradient former. Protein markers or fractions (0.2-0.3 ml) adjusted to 10% glycerol, 300 mM KCl were layered on 12 ml gradients and centrifuged for 40 hr at 40,000 rpm at 4°C (SW40.ti rotor). Fractions were assayed for ISGF3 activity by gel shift. ISGF3α and ISGF3γ activity was detected by *in vitro* complementation of fractions with crude or enriched fractions of ISGF3γ and ISGF3α, respectively, and gel shift assay. Marker fractions were resolved on 10% SDS-PAGE and stained with Commassie.

For sedimentation analysis of ISGF3-ISRE complexes a 15X standard DNA-binding reaction with labeled ISRE or unrelated oligonucleotide was loaded on gradients as described above, except centrifugation was for 24 hr. Fractions were assayed directly on low ionic strength polyacrylamide gels, or assayed by *in vitro* complementation and gel shift analysis. Fractions were concentrated using Centricon 30, recovered protein resolved on 7.5% SDS-PAGE, and stained with silver for analysis of protein composition of gradient fractions.

Copper-Phenanthroline Footprinting

Gel shift analysis was performed using uniquely end-labelled ISRE DNA as probe. Following electrophoresis, the entire shift gel was treated with 1,10-phenanthroline-copper ion as described (Kuwabra and Sigman, 1987) with the following modifications. Reactions were performed at 37°C for 5 min and 1,10-phenanthroline, copper sulfate and DTT were added to final concentrations of 1 mM, 0.2 mM and 2 mM, respectively. Gels were autoradiographed and DNA recovered from excised regions corresponding to nuclear ISGF3, cytoplasmic ISGF3 and ISGF3γ was resolved on an 8% sequencing gel.

Off-rate determination

Off-rate analysis was performed essentially as described (Hardy et al., 1989) using enriched cytoplasmic ISGF3α, ISGF3γ, and nuclear ISGF3 fractions. Formation of DNA-protein complexes reached equilibrium by 20 min, at which time a 500-fold molar excess of unlabeled ISRE oligonucleotide was added, and at serial time points aliquots were analyzed by gel shift. The intensity of bands was quantitated by laser densitometry (LKB Ultroscan XL) of autoradiographs exposed without intensifying screens.

Renaturation of ISGF3γ

A 200-fold enriched ISGF3γ fraction was concentrated using Centricon 30, and resolved on 10% SDS-PAGE which was pre-run with 0.1 mM sodium thioglycolate. Using Rainbow protein markers (Amersham) as a guide, gel regions were excised, protein was eluted by "crush and soak", supernatants subjected to acetone precipitation, and pellets resuspended in 40 µl column buffer (as above) containing 100 mM KCl, 0.1% NP-40 and 6 M guanidine-HCl. After eluates were incubated for 40 min at room temperature, guanidine-HCl was removed with G-25 spun columns equilibrated in the same buffer without guanidine-HCl. Following an additional 2 hours at room temperature, ISGF3γ activity was assayed by *in vitro* complementation.

Photoaffinity crosslinking

A uniformly radiolabeled 5-azido-deoxyuridine substituted (kindly provided by Dr. R.K. Evans) ISRE probe was incubated with fractions under standard conditions and DNA-protein complexes resolved by gel shift assay (Woody et al., 1988; Evans et al., 1986). The intact gel was exposed to ultraviolet irradiation for 5 min on ice in a Stratalinker2400 (Stratagene) and autoradiographed. Identified ISRE complexes were excised, incubated for 15 min at 65°C in sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 5% β-

mercaptoethanol, 2.3% SDS), rinsed twice with stacking buffer (62.5 mM Tris-HCl [pH 6.8], 0.1% SDS), protein resolved by direct elution onto 8% SDS-PAGE, and visualized by autoradiography. ^{14}C-labeled protein markers were loaded on neighboring lanes for size determination.

Preparative gel shift analysis

For the analysis of polypeptides present in the ISGF3-ISRE and ISGF3γ-ISRE complexes a 7X DNA-binding reaction including a total of 175 ng of ISRE or unrelated oligonucleotide was resolved by standard gel shift analysis. Specific ISRE complexes or a parallel region of a control lane were excised following autoradiography, gel slices were incubated 15 min at 65°C in sample buffer, and rinsed twice with stacking buffer. Polypeptides were directly electroeluted onto 8% SDS-PAGE and detected by silver stain.

Chapter 7

Summary and Future Directions

Summary

The series of experiments that constitutes this thesis contributes to the growing understanding of signal transduction and gene regulation in response to cell surface ligands. For the type I IFN system, an informative, yet incomplete model of the cellular regulatory strategy employed has been developed. In forming this model several questions have been addressed, including those set out in Chapter 1.

What are the cis-regulatory elements which are ultimate targets for signal transduction?

The ISRE, a conserved regulatory element present in all type I IFN responsive genes is both necessary and sufficient for IFN-dependent transcriptional response (Chapter 2; and Reich and Darnell, 1989). This nuclear component of the IFN signalling system is a terminal target of occupied-receptor initiated signal transduction. While the role of the ISRE in transcriptional activation is clear, it has not been possible, for technical reasons, to attribute the regulated decline in ISG expression to the ISRE, or any other specific sequence in ISG promoters.

What are the trans-acting factors that mediate IFN-dependent transcriptional control through the ISRE?

Three nuclear DNA-binding proteins specific for the ISRE have been identified and characterized (Chapter 2). Extensive analysis of the two IFN-induced factors, ISGF2 and ISGF3, has provided strong support for the positive regulatory role of ISGF3 (Chapters 3 and 4). Analysis of ISGF2, on the other hand, has failed to implicate this factor in any aspect of ISG regulation (Chapters 2-4; Pine et al., 1990). Furthermore, the constitutive ISRE-binding factor, ISGF1, has not received much attention and may, in fact, play a central role in all phases of ISG regulation. The existence of multiple ISRE-binding proteins suggests that complex regulatory interactions between these factors may operate. However, in all situations studied, the level of ISG expression in response to type I IFN was apparently a function of intracellular ISGF3 concentration (Chapter 2 and 3; Levy et al., 1990). The ostensible simplicity of ISG regulation is, perhaps, unexpected and suggests two alternative explanations. First, ISG regulation is indeed complex and is ultimately determined through the interactions of multiple ISRE-binding proteins, but due to the characteristics of the experimental system (tissue culture cells stimulated with high levels of specific ligand in the absence a variety of physiologically important inputs) this complexity is not observed. Second, ISG regulation is completely dependent upon the intracellular concentration

of ISGF3, and the binding of additional factors to the ISRE is an in vitro phenomenon which has no relevance in vivo. Clarification of these issues will depend on the application of techniques allowing the description of ISRE occupancy in vivo under various conditions.

The characteristics of ISG induction point to two aspects of regulation: positive regulation through pre-existing cellular proteins and negative regulation through newly synthesized proteins. As discussed, ISGF3 is strongly implicated as the positive regulator of ISG induction acting at the ISRE. This factor is post-translationally activated in a transient manner, as is ISG expression. Therefore, it is possible that down-regulation of ISGs is mediated at the level of ISGF3 inactivation, and does not involve additional ISRE-binding factors. Contrary to this idea is the observation that protein synthesis inhibitors have little effect on the IFN-induced accumulation and decline of ISGF3, whereas ISG expression is maintained at a high level in the presence of protein synthesis inhibitors. This suggests that a decline in ISGF3 levels is not sufficient for the decline in ISG expression and that labile factors must also be involved. As yet, little information regarding the nature of ISGF3 inactivation or any other element of ISG down-regulation is available, and therefore we cannot address this aspect of ISG regulation.

What is the nature of intracellular messengers in the type I IFN system?

As discussed, no evidence exists implicating the "classical" second messengers in IFN-stimulated signal transduction. However, the observation that ISGF3 is activated in the cytoplasm of stimulated cells and subsequently translocates to the nucleus (Chapter 5) identifies this DNA-binding factor as an important intracellular messenger involved in transmitting cell surface signals across compartmental boundaries. ISGF3 is detectable in the cytoplasm within 1 min of treatment and therefore represents an early target for ligand-receptor initiated signalling. The multifunctional nature of ISGF3 as an intracellular messenger and transcriptional activator is reflected in its multisubunit composition. ISGF3 is apparently a heterotetramer with distinct subunits carrying out distinct functions. At least one of a group of three polypeptides, designated ISGF3α, is a target for receptor initiated post-translational modification. Once active, ISGF3α undergoes facilitated nuclear translocation. The fourth subunit of ISGF3, designated ISGF3γ, is a weak ISRE-binding protein present in both the cytoplasm and nucleus of untreated cells. Upon nuclear accumulation, ISGF3α associates with ISGF3γ forming the ISGF3 complex which has high affinity for the ISRE. Therefore, it seems likely that allosteric modulation of ISGF3γ by ISGF3α results in enhanced ISRE

affinity. In the case of the type I IFN system, a latent cytoplasmic transcription factor subunit is an early target for signal-dependent activation, and subsequently transmits cell surface information across subcellular boundaries to mediate specific transcriptional response.

How is specificity maintained in the response to ligand-receptor interactions?

The activation of ISGF3α by ligand-receptor interaction is detectable in the cytoplasm within 30 sec of stimulation (Chapter 5). ISGF3α is both an early target for intracellular signalling and an intracellular messenger, and with the production of this protein messenger the specificity of cellular response is ensured. By virtue of associated sequence-specific DNA-binding activity, the transcription factor subunit ISGF3α represents a highly specific intracellular messenger mediating the transcriptional response of a limited set of genes containing the ISGF3 binding site (ISRE). Therefore, the utilization of transcription factors, or their subunits, as intracellular messengers confers a high degree of specificity on cellular response to a particular ligand-receptor pair. In several characterized systems, such as NF-κB, *dorsal*, and the glucocorticoid receptor, this highly specific signal transduction strategy is employed (see Chapters 1, 5, and 6 for discussion).

An important question remains unanswered. How is ISGF3α

activation coupled to receptor occupancy? In other words, what is the event, or series of events, which occur within 30 sec of ligand binding that mediate ISGF3α activation, and what is the nature of post-translational activation of ISGF3α? While it is not yet possible to answer these questions, the described characteristics of the type I IFN system may provide clues. Since the "classical" second messengers systems are apparently not involved in this signal transduction pathway, the cellular mediators of IFNα signalling must be distinct from the well described effectors such as protein kinase C, cAMP-dependent protein kinase, and G-proteins. The primary structure of the IFNα-receptor reveals no recognizable motif characteristic of a known protein structure or activity (Uze et al., 1990). In fact, expression studies suggests that fully functional receptor requires additional interacting polypeptides. These accessory proteins could include additional transmembrane IFNα-binding proteins, integral membrane proteins having intrinsic enzymatic activity involved is signalling, or associated cytoplasmic proteins which directly interact with the receptor and through enzymatic or nonenzymatic action mediate signalling. Perhaps the simplest scenario is the following: ISGF3α (or one of the ISGF3α subunits) directly interacts with the IFNα-receptor or an associated protein, and in this way is sequestered at the plasma membrane in

an effectively inactive state. Upon ligand binding, a conformational alteration of the IFNα-receptor complex facilitates disassociation and release of ISGF3α as a soluble cytoplasmic activity which is competent for nuclear translocation and association with ISGF3γ to form ISGF3.

An example of this type of regulatory scheme is found in signal transduction through the CD4 or CD8 receptors. In this system, T cell activation involves the engagement of T cell antigen receptor, antigen bound to a major histocompatibility complex molecule, and CD4 or CD8 receptor. The cytoplasmic domains of CD4 and CD8 appear to be physically associated with p56^{lck}, a *src*-related protein tyrosine kinase. Engagement of the CD4 or CD8 receptors triggers disassociation of p56^{lck}. The soluble cytoplasmic protein has enhanced kinase activity as well as access to previously unavailable substrates. While the *src* family of proteins is quite conserved in the C-terminus, it is interesting to note that the region of p56^{lck} required for association with CD4 and CD8 is an N-terminal region that is not conserved between *src*-related proteins. This observation suggests that individual members of the *src* family, with unique N-termini, may directly interact with specific integral membrane receptor, and in this way mediate specific intracellular responses to a particular ligand (for review: Robey and Axel,

1990). It is possible that in many cases the direct interaction of receptors and effector molecules accounts for specific signal transduction, rather than the gross modulation of a limited group of small molecule second messengers.

Future directions

The goal of this project was the complete description of the cellular events which integrate IFNα binding at the cell surface with transcriptional activation of ISGs. At this point there remains a window of 30 sec following the initiation of intracellular signalling which is not understood. In order to describe these early events it will be necessary to purify ISGF3 to homogeneity, prepare specific antisera to each polypeptide subunit, and to isolate clones corresponding to each of these proteins. With these reagents available it will be possible to identify the protein(s) which is post-translationally modified by ligand binding. A description of the exact type of modification occurring should also be possible. Through this analysis, a reconstruction of the cellular events initiated at the cell membrane resulting in ISGF3α activation will eventually allow a complete description of signal transduction in response to IFNα, perhaps revealing a generally employed cellular strategy.

An additional approach is available through the analysis of the cloned IFNα-receptor and associated proteins. For example, the analysis of receptor associated proteins through immunoprecipitation under various conditions may identify important components of the signalling pathway. In particular, the association of an ISGF3α subunit with the cytoplasmic domain of the receptor could be tested in this way.

Several additional aspects of ISGF3 function remain to be addressed: 1) What is the ISGF3γ structure that mediates ISRE binding? 2) How does the interaction of ISGF3α with ISGF3γ lead to increased DNA-binding affinity? 3) Which subunit of the ISGF3 complex mediates transcriptional activation? 4) In what way does ISGF3α interact with the cellular nuclear translocation machinery? 4) What is the nature of ISGF3 inactivation during the later stages of ISG induction? With the appropriate molecular reagents in hand, an understanding of these important issues of ISGF3 structure-function and their relationship to ISG regulation will become possible.

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