REGULATION OF THE HUMAN INTERFERON-ß PROMOTER

by

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ABSTRACT

Human type I interferons offer a relevant system to examine cell-specific inducible gene expression. Interferon genes are transcriptionally activated in a variety of cell types following induction by synthetic double-stranded RNA (poly I:C) and viruses. A transient expression system was developed which permits regulated expression of different IFN-CAT (chloramphenicol acetyltransferase) hybrid genes in human cells. Using this system *in vivo* competition assays identified positive and negative cellular factors interacting with the IFN-ß promoter. A factor that recognizes negative upstream regulatory sequences was also identified in uninduced myeloid cell extracts. Complementary studies demonstrated that transcription of the IFN-ß gene *in vitro* could be inhibited by a 44 bp synthetic oligonucleotide corresponding to the interferon regulatory element (IRE). This element is comprised of two genetically distinct positive regulatory domains, PRDI and PRDII, that are essential for maximal induction by virus or poly I:C. Binding and competition analysis showed that the PRDI element interacts with a factor(s) present in uninduced and virus-induced Hela extracts. The DNA-binding specificity of the PRDI factor(s) is characteristic of proteins recently shown to be involved in IFN-stimulated gene transcription. The PRDII domain is unrelated to PRDI, but shares 80% nucleotide homology with the NF-κB binding site in the immunoglobulin kappa enhancer. PRDII was found to interact with an NF-κB-like activity in both lymphoid and non-lymphoid cell extracts. An HIV-1 enhancer oligonucleotide containing two repeated κB elements was interchangeable with PRDII in the gel retardation assay. UV-crosslinking analysis further revealed that several distinct inducible and constitutive proteins bind specifically to PRDII in different cell types, and suggests that multiple factors may interact with this element to regulate IFN-ß transcription. Taken together, these studies demonstrate that distinct signal transduction pathways common to a variety of immunoregulatory and IFN-stimulated genes, converge to mediate expression of the IFN-ß promoter.

RESUME

L'interféron humain de type I représente un système pertinent pour l'étude de l'expression de gènes, cellule-spécifique et inductible. Les gènes codant pour l'interféron sont transcriptionnellement activés dans divers types cellulaires, après induction par des ARN double-brins synthétiques ou par des virus. Un système d'expression transitoire a été développé pour permettre l'expression régulée de différents gènes hybrides IFN/CAT (chloramphenicol acetyltransferase). Ce système a été utilisé pour des expériences de compétition *in vivo*, qui ont permis l'identification de facteurs cellulaires positifs et négatifs interagissant avec le promoteur IFN-ß. De même, un facteur reconnaissant des séquences régulatrices négatives en amont du promoteur a été identifié dans des extraits de cellules myéloïdes non induites. Des études complémentaires démontraient que la transcription *in vitro* du gène IFN-ß pouvait être inhibée par un oligonucléotide synthétique de 44 bp correspondant à l'IRE (interferon regulatory element). Cet élément comprend deux domaines régulateurs positifs génétiquement distincts, PRDI et PRDII, qui sont essentiels pour une induction maximale par le virus ou le poly I:C. Des expériences de fixation et de compétition ont montrées que l'élément PRDI intéragit avec un facteur(s) présent dans des extraits de cellules Hela non induites ou induites par le virus. La spécificité de fixation à l'ADN de ce facteur est caractéristique des protéines impliquées dans l'expression de gènes controllés par l'interféron. Le domaine PRDII, distinct de PRDI, présente 80% d'homologie avec le site de fixation NF-κB, présent dans la séquence activatrice du gène immunoglobuline kappa. Une activité de fixation à PRDII ressemblant à celle de NF-κB, a été identifiée dans des extraits de cellules lymphoïdes et non lymphoïdes. Un oligonucléotide qui représente la région 'enhancer' du VIH-1 comprenant deux élements κB en tandem, pouvait remplacer PRDII dans les expériences de retardement de migration sur gel. Les expériences d'irradiation aux ultraviolets des complexes protéines-ADN (UV cross-linking) ont révélées que plusieurs protéines constitutives et inductibles, pouvaient intéragir de façon spécifique avec PRDII dans différents types cellulaires. Ceci suggère qu'une multitude de facteurs pourraient intéragir avec cet élément pour réguler la transcription de l'IFN-ß. De façon globale, ces études montrent que des voies distinctes de transduction, communes pour plusieurs gènes immuno-régulateurs et gènes stimulés par l'IFN, convergent pour médier l'expression du promoteur IFN-ß.

To my wife Chris and my parents for their love, support and endless patience

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PREFACE

In accordance with the guidelines concerning thesis preparation, I have exercised the option of writing the experimental portion of this thesis (chapters 2 to 6, inclusive) in the form of original papers. This provision reads as follows: " The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text, of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation." I have therefore included, as chapters of this thesis, the texts of four original papers which have been accepted for publication, and one original manuscript recently submitted for publication. Each chapter includes its own Abstract, Introduction, and Results/Discussion section. A separate Materials and Methods section is included in chapters 4-6, where as in chapters 2 and 3 this section is integrated into the figure legends and text. A General Introduction and Discussion to the thesis has also been added. In order to bridge connecting papers chapters 3-6 each contain a preface. References for all chapters are grouped at the end of the thesis.

The manuscripts, in order of their appearance in the thesis are:

1. Xanthoudakis, S., Alper, D., Hiscott, J. (1987). Transient expression of the beta interferon promoter in human cells. Mol. Cell. Biol. 7: 3830-3835.

2. Xanthoudakis, S., Hiscott, J. (1988). Modulation of interferon gene transcription by positive and negative cellular factors. Biochem. Biophys. Res. Comm. 154: 1338-1344.

3. Xanthoudakis, S., Hiscott, J. (1987). Identification of a nuclear DNA-binding protein associated with the interferon-ß upstream regulatory region. J. Biol. Chem. 262: 8298-8302.

4. Xanthoudakis, S., Cohen, L., Hiscott, J. (1989). Multiple protein-DNA interactions within the human interferon-ß regulatory element. J. Biol. Chem. 264:1139-1145.

5. Xanthoudakis, S., Hiscott, J. (1990). Analysis of protein-DNA interactions within the PRDII domain of the interferon-ß promoter reveals distinct constitutive and inducible complexes. (submitted).

The candidate was responsible for all the research described in chapters 2 to 6 with the following exceptions:

Deborah Alper contributed equally to the experiments outlined in figures 1, 4 and 5 of chapter 2.

Lucie Cohen performed the experiment in figure 1 of chapter 5. Lucie also assisted with preliminary development of the *in vitro* transciption assay used in figure 7 of chapter 5.

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LIST OF ABBREVIATIONS

BSA	:	Bovine serum albumin
BUdR	:	Bromo-deoxyuridine
CAT	:	Chloramphenicol acetyltransferase
EMSA	:	Electrophoresis mobility shift assay
FCS	:	Fetal calf serum
HAU	:	Hemaggultinating units
IFN	:	Interferon
rIFN	:	Recombinant interferon
IRE	:	Interferon regulatory element
IRF	:	Interferon regulatory factor
ISG	:	Interferon-stimulated gene
ISGF	:	Interferon-stimulated gene factor
ISRE	:	Interferon-stimulated response element
LPS	:	Lipopolysaccharide
NDB	:	Nuclear dialysis buffer
NRD	:	Negative regulatory domain
NTP	:	Nucleoside triphosphate
dNTP	:	Deoxynucleoside triphosphate
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PMA	:	Phorbol myristate acetate
POLY I:C	:	Poly inosine : cytosine
PRD	:	Positive regulatory domain
TK	:	Thymidine kinase
TPA	:	Tumor promoting agent
UTR	:	Untranslated region
WCE	:	Whole cell extract

CHAPTER 1

GENERAL INTRODUCTION

1.1 AN OVERVIEW OF THE INTERFERON SYSTEM

Interferons comprise a diverse group of inducible cellular proteins with a wide range of antiviral, antiproliferative and immunoregulatory effects. The molecular mechanisms involved in interferon induction and interferon action have intrigued biochemists since their discovery more than three decades ago. Hallmark experiments by Issacs and Lindenmann provided the first indication that viral interference was mediated by a soluble cell secreted factor (1). The antiviral activity, termed interferon, was detected in the supernatants of virus-infected avian membrane cultures and found to be both protease-sensitive and nuclease-resistant (2). Since then, many studies aimed at characterizing interferons and their biological activities have contributed to our current understanding of the interferon system.

Several components of this system are schematically illustrated in figure 1. The response can be dissociated into distinct biochemical events that relate to either IFN activation or IFN action. The pathway leading to interferon activation is initiated by an interaction between the inducer and the target cell. Upon stimulation an intracellular signal is rapidly transduced to the nucleus resulting in the induction of specific IFN genes. The transcriptional response is transient and the mRNA produced encodes a precursor polypeptide that undergoes post-translational processing to generate a modified biologically active form of the protein. Following secretion the mature protein binds to its cognate receptors on neighbouring cells and exerts its numerous biological effects. Although individual aspects of the model have been intensely investigated, several questions regarding the underlying molecular mechanisms controlling these events have yet to be answered. For example, what is the nature of the transducing signal responsible for

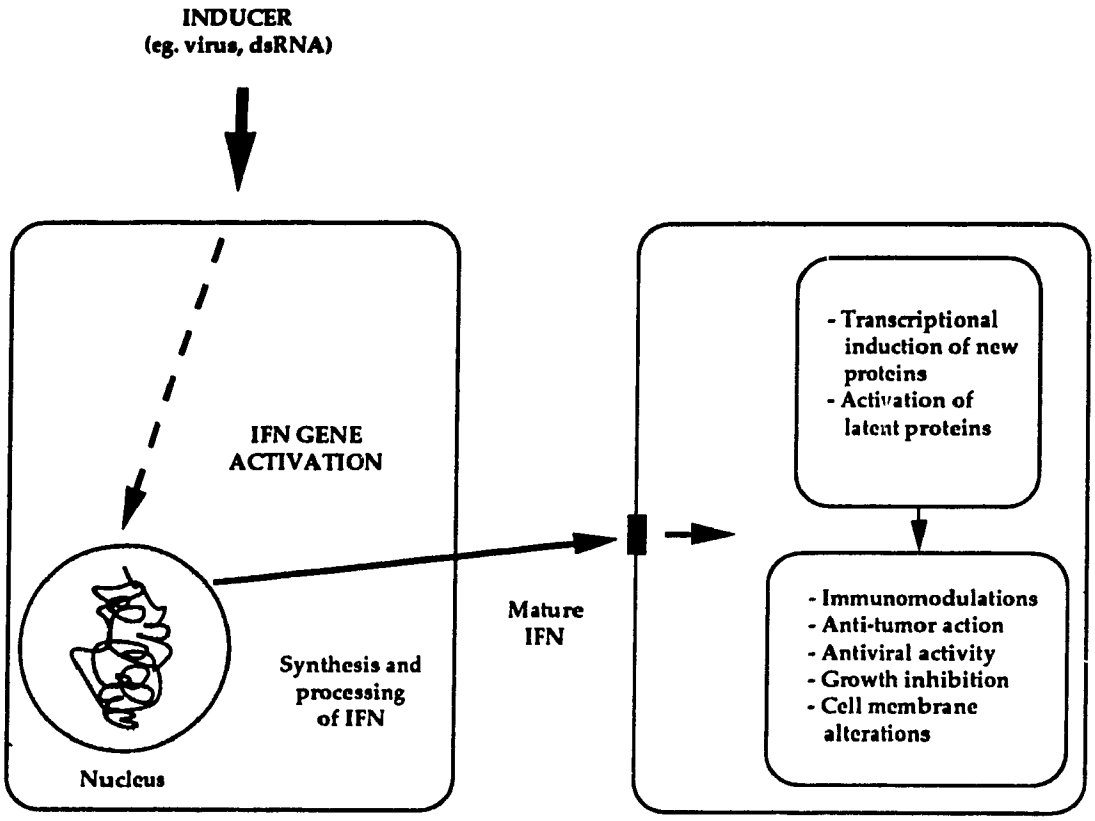


Figure 1. Schematic diagram of the interferon system

activation of different IFN genes? How are interferons regulated at the level of the gene, often in a cell specific manner? How do interferons transcriptionally stimulate the expression of other cellular genes? Recent advances in recombinant DNA technology have permitted researchers to address these questions with some degree of success. The cloning of various human and murine IFN genes has furnished the necessary reagents for these types of investigations.

The work presented in this thesis has focused on characterizing the biochemical and genetic requirements for IFN-ß promoter activation. However, in order to convey a general understanding of interferon biology, a comprehensive overview of the entire system is provided in the remainder of the introduction.

1.2 CHARACTERIZATION OF INTERFERONS

Interferons (IFNs) can be classified into three antigenically distinct groups termed α, ß and γ. Antisera raised against all three types of interferon do not cross-react and have been shown to augment viral replication both *in vitro* and *in vivo*. In addition, antibody neutralization inhibits the antiviral and non-antiviral effects of interferon alike, suggesting that the numerous biological effects of interferons are mediated by a common or closely related antigenic domain on the proteins. The type and quantity of interferon produced usually depends on the cell type as well as the inducing stimulus. Generally, IFN-α and IFN-ß (or type I acid-stable interferons) are synthesized by leukocytes and fibroblasts, respectively, whereas lymphoid cells mainly synthesize IFN-γ (or type II acid-labile interferon). In some cases, however, there may be overlap in the profile of interferon expression. A given cell type

can be stimulated to produce a mixture of interferons, although the relative concentration of each species may vary (3, 4, 5).

In contrast to IFN-ß and IFN-γ, IFN-α is represented by a family of immunologically cross-reactive proteins (Table 1) (6, 7). Two distinct IFN-α sub-families, IFN-α_I and IFN-α_{II}, have been identified in the human, murine and bovine genomes and are believed to be present in most mammals (7-19). The IFN-α_I sub-family consists of at least 20 structurally related non-allelic genes, sharing 70-90 % nucleotide homology across a 165-166 amino acid coding sequence that specifies the mature protein (7-15). The IFN-α_{II} sub-family comprises approximately 6-7 structurally related genes encoding mature proteins of 172 amino acids (7, 16). Both subfamilies localize to human chromosome 9, lack intervening sequences and are coordinately expressed after induction by virus (15, 16, 20-22). At least two IFN-α_I genes appear to have evolved as a result of unequal crossover or gene conversion between members of the same subfamily (23, 24). Several of the IFN-αs represent pseudogenes which are transcriptionally inert, or express non-functional proteins because of chromosomal rearrangement or point mutations (7, 24, 25).

Human IFN-ß is specified as a 166 amino acid mature protein by a single intronless gene located on chromosome 9, and shares 30-40 % nucleotide homology with the IFN-αs (26-36). Multiple and unique IFN-ß genes have been described in other vertebrate species (19). An intron-containing IFN-ß-like gene, IFN-$ß_2$, has also been identified, but shares little nucleotide or amino acid homology with IFN-ß. Although IFN-$ß_2$ is inducible and serologically related to IFN-ß, it possesses little antiviral activity and has biological functions that are more characteristic of interleukins than interferons (37, 38).

Table 1. The human interferon multigene family

	IFN-α	IFN-ß	IFN-γ
Number of members	> 20	1	1
Chromosomal location	9	9	12
Structure	Intronless	Intronless	3 Introns
Active protein	165-172	166	146
Signal peptide	23	21	20
Glycosylation	≥ 3	Yes	Yes
Major biological activity	Antiviral	Antiviral	Immune regulation
Inducers	Viruses Antigens	Viruses dsRNA	Mitogens Antigens Immune stimuli
Major producer cell type	Macrophages B-lymphocytes	Fibroblasts Epithelial cells	T-lymphocytes

IFN-γ, a 146 amino acid mature polypeptide is coded by a single gene containing 3 intervening sequences and localizes to chromosome 12. This gene shares almost no nucleotide homology with the α- or ß-IFNs, and unlike the latter classes has yet to be identified in non-mammalian vertebrates (39-42). All three classes of interferon genes encode a short 20-23 amino acid signal polypeptide that must be cleaved prior to secretion of the mature protein (7, 15, 28, 40).

Recombinant interferons (rIFNs) can be efficiently expressed in several vector host systems. Synthesis of IFNs in bacteria and yeast has made possible the purification of large-scale quantities of the proteins. Replacement of the DNA sequences specifying the signal polypeptide with bacterial or bacteriophage regulatory sequences (ie. trp, lac, lambda promoter/operator) and ribosome binding sites, helped facilitate the production of high levels of recombinant interferon in E. coli (15, 28, 40, 44). Glycosylated forms of the proteins have been generated by expressing recombinants in a eukaryotic cells. In yeast, this was accomplished by linking interferon coding sequences to the Saccharomyces cerevisiae alcohol dehydrogenase or glyceraldehyde dehydrogenase promoters (43, 44). Yeast expression vectors for secretion of human interferons have also been described (44). In mammalian cells, constitutive expression of rIFN was achieved by fusing IFN structural genes to viral promoters (15, 40, 44, 45). In this host system rIFN titers could be significantly augmented by co-amplifying a linked dihydrofolate reductase (DHFR) gene through methotrexate selection (45).

Prokaryotic and eukaryotic rIFN is biologically active *in vitro* and *in vivo*. The cross-species antiviral activity, antigenicity and physiochemical properties of E. coli and yeast derived IFNs are indistinguishable from those of their natural counterparts (28, 40, 43, 44, 46 47). For instance, recombinant

and leukocyte-derived IFN-α are comparable with regard to a number of non-antiviral activities including enhancement of NK cell-mediated cytotoxicity, augmentation of antibody dependent cell-mediated cytotoxicity, and inhibition of cell proliferation (48). *In vivo*, natural and E. coli produced IFN-α display similar antiviral activity, and at least in rhesus monkeys the bacterial form appears to be less toxic (49). Finally, novel interferons have been created by fusing the protein-coding segments of different rIFN-α genes. The rIFN-α1/α2 chimeras have antiviral properties that are distinct from either of the parental proteins, and are in some cases more potent (50, 51).

1.3 INTERFERON INDUCERS

Interferons can be induced in most vertebrates by a wide spectrum of natural and synthetic agents which include: members of most major animal virus groups; certain fungi, bacteria, protozoans and their products; natural or synthetic single and double-stranded polyribonucleotides; anionic polymers; low molecular weight compounds; mitogens, antigens and specific immune responses (52). With regard to structure, the myriad of inducing agents is so diverse that it is almost impossible to identify a common feature that accounts for each of their activities. This has lead to the proposal that multiple and possibly related signal transduction pathways act to stimulate interferons.

Characterization of inducers on the basis of strength is at best tentative because a particular inducer may work more efficiently *in vivo* than *in vitro* (or vice-versa), or in certain cell types but not others. Poly I:C for example is a strong inducer of fibroblast interferon in epithelial cells, but has no detectable effect on cells of the monocytic lineage (52). Adenoviruses fail to induce

interferons in cultured monkey, rabbit, human, and hamster cells, but can do so in chicken cells or whole hamsters if the virus is administered intravenously (53, 54). Thus like interferons themselves, inducers can be better classified in terms of host range rather than strength.

With viral inducers the initial cell surface interaction between the virus and the target cell is not an integral part of the induction response (55). Interferons can be stimulated with certain infectious naked RNAs, indicating that viral entry and uncoating do not in themselves represent critical events (56). In the case of polyribonucleotides, the molecules are believed to act intracellularly. Liposome and DEAE-mediated transfer of poly I:C into cells can significantly augment induction (57, 58). Similarly, low molecular weight compounds which have been shown to increase membrane permeability, act to enhance poly I:C induction without altering the initial cell surface interaction (59, 60).

The capacity of double-stranded (ds) RNA to activate interferons raised speculation about whether the formation of dsRNA intermediates during the life cycle of the virus was the basis of viral induction. However, investigations that have focused on the requirement for viral dsRNA and/or viral replication have been controversial. With certain viruses the input virion RNA is sufficient to trigger induction (61, 62), while with others early replicative events are required to see an effect (63, 64). For instance, ultraviolet inactivation of Newcastle Disease Virus RNA polymerase concomitantly results in a loss of IFN inducing potential (65). Similar observations have been made in murine L cells with ultraviolet-treated reoviruses; however in the human and chick cell systems the input reovirus RNA seems to be enough to induce interferon (55, 66). Some replication-defective single-stranded RNA viruses that do not package a virion-associated

RNA transcriptase, still maintain their inducing ability (62, 67). Specific strains of encephalo-myocarditis virus which fail to transcribe their RNA in interferon-treated cells, are more potent inducers than viruses which retain this function (56). Consequently, in these studies it is not known whether some secondary or tertiary stucture inherent to single-stranded RNA provides the putative dsRNA inducing signal (56, 62, 67).

A number of reports have argued that replication per se may be insufficient to cause induction. Work with temperature-sensitive strains of Sindbis virus has shown that at non-permissive temperatures, mutants remain replication competent, but are incapable of inducing interferon (68). This would indicate that additional viral processes are necessary for induction to occur. Additionally, interferon induction by adenoviruses can be abolished by exposing the virions to proteases without affecting infectivity (54, 69). Although some structural virion component may serve an inducing role in these viruses, efforts to isolate such an activity have failed and argue against this proposal (54).

1.4 INTERFERON INDUCTION

The induction of interferons is regulated by a transcriptional mechanism. Interferons cannot be induced in enucleated cells or in cultures that are treated prior to induction with inhibitors of mRNA synthesis (70-72). The kinetics of the induction response are transient and can be separated into two phases: the lag phase which represents a variable period of transcriptional latency when the inducer is bound, absorbed and processed by the cell; and the production phase which is characterized by the onset and eventual decline of transcription, translation, and secretion of the active protein (figure 2) (72).

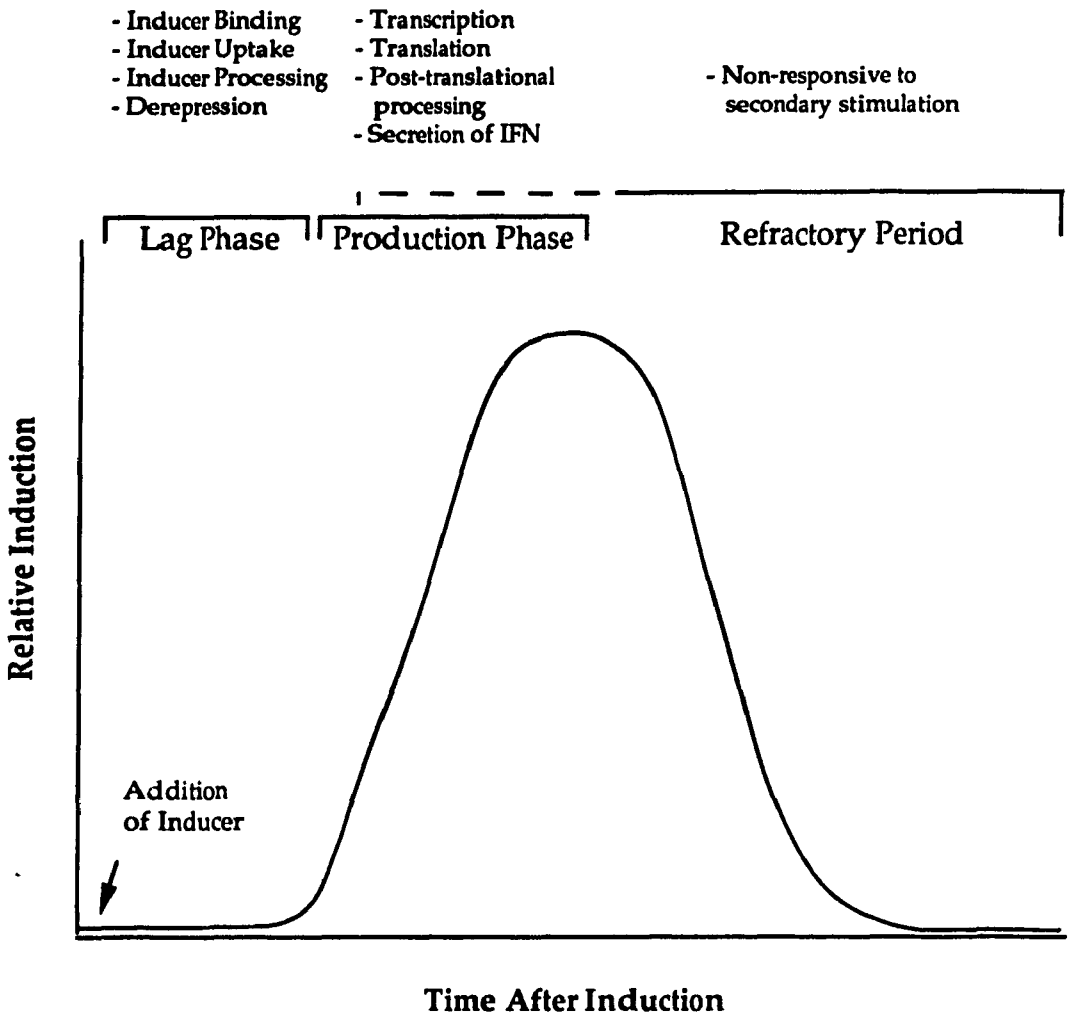


Figure 2. Kinetics of interferon induction. The graph illustrates an interferon induction curve following treatment with an inducing agent. Some of the information from this figure was reproduced from ref. (22).

Termination of IFN synthesis is characterized by a refractory period during which re-stimulation fails to elicit a secondary response (73). The duration of the lag phase and the magnitude of the production phase vary considerably depending on the inducer-cell combination. In murine kidney cells the synthesis of IFN protein begins almost immediately following treatment with poly I:C, but is preceded by a four hour lag period if the same cells are infected by virus or if instead L cells are examined. The differences in lag time may reflect alternate usage of a multistep induction pathway in which the number of steps required are characteristic for each cell type and inducer. According to this notion, poly I:C could circumvent a series of time-dependent steps in murine kidney cells that are otherwise necessary for induction by viruses (74). Multistep pathways would potentially offer more targets for inhibition. This may account for the increased sensitivity of virus versus poly I:C inductions to treatment with actinomycin D (75, 76).

Despite certain negative effects, metabolic inhibitors of protein and RNA synthesis can also dramatically enhance IFN induction if administered to the culture at the appropriate time (77). The effect known as 'superinduction' is believed to be mediated by both a transcriptional and post-transcriptional mechanism. An accentuated response is observed when cycloheximide is added prior to the peak of normal IFN production and then removed before protein synthesis terminates. This effect does not materialize if cycloheximide is added after the peak of IFN synthesis, indicating that the events involved in terminating induction are initiated sometime before this point. The superinduction effect can be further enhanced if actinomycin D is administered to the culture during the shutoff phase of the response (77). One possibility is that such inhibitors repress translation and transcription of a repressor protein which acts to inhibit IFN synthesis. The mode of control

could be either post-transcriptional or post-translational. The latter mechanism seems unlikely since enhanced IFN production correlates with the inhibition of protein synthesis and not protein degradation (78, 79). The data is more consistent with a model in which the putative repressor acts to post-transcriptionally regulate the decay of IFN message. This is supported by reports which demonstrate that the levels of translatable IFN mRNA are moderately higher in cells that have been superinduced relative to those treated with inducer alone (80, 81 82). The half-life of mRNA from poly I:C-treated human FS-4 fibroblasts increases from 18 minutes to 49 minutes if the cells are co-induced with cycloheximide. Co-induction with poly I:C, cycloheximide and actinomycin D further increases the half-life of the message to 68 minutes (81). Raj and Pitha found that in poly I:C-treated fibroblasts, IFN mRNA levels were maximal at 3 hours after induction, while in poly I:C/cycloheximide-induced cells the mRNA accumulated for up to 11 hours, with a corresponding 5-10 fold increase in the message during this period. Within 60 minutes after the removal of cycloheximide, IFN protein synthesis resumed and the transcripts from induced and superinduced cells were reported to have comparable half-lives. The transcripts isolated under both inducing conditions were full-length at 3 hours post-induction, but those detected at later times in the poly I:C/cycloheximide-treated cells were found to be progressively shorter (82).

Experiments with cloned IFN genes have shown that replacement of the IFN-ß coding and 3' untranslated regions (3' UTR) with reporter sequences specifying stable transcripts, prolongs the decay of induced reporter message (83). This is consistent with studies indicating that the 3' UTR of a number of transiently expressed cytokine, lymphokine and proto-oncogenes contains an AT-rich concensus sequence ATTTA (AUUUA in the RNA) which mediates

cytoplasmic instability of the transcript (84). Homologous AT-rich motifs are present in the 3' UTR of various IFN genes. It is speculated that recognition of the AUUUA concensus by a specific RNA endonuclease is responsible for degradation of the transcript. The instability can be relieved by treatment of the cells with cycloheximide. Cycloheximide would act to block synthesis of this putative RNAase and thereby increase the half-life of the message (84).

The studies described above clearly support a model in which enhancement of interferon expression by different metabolic inhibitors is dependent on post-transcriptional control. However, gene transfection experiments using chimeric human IFN-ß promoter-CAT (chloramphenicol acetyltransferase) plasmids further indicate that superinduction is in part mediated by a transcriptional mechanism. In human 293 epithelial cells, promoter-mediated induction of a linked CAT gene can be enhanced 7 fold by co-inducing the cells with virus and cycloheximide. Cycloheximide alone is unable to induce CAT expression in this system (85). In addition, mutational analysis of the promoter has shown that the DNA sequences responsible for induction and superinduction are indistinguishable (86). Since the human IFN-ß promoter is thought to be both negatively and positively regulated, the transcriptional effect of cycloheximide might be to inhibit the synthesis of a repressor and prolong activation by positive trans-acting factors (see 1.8 and discussion).

1.5 INTERFERON PRIMING

Exposure of cells to interferon induces a hyporesponsive state during which the cells are refractory to induction (73). However, interferon pretreatment can also augment the primary induction response by an effect known as priming (87). Murine L cells normally fail to produce interferon following stimulation by poly I:C, unless DEAE-dextran is also present (88). The requirement for DEAE-dextran can be circumvented by priming the cells with interferon. In general, interferon-treated cells produce more interferon than untreated cells following induction (87, 89-93). Priming in several cell systems has also been found to alter the kinetics of the induction response. The induction curve is shifted so as to reduce the lag period between the addition of inducer and the onset of interferon synthesis (89, 94). This is consistent with the observation that in primed cells the induction response becomes resistant to inhibition by actinomycin D more rapidly than in unprimed cells (89).

Fujita et al. reported that priming stimulates the production of interferon mRNA in polyI:C-induced L cells relative to unprimed cells, whereas virus induction diminishes synthesis under these conditions (93). On the other hand, Abreu and Stewart found that the net amount of induced message and the half life of the mRNA were indistinguishable between primed and unprimed L cells (89). However, they did observe that the levels of secreted interferon were considerably more elevated after priming. These authors concluded that priming was mediated by enhanced translation of IFN message, and proposed that priming at the cellular level may involve modification of the translational machinery. Another possibility is that interferon mRNA in primed cells is presented to the ribosome in a more

translatable form, perhaps through an association with some cellular factor. It has been recently demonstrated that the 3' UTR of human IFN-ß mRNA has an inhibitory effect on translation *in vitro*. Replacement of the IFN-ß 3' UTR with a ß-globin 3' UTR increased translatability of IFN-ß message in frog oocytes and rabbit reticulocyte lysates. Furthermore, the IFN-ß 3' UTR was found to drastically reduce the translatability of a chicken lysosomal RNA when substituted downstream of this gene (95). It is tempting to speculate that in certain systems priming somehow relieves the inhibitory effect of the 3' UTR, thereby permitting the message to be more efficiently translated.

Several studies have shown that priming causes a marked increase in interferon gene activity upon induction. Pretreatment of Hela or tk143 cells with interferon prior to poly I:C or virus induction, results in a 20-100 fold enhancement in the level of induced IFN-ß mRNA (96). Hiscott and co-workers demonstrated that IFN priming of monocytic U937 cells before virus infection caused a 5-20 fold stimulation in steady state levels of mRNA corresponding to the IFN-α1, IFN-α2, and IFN-ß genes (85). This was accompanied by a three fold stimulation in the amount of antiviral activity in the cell culture supernatants. Chase experiments with actinomycin D determined that the priming effect was not due to stabilization of the mRNA, but rather enhanced transcription. They also found that hybrid IFN-ß-promoter/CAT gene templates were 5 fold more active in primed/virus-infected 293 cells, than in cells treated with virus alone. This effect was entirely mediated by the promoter since their constructs did not contain any IFN-ß coding or 3' non-coding sequences (85). These findings are supported by studies which show that individual domains of the IFN-ß promoter are interferon inducible, as are the genes encoding the transcription factors which interact with these domains (see discussion).

1.6 INTERFERON ACTION

Type-I interferons (IFN-α, IFN-ß) and type-II interferons (IFN-γ) share many of the same biological effects, but act via distinct receptors. IFN-α and IFN-ß compete for binding to a common low abundance (2-5 x 10^3/cell) receptor encoded by chromosome 21 in humans and chromosome 16 in mice. Antibody neutralization of type-I interferon receptors concomitantly inhibits the binding and biological activity of IFN-α and IFN-ß, but not IFN-γ. Human IFN-γ interacts with a more abundant cell surface receptor (50-70 x 10^3/cell) encoded by chromosome 6 (97-99).

Following the initial cell surface interaction type I and II interferons are rapidly internalized via receptor-mediated endocytosis (98). However, it is not clear whether internalization is necessary to elicit a biological effect. Microinjection of type-I interferons into cells precludes development of the antiviral state, implying a requirement for the ligand-receptor interaction (100). On the other hand, type II interferon has been shown to activate macrophages when introduced into the cells via liposome vesicles or if overexpressed from transfected plasmids that specify a non-secretable form of the protein (101, 102). There are also reports which suggest that type I and II interferons translocate to the nucleus and interact with specific receptors on the nuclear membrane (103-104). More recent studies indicate that intracellular signals originate in the cytoplasm (see 1.7), although classical second messengers such as phosphatidyl-inositol metabolism, diacylglyerol, Ca^{++}, cAMP, cGMP and protein kinase C do not appear to be involved in the triggering process (6, 98).

Irrespective of the pathway the end result is clear; exposure of cells to interferon results in the transcriptional activation of no less than 20-25 genes

(Table 2) (105). A number of interferon-inducible genomic and cDNA clones have now been isolated. Several of these have been given a numerical designation only because a biological activity has not been assigned to them. Others specify proteins that mediate the antiviral, antiproliferative and immunoregulatory effects of interferon, including: B2-microglobulin, major histocompatibility antigens, thymosin B4, metallothionein-IIa, 2'-5' oligoadenylate synthetase, dsRNA-dependent protein kinase and the Mx protein (105). The following sections describe some of what is known about the diverse biological activities associated with interferons.

A. The Antiviral Activity

Two of the genes which are rapidly induced by interferon encode enzymes that are involved in establishing the antiviral state. These proteins, 2'-5' oligoadenylate synthetase (OASE) and dsRNA-dependent protein kinase (also termed dsI, DAI, PI and PKds), initiate a cascade of events that act to inhibit viral multiplication through distinct metabolic pathways (figure 3). Once induced, OASE and DAI are activated by a reversible non-covalent interaction with double-stranded RNA (5, 6). Multiple forms of OASE have been identified in several species (6). In humans, differential 3' splicing of primary OASE transcripts gives rise to a 1.6 kb and 1.8 kb mRNA encoding proteins of 41.5 kD and 46 kD (106,107). OASE utilizes ATP to catalyze the formation of short oligoadenylates linked by 2'-5' phosphodiester bonds (108). At the moment the only known function of these oligoadenylates is to activate a latent endoribonuclease activity (RNAase L) found in both interferon-treated and untreated cells (109). RNAase L exists as an 80-85 kD protein and cleaves natural and synthetic single-stranded RNA on the 3' side

Table 2. Interferon-induced cellular genes *

Name of clone or gene	RNA size (kb)	Protein size (kDa)	Function	Induction
(2'-5')oligo A synthetase	3.6	100, 67	dsRNA dependent synthesis of ppp(A2p)nA activator of RNAase L	α, β > γ
E18	1.8	46		
E16	1.6	40		
C56 pIF-2	2.0	56	Poly I:C binding function unknown	α, β > γ
pIF-1	2.9	42 58		
1-8 gene family	0.8	?		α, β
6-16	1.0	13		α, β
MT-IIa	0.5	7	Metallothionein metal detoxification	IFN, Zn Dex
Thymosin ß4	0.8	(5.25)	Induces terminal transferase in B-cells	
6-26		16		
HLA-A,B,C	1.8	44	Heavy chain of histocompatibility class I antigens	γ > α, β
ß2-microglobulin	0.9	14	Small invariant HLA class I chain	γ > α, β
HLA-DRα	1.3	34	HLA class II heavy and light chains	γ >> α, β
HLA-DRß		29		
pIF-γ-31	1.5	12.4	Excreted as 10K platelet factor 4	γ >> α
GBP	4.0	67	GTP, GDP binding	α, β, γ
15 kD protein	0.7	15	Cytoplasmic protein	α, β
Murine Mx	3.5	72	Specific inhibition of influenza virus	α, β

* Reproduced from ref. (105)

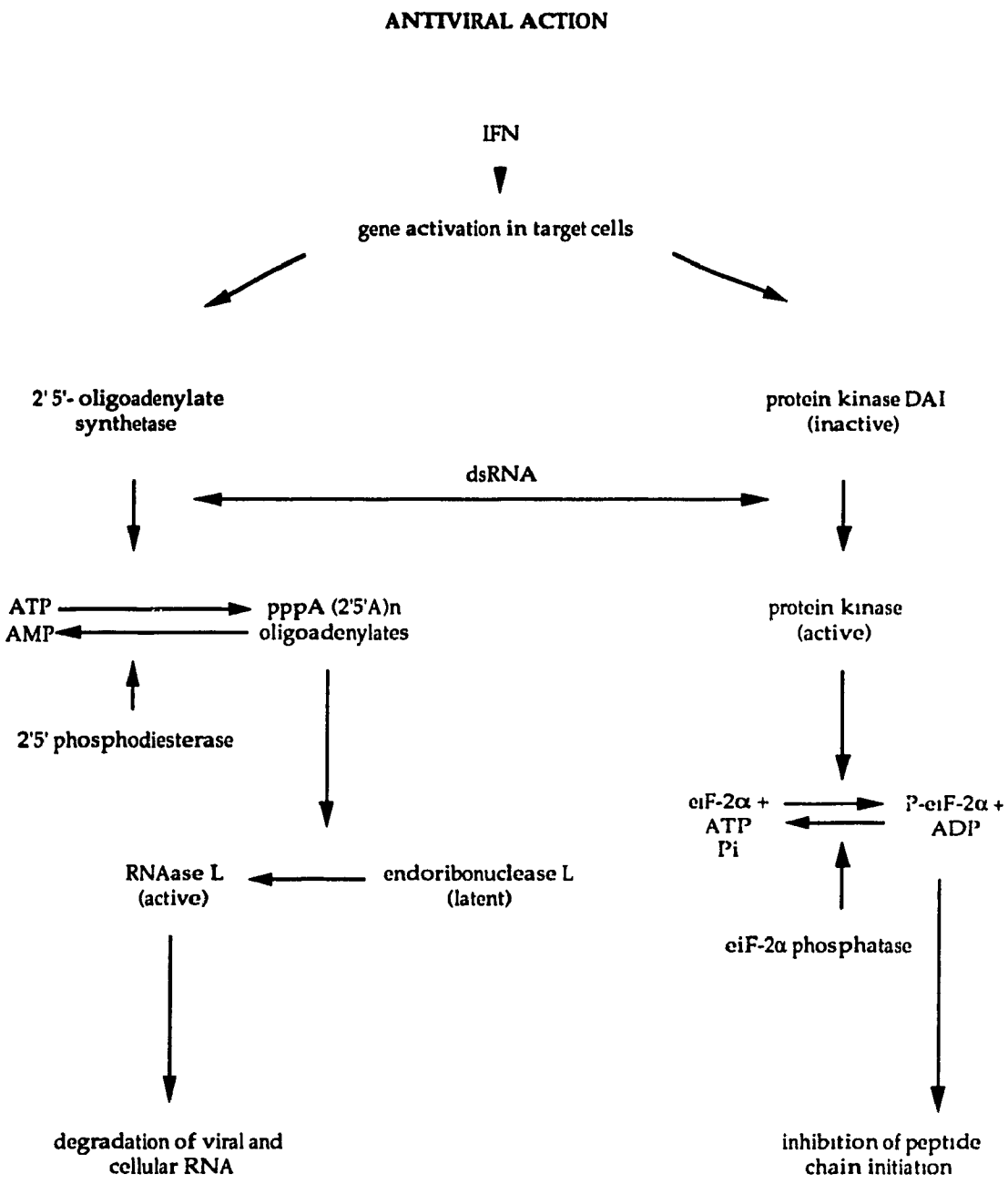


Figure 3. The antiviral cascade. The diagram illustrates the enzymatic pathways which mediate the antiviral activity of interferons.

of uridine dinucleotides (ie. UA, UG, UU) (109, 110). This endoribonuclease is not selective for viral RNA and can degrade cellular RNA (ie. rRNA) with equal efficiency (111). It is thought that local activation of OASE by ribosome associated viral dsRNA may be responsible for preferential degradation of viral transcripts (112).

RNAase L activity is indirectly modulated by a 40 kD protein, 2'phosphodiesterase (2'Pdi), which acts to degrade the oligoadenylates synthesized by OASE. 2'Pdi degrades the OASE product at the 2'phosphodiester bond and has also been shown to cleave the CAA terminus of tRNA molecules (113). The activity of 2'Pdi can be stimulated up to 4 fold with high concentrations of interferon in certain cell lines, although in general, interferon-treated and untreated cells possess comparable levels of 2'Pdi activity (114, 115).

The second pathway in the antiviral cascade is triggered by the induction of the dsRNA-dependent protein kinase (DAI). DAI mediates phosphorylation of the α-subunit of eukaryotic translation initiation factor (eIF-2α). Translation is inhibited because phosphorylated eIF-2α is unable to recycle for subsequent rounds of protein chain initiation(116, 117). The activity of eIF-2α can be restored through a dephosphorylation reaction which is catalyzed by a cellular protein designated eIF-2α phosphatase. EIF-2α phosphatase has been purified from rabbit reticulocyte lysates as a dimeric complex with subunits of 36 kD and 60 kD (118).

The dsRNA-dependent protein kinase was also recently purified to homogeneity and the cDNA encoding this protein has been cloned (119, 120). Earlier studies suggested that the kinase exists as a heterodimer comprised of a 68 kD and 48 kD component. The 48 kD protein was believed to phosphorylate the 68 kD protein, which in turn acted to phosphorylate eIF-2α

(121). However, subsequent analysis revealed that the 48 kD protein arises from the 68 kD protein through proteolytic cleavage resulting during the course of purification (122). More recent reports indicate that the enzyme purifies as 68-70 kD protein with dual kinase activity and that a single molecule of dsRNA interacts with a homodimeric form of the kinase (119, 122). According to the current model one subunit in the complex phosphorylates the adjacent subunit and in doing so alters the specificity of the phosphorylated protein. The phosphorylated form of the kinase is no longer capable of recognizing other kinase molecules, but can efficiently phosphorylate eIF-2α (119, 122).

Activation of the kinase requires at least 50 bp of dsRNA structure and is antagonized by adenovirus VAI RNA (119, 123, 124). Mutant strains of adenovirus that are unable to transcribe their VAI RNA late in infection are more sensitive to the antiviral effects of interferon (123, 125). The viral RNA interacts with the enzyme, but because of significant secondary structure is unable to stimulate auto-kinase activity (119, 124). Consequently, the activatable supply of kinase is reduced and eIF-2α is not inhibited. However, VAI RNA does not prevent activated DAI from phosphorylating eIF-2α. If dsRNA is added to an extract containing latent kinase, phosphorylation is not precluded by subsequent addition of VAI RNA, although simultaneous addition of both RNAs does inhibit phosphorylation (123).

B. The Antiproliferative Activity

Interferons are members of a complex network of cytokines (interleukins, tumor necrosis factor, hematopoietic growth factors) that are responsible for maintaining cellular homeostasis. In addition to their well characterized antiviral activity interferons play an important role in the regulation of cell growth and differentiation (126). The antiproliferative effects of interferons have been repeatedly demonstrated since Paucker and colleagues first reported that interferons could act to restrict cell growth in culture (127, 128). Subsequent investigations showed that interferon treatment prevented cell cycling and inhibited the burst of DNA synthesis which normally accompanies mitogenic stimulation of cells (129). Moreover, interferon pretreatment of quiescent fibroblasts was found to inhibit the accumulation of certain growth factor-induced secretory proteins (130).

Interferons may exert their antiproliferative effects by regulating proto-oncogene expression. The accumulation of c-fos and c-myc transcripts in response to serum or PDGF stimulation is antagonized by interferon and results in the inhibition of the G_0 to S transition (131-135). Daudi lymphoblastoid cells are highly sensitive to minute concentrations of interferon. In this system interferon causes a 4-7 fold decrease in induced levels of steady-state c-myc message via an accelerated rate of RNA degradation (133, 134). Several IFN-resistant Daudi variants which fail to down regulate c-myc message and grow continuously in the presence of interferon have been isolated (133, 136). These cells possess normal interferon receptors and maintain other interferon-mediated intracellular responses (132, 137). Somatic cell fusion of IFN-resistant with IFN-sensitive cells restores the sensitive phenotype (ie. hybrids are able to respond to interferon

treatment and reduce the levels of c-myc RNA) (138), implying that in resistant cells the down-regulation of c-myc RNA is not masked by a suppressive factor(s), but rather is due to the absence of a factor(s) which mediates sensitivity to interferon.

Some evidence suggests that the negative growth effects of interferons are related to their antiviral activity, and that the 2'-5' oligoadenylate synthetase (OASE)-RNAase L pathway may be involved. The concentration of OASE was reported to increase during interferon-induced hematopoietic cell differentiation, while a decrease in OASE was found to accompany the onset of growth in certain cell lines (139, 140). Higher levels of OASE have been detected in the nuclear compartment of growth arrested cells (140). Furthermore, cell-cycle progression of serum-stimulated quiescent fibroblasts, and DNA synthesis in lectin-stimulated lymphocytes is precluded by the addition of 2'-5' oligoadenylate (140, 141). It is therefore conceivable that post-transcriptional inhibition of growth related cellular RNAs (possibly proto-oncogene RNA) is mediated by RNAase L, even though this has not been established. Interestingly, IFN-resistant Daudi cells which fail to respond to the negative growth effects of interferon are still able to induce OASE, but not the 6-16, 9-27 or 1-8 genes which are normally co-induced by interferon (142). In these cells, however, direct impairment of RNAase L activity may account for their ability to escape interferon inhibition. In addition, the 6-16, 9-27 and 1-8 gene products might themselves be involved in regulating cell growth, given that a biological function has yet to be assigned to these proteins. Ultimately, the precise role of the enzymes in the 2'-5' oligoadenylate synthetase-RNAase L pathway, as well as other interferon-induced proteins in controlling cell growth remains to be determined.

C. Immunoregulatory Effects of Interferons

The importance of interferons as elements of both the innate and humoral immune system has long been recognized. IFNs represent major immunoregulatory cytokines, with functions that include macrophage and NK cell activation; stimulation of differentiation and maturation of various immunocytes; and induction of histocompatibility antigens in most cell types (143).

One of the first recognized effects of interferons was their ability to augment morphological differentiation and phagocytic activity of macrophages (143, 144). Interferon-induced enhancement of foreign antigen uptake is partly due to increased Fc receptor-mediated phagocytosis (144). Interferon-primed and antigen-presenting macrophages possess reduced proliferative potential and can selectively bind tumor cells. IFN-γ is required for establishing tumoricidal competence in these cells, and synergizes with plant mitogens to induce expression of tumor necrosis factor (TNF) (145).

Interferons can act to inhibit or enhance primary antibody responses to T-dependent and T-independent antigens, both *in vitro* and *in vivo*. Whether the effect is suppressive or augmentive depends in part on the time at which interferon is administered. If the cells are exposed to interferon simultaneously or subsequent to the antigenic challenge, the antibody response is enhanced, while pretreatment of lymphocyte cultures before antigen stimulation causes suppression of the antibody response. Furthermore, when interferon acts in an inhibitory capacity, T-dependent B cell responses are more sensitive to suppression than T-independent B cell responses. The simplest model which best fits this data suggests that interferon pretreatment represses B cell activation by exerting anti-

proliferative effects on B cells, but augments the B cell response to antigen later by inhibiting the proliferation of suppressor T-lymphocytes (143).

Many of the immunoregulatory effects of interferons relate to induced alterations in cell-surface molecules on hematopoietic cells. Exposure of lymphocytes, monocytes and macrophages to interferon results in a dose-dependent increase in IgFc receptors on the cell membrane (146-148). Additionally, interferons have been demonstrated to transcriptionally stimulate expression of major histocompatibility antigens (MHC class I & II) and ß2-macroglobulin (ß2m) on both immune and non-immune cell types (149-154). With respect to this function, IFN-γ is considerably more potent than either IFN-α or IFN-ß (153). Klar and Hammerling recently showed that IFN-γ also mediates cytoplasmic assembly of MHC class I heavy chains with ß2m after induction. In the absence of IFN-γ the MHC class I and ß2m proteins accumulate intracellularly, but do not associate (154). Interferon-induced enhancement of MHC expression provides an alternate mechanism by which to clear an infecting virus. Cell surface expression of viral antigens in association with MHC class I can render an infected cell susceptible to the cytolytic activity of T_{cyt}-lymphocytes. Similarly, the anti-tumor activity of interferons may be mediated through the induction of MHC class I molecules in association with tumor specific antigens (155).

There is some data to suggest that interferon-mediated induction of histocompatibility antigens is related to the development of various autoimmune disorders. Several studies reported high IFN serum concentrations in patients with autoimmune disorders; during periods of remission these levels were greatly reduced (156, 157). In addition, peripheral blood monocytes from individuals with autoimmune disease contain elevated levels of 2'5'-oligoadenylate synthetase; however, the levels of this

enzyme do not consistently correlate with the presence of interferon (158). Early studies demonstrated that interferon treatment could accelerate progression of autoimmune disease in mice (159). It is known that MHC class II expressing cells are capable of eliciting an immune reaction by presenting antigen to T-helper lymphocytes (160). Overexpression of MHC class II antigens has been associated with various murine autoimmune diseases (161, 162). Given that IFN-γ can induce MHC class II antigens in cells that do not normally express this marker (152), it is possible that some autoimmune disorders involve an interferon triggered immune reaction against a self antigen.

Interferons are believed to play a major role in regulating non-specific cell mediated immunity. The cytolytic activity of natural killer (NK) cells is significantly enhanced by all three classes of interferon (IFN-α, ß, γ) (163-165). This effect is especially important during early neonatal development when the immature immune system is not capable of mounting a full thymic-dependent response (164). Antibody neutralization of interferon diminishes NK-mediated cytotoxicity *in vivo* (164). Interferon-activated NK cells display enhanced secretion of cytolytic factors (ie. perforin), accelerated lytic ability, increased adherence and wider target range (163-165). Activation requires de novo protein synthesis and can be mimicked by treatment with 2'5'-oligoadenylate, suggesting possible involvement of the antiviral cascade (166).

Paradoxically, the effect of interferon on NK targets is antagonistic to that observed on NK cells themselves. Cells exposed to interferon become resistant to cytopathic NK activity (163-165). NK cells are capable of binding to interferon-treated cells, but fail to release cytolytic factors (167). The protective action of interferon correlates with increased cell surface expression of sialic acid containing molecules (168). Histocompatibility antigens are interferon-

inducible sialoglycoproteins; according to some reports resistance to cell lysis is concomitant with elevated MHC surface antigens. Reciprocally, cells that fail to express MHC antigens in response to interferon treatment are more susceptible to lysis by NK cells. These cells, however, are still able to mount an antiviral response (169).

1.7 REGULATION OF INTERFERON-STIMULATED GENES

A. Mechanisms of Interferon-mediated Gene Activation

Transcriptional activation of IFN-stimulated genes (ISG54/-15, 2'5' OASE, MHC class I, 6-16, and the 1-8 gene family) occurs within minutes following the addition of ligand. In many cases, induction is dependent on 13-15 bp consensus element (ISRE) located within the 5' flanking region of these genes (170-178). Synthetic ISRE oligonucleotides or restriction fragments containing intact ISRE motifs are able to confer inducibility to heterologous promoters in transient expression assays (170-174, 176-180). Multimerization of natural or synthetic ISREs corresponding to the human 2'5' OASE promoter increases inducibility of the transfected gene by 2-6 fold (174). Furthermore, mutation of ISRE sequences derived from various IFN-stimulated genes abolishes IFN-mediated transcriptional activity *in vivo* (170, 172, 180-182).

Several studies have recently identified DNA-binding proteins that interact with the ISRE (170, 171, 173, 174, 178, 180-183). Most of these ISRE binding factors fall into 3 categories: those which bind DNA constitutively (170, 173, 174, 178, 180, 181); those which bind rapidly in response to IFN treatment and do not require protein synthesis (ie. ISGF3, factor E) (170, 180-182); and those

which are induced to bind at later times, but require protein synthesis (ie. ISGF2, factor L) (170, 180, 182). In general, the DNA-protein interactions of all three classes of factors are restricted to boundaries of the ISRE, although in at least one case sequences 5' to the ISRE are required for a stable interaction (180).

Transcriptional activation of the ISG54/-15 and 6-16 genes correlates with inducible binding of ISGF3 and factor E, respectively. These activities have been extensively characterized by different groups, but probably represent the same protein (170, 180, 182, 184, 185). ISGF3 is undetectable or present in minute amounts in IFN-resistant cells which do not support ISG transcription, and fail to develop the antiviral state (186). Mutations in the ISRE that prevent ISGF3 binding *in vitro*, also inhibit transcriptional activity *in vivo* (182). Similar correlations between factor binding and expression have been observed with the ISREs of the 6-16 and MHC class I genes (180, 181, 183).

Binding of ISGF3 and factor E is stimulated by IFN-α in the presence of cycloheximide, indicating that the proteins exist in a latent form prior to induction (170, 180). Both factors are cytoplasmically activated within 30-60 seconds after IFN-α treatment, and then translocate to the nucleus where they interact specifically with the DNA (184, 185). Mature ISGF3 exists as a heterodimeric complex. A series of elegant extract mixing experiments showed that prior to translocation, the α-component of ISGF3 (ISGF3α) associates stoichiometrically with a second component designated ISGF3γ (184). ISGF3γ is inducible by IFN-γ in Hela cells, but appears to be constitutively active in FS2 fibroblasts and Daudi lymphoblastoid cells. Protein alkylation of ISGF3γ precludes assembly of ISGF3, and treatment of the cells with sodium flouride (NaF) results in cytoplasmic accumulation of

the complex. The mechanism of inhibition by NaF is not known, but may be related to the impairment of some process that involves shuttling of phosphoryl groups (184). Photo- affinity cross-linking studies indicate that the active form of factor E may also consist of multiple subunits (Dale, T. C., unpublished observation)

Purified ISGF3 is able to stimulate ISRE-dependent transcription *in vitro* (187). It is not clear if each component in the complex makes contact with the DNA or if both are required for transcriptional activation. The purified α and γ subunits resolve on SDS polyacrylamide gels as proteins of 50 kD and 100 kD, respectively (187, 188). Mixing experiments with glycerol gradient fractions suggest that binding of ISGF3 is dependent on complex formation (Kessler, D. S., personal communication). However, denaturation-renaturation studies indicate that proteins in the 50 kD range are sufficient for activity (187). Whether ISGF3γ is required for binding and transcription, or whether it merely serves a carrier function in intact cells remains to be determined.

The shutoff of ISRE-mediated transcription, correlates with the induction of a distinct set of temporally delayed proteins whose binding activity is dependent on de novo protein synthesis (170, 180). One of these factors, ISGF2, has been purified to homogeneity from Hela cells and has an apparent molecular weight of 56 kD (189). A related activity in Hela cells, factor L, binds to the 6-16 gene ISRE with similar induction kinetics (180). Although the cloning of ISGF2 has not been published, there are reports that the gene encoding ISGF2 is transcriptionally activated following treatment of cells with IFN-α or IFN-γ (189). The recognition sequences of ISGF2 are encompassed within the region required for ISGF3 binding and the proteins make similar

contacts with the DNA (182). Analysis of purified ISGF2 suggests that a single molecule can directly interact with the ISRE (189).

Detection of factor L and ISGF2 only during the shutoff phase of transcription implies that these proteins may be involved in mediating repression of ISRE-dependent transcription (170, 180). How then is repression mediated under basal conditions when these proteins do not appear to act? The simplest explanation is that ISRE-dependent transcription does not occur in the absence of positive trans-acting factors. An alternate possibility proposed by Stark and co-workers is that constitutive ISRE binding factors may be responsible for maintaining repression in uninduced cells. This notion is based on studies showing that the constitutive (C) and late (L) factors bind similarily to the 6-16 ISRE, but in a manner that is distinct from factor E (180). However, differential binding of the putative ISRE repressor(s) or activator(s) may be unique to this system. ISGF2 and ISGF3 interact with a common set of sequences in the ISG54/-15 ISREs. Saturation point mutagenesis of these elements failed to identify mutations which could selectively abolish ISGF2 binding without diminishing ISGF3 binding (182). Similarly, gel retardation and/or chemical footprinting analysis of the MHC class I and 2'5' OASE ISREs indicate that both the constitutive and inducible DNA-binding factors interact with the same sequences (173, 181). Several candidate cDNA clones encoding constitutive and inducible ISRE binding factors have been reported (189-191). Analysis of these clones will help clarify their role as activators/repressors in the signal transduction pathway.

B. Signal Transduction of Type I Versus Type II Interferons

Differential responses to interferons (α, ß, γ) have been noted with several IFN-stimulated genes. IFN-γ stimulation has little or no effect on the expression of the 6-16, ISG54 and ISG15 genes, while IFN-α and IFN-ß act as potent inducers of these templates (170, 171). Reciprocally, MHC class II genes are mainly responsive to IFN-γ (105, 192). A subset of genes including 2'5'OASE, GBP, MHC class I genes, and the 1-8 gene family are highly inducible by all three classes of interferon (172-178, 181, 183). Preferential activation of some genes by one type of interferon versus another suggests that interferons may mediate their effects via distinct signal transduction pathways. Similarly, the absence of a biased response in other cases indicates that the pathways might overlap.

IFN-γ stimulates ISGF3γ (in Hela cells) and ISGF2, both of which are involved in mediating induction by IFN-α (184, 189). These components may serve some dual role by also modulating IFN-γ specific signals. It is of interest that the differential response of the 6-16 gene to type I versus type II interferons can be eliminated by artificially manipulating the 5' regulatory sequences. In the context of its natural promoter the 6-16 ISRE is activated by IFN-α, but not IFN-γ; this constraint is relieved when the ISRE is placed upstream of a heterologous promoter (172). A similar effect is observed if the intact human 6-16 gene is stably transfected into murine L cells (Porter, A. C. G. and Kerr, I. M., unpublished observations). Thus, the capacity for signal overlap appears to exist in this system, but additional controls may be superimposed which mediate specificity for type I interferon.

Evidence for differences between the IFN-α and IFN-γ signal transduction pathways comes from expression studies on the guanylate-binding protein

(GBP) gene (193, 194). The shutoff of IFN-α-induced GBP transcription occurs within 6 hours following activation, whereas high level expression continues for at least 24 hours after the addition of IFN-γ. This delay in repression is a dominant phenomenon and is not attributable to the utilization of alternate mRNA initiation sites or to treatment with sub-optimal concentrations of interferon. Co-treatment of cells with a mixture of IFN-α/γ results in shutoff kinetics that are characteristic of the IFN-γ response. Under these conditions delayed repression of the ISG54 gene is also observed. The overriding effect is partly due to the extended maintenance of mature ISGF3. However, cells treated with only IFN-γ do not contain ISGF3 binding activity, presumably because the ISGF3α component is not activated. In addition, once IFN-α-mediated expression ceases, the gene is refractory to secondary stimulation by IFN-α, but not IFN-γ. Together with the observation that IFN-α and IFN-γ act to synergistically stimulate GBP transcription, these findings suggest that IFN-γ-mediated activation and prolongation of GBP transcription is due to a γ-specific factor(s) (193).

1.8 REGULATION OF INTERFERON-ß TRANSCRIPTION

Interferons provide an excellent model to study cell-specific inducible gene expression. The genes are transcriptionally activated in response to induction by virus or synthetic double-stranded RNA. As with many eukaryotic gene systems a reverse genetics approach has been used to analyse the genetic and biochemical requirements for IFN-ß transcription. Induction is mediated by sequences present in the 5' flanking region of the gene. Cloned IFN-ß templates are appropriately regulated in heterologous cells following chromosomal integration with selectable markers (195-198) or introduction

on viral vectors that are transiently (199, 200) or stably maintained in an extrachromosomal form (201-203).

The cis-acting requirements for IFN-ß gene induction were delineated by genetic analysis of truncated promoter templates (199, 200, 204-207). Zinn et al. used BPV-derived vectors to deliver a series of deletion mutants into murine C127 cells (204). Expression in this system was measured at the RNA level using a quantitative nuclease mapping assay. Analysis of different 5' promoter deletions identified a negative regulatory element in the region between -210 and -107; removal of this element resulted in significantly higher basal expression and also slightly augmented the induced levels of IFN-ß message. Progressive deletion down to -77 did not severely impair transcription, whereas deletion to -73 drastically reduced the level of induced activity, suggesting that in C127 cells the 5' boundary for efficient inducibility localizes to the region between -77 and -73 (204). The data was qualitatively the same regardless of whether the deleted templates were introduced into cells on a modified BPV vector that lacked known viral enhancer sequences, or if integrated into the host chromosome (205). A parallel study by Fujita et al. found that in murine L929 cells, the 5' boundary for induction mapped to the region between -115 and -103. In their study deletion to -91 significantly decreased inducibility, while deletion to -74 completely precluded transcription (206). An extended 5' sequence requirement to -111 in murine L cells was independently confirmed by Dinter and co-workers (207). Given that the above studies used similar delivery systems to test a series of deletion mutants, it is likely that the observed variability in delineating the 5' end-point for induction was due to differences in cell type rather than experimental design.

Induction of the IFN-ß promoter is not dependent on the sequences around the TATA box and the CAP site. The region downstream of -37 is insufficient to render the SV40 enhancer inducible. Nucleotide sequences between -19 to +277 can be removed without deleteriously affecting inducibility (204, 206). A 42 bp promoter fragment (-77 to -37) designated the interferon regulatory element (IRE), represents the minimal sequence requirement for induction in C127 cells, and possesses several characteristics of an inducible enhancer (figure 4) (205). The IRE confers inducibility to a heterologous -39 thymidine kinase (tk) basal promoter linked to an IFN-ß or human growth hormone (HGH) structural gene; it acts in an orientation independent manner on a relatively inactive -40 IFN-ß deletion mutant (~2 fold inducible) or an intact -116 tk promoter-IFN-ß structural gene fusion; and confers inducibility when placed 800 bp upstream of the -116 tk promoter, or several hundred base pairs downstream (in either orientation) or upstream of a weakly inducible (~5 fold) -73 deletion mutant. However, despite its range of activities on a heterologous promoter the IRE displays less flexibility in the context of its own basal promoter as it is unable to act at a distance on a -40 IFN-ß deletion mutant (205). Similarly, DNA fragments with extended 5' flanking sequences (-123 to -37) function in either orientation within 10 bp of a -37 deletion mutant, but not if placed further upstream or downstream of the gene (206). Analogous studies in the IFN-α1 system indicate that a single copy of the VRE (virus responsive element) is unable to confer activity when placed downstream of a ß-globin gene or within the first intron, although tandem copies at these positions give 3-4 fold inducibility (208).

The intact IRE reversibly represses the constitutive activity of a -105 tk promoter when the two are juxtaposed (209). Mutational analysis of the 3' IRE sequences revealed that the region between - 55 and -37 constitutes a negative

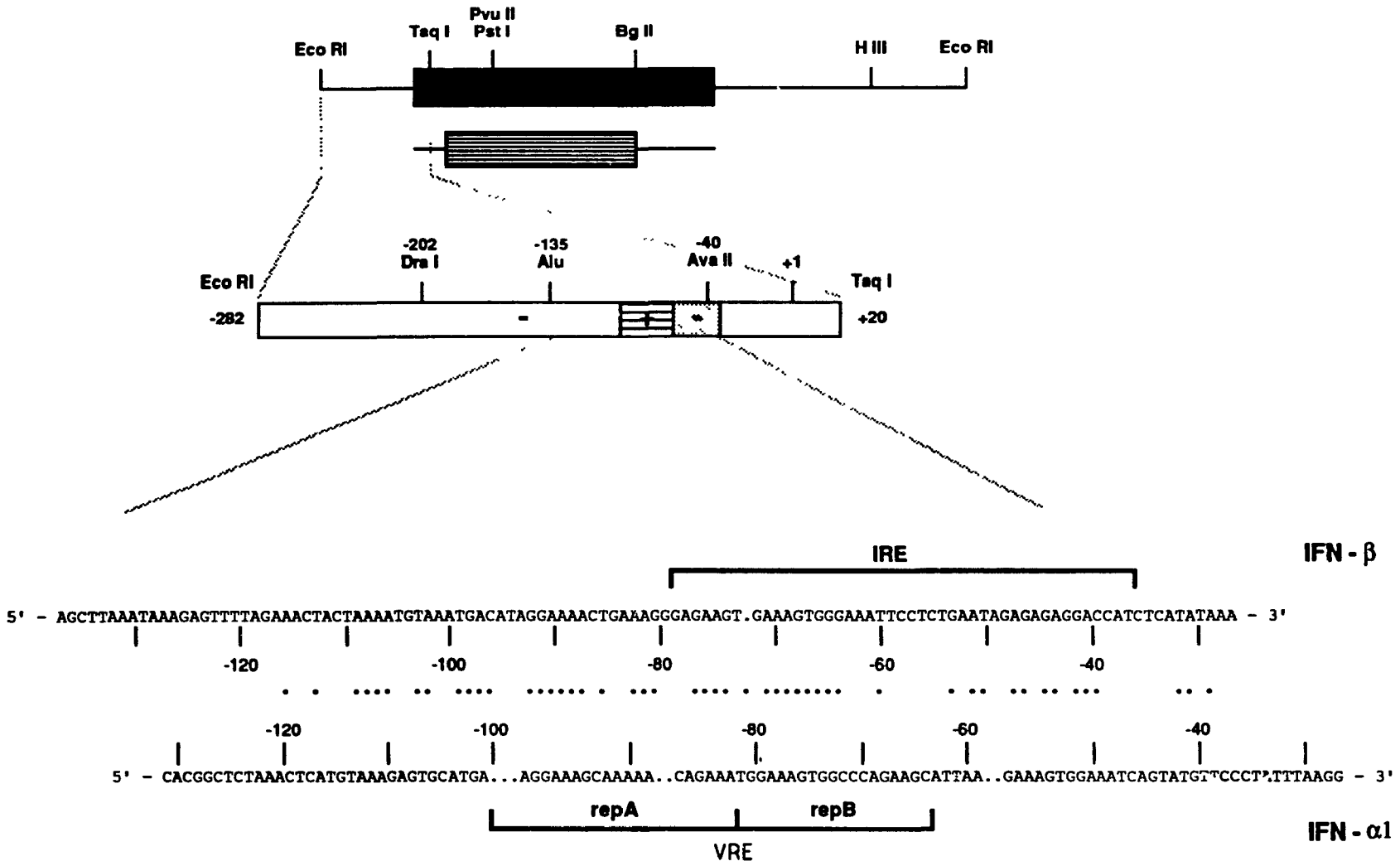


Figure 4. Schematic of the IFN-ß 5'-flanking region. The 1.8 kb Ecor I fragment containing the gene (solid box) is shown. The mRNA transcript and protein coding region (lined box) are shown below the gene. The Ecor I-Taq I promoter fragment containing the positve (+) and negative (-) regulatory elements is expanded, as are the nucleotide sequences between the Ava II and Alu I sites. The IFN-α1 regulatory sequences are aligned below the IFN-ß sequences. The interferon regulatory element (IRE) and the virus responsive element (VRE) are indicated by brackets. The figure is based on Ref. (204, 205, 208).

regulatory element. Removal of these sequences increases the basal level of transcription and thereby decreases overall inducibility of the gene. In the absence of the negative domain the remaining IRE seqences between -77 to -55 act in a positive manner and confer a high level of constitutive activity to the IFN-ß TATA box (-40 deletion mutant), the tk TATA box and a -73 deletion mutant at a distance of 100 bp. However, these sequences still mediate 9 fold inducibility on a -40 deletion mutant, suggesting that partial negative activity may still be associated with the remaining region, or that some of the transcription factors which interact with these sequences remain subject to modulation by the inducing stimulus (209). Superimposition of an inducible activity onto a constitutive activity may be due to potentiation of a constitutive factor(s) by the inducing signal. Alternatively, the constitutive and inducible activities may be mediated by distinct factors. Thus, transcriptional activation may involve derepression of a constitutive activity, or derepression of a constitutive activity in concert with an inducible activity.

1.9 SPECIFIC RESEARCH AIMS

The primary objective of the work presented in this thesis has been to identify specific components of the signal transduction pathway involved in mediating human IFN-ß gene induction. In contrast to other studies we have chosen to conduct our investigations exclusively in the homologous cell system. To date, the project has focused on two main aspects of IFN molecular biology : (1) the cell-specific expression of cloned IFN-α and IFN-ß genes in human cells; and (2) the role of trans-acting proteins in the regulation of IFN-ß transcription. The transient expression system described in chapter 2 of the thesis has proved useful in our efforts to delineate the genetic and biochemical requirements for promoter activation. As a complementary strategy we sought to identify and characterize soluble factors interacting with both the positive and negative regulatory domains of the promoter. These approaches have enabled us to examine the regulation of the human IFN-ß gene in the uninduced and induced state.

CHAPTER 2

TRANSIENT EXPRESSION OF THE BETA INTERFERON PROMOTER IN HUMAN CELLS

ABSTRACT

A human transient expression assay has been utilized to examine the inducible transcriptional activation of the IFN-ß and IFN-α1 promoters in a homologous cellular environment. Use of 293 cells, an adenovirus DNA-transformed human embryonic kidney line, permitted Sendai virus inducible expression of IFNß-CAT hybrid genes. Introduction of the simian virus 40 (SV40) enhancer 5' or 3' to the IFN-CAT gene increased basal (uninduced) levels of CAT activity; in one construct the SV40 enhancer-IFN-ß regulatory region combination increased the induced CAT activity 50-100 fold, suggesting that this may be a generally useful inducible enhancer-promoter combination. No expression from the IFN-α-CAT hybrid gene was detected in 293 cells, indicating that human epithelial cells lack a factor required for expression of the IFN-α promoter. However, when the IFN-α regulatory region was combined with the SV40 enhancer a low level of inducible CAT activity was detected in the human transient system.

INTRODUCTION

Human interferons represent a unique class of inducible cellular proteins with a wide range of antiviral, antiproliferative and immune regulatory activities (18,29). Interferons (IFNs) are classified into three antigenically distinct groups, designated IFN-α, IFN-ß and IFN-γ. The alpha IFNs are encoded by two multigene families, IFN-α_I, consisting of at least 20 distinct genes or pseudogenes, and IFN-α_{II}, encoded by at least 6-7 genes (4,18). IFN-ß is encoded by a single gene (25), although a related, intron-containing IFN-$ß_2$ gene has been described (16); IFN-γ is encoded by a single, intron-containing gene (11). Induction of many cell types with virus, synthetic polyribonucleotides or bacterial antigens leads to the secretion of IFNs (29); in general, fibroblastic and epithelial cells synthesize IFN-ß, while peripheral blood leukocytes, lymphoid and myeloid cell lines produce a heterogenous mixture of IFN-α and IFN-ß as a consequence of differential gene transcription (14).

Stable introduction of the human IFN-α1 or IFN-ß gene into murine fibroblastic cell lines by permanent transformation has defined the cis-acting elements involved in IFN inducibility. A 46 nucleotide IFN-α1 promoter fragment from position -109 to -64 conferred virus inducibility upon a heterologous rabbit ß-globin gene (20,26,28). The sequences involved in poly(rI-rC) activation of the human IFN-ß gene in mouse C127 cells are located -37 to -77 relative to the mRNA cap site and display the characteristics of an inducible enhancer element (8,33); a study involving the use of mouse L929 cells identified a different IFN-ß promoter fragment, spanning -115 to -37 relative to the mRNA CAP site, required for virus inducibility (6). Recently, an elegant study has demonstrated that the IFN-ß enhancer element is negatively regulated and has identified regions of the IFN-ß promoter to

which putative regulatory factors bind (7,34). In addition to sequences required for transcriptional activation at the 5'-flanking end of the gene, sequences at the 3'-untranslated end of the gene and mRNA are involved in RNA stability and turnover (23,24,27).

Studies on the characterization of cis-acting sequences involved in human interferon gene regulation have been carried out almost exclusively in murine fibroblastic cells co-transformed with human IFN genes and a selectable marker (3,6-8,13,20,25,28,33,35). The murine and human fibroblast cell systems, however, differ fundamentally in that following virus infection in mouse cells, IFN-α and IFN-ß mRNAs and proteins are synthesized in an approximately equimolar mixture (15,32), whereas in human fibroblasts, virus infection and dsRNA treatment result in predominantly IFN-$ß_1$ synthesis (14,27). Therefore with these differences in mind, we set out to develop a human transient expression system to examine the transcriptional activation of IFN-α and IFN-ß genes in a homologous cellular environment and to ask if different IFN genes are transcriptionally activated by common regulatory factors or if expression of the transfected genes reflects the specificity of the human cell.

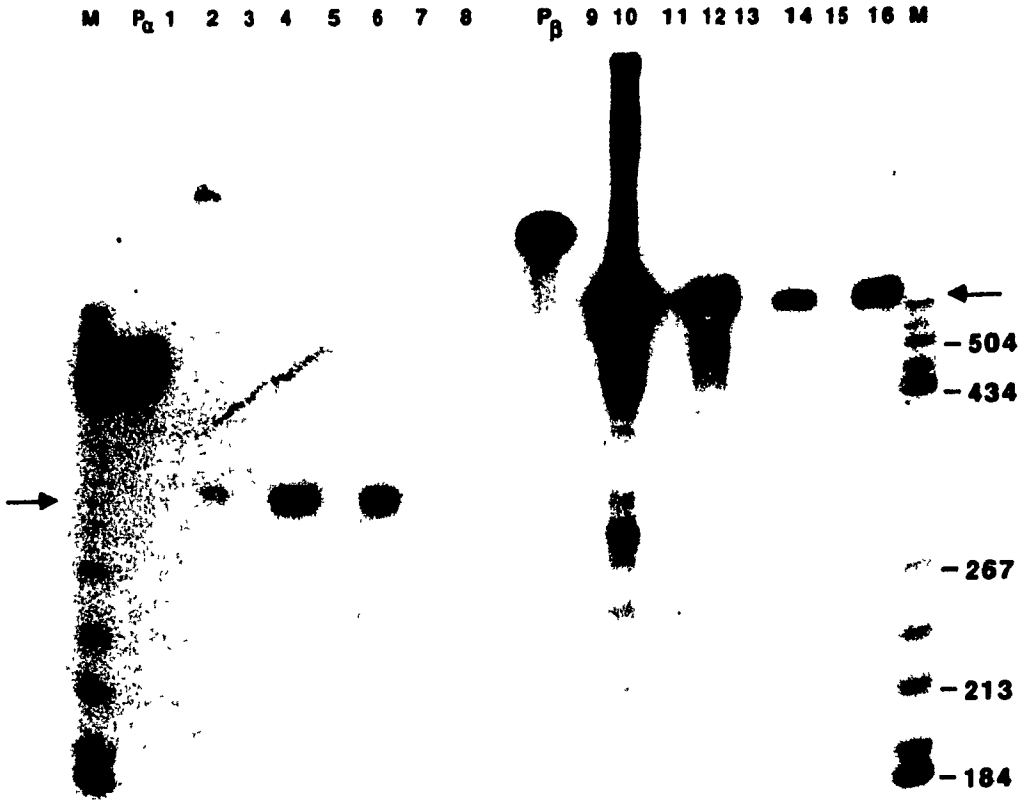
RESULTS AND DISCUSSION

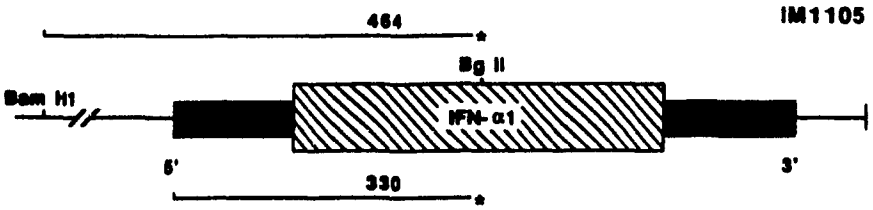
Production of Endogenous IFN mRNA in Human Cells. To assess the expression of endogenous IFN genes in human cell lines following induction by Sendai virus, total RNA was isolated from various human cell lines at 5.5 hours after induction and hybridized with ^{32}P end-labeled IFN-ß and IFN-α1 probes (Fig. 1). Analysis of RNA from Sendai virus-induced MG63, HeLa and 293 fibroblasts (Fig. 1; see also Fig. 4) and two human myeloid cell lines, U937 and KG-1, identified IFN-ß transcripts in all cells examined, but at widely variant levels; in addition, IFN-α1 was synthesized in U937 and KG-1 cells. No IFN-α1 was detected in HeLa or 293 cells and the amount of IFN-α1 in MG63 cells was just above the level of detectability (about 5 copies/cell). Thus although fibroblastic or epithelioid cells produced variable amounts of IFN-ß in response to Sendai virus infection, IFN-α1 RNA was either not detectable (HeLa, 293) or present at very low levels (MG63) in these cell types. The myeloid cells, on the other hand, synthesized both IFN-α1 and IFN-ß RNA following virus induction. Introduction of the IFN-ß and IFN-α1 promoters into 293 cells will therefore permit a comparison of the expression of genes with fibroblastic/epithelial (IFN-ß) or hematopoietic (IFN-α1) cell specificity in a human epithelial environment.

Enhancer-Mediated Expression of IFN-CAT Plasmids in Human Fibroblasts. The parental chloramphenicol acetyltransferase (CAT) plasmids were constructed by removing either the entire simian virus 40 (SV40) promoter-enhancer combination (SV_0CAT) or most of the SV40 72 base-pair (bp) repeats (SV_1CAT) with the exception of 22 bp of one repeat, as described previously (9). Expression of CAT activity in SV_1CAT-transformed 293 cells was only about 4% of the level observed in SV_2CAT-transformed cells (see

Figure 1. Production of Endogenous IFN-α1/IFN-ß mRNA in Human Fibroblastic and Myeloid Cells after Sendai Virus Induction. All cell cultures were grown at 37°C in RPMI-1640 supplemented with 5-10% fetal calf serum and antibiotics. Virus inductions were carried out with Sendai virus (produced in 11-14 day old chick eggs) at a concentration of 2000 HAU/ml for 90 min. Cells were then washed with PBS and incubated in complete medium. Total cellular RNA was isolated from cells at 5.5 hours after infection by an SDS-proteinase K-phenol method (14), followed by RNAase-free DNAase digestion.

The probes for mapping IFN-α1 and IFN-ß transcripts are indicated in the diagrams of the human IFN genes. Protein coding sequences are represented by the hatched boxes; non-translated 5' and 3' regions are indicated by solid boxes and bacterial sequences by solid lines. The distance in nucleotides between the ^{32}P-labeled 5' end of the probe (marked with an asterisk) and the 3' end is indicated above the IFN gene. The distance in nucleotides between the 5' end of the probe and the CAP site is indicated below the IFN gene. Total cellular RNA was isolated from uninduced (odd numbered lanes) and induced (even numbered lanes) cells at 5.5 hours after initial Sendai virus infection and analyzed for IFN-α1 mRNA (lanes 1-8) or IFN-ß mRNA (lanes 9-16) by S1 mapping (14). Lanes: P_{α}, IFN-α1 464 bp probe alone; 1,2 - MG63 cell RNA; 3,4 - KG-1 cell RNA; 5,6 - U937 cell RNA; 7,8 - HeLa cell RNA; P_{β}, IFN-ß 922 bp probe alone; 9,10 - MG63 cell RNA; 11,12 - KG-1 cell RNA; 13,14 - U937 cell RNA; 15,16 - HeLa cell RNA; M, marker of pBR322 digested with HaeIII and labeled with γ-^{32}P-ATP.


M
Pα
1
2
3
4
5
6
7
8
Pβ
9
10
11
12
13
14
15
16
M
504
434
267
213
184


464
IM1105
Bg II
Bam H1
IFN-α1
5'
3'
330

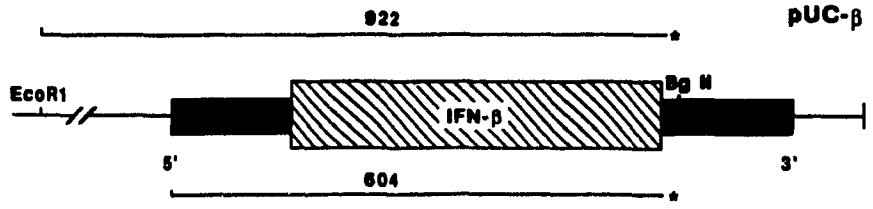

922
pUC-β
EcoR1
Bg II
IFN-β
5'
3'
604

Fig. 5); conversion of ^{14}C-chloramphenicol with lysates from SV_0CAT transfected cells was only 2% of the control level. Promoter fragments from IFN-ß and IFN-α1 were subcloned into SV_1CAT and SV_0CAT vectors (Fig. 2) and in some plasmids, the NcoI-PvuII 233 bp SV40 enhancer-containing fragment (2,12,22), was placed 5' or 3' to the SV_0ß-CAT hybrid gene in either the NdeI or Bam H1 site, respectively (Fig. 3). The transcriptional activity of the SV40-IFNß enhancer-promoter combinations was analyzed in 293 cells after they had been transfected with the hybrid genes, induced with Sendai virus at 20 h after transfection, and harvested for CAT assay 20 h later. In this experiment SV_2CAT and SV_0ß transfected lysates (170 µg) produced a 0.96 and 41.7 relative induction, respectively, although the absolute conversion of ^{14}C-chloramphenicol by induced lysates differed by a factor of 5.6 (28% versus 5%, respectively). The insertion of the SV40 enhancer either 5' or 3' to the IFNß-CAT hybrid gene increased basal CAT expression 2.5-7.5 fold. After virus induction, relative CAT activity was increased 95 fold in $SV5^+$ß transfected lysates; insertion of the SV40 enhancer in either the opposite (-) orientation at the 5' site, or in its normal (+) orientation at the 3' site produced only a 12 fold relative induction. Absolute conversion values for $SV5^+$ß and SV_2CAT lysates (66.5 and 28.1%, respectively) suggested that transcription from the $SV5^+$ß promoter was 2.4 times the rate from the SV40 promoter. Taking into consideration the cumulative synthesis over 62 hours of CAT enzyme in SV_2CAT transfected cells and the induced CAT synthesis over 24 hours in $SV5^+$ß cells, it appeared that the highly inducible $SV5^+$ß enhancer-promoter combination functioned about 5 times better in human 293 cells than the SV40 enhancer-promoter alone.

Figure 2. Construction of IFN-CAT Plasmids. The structures of pSV_2CAT, pSV_1CAT and pSV_0CAT (9) are illustrated; IFN-ß and IFN-α1 promoter fragments (illustrated below the SVCAT plasmids) were inserted into SV_1CAT and SV_0CAT vectors by blunt end ligation. The IFN-ß EcoR1-Taq1 fragment and the IFN-α1 BamH1-Hinf I fragment from IM1105 were cloned into SV_0CAT in both orientations; the same IFN-ß fragment and the IFN-α1 BamH1-Sau961 fragment were cloned into SV_1CAT in both orientations. The NdeI and Bam H1 sites were used to insert the SV40 NcoI-PvuII 233 bp fragment at the 5' end and 3' end of the IFN-CAT hybrid genes, respectively. The solid box represents the CAT enzyme coding sequences; the SV40 promoter and 72 bp repeats are indicated by the smaller open box and the two hatched boxes, respectively. Transcription initiation sites and direction of transcription are indicated by the arrows on the SV40 or IFN promoter. Bacterial sequences including origin of replication and location of ß-lactamase gene are shown by solid lines, ori, and AMP, respectively. The translation initiation codon for the CAT protein (AUG) is the first initiation codon in the IFN-CAT hybrid genes.

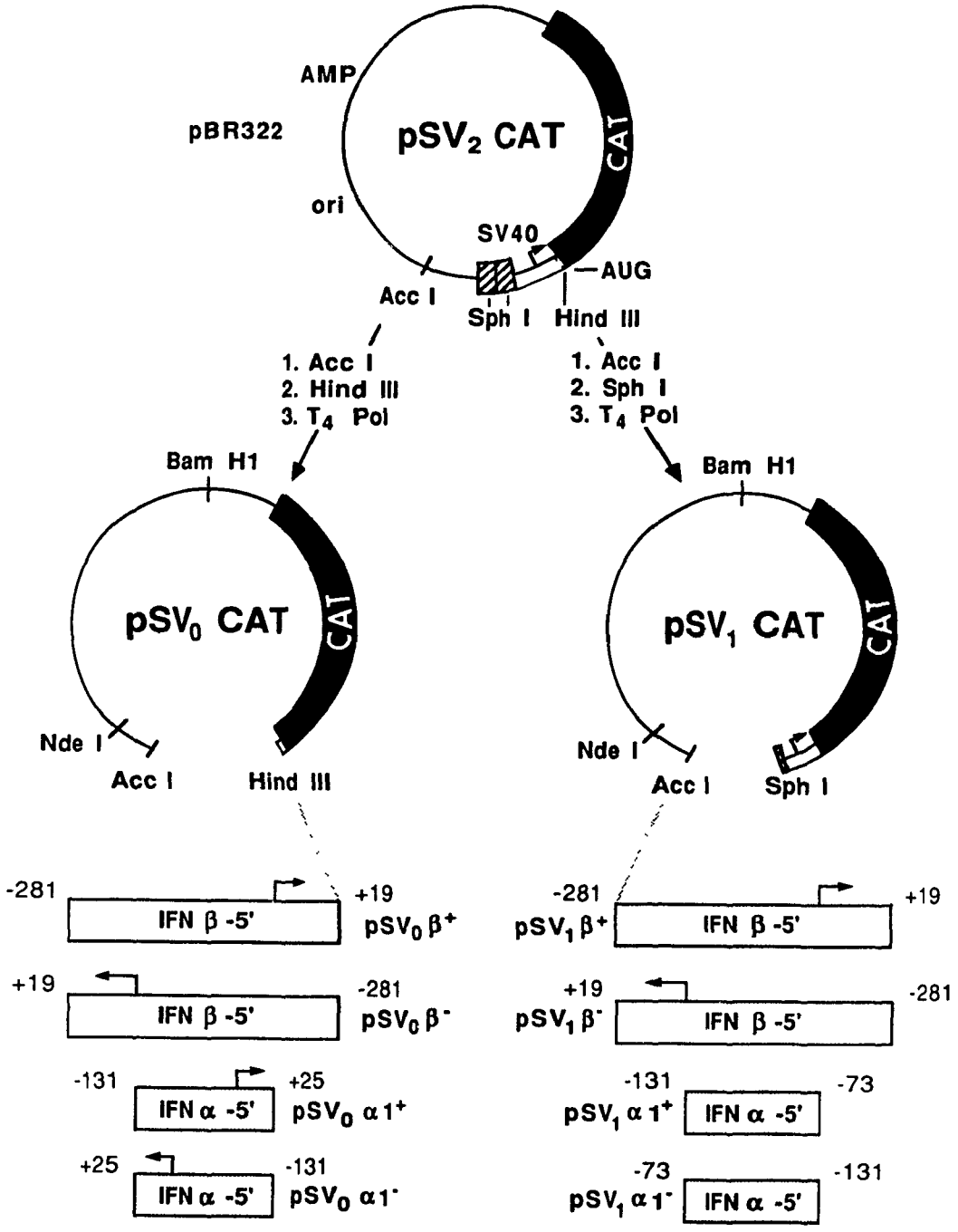
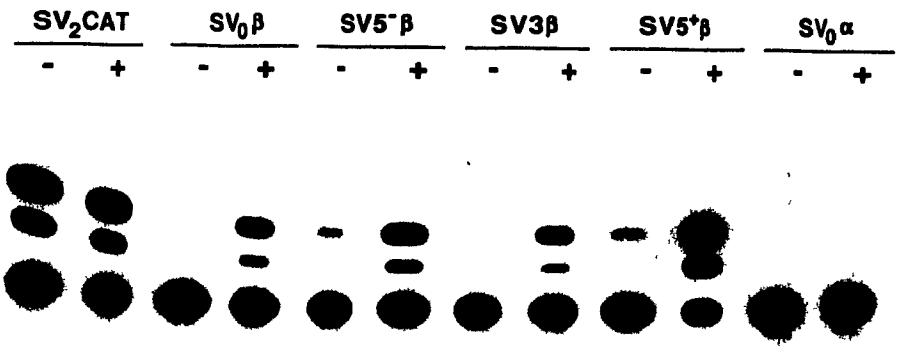
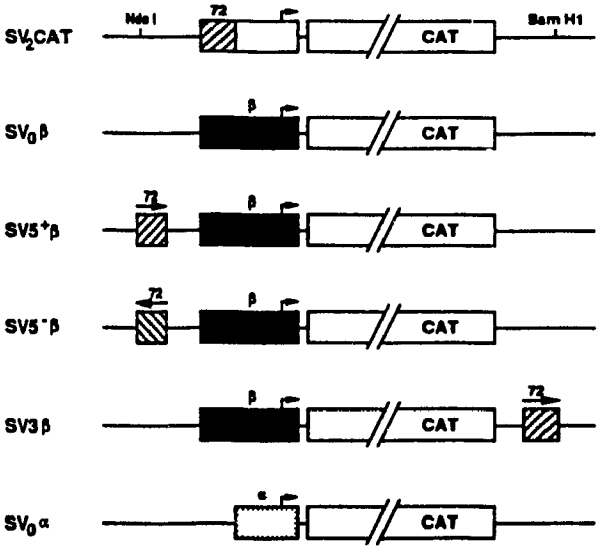

AMP
pBR322
pSV2 CAT
CAT
ori
SV40
AUG
Acc I
Sph I
Hind III
1. Acc I
2. Hind III
3. T4 Pol
1. Acc I
2. Sph I
3. T4 Pol
Bam H1
pSV0 CAT
CAT
Nde I
Acc I
Hind III
Bam H1
pSV1 CAT
CAT
Nde I
Acc I
Sph I
-281
IFN β -5'
+19
pSV0 β+
+19
IFN β -5'
-281
pSV0 β-
-131
IFN α -5'
+25
pSV0 α1+
+25
IFN α -5'
-131
pSV0 α1-
-281
pSV1 β+
IFN β -5'
+19
+19
pSV1 β-
IFN β -5'
-281
-131
pSV1 α1+
IFN α -5'
-73
-73
pSV1 α1-
IFN α -5'
-131

Figure 3. Inducible Expression of IFN-CAT Hybrid Genes in 293 Cells and the Influence of the SV40 Enhancer. The illustrated IFN-CAT hybrid genes were transfected into subconfluent 293 cells using the calcium phosphate method (10) and 20 hours after transfection were either mock induced or infected with Sendai virus for 90 minutes. Cell lysates were prepared 20 hours later and 170 μg of protein (Biorad protein assay) was analyzed in a 60 minute CAT enzyme assay (9). The acetylated products of chloramphenicol were separated by thin layer chromatography and visualized by autoradiography. The percent conversion was measured by counting the total radioactivity in 1- and 3-acetylchloramphenicol and dividing by the total radioactivity in the lane. Relative induction was determined by dividing percent conversion in the induced sample by that in the mock sample. Samples were analyzed in duplicate in two to four separate experiments. The schematic diagram illustrates the plasmids used in the experiment and the adjacent table summarizes the percent conversion and relative induction. -, mock induced samples; +, Sendai-virus induced samples. The SV40 233 bp enhancer is indicated by the hatched box and arrows indicate its relative orientation. The IFN-ß and IFN-α1 promoter fragments are identical to those of Figure 2.



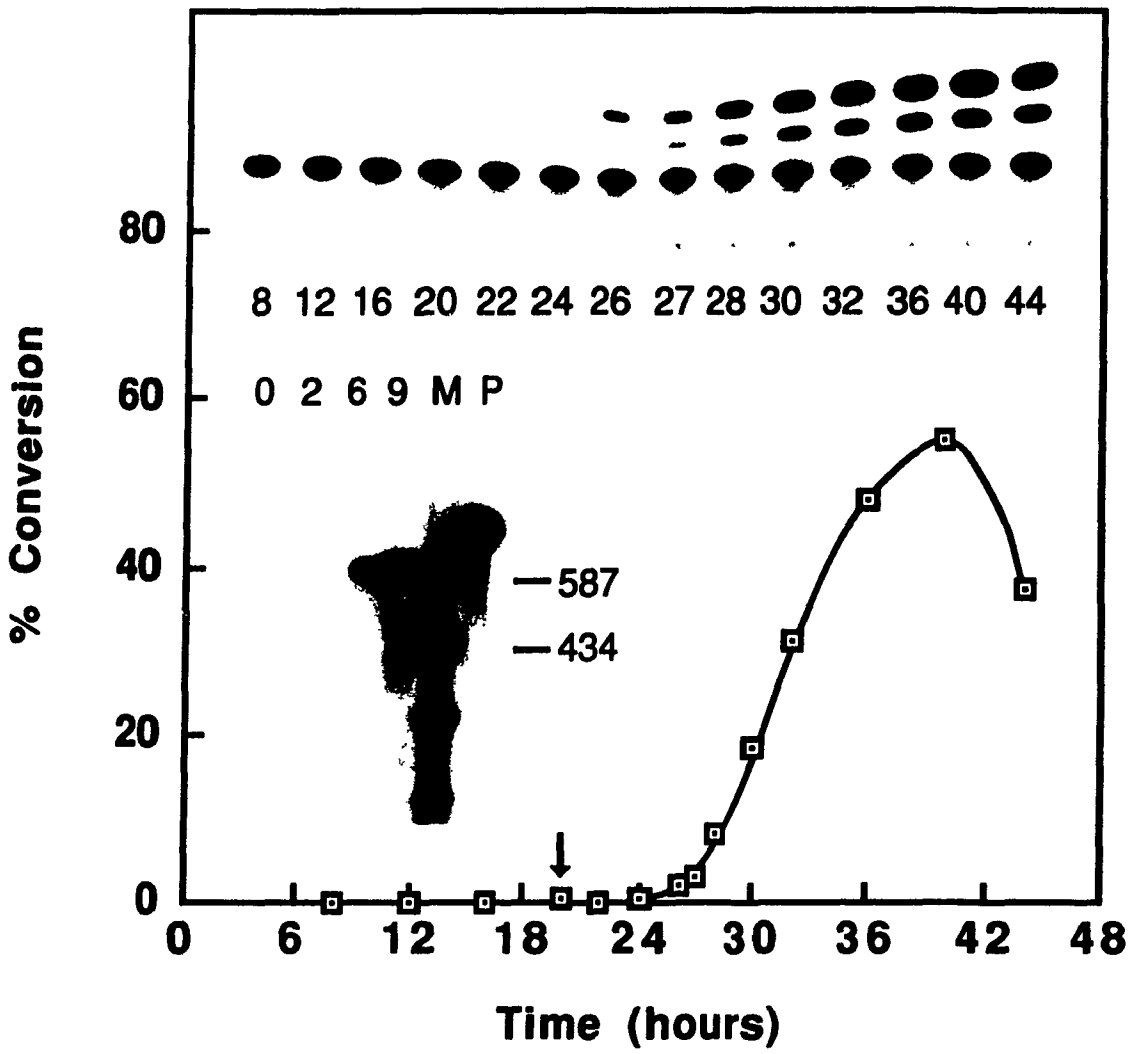


	% CONVERSION -	% CONVERSION +	RELATIVE INDUCTION
SV_2CAT	29.4	28.1	0.96
$SV_0\beta$	0.12	5.0	41.7
$SV5^+\beta$	0.7	66.5	95.0
$SV5^-\beta$	0.9	11.4	12.7
$SV3\beta$	0.3	3.5	11.7
$SV_0\alpha$	0.08	0.07	0.88

Kinetics of SV5ß Inducibility. The kinetics of expression of the IFN-ß promoter linked to the SV40 enhancer was examined in 293 cells relative to the endogenous IFN-ß promoter. Cells were transfected with SV5^{+}ß hybrid plasmid, infected with Sendai virus 20 hours later, and assessed for accumulation of CAT activity at different time periods after induction (Fig. 4). By 6 hours after virus infection CAT enzyme began accumulating, and the amount steadily increased during the next 14 hours. The drop at 44 hours may be due to degradation of accumulated CAT enzyme as a result of virus infection. Endogenous IFN-ß mRNA was not measurable at 2 hours after induction, but was identified by S1 mapping at 6 and 9 hours (Figure 4, inset). Analysis of transcripts synthesized from the SV5^{+}ß plasmid demonstrated that before virus induction, mRNA was initiated downstream of the SV40 promoter fragment, which includes the enhancer and Spl binding sites; after induction however, transcripts which initiated at the authentic IFN-ß CAP site were also identified (S.X. and D.A., unpublished). CAT activity in SV_2CAT transfected cells was first detectable at 8 hours after transfection, accumulated in a linear manner, and was not affected by Sendai induction (data not shown), as expected for a constitutive enhancer-promoter combination. Therefore, the IFNß-CAT construct was expressed with kinetics similar to that of the endogenous human IFN-ß gene in 293 cells, and once the promoter was activated, CAT activity accumulated for about 14 hours.

Transfected IFN-α1 Promoter is not Expressed in the Transient Assay. No detectable CAT enzyme activity was observed in $SV_0\alpha$ transfected 293 lysates, either before or after virus induction (Fig. 3). The influence of the SV40 enhancer upon the inducibility of the IFN-α1 regulatory element was examined relative to similar IFNß-CAT constructs in 293 cells by subcloning

Figure 4. Kinetics of Activation of the IFN-ß-CAT Hybrid Gene. The SV5^{+}ß plasmid was transfected into 293 cells and analyzed for the accumulation of CAT activity at various times after transfection and following virus infection. The induction of the endogenous IFN-ß gene (shown in the inset) was analyzed by S1 mapping of total RNA (100 µg) using the ^{32}P end-labeled probe described in Fig. 1. The arrow indicates the time of Sendai virus infection (hours after transfection).

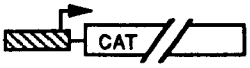
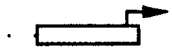
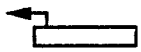
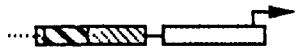
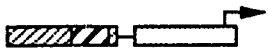
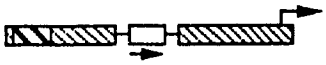
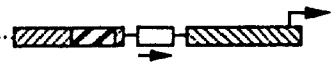

80
60
40
20
0
% Conversion
8 12 16 20 22 24 26 27 28 30 32 36 40 44
0 2 6 9 M P
587
434
0 6 12 18 24 30 36 42 48
Time (hours)

the SV40 enhancer adjacent to the $SV_0\alpha$-CAT and $SV_1\alpha$-CAT hybrid genes (Fig. 5). After induction, the $SV5^+ß$ plasmid converted about 5 times more chloramphenicol than SV_2CAT and produced a 54-fold relative induction; $SV_0ß$-CAT displayed 57-fold relative inducibility. Curiously, the construct with the IFN-ß promoter in the opposite orientation was also 16-fold inducible, although the absolute conversion was about 4-fold lower than SV_2CAT. It has not been determined what comprises the transcriptional start site of this plasmid.

The juxtaposition of the SV40 enhancer fragment and the IFN-α regulatory element produced some inducible CAT activity with the $SV5^+\alpha^+$ and $SV5^-\alpha^+$ plasmids (4.5 and 7.0, respectively), but the percent conversions were 4-5 times lower than SV_2CAT. Despite the low level of inducible IFN-α expression, these results indicate that the SV40 enhancer can complement the IFN-α element to overcome the block to transcriptional activation in 293 cells.

The choice of 293 cells in the development of a human transient system to analyze the inducible expression of the interferon promoter provides the advantage that transfected DNA is unusually stable in these cells (1) and that IFN-ß is the predominant species synthesized after virus induction. Analysis of IFNß-CAT promoter activation in 293 cells indicates that the transfected gene is expressed with kinetics similar to those for the endogenous IFN-ß gene. Nonetheless, since 293 cells are transformed by adenoviral DNA and produce Ela proteins, it is necessary to consider interference with transfected gene expression by Ela; transfection studies, for example, have demonstrated that transcription from the SV40 early promoter is repressed by adenovirus Ela proteins (17,19,30). In contrast, with this strain of 293 cells it has been found that SV40 T antigen is efficiently expressed as determined by indirect

Figure 5. Comparison of IFN-ß and IFN-α1 Inducible Expression in 293 Cells. The plasmids illustrated on the left side of the Figure were transfected into 293 cells, and Sendai inducible CAT activity was analyzed as described in Figure 3; the results are expressed as percent conversion and relative inducibility of induced and uninduced duplicate samples. The arrows above the boxes indicate transcription initiation sites. The dark box represents the IFN-ß regulatory region; the stippled box represents IFN-α1 promoter sequences; the hatched box represents the SV40 Nco I-Pvu II 233 bp fragment containing the enhancer element (broad stripe). The relative orientation of SV40 enhancer is indicated by the location of enhancer box; the IFN-α1 enhancer element (-131 to -73) and orientation is indicated by the stippled box and the arrow beneath the box.

Plasmid		% Conversion Uninduced	% Conversion Induced	Relative Inducibility
SV_2CAT		5.7	6.2	1.1
SV_1CAT		0.2	0.3	1.5
SV_0CAT		0.1	0.1	1.0
$SV5^+\beta^+$		0.6	32.4	54.0
$SV_0\beta^+$		0.2	11.4	57.0
$SV_0\beta^-$		0.1	1.6	16.0
$SV_1\beta^+$		0.2	1.0	5.0
$SV_1\beta^-$		0.2	0.3	1.5
$SV_0\alpha^+$		0.1	0.1	1.0
$SV_0\alpha^-$		0.1	0.1	1.0
$SV5^+\alpha^+$		0.2	0.9	4.5
$SV5^-\alpha^+$		0.2	1.4	7.0
$SV_1^+\alpha^+$		0.1	0.1	1.0
$SV_1^+\alpha^-$		0.1	0.1	1.0
$SV_15^+\alpha^+$		0.2	0.5	2.5
$SV_15^-\alpha^+$		0.2	0.7	3.5

immunofluorescence, indicating that repression of the SV40 early promoter may not be absolute (J.Hiscott, unpublished data). The combined negative regulation imposed by the IFN-ß promoter (7), together with repression of the SV40 enhancer in the uninduced state, may contribute to the low background activity and high inducibility of the SV40-IFNß enhancer-promoter combination.

To augment the expression of IFN-CAT hybrid genes, the SV40 enhancer was juxtaposed with the IFN-ß promoter and, in effect, the influence of a strong constitutive enhancer upon an inducible enhancer was assessed by these experiments. The evidence presented here supports the idea that negative regulation imposed by the IFN-ß promoter in the uninduced state is dominant over the SV40 constitutive enhancing influence; nonetheless the SV40 enhancer was able to modestly increase the basal uninduced level of expression from the IFN-ß promoter (2-10 fold). After induction by virus, the presence of the SV40 enhancer increased the absolute and relative induction of CAT expression, but in an orientation and position dependent manner.

Human 293 cells contain the necessary trans-acting factors to produce inducer specific activation of the IFN-ß gene; however the lack of expression of the IFN-α-CAT hybrid gene indicates that a distinct factor(s) not present in 293 cells is required for activation of a myeloid cell specific IFN-α1 promoter. The IFN-α1 promoter contains all the sequence requirements for regulated expression in mouse fibroblasts (20,26,28) and yet is not active extrachromosomally in 293 cells. The endogenous IFN-α promoter is also not activated by Sendai virus induction in these cells, reflecting a fundamental difference between the human and murine systems, in which both IFN-α1 and IFN-ß are transcriptionally activated by virus induction. A human transient expression system will provide a convenient means to examine *in*

vivo interactions between common regulatory factors required for transcriptional selectivity, distinct IFN-binding proteins, and the DNA sequences involved in inducible IFN gene expression.

CHAPTER 3

MODULATION OF INTERFERON GENE TRANSCRIPTION BY POSITIVE AND NEGATIVE CELLULAR FACTORS

PREFACE

The control of eukaryotic promoters is dependent on interactions between trans-acting proteins and cis-acting regulatory sequences. In the case of the IFN-ß gene positive and/or negative DNA-binding factors may contribute to inducibility of the promoter. The experiments in the following chapter have relied on the use of the transient expression system (chapter 2) to functionally characterize transcription factors involved in IFN-ß gene induction. To this end, a series of *in vivo* competition experiments was conducted in which an IFN-ß test gene was cotransfected into 293 cells, together with an excess of different competitor plasmids harboring homologous or heterologous promoter sequences. We reasoned that competitive depletion of the required transcripton factors might result in either increased or decreased transcription of the promoter, depending on whether they were acting in a positive or negative manner. By using different IFN-ß promoter fragments as competitor templates, and also sequences corresponding to other eukaryotic promoters, we sought to localize the site of interaction and determine its specificity.

ABSTRACT

In vivo competition experiments were designed to identify the role of trans-acting cellular factors in the virus-inducible activation of the interferon-ß promoter. Co-transfection of a constant amount of IFN-ß/CAT test gene and increasing amounts of competitor DNA containing different IFN regulatory domains into human epithelioid 293 cells identified a low abundance, positive cellular factor(s) that interacts with the IFN regulatory region. Depletion of the factor decreases virus-induced and constitutive level expression of the IFN-ß promoter, and also partially inhibits expression from the SV40 early promoter. Negative regulatory effects produced by factors interacting with the IFN upstream region (-135 to -202) and with the SV40 enhancer were also observed.

INTRODUCTION

Regulation of eukaryotic transcription is mediated by complex interactions between promoter sequences and cellular factors (1,2). Activation of the human Type 1 interferon genes (IFN-α and IFN-ß) provides a relevant model to examine cell specific, inducible gene expression since multiple IFN genes can be activated in response to a variety of natural and synthetic agents, including viruses, synthetic polyribonucleotides and foreign antigens (3,4,5). In general, peripheral blood leukocytes and leukemic cell lines synthesize a mixture of alpha interferons (IFN-α) that are encoded by a multigene family, while fibroblasts and epithelial cells produce predominantly IFN-ß, which is derived from a unique gene (6,7). Type 1 IFN gene activation is controlled by inducible enhancer elements, which contain overlapping positive and negative regulatory domains (8-12). Because the induction of IFN expression is not blocked by protein synthesis inhibitors, it is thought that factors required for IFN induction pre-exist in the cell and are post-translationally modified as a consequence of the induction phenomenon (13). To characterize further the requirements for interferon gene expression, *in vivo* experiments were designed to identify the role of putative trans-acting cellular factors in IFN-ß gene activation. We asked whether negative or positive regulatory factors interacted with the IFN-ß regulatory element (IRE), and if these factors were common to other constitutive or inducible enhancer domains. Our experiments demonstrate that the rate limiting proteins for IFN-ß expression *in vivo* are positive acting factors; negative regulation is imposed by the upstream (-100 to -200) region.

MATERIALS AND METHODS

Plasmids. The isolation of different DNA fragments used in this study and the construction of IFN/CAT hybrid plasmids have been described in detail (14,15).

Transfections and Virus Induction. Human 293 cells were originally derived by transformation with adenovirus DNA. Cells (2-4 x 10^6 cells/100mm dish) were transfected by the calcium phosphate procedure (16) with a total of 30 μg of plasmid DNA: 2 μg of test gene, a variable amount of specific competitor DNA (indicated in the experiment), and non-specific pUC8 plasmid DNA, added to a final of 30 μg total DNA. After 4-6 hours, the transfection medium was removed and the cells were incubated in RPMI 1640 containing 5% fetal calf serum (FCS). At 24 hours post-transfection, the cells were induced with Sendai virus (500 HAU/ml) for 2 hours; cell monolayers were then rinsed with PBS, and incubated at 37°C with RPMI 1640 containing 5% FCS. The cells were analyzed for CAT enzyme activity 18-20 hours after virus induction.

CAT Assays. Analysis of chloramphenicol acetyltransferase activity was performed at 42-44 hours after transfection according to the procedure of Gorman *et al* (17). A constant amount of protein from different samples was used in each analysis, as determined beforehand by the Bio-Rad protein assay. The percent conversion of [^{14}C]-chloramphenicol to acetylated chloramphenicol was quantified by scintillation counting in Aquasol (New England Nuclear).

RESULTS AND DISCUSSION

A human transient expression system has been described (14) that permits accurate, virus-inducible expression of the IFN-ß regulatory element linked to the chloramphenicol acetyltransferase (CAT) reporter gene. Using the transient expression system, *in vivo* competition experiments were designed to identify positive or negative trans-acting interactions important for IFN-ß gene activation. A constant amount of test gene template (SV5ß) was transfected into the human epithelioid 293 cell line together with increasing amounts of competitor DNA containing different IFN-ß regulatory domains (Figure 1). The schematic diagram illustrates the structure of SV5ß and the ß163 competitor plasmid, containing the Dra I-Ava II fragment derived from the IFN-ß 5'-flanking region (-202 to -40, relative to the mRNA CAP site) subcloned into the Sma I site of plasmid pUC8. The ß163 fragment contains the IFN-ß regulatory element (IRE, -77 to -37) required for virus inducible transcription, but does not span the TATA region or mRNA CAP site (8,9). If limiting concentrations of cellular factors required for IFN-ß gene expression are present in transfected cells, then increasing amounts of a competitor plasmid capable of binding trans-acting factors should modulate reporter gene activity. Removal of negative regulatory factors involved in repression of the IFN-ß gene would result in higher CAT levels in uninduced cells, while depletion of positive acting proteins would yield decreased CAT levels in virus induced-cells.

The results of *in vivo* competition between the SV5ß test gene and the ß163 competing plasmid are illustrated in Figure 2. In the absence of virus induction the IFN-ß/CAT hybrids were expressed at low constitutive levels (0.4% conversion), while after virus infection, a 50-100 fold relative induction of CAT levels was detected in 293 cells (14). When co-transfection was

Figure 1. Schematic Outline of the In Vivo Competition Assay. A constant amount (2 μg) of the SV5ß test gene was transfected into 293 cells together with variable amounts of competitor DNA (0-30 μg) representing different domains of the IFN promoter (illustrated is the ß163 plasmid). CAT activity was determined in Sendai-induced or mock-induced transfected cells.

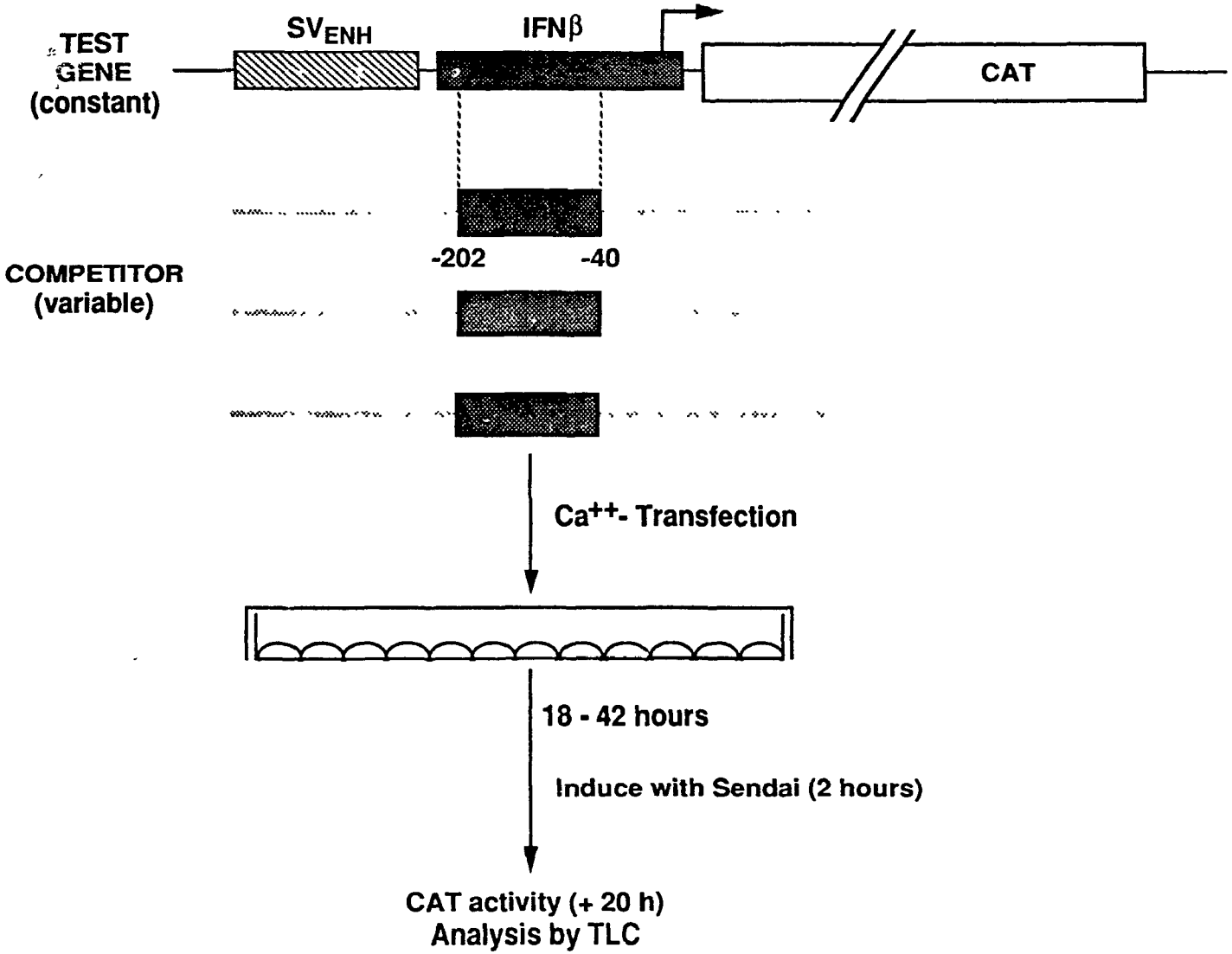
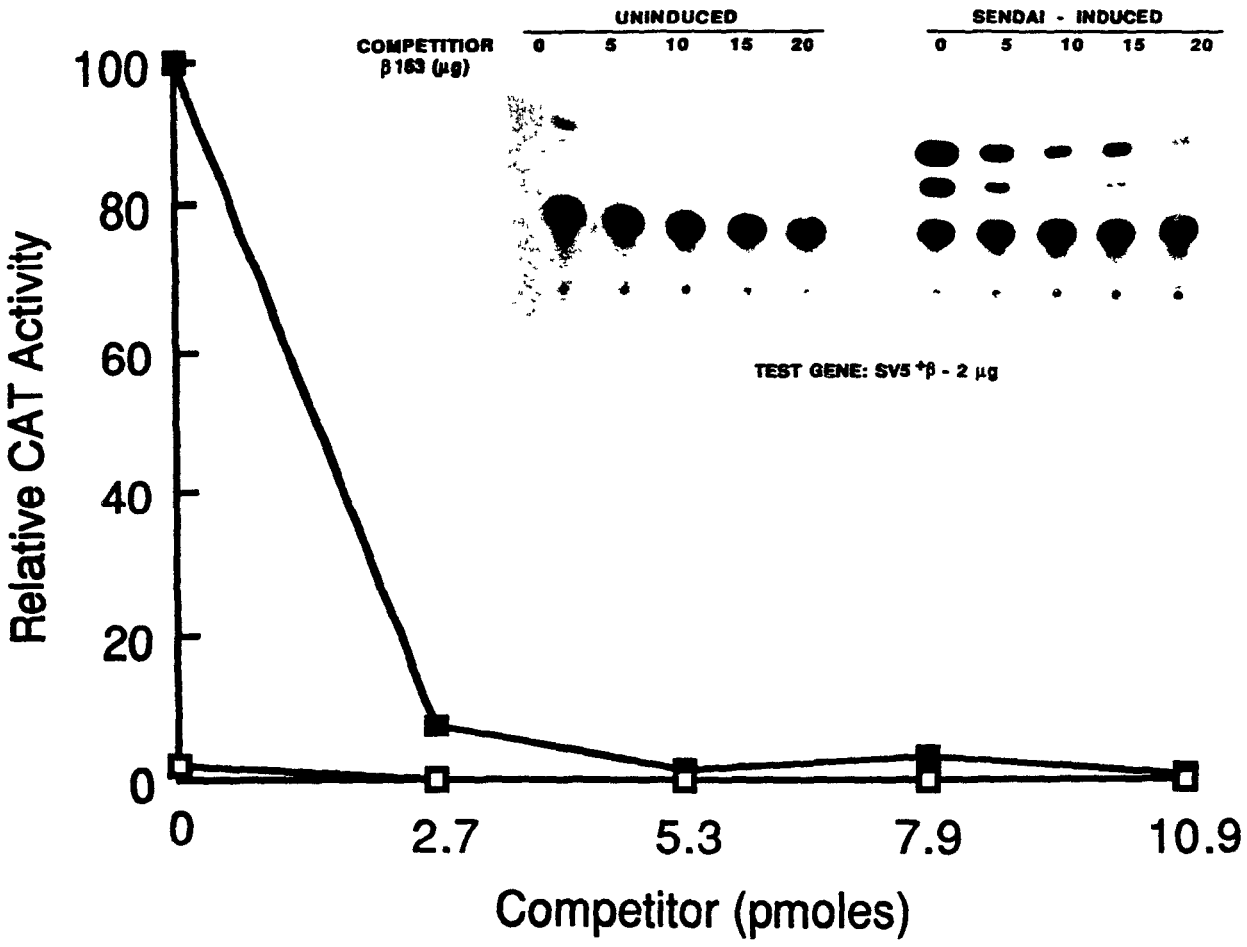

TEST GENE (constant)
SV$_{ENH}$
IFNβ
CAT
-202
-40
COMPETITOR (variable)
Ca^{++}- Transfection
18 - 42 hours
Induce with Sendai (2 hours)
CAT activity (+ 20 h)
Analysis by TLC

Figure 2. In Vivo Competition for SV5ß Expression by ß163. Cells were transfected and CAT assays performed as described in the Methods. CAT levels in uninduced (□) and Sendai virus-induced (■) cells in the absence or presence of ß163 DNA was determined and expressed as a percent of CAT activity obtained in virus-induced cells (34% conversion) in the absence of specific competitor DNA. The inset illustrates a representative determination of CAT activity and the effect of increasing amounts of competitor DNA. 1 Ac, 3 Ac - acetylated form of [^{14}C]-chloramphenicol; CAM- substrate [^{14}C]-chloramphenicol.


UNINDUCED
SENDAI - INDUCED
COMPETITOR
β163 (μg)
0 5 10 15 20
0 5 10 15 20
TEST GENE: SV5 +β - 2 μg
Relative CAT Activity
100
80
60
40
20
0
0
2.7
5.3
7.9
10.9
Competitor (pmoles)

performed with increasing amounts of ß163 plasmid, a 10 fold decrease in CAT activity was observed following virus induction when 2.7 fmoles of ß163 competitor DNA was used; a 50 fold decrease was seen with 10.9 pmoles of ß163 competitor. This experiment suggested that the ß163 fragment interacted with a low abundance, positive cellular factor(s) required for IFN-ß inducibility. In uninduced cells, competition with the ß163 fragment did not increase the basal level of SV5ß expression as would be expected if a negative regulatory protein was removed from the IFN-ß promoter (Figure 2). The low level of basal CAT activity in fact decreased to below detectable levels (0.1% conversion), suggesting that competition was depleting a positive rather than negative factor.

The capacity of other DNA domains to modulate test gene expression *in vivo* was also investigated (Figure 3). Plasmids containing the SV40 enhancer (a 233 bp Nco I-Pvu II fragment), the IFN-α1 promoter element (-131 to -71), and two IFN-ß specific fragments which subdivided ß163 into distal ß67 (-202 to -135) and proximal ß96 (-135 to -40) fragments were co-transfected into 293 cells together with SV5ß. Virus-inducible CAT activity was decreased by ß163 and by ß96, both of which contain the IRE. Conversely, increasing amounts of the IFN-α1 promoter fragment and the ß67 fragment increased CAT levels 2-3 fold, implying that these fragments partially removed negative regulatory factors. The most striking effect was observed with the SV40 enhancer which increased CAT activity 5 fold when 10 µg of competitor plasmid was used.

When the SV_2CAT plasmid (14,17) was used as test gene, competition with the ß163 fragment decreased (2.5 to 5.0 fold) the relative CAT levels driven by the SV40 early promoter (Figure 4). This partial inhibition of SV40 early promoter function by ß163 suggests that a positive trans-acting factor required

Figure 3. Competition for Trans-acting Factors by Different Promoter Domains. Plasmid SV5ß was co-transfected with competitive DNA as described in the Methods. CAT activity in each sample (C) is expressed as a percent of the value obtained in virus-induced cells in the absence of specific competitor DNA (Co). Symbols for the different competitor DNAs are shown in the inset.

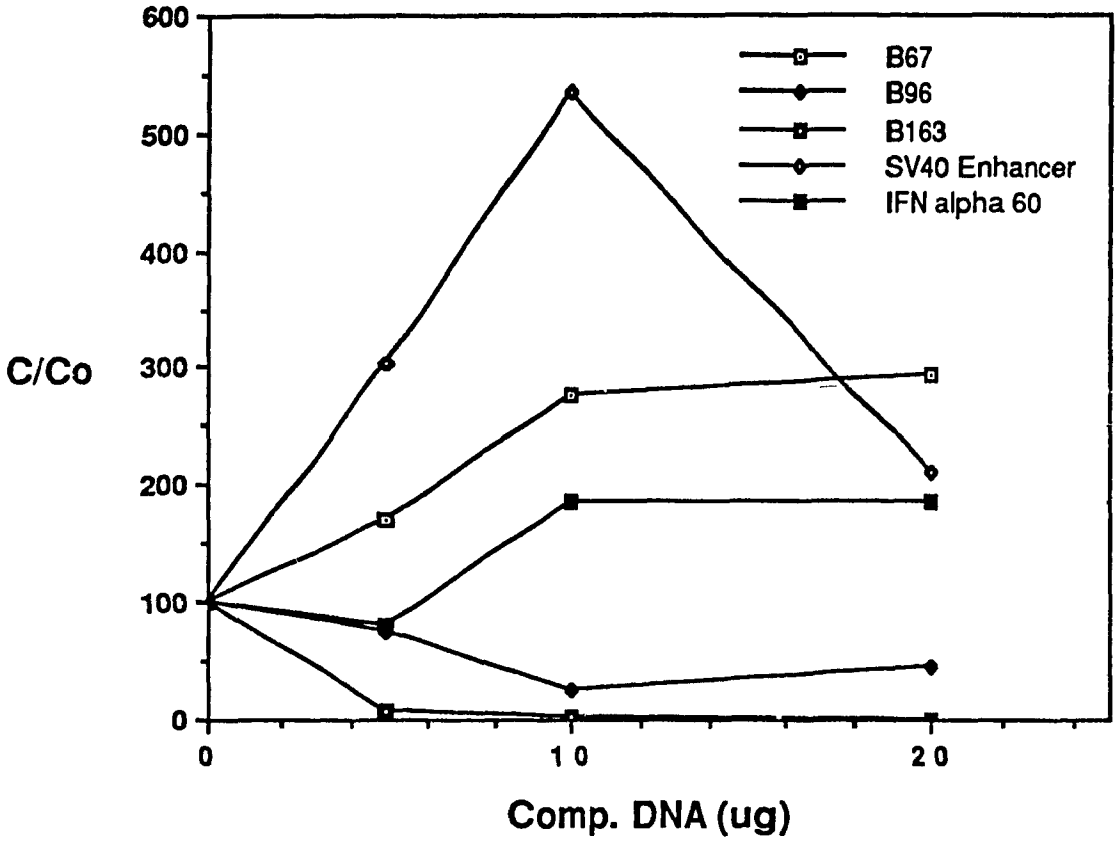
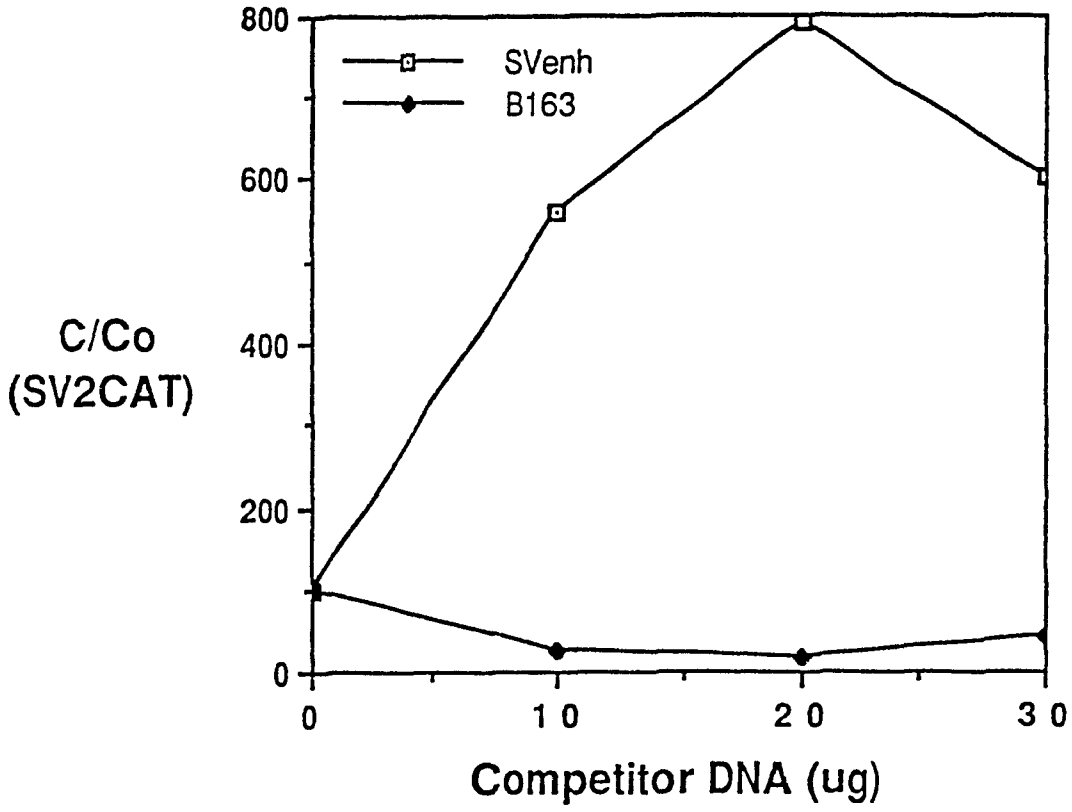

600
500
400
300
200
100
0
C/Co
0
10
20
Comp. DNA (ug)
B67
B96
B163
SV40 Enhancer
IFN alpha 60

Figure 4. In Vivo Competition for SV2CAT Expression by ß163 or the SV40 Enhancer. Plasmid SV_2CAT (2 μg) was co-transfected with variable amounts of plasmid containing ß163 or the SV40 enhancer fragment. At 48 hours after transfection, CAT activity was determined. The value C/Co is described in Figure 3.


800
600
400
200
0
SVenh
B163
C/Co
(SV2CAT)
0
10
20
30
Competitor DNA (ug)

for SV40 early promoter activity may interact with the IFN-ß regulatory domain. In contrast, competition using increasing amounts of the SV40 enhancer fragment augmented CAT activity from the SV40 promoter by 5 to 7 fold. Since it has previously been shown in 293 cells that adenovirus Ela gene products repress the SV40 enhancer (18,19), these experiments demonstrate that repression of the SV40 early by Ela is reversed by competitive depletion of cellular factors interacting with the SV40 enhancer.

These studies have indicated the presence of a low abundance, positive cellular factor(s) that interacts with the IFN-ß regulatory region. Competition of the factor decreases virus-induced and constitutive level expression of the IFN-ß promoter, and also partially inhibits expression of the SV40 promoter. This human protein may be similar to a simian factor identified in CV-1 cells (20). The *in vivo* competition results correlate well with results obtained *in vitro* using gel retardation assays which indicate that a 10-100 fold molar excess of competitor DNA corresponding to -79 to -64 of the IFN-ß promoter removes a factor that binds to the core of the IFN-ß enhancer (data not shown). Co-transfection with the IFN-ß upstream region (-135 to -202) and the IFN-α1 promoter (-131 to -71) produced a 2-3 fold increase in test gene expression, supporting the idea that a negative cis-acting element is present immediately upstream of the virus-inducible enhancer (15,21,22). Sequence homologies between the IRE and other cellular and viral enhancers (9) imply that common trans-acting factors may interact with multiple eukaryotic enhancer motifs. Transcriptional control would thus be specified by distinct combinations of ubiquitous trans-acting factors with unique tissue-specific or inducible proteins.

CHAPTER 4

IDENTIFICATION OF A NUCLEAR DNA-BINDING PROTEIN ASSOCIATED WITH THE INTERFERON-ß UPSTREAM REGULATORY REGION

PREFACE

The *in vivo* competition studies described in chapter 3 suggest that both positive and negative regulatory factors interact with the IFN-ß promoter. In order to characterize the biochemical requirements for IFN-ß regulation more directly, experiments were undertaken to probe nuclear extracts for the presence of sequence-specific DNA-binding proteins recognizing the IFN-ß regulatory region. Protein-DNA interactions were examined using an electrophoretic mobility shift assay. This procedure is based on the observation that soluble transcription factors can form stable complexes with radiolabeled DNA fragments in solution. The complexed and unbound forms of the probe DNA are readily distinguished after resolution on native polyacrylamide gels. As with the *in vivo* competition experiments, protein-DNA interactions can be inhibited and further localized by including in the reaction mixture, a molar excess of unlabeled competitor DNAs specifying different promoter domains. Competition experiments of this nature are also useful in determining the specificity of the interaction with respect to both the probe DNA and the competitor template.

ABSTRACT

Nuclear protein extracts were prepared from uninduced myeloid leukemic KG-1 cells and analyzed for interferon-specific DNA-binding by a gel electrophoresis DNA-binding assay. A protein was detected that bound specifically to a 163 base pair upstream region of the IFN-ß promoter. A series of competition studies were performed to assess whether this was a general DNA-binding protein; binding of this factor to a radiolabeled ß163 fragment was only diminished by an excess of cold unlabeled ß163 or a 67 base pair fragment (ß67) derived from ß163. Other promoter and enhancer transcriptional domains including the 1) c-fos serum responsive element, 2) c-fos promoter, 3) SV40 enhancer, 4) IFN-αi promoter, and 5) plasmid pAT153 did not compete effectively for binding of the protein. These results indicate that this protein is not a general enhancer- or promoter-binding factor and may be unique to IFN-ß. Analysis of ß67 DNA sequences revealed no homology to known recognition sequences for the Spl or CTF transcription factors.

INTRODUCTION

The interferons constitute a group of unique inducible cellular proteins primarily involved in cellular defense against viral infection (1,2). The human interferon family can be subdivided into three antigenically distinct classes termed α, ß, and γ. IFN-α is represented by a multigene family composed of at least twenty nonallelic members, whereas IFN-ß and IFN-α are encoded by single genes (3-6). The type and relative quantity of interferon produced is cell-specific and is dependent on the nature of the inducing stimulus (7-10).

Although quiescent under normal physiological conditions, interferon genes are transcriptionally activated and transiently expressed following induction. The cis-acting DNA sequence elements responsible for transcriptional activation in mouse fibroblasts, as defined by *in vitro* mutagenesis (11-15), include a short motif located -77 to -37 relative to the mRNA start site which imparts viral inducibility upon the natural or a heterologous promoter in mouse C127 cells (15); a related study using L929 cells has defined the IFN-ß inducibility sequences to the region -115 relative to the CAP site (13). These DNA regions define an inducible enhancer element that is negatively regulated in the uninduced state (15,16); *i.e.* transcription is repressed in the uninduced state and the block to transcription must be relieved before gene expression occurs.

Less well characterized is the IFN-ß region located upstream of -100 relative to the mRNA CAP site. In C127 cells removal of this DNA segment increases basal IFN-ß transcription in the uninduced state (11). Thus, the DNA sequences in the IFN-ß -100 to -200 region also appear to function in negative regulation.

In order to understand the molecular mechanisms that control IFN-ß transcription, the genetic information gained through deletion analysis must be supplemented by studies that address the trans-acting biochemical requirements for induction. Recent studies have described detailed interactions of sequence-specific DNA-binding proteins with the 5' regulatory elements of several genes including the SV40 early gene, the herpes simplex thymidine kinase gene, and the inducible heat-shock gene (HSP70) (17-21). In this study, we have examined the possibility that cellular factors might interact with the upstream sequences of the IFN-ß gene to mediate transcriptional repression. Nuclear protein extracts were prepared from the human myeloid leukemic KG-1 cell line (22), and analyzed for the presence of proteins with specificity for several eukaryotic promoters. Using a gel electrophoresis DNA-binding assay, we have identified a nuclear protein which can selectively bind to an upstream region of the IFN-ß gene.

METHODS AND MATERIALS

Cells and Cell Culture: The established myeloid leukemic KG-1 cell line, derived from a patient with erythroleukemia, was grown at 37°C in mass spinner suspension (4 liters) to a final density of 10^6 cells/ml. Cultures were gradually scaled up with a 1:2 split and fed with RPMI-1640 supplemented with 5-10% fetal bovine calf serum, glutamine, and antibiotics. The cells were harvested by low speed centrifugation (1200 rpm) in an IEC centrifuge and the pellet washed twice with phosphate-buffered saline. A final packed cell volume of approximately 2-3 ml was used for preparing nuclear extracts immediately after harvesting, or was frozen in liquid N_2 and stored at -70°C until further use.

Preparation of Nuclear Extracts: Nuclear extracts were prepared according to the method of Dignam *et al.* (23) with a few modifications. The cell homogenate containing intact nuclei was layered over a 3 ml sucrose cushion containing 2 M sucrose, 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol. Nuclei were pelleted by centrifugation in a Sorval HB-4 rotor at 10,000 rpm for 60 min at 4°C and then harvested. When required, nuclear extracts were concentrated using a Minicon B-15 concentrator (Amicon). All manipulations were performed in a 4°C cold room and whenever possible on ice. The extract was aliquoted, frozen in liquid N_2 or a dry ice-ethanol bath, and stored at -70°C.

Preparations of Probes

IFN-ß Fragments. The human 5'-flanking 300-base pair (bp) EcoRI-TaqI frament was cleaved from plasmid pUCß, digested with AvaII and DraI to yield a 163 bp promoterless DraI-AvaII fragment, treated with T_4 polymerase

to yield blunt-end DNA fragments, subcloned into the SmaI site of pUC8 and designated pUCß163. The intact IFN-ß fragment was retrieved by digestion of the flanking pUC8 polylinker sequences with EcoRI and BamHI, and then further cleaved by AluI to generate two subfragments containing 67 bp (ß67) and 96 bp (ß96) of IFN-ß DNA. To avoid confusion about the precise 5' and 3' boundaries of the different IFN-ß fragments with respect to the restriction map of the endogenous gene, the linker-containing fragments are referred to as ß163 (DraI-AvaII), ß67 (DraI-AluI), and ß96 (AluI-AvaII) in the text.

IFN-α1. The 5'-flanking 65 bp BamHI-Sau96I fragment of the human IFN-α1 gene was isolated from plasmid IM1105 (30).

c-FOS Fragments. The 373 bp PstI fragment of the human c-fos 5'-flanking region was cleaved with ApaI to generate FOS-138, a PstI-ApaI subfragment containing the serum-responsive elements, and FOS-PRO-235, an ApaI-PstI subfragment containing the c-fos promoter.

SVENH233. The 233 bp NcoI-PvuII enhancer fragment was cleaved from plasmid pSV_2CAT and subcloned into the pUC8 SmaI site to generate plasmid pUCSV.

pAT145. The pBR322 145 bp HinfI fragment was isolated from plasmid pAT153, a derivative of plasmid pBR322 containing a deletion of the nucleotide sequences between 1644 and 2349. In all subcloning experiments blunt ends were generated by treating the DNA with T4 polymerase (Pharmacia P-L Biochemicals).

Probes were prepared by treatment of the various DNA fragments with calf intestinal alkaline phosphatase and then 5'-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Gel Electrophoresis Assay: A constant amount of [γ-^{32}P]ATP end-labeled DNA probe (0.66 ng) was combined with a large excess (6 μg) of the alternating copolymer, poly(dI-dC)•(dI-dC), (Pharmacia P-L Biochemicals) and varying amounts of KG-1 nuclear extract (as described in the figures). All reactions were brought to a final volume of 10-20 μl with nuclear extract buffer D (23) and incubated at 30°C for 30 min. The assay parameters for band competition were identical except that the amount of KG-1 nuclear extract was held constant (9.2 μg) and increasing amounts of various competitor fragments (0,2,5,10, and 20 ng) were added to the reaction mixture. Following incubation, 1 μl of bromphenol blue dye was added to each reaction, and the samples were immediately loaded on a native 4% Tris-glycine (25 mM Tris, 195 mM glycine, pH 8.5) polyacrylamide gel. Electrophoresis was performed at room temperature at 8.3 V/cm until the bromophenol blue dye had migrated a distance of approximately 9 cm. The wet gels were blotted to Whatman 3MM chromatographic paper, dried and autoradiographed.

RESULTS AND DISCUSSION

The gel electrophoresis DNA-binding assay used in these studies is based on the observation that proteins bound to DNA fragments retard DNA migration through low ionic strength polyacrylamide gels when compared to uncomplexed DNA (24-26). The binding of nonspecific proteins to DNA is eliminated by the inclusion of a vast (about 10,000-fold) excess of the alternating copolymer poly(dI-dC)•(dI-dC) in the DNA-binding reaction. Specific end-labeled DNA fragments are used to probe the protein extract for sequence-specific binding.

In this study, the IFNß 5'-flanking DraI-AvaII 163 bp fragment was isolated from pUCß163 plasmid and cleaved to generate the DraI-AluI 67 bp fragment and the AluI-AvaII 96 bp fragment (Fig. 1). These fragments were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase and used in a band retention assay together with variable amounts of KG-1 nuclear extract, usually 0-9.2 µg protein, poly (dI-dC)•(dI-dC) as nonspecific competitor nucleic acid (6 µg), and as indicated in individual experiments, 0-20 ng of unlabeled (cold) competing DNA fragment. The radiolabeled fragment was present in the reactions at 0.66 ng. Other fragments used in these experiments included 1) pAT-145, a 145 bp Hinf I fragment from plasmid pAT153; 2) SVENH-233, the NcoI-PvuII fragment encompassing the SV40 enhancer and five G-C rich tandem repeats (27,28); 3) FOS-138, the PstI-ApaI fragment containing the human c-fos serum responsive elements (29); 4) FOS-PRO 235, the ApaI-PstI c-fos promoter fragment (29); and 5) IFN-α1, the BamH1-Sau96 IFN-α1 65 bp fragment containing sequences previously shown to confer inducibility in mouse L929 cells (30).

The ß163 and pAT-145 fragments were end-labeled and used to probe the KG-1 extract for specific DNA-binding factors (Fig. 2). In the absence of

Figure 1. Schematic of the Interferon-ß Gene and Promoter Fragments used in the Band Retention Assay. The 1.8-kilobase pair EcoR1 fragment containing the gene (solid box) for IFN-ß is shown; below the gene the mRNA transcript and protein coding region (hatched box) are illustrated. The EcoR1-Taq1 300 bp promoter fragment is expanded below the structural gene and indicates restriction enzymes used to generate fragments used in this study. The hatched and stippled boxes, together with the - and + symbols represent cis-acting DNA elements involved in IFN-ß regulation (based on refs. 11 and 16).

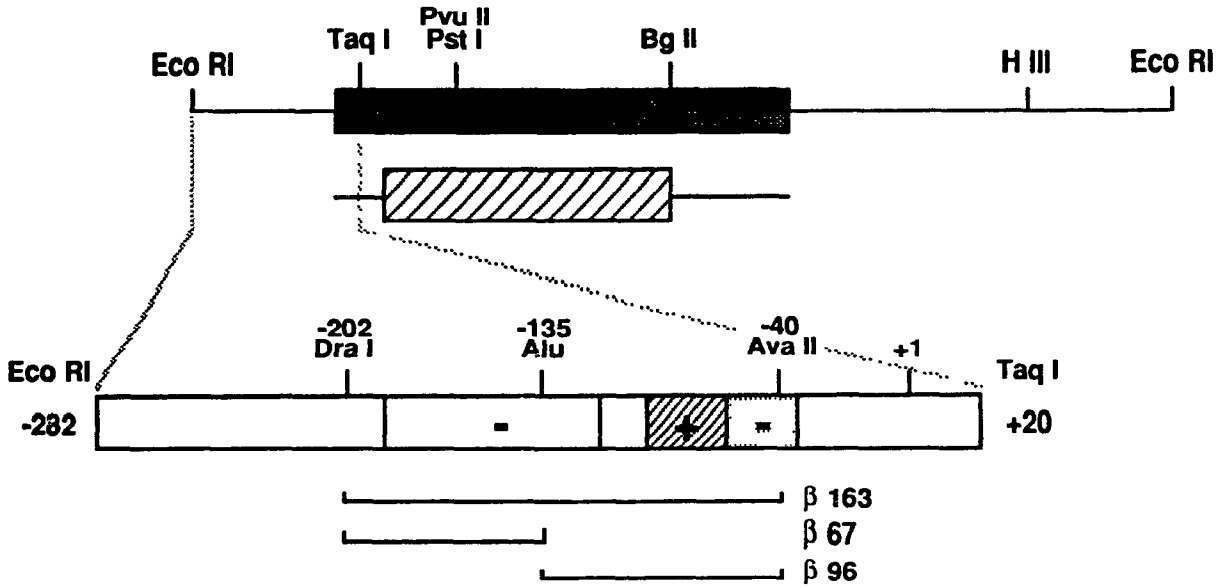
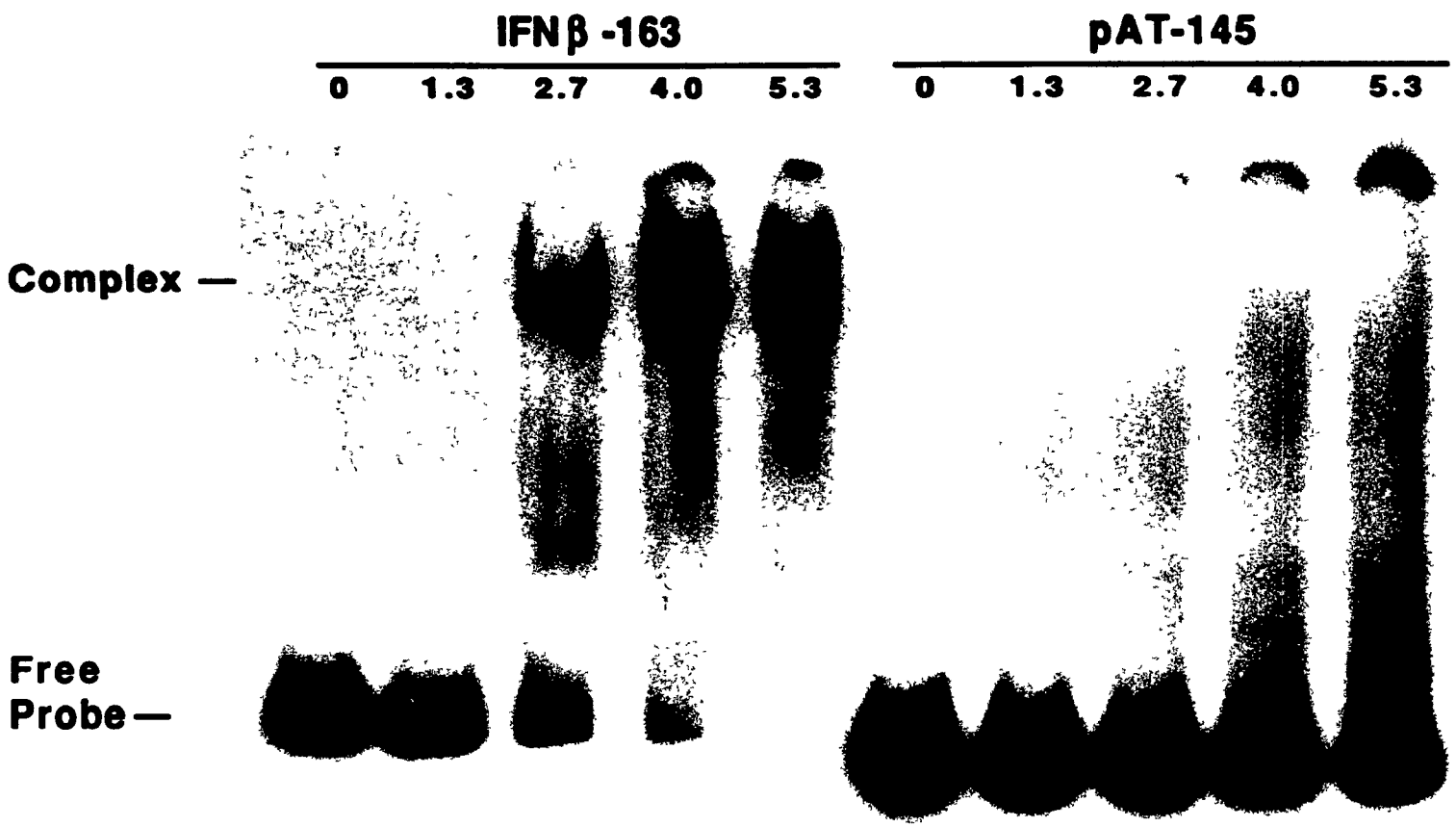

Eco RI
Taq I
Pvu II
Pst I
Bg II
H III
Eco RI
-202
Dra I
-135
Alu
-40
Ava II
+1
Eco RI
Taq I
-282
+20
β 163
β 67
β 96

Figure 2. Detection of Interferon-ß DNA-Binding Proteins in KG-1 Nuclear Extracts. The ^{32}P-labeled ß163 and pAT145 fragments (0.66 ng) were incubated with 6 µg of poly (dI-dC)•(dI-dC) and increasing amounts of nuclear extract (µg), indicated above the gel lane. Samples were electrophoresed through a low ionic strength 4% polyacrylamide gel, dried down and exposed to autoradiographic film.

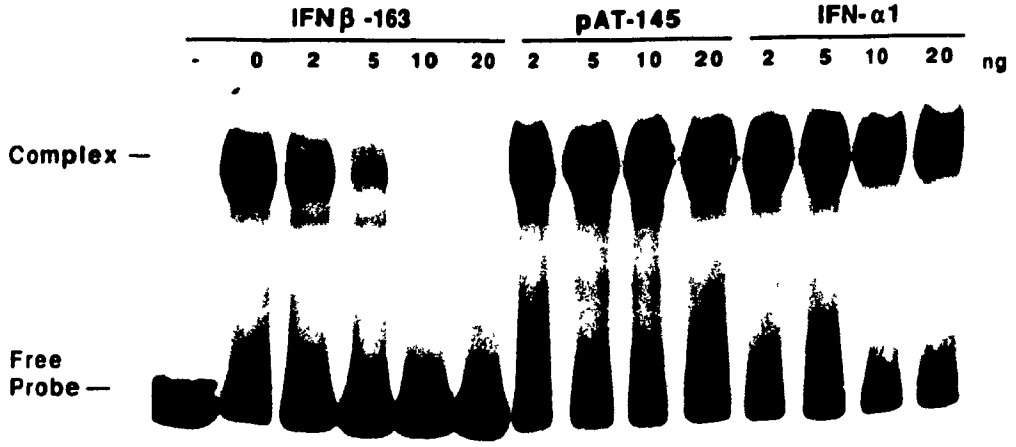

IFNβ -163
pAT-145
0
1.3
2.7
4.0
5.3
0
1.3
2.7
4.0
5.3
Complex —
Free
Probe —

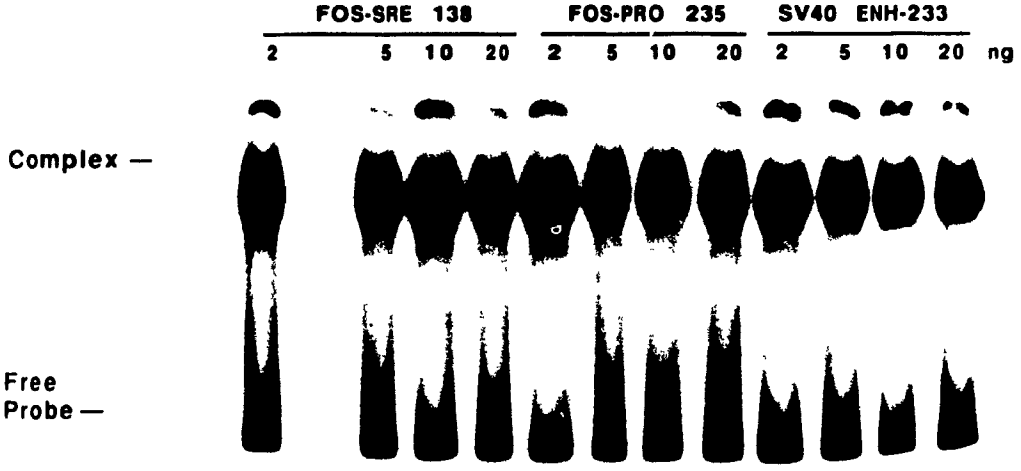
protein, the two fragments essentially co-migrated in the gel; however, with increasing protein concentration a band of lower mobility was observed with ß163 only. Treatment of the binding reactions with proteinase K caused the fragment-factor complex to co-migrate with free probe (data not shown), indicating that the complex represented protein-DNA interactions. To determine whether the protein(s) complexed with the interferon-ß promoter fragment represented a specific or nonspecific promoter binding activity, a series of competition experiments was performed in which the amount of labeled ß163 was held constant (0.66 ng) and increasing amounts of cold competing promoter and/or enhancer DNA fragments (0-20 ng) were incubated in the binding reaction. As illustrated in Fig. 3, the only restriction fragment capable of competing with labeled ß163 for binding was cold ß163 fragment. Interestingly, the IFN-α1 fragment was ineffective in competing for binding activity as were the c-fos, SV40 and pAT fragments. Based on the transcriptional domains encompassed by these fragments, it is concluded that the DNA-binding protein is not specific for these functional elements, nor is it capable of recognizing the IFN-α1 upstream sequences. These observations suggest that the KG-1 extract contains one or more factors with a much higher affinity for the IFN-ß regulatory region than for other promoter- or enhancer-containing restriction fragments, including IFN-α1. Consequently, this IFN-ß DNA-binding protein has been designated KGß.

Competition experiments were performed with radiolabeled ß163 (0.66 ng) and cold competitor fragments, ß67 and ß96 (0-20 ng) derived from ß163 (Fig. 1), to further localize the DNA-binding domain. As illustrated in Fig. 4, the ß67 fragment competed effectively for KGß. A 30-fold excess of competing ß67 fragment dissociated 90% of the KGß-ß163 complex, whereas the same excess of ß96 fragment decreased the amount of complex by only 50%. This result

Figure 3. Competition Between IFN-ß163 and Different Promoter/Enhancers for DNA Factor Binding. Labeled ß163 (0.66 ng) was added to 6 µg poly (dI-dC)•(dI-dC) and various amounts of unlabeled DNA fragments. The DNA mixture was incubated with 9.2 µg of nuclear protein at 30°C for 30 min, followed by electrophoresis as described under "Methods and Materials". The amount (nanograms) and type of competing DNA are indicated above the gel lanes.

Competition Between IFNβ-163 and Different Promoter/Enhancers for DNA Factor Binding





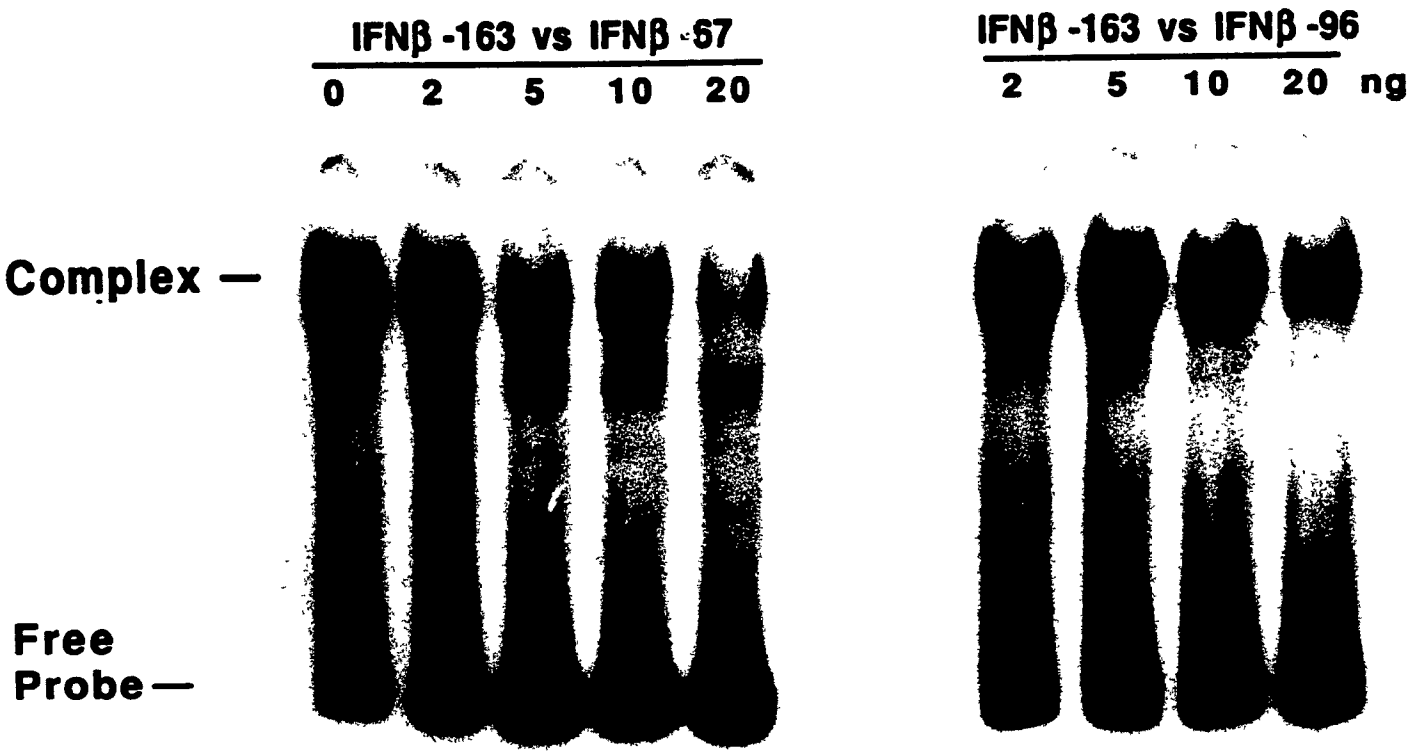
^{32}P-IFNβ-160 - 0.66 ng
poly dI:dC - 6 µg
KG-1 extract - 9.2 µg

raised two possibilities: 1) ß163 bound multiple factors and ß67 competed effectively for one of these putative factors, while ß96 competed for a distinct factor or 2) Alu cleavage of ß163 which generated ß67 and ß96 disrupted a unique KGß binding site, and in so doing, resulted in two fragments with different portions of the recognition sequence (and hence different affinities) for the same protein.

Recently, Zinn and Maniatis (31) used *in vivo* DNAase I footprinting to detect factors binding to the -94 to -168 and -38 to -68 region of the IFN-ß gene. These domains correlate closely with the 5' and 3' boundaries of functionally distinct negative/positive regulatory elements delineated by deletion analysis in other studies; removal of negative regulatory elements resulted in higher basal levels of gene activity (11,16). These authors have presented a model suggesting that specific factors repress IFN-ß transcription prior to induction. Subsequent to induction, a positive transcription factor(s) would act to alleviate repression by an unknown mechanism. This proposal is reminiscent of negative regulatory mechanisms controlling transcription in bacteria (32), yeast (33, 34), and mammalian cells (35-38). In yeast, the MATα2 locus of α-mating type cells encodes a negative regulatory protein that represses a-mating type-specific genes by binding to a 34 bp domain located 135 nucleotides upstream of the CAP site (33,34). Other examples of repression include adenovirus Ela-mediated repression of the SV40 and immunoglobulin gene enhancers (35-37), and the repression of the SV40, but not the RSV enhancer elements in embryonal carcinoma cells (38).

Whereas models for negative eukaryotic transcription regulation have not been studied in great detail, several reports have characterized the interaction of positive trans-acting factors with a variety of viral and cellular eukaryotic

Figure 4. Competition for ß163 DNA-Binding by ß67 and ß96. The conditions and experimental procedure used are identical to Fig. 3 except that the competing DNAs were ß67 and ß96 fragments.



^{32} P-IFNβ -160 - 0.66 ng
poly dI:dC - 6 μg
KG-1 extract - 9.2 μg

templates. The purified HeLa transcription factor Sp1 binds cooperatively to the G-C hexanucleotide repeats of the SV40 early promoter, and to two similar elements in the distal promoter of HSV thymidine kinase (tk) gene (19,20). A biochemically distinct transcription factor, CTF, binds to the CCAAT pentanucleotide homology that is flanked by the Sp1 binding elements in the HSV-tk gene (20,21). DNAase I footprinting and band retention assays have demonstrated common and cell-specific proteins interacting with the enhancer domains of SV40 (18), immunoglobulin (40-42), c-fos (43,44), and adenovirus genes (26,39). It is therefore likely that some negative regulatory mechanisms will involve not only relief of repression by removal of repressor molecules, but also positive interactions between common and unique transcription proteins recognizing adjacent or overlapping DNA sequences. Binding of specific factors may impose conformational changes in the DNA that permit general transcription factors and RNA polymerase II to interact at the promoter.

Using an *in vitro* band retention assay and nuclear extracts from a myeloid leukemic cell line, we have identified an interferon-ß specific protein that interacts with the -135 to -202 region; band competition analysis has demonstrated that the DNA-binding protein is not a general promoter- or enhancer-binding factor. Since the putative factor fails to recognize the regulatory sequences of IFN-α1, we suggest that the regulation of different interferon genes may in part require distinct factors. It is tempting to speculate that KGß represents a nuclear protein responsible for repression of interferon-ß gene transcription in the uninduced state.

CHAPTER 5

MULTIPLE PROTEIN-DNA INTERACTIONS WITHIN THE HUMAN INTERFERON-ß REGULATORY ELEMENT

PREFACE

A gel electrophoresis mobility shift assay (EMSA) was used to demonstrate protein-DNA interactions specific for an upstream region of the IFN-ß promoter (chapter 4). Although we have no direct evidence that the factor(s), KGß, is a repressor protein(s), this possibility is suggested by *in vivo* competition experiments (chapter 3) and deletion studies that implicate the sequences in the -100 to -200 region in negative regulation (Zinn, K. et al. 1983. Cell 34: 865-879). *In vivo* competition analysis also indicated that sequences downstream of -135 interacted with positive trans-acting proteins. This downstream region encompasses the interferon regulatory element (IRE), which represents the minimal inducibility sequence requirement as defined in murine C127 cells (Goodbourn, S. et al. 1985. Cell 41: 509-520). In order to characterize protein-DNA interactions within the IRE, a synthetic radiolabeled IRE oligonucleotide was used to examine factor binding in uninduced and virus-induced Hela nuclear extracts. Binding to the intact IRE was further localized by EMSA competition experiments using short oligonucleotides spanning this region as well as sequences upstream. Experiments were also designed to determine if IRE binding factors present in Hela nuclear extracts were required for specific transcription of an IFN-ß template *in vitro*.

ABSTRACT

High efficiency nuclear protein extracts were prepared from uninduced and recombinant interferon (IFN)-α2-primed Sendai virus-induced HeLa S3 cells and analyzed for DNA-binding proteins specific for the human interferon-ß regulatory element (IRE). Analysis of protein-DNA interactions by a gel electrophoresis DNA-binding assay resolved three complexes (designated A,B, and C) specific for the IRE probe (-79 to -36) in uninduced and induced cells. Competition studies using greater than a 100-fold molar excess of unlabeled DNA fragments representing different IFN-ß promoter sequences localized the DNA-binding domain to the sequences -79 to -64 (P1 probe), a region previously shown to be essential for virus-inducible gene expression. Other adjacent IFN-ß segments including the negative regulatory element (-65 to -30) as well as the SV40 enhancer, c-fos serum-responsive element, and IFN-α1 promoter fragment (-131 to -71) were unable to compete for the IRE binding protein(s). The 16 base pair enhancer core (P1 probe) specifically bound similar amounts of protein from uninduced and induced extracts; a synthetic tetrahexamer, AAGTGA, competed efficiently for binding to the P1 region. Competition studies indicated that adjacent upstream IFN-ß DNA sequences -94 to -78 (P5) also contained a sequence motif capable of binding the P1 protein. *In vitro* transcription of the IFN-ß template in HeLa nuclear extracts was partially inhibited when increasing amounts of competitor IRE fragment were added to the reaction. These results demonstrate that multiple DNA sequence motifs within the IFN-ß regulatory element interact to bind transcription regulatory proteins which are present in normal and virus-infected HeLa cells.

INTRODUCTION

Spatial and temporal interactions of protein factors with multiple cis-acting DNA regulatory elements play a critical role in the regulation of constitutively expressed, tissue-specific, and inducible RNA polymerase II transcribed genes (reviewed in Ref. 1). Numerous transcription factors from all three gene classes have been characterized, and in some cases, purified based on affinity to their cognate DNA-binding domains (2-6). A subset of transcription factors appears to overlap with members of all three groups: for example, activating protein 1 (AP-1) interacts with promoter elements adjacent to the simian virus 40 (SV40), metallothionein, collagenase, and stromelysin genes, all of which contain the AP-1 consensus binding motif ATGACTCTT (7-9); recognition sequences (GGGCGG) for the Sp-1 protein are found adjacent to several genes (10); the CCAAT-related sequence motifs present in the 5'-flanking region of many genes interact with different factors exemplified by CTF/NF-1 and COUP (3,11,12). In addition, proteins that lack DNA-binding specificity but contribute to transcriptional activation via protein-protein interactions have also been identified (13).

The interaction of the DNA-binding protein, NF-κB, with the immunoglobulin κ light chain gene enhancer sequence (GGGACTTTCC) is of particular interest as a model of tissue-specific gene expression (14-19). Although NF-κB is binds constitutively in mature B cells, DNA-binding activity can be induced in pre-B cells by lipopolysaccharide, and correlates with lymphoid-specific activation of light chain gene transcription (17). Recent studies indicate that post-translational modification of NF-κB is required for DNA-binding (18,19). Similarly, a repertoire of promoter binding factors associated with other inducible genes such as c-fos (20-23), metallothionein (7-9), and HSP70 (6,24,25) have been identified. These

observations indicate that the spatially defined arrangement of a limited number of cell-specific and ubiquitous transcription factors along the DNA helix, and post-translational modification of these proteins, is important in eukaryotic transcription regulation.

The virus-regulated induction of type 1 (IFN-α and IFN-ß) interferon promoters likewise provides a model to study inducible cell-specific gene expression. Multiple cis-acting DNA sequence motifs located about 100 nucleotides upstream of the mRNA start site comprise an inducible enhancer element (26-30) with overlapping positive and negative regulatory domains (31). The type of interferon produced following virus infection is determined by the host cell (32-34). Therefore, the pattern of protein-DNA interactions contributing to IFN gene activation may reflect characteristics of both cell-specific and inducible gene regulation. Protein-DNA contacts around the IFN-ß promoter have been examined using *in vivo* DNase I footprinting, and in fact, different factor binding patterns have been observed before and after induction (35). Recently, Keller and Maniatis (36) identified three factors that bind specifically to two domains within the interferon-ß regulatory element (IRE), one of which is present only in extracts from poly(I)-poly(C) induced cells.

The present study further characterizes the interactions of nuclear proteins from uninduced and virus-induced HeLa S3 cells with the IRE. The IFN-ß enhancer core element between -79 and -64 (P1) was found to bind a factor in similar amounts from uninduced and induced extracts; the adjacent upstream sequences -94 to -78 also bound the factor. A synthetic tetrahexameric sequence of the type AAGTGA (P3) competed efficiently for P1 binding activity. The functional role of protein-DNA interactions involved in IFN-ß gene expression was demonstrated by inhibition of *in vitro*

transcription of an IFN-ß template when competed with an excess of the IRE fragment.

MATERIALS AND METHODS

Induction of HeLa Cells and Preparation of Nuclear Extracts. HeLa S3 suspension cultures were grown to a density of 10^6 cells/ml in RPMI 1640 medium supplemented with 5% fetal calf serum and antibiotics. High efficiency nuclear extracts were prepared from 10 liters of uninduced HeLa S3 cells following the protocol described by Shapiro et al. (37). The induced extract was prepared from the same volume of cells following priming/induction. Briefly, cells were treated for 18 h with 1000 IU/ml of recombinant IFN-α2 (a kind gift from Schering Canada), pelleted by centrifugation at 1200 rpm for 15 min, resuspended in a volume of 1 liter in complete medium, and infected with Sendai virus (200 hemagglutinating units/ml) for 2 h. At the end of the induction period, cells were processed for nuclear extract as described (37). The nuclear extracts (final protein concentration 15-20 mg/ml) were quickly frozen in liquid nitrogen and stored in aliquots at -70°C. Crude aliquots of 20-40 mg of nuclear protein were assayed for P1 DNA-binding activity.

The extracts were also fractionated on a 7 ml bed of heparin-Sepharose (Pharmacia LKB Biotechnology Inc.); the sample (20 mg) was applied in nuclear dialysis buffer (NDB) (37) and the column was eluted stepwise with buffers (14 ml) containing NDB and 0.1-0.8 M KCl. Fractions from the different step elutions were pooled and assayed for P1 DNA-binding activity.

RNA Isolation and S1 Analysis. Total cellular RNA was isolated from uninduced, Sendai virus-induced (200 hemagglutinating units/ml), and IFN-α2-primed (1000 IU/ml, 18 h) - Sendai virus-induced HeLa S3 cells at different times after induction according to a modified guanidinium isothiocyanate

procedure (38). IFN-ß-specific mRNA was measured with a 5'-[γ-^{32}P]ATP-labeled BglII-EcoRI 922-bp probe as previously described (32).

Preparation of IFN Probes. DNA fragments comprising ß96, the c-fos serum-responsive element, the SV40 enhancer, and the IFN-α1 60-bp fragment have been described previously (39). Synthetic oligonucleotides spanning the IFN-ß regulatory element between nucleotides -30 and -132 (see Fig. 2) were prepared by a Pharmacia LKB Biotechnology Inc. gene assembler and purified by separation on a 10% polyacrylamide gel followed by Sephadex G-25 chromatography. Complementary strands were annealed and repurified by gel electrophoresis to separate single- from double-stranded material.

Fragments were 5' end-labeled using [γ-^{32}P]ATP (ICN, 3888 Ci/mmol) and polynucleotide kinase (Pharmacia LKB Biotechnology Inc.). Incorporation of label was monitored by measuring radioactivity in trichloroacetic acid-insoluble material.

Gel Electrophoresis DNA-Binding Assay. The gel retardation assay was performed as previously described (39) with modifications; probe DNA (30,000-50,000 cpm, 0.1 ng) was incubated with 10 µg of poly(dI-dC) as nonspecific competitor and 0-16 µg of nuclear extract as described in individual experiments. Unlabeled specific competitor DNAs were used at 0-80 ng as described. Reactions were carried out at 22°C for 30 min in nuclear dialysis buffer (NDB: 20 mM HEPES, pH 7.9, 5% glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM dithiothreitol) and immediately loaded on a 6% native polyacrylamide gel (60:1 cross-link) prepared with Tris-glycine (25 mM Tris, 195 mM glycine, pH 8.5). The gels were run at 150 V for 1.5-2.5 h, dried and exposed to autoradiographic film at -70°C. The relative intensities of the

protein-DNA complexes were measured using the LKB 2200 scanning laser densitometer; scans were integrated using the LKB 2202 Gelscan software package.

***In Vitro* Transcription.** Transcription reactions were carried out as described (37) with modifications. All 4 nucleoside triphosphates were unlabeled; each reaction (20-25 µl total volume) was preincubated with 1 µg of poly(dI-dC) for 10 min at room temperature before addition of the specific pUCß or pwtSV template (250 ng). For competition assays synthetic IRE (20 and 60 ng) was added to the reaction after the initial preincubation with poly(dI-dC) and incubated for an additional 15 min at 30°C prior to the addition of the pUCß or pwtSV template. The reactions were terminated and the RNA was harvested by a small scale guanidinium isothiocyanate procedure (38). Transcripts were hybridized to 5' end-labeled probes, either the 922-bp BglII-EcoRI IFN-ß probe or the 423-bp AvaII-KpnI SV40 early region probe, and analyzed by S1 mapping.

RESULTS

Factor Binding to the IRE. We sought to analyze the regulatory DNA-protein interactions responsible for induction of the interferon promoter in extracts prepared from uninduced and Sendai virus-induced HeLa S3 cells. Since priming of certain cell lines with IFN dramatically increases the yield of virus-inducible IFN mRNA and protein (40), the transcription of the endogenous IFN-ß gene was initially examined in Sendai virus-induced and IFN-α2-primed Sendai-induced HeLa S3 cells. Pretreatment of the cell population with IFN-α2 increased the virus-induced amount of IFN-ß mRNA and altered the induction kinetics of the IFN-ß gene significantly (Fig. 1). The peak of IFN-ß mRNA in primed cultures was seen at 4 h after virus induction as compared to 6 h in unprimed HeLa S3 cells. Based on the induction results, nuclear extracts were prepared from uninduced and IFN-α2-primed Sendai virus-infected cultures at 3 h after induction. When uninduced or virus-induced extracts were probed using the end-labeled 44 bp IRE oligonucleotide (Fig. 2), several discrete complexes were identified (Fig. 3). Addition of a 20-100-fold molar excess of unlabeled IRE DNA, but not a 75 bp fragment derived from plasmid pAT153, was able to compete specifically for three of these complexes. The slowest migrating complex was termed complex A, while the fastest moving complexes were designated B and C (Fig. 3). A complex (NS) that migrated more slowly than complexes B and C was not competed by any fragments used in this study and was therefore considered to be a nonspecific complex. No differences in complex formation were observed between the induced and uninduced extracts, demonstrating that at least some of the factors interacting with the IFN-ß IRE are present prior to virus infection.

Figure 1. Induction of IFN-ß mRNA in IFN-α2-primed, or Unprimed, Sendai Virus-infected HeLa S3 Cells. Suspension cultures of HeLa S3 were pretreated with IFN-α2 (1000 IU/ml, 18 h) or left untreated prior to Sendai virus infection (200 hemagglutinating units/ml). Total RNA was isolated from 10^8 cells and analyzed by S1 mapping using the 5' end-labeled 922-bp BglII-EcoRI IFN-ß probe (Pß). The IFN-ß DNA-RNA 604 bp hybrid signal is indicated by the symbol ß; the radioactivity (^{32}P counts/min) in the IFN-ß DNA-RNA signal was cut from the gel and quantified. (•), ^{32}P counts/min from primed induced cells, (o), ^{32}P counts/min from unprimed induced cells. M, pAT153 digested with HaeIII and end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. Lanes 1-9, RNA isolated from unprimed Sendai virus-induced HeLa S3 cells at 0,1,3,2,4,6,8,10, and 12 h, respectively; lanes 10-18, RNA isolated from IFN-α2-primed Sendai virus-induced HeLa S3 cells at 0,1,2,3,4,6,8,10, and 12 h, respectively; lanes 19 and 20, RNA isolated from uninduced and 4-h induced MG63 cells. Note that in lanes 3 and 4 the timed samples were reversed.

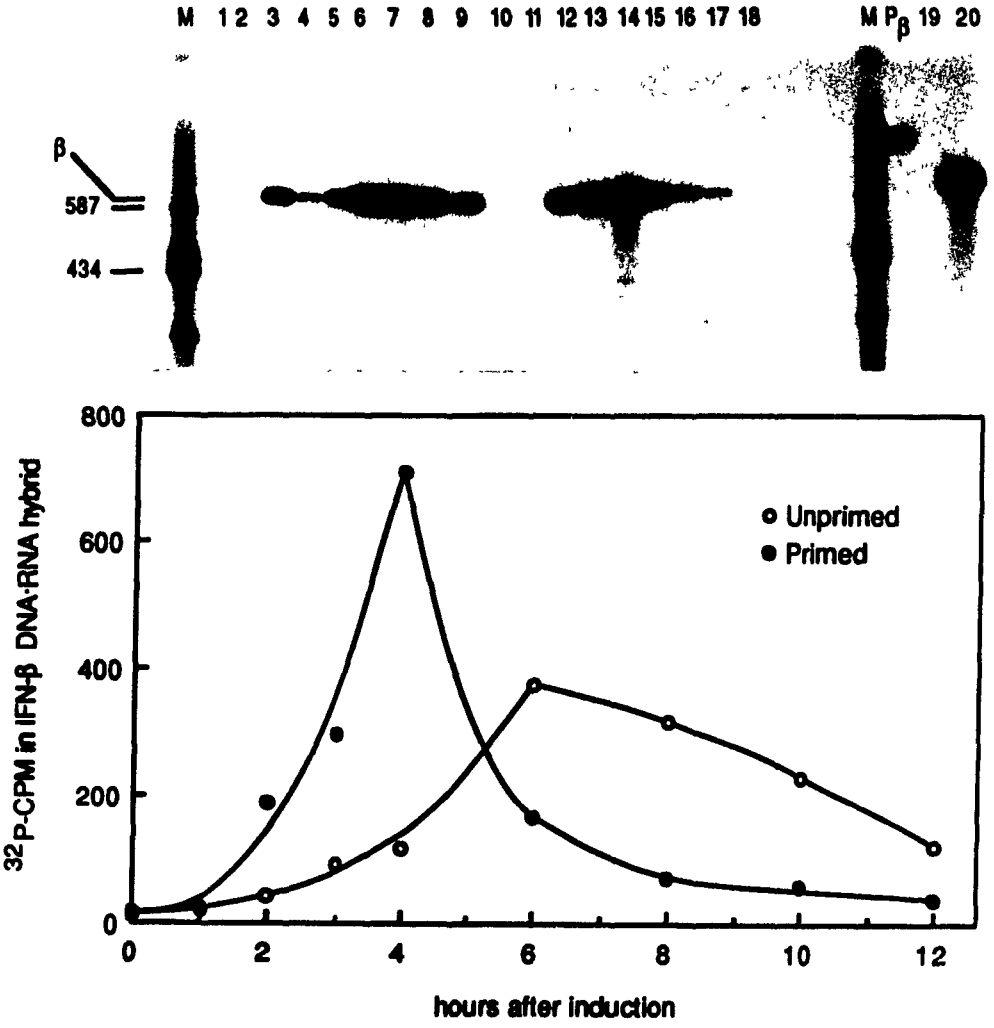
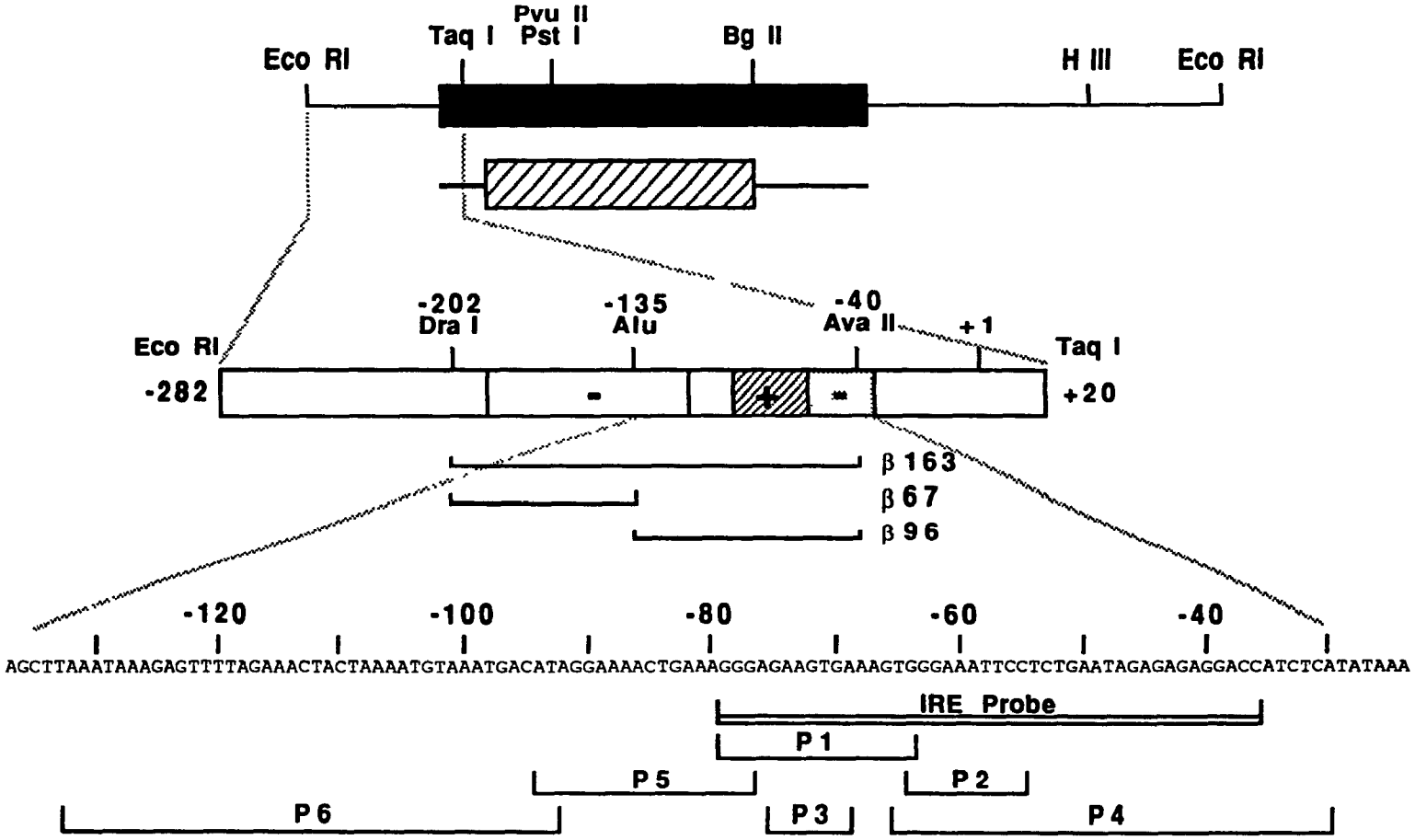

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M Pβ 19 20
β
587
434
800
600
400
200
0
0 2 4 6 8 10 12
32P-CPM in IFN-β DNA·RNA hybrid
hours after induction
Unprimed
Primed

Figure 2. Schematic of the Human Interferon-ß Gene and the Promoter Domains used in the Band Retention Assay. The 1.8-kilobase pair EcoRI fragment containing the gene for IFN-ß (solid box) is shown; below the gene the mRNA transcript and protein coding region (hatched box) are illustrated. The EcoRI-TaqI 300 bp promoter fragment is expanded below the structural gene and indicates restriction enzymes used to generate subfragments (39). The hatched and stippled boxes together with the (-) and (+) symbols represent cis-acting DNA elements involved in IFN-ß regulation (based on Refs. 26,28, and 35). The synthetic oligonucleotide probes used in this study are illustrated below the IFN-ß nucleotide sequence between -137 and -24.


Pvu II
Taq I
Pst I
Bg II
Eco RI
H III
Eco RI
-202
Dra I
-135
Alu
-40
Ava II
+1
Eco RI
Taq I
-282
+20
β 163
β 67
β 96
-120
-100
-80
-60
-40
AGCTTAAATAAAGAGTTTTAGAAACTACTAAAATGTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCTGAATAGAGAGAGGACCATCTCATATAAA
IRE Probe
P1
P5
P2
P6
P3
P4

Competition for Factor Binding by Different Regulatory Domains. To assess whether the factors interacting with the IRE probe represented specific IFN-ß binding proteins or more generalized transcription factors, a series of competition studies were performed using DNA fragments representing different domains of the IFN-ß promoter, as well as the regulatory domains of the SV40 enhancer, the c-fos enhancer, and the IFN-α1 promoter. The results of these competition studies are summarized in Fig. 4. A 16 bp synthetic oligonucleotide representing IFN-ß sequences between -79 and -64 competed effectively for proteins interacting with the IRE probe (Fig. 4) as did unlabeled IRE fragment; 20-100-fold molar excesses of unlabeled IFN-ß promoter fragments represented by P4 (-65 to -30), P5 (-94 to -78), and P6 (-132 to -92) did not diminish factor binding to the IRE. The c-fos, SV40, and IFN-α1 fragments also failed to compete for the factor binding to the IRE (data not shown).

Several conclusions can be drawn from these experiments: 1) the major protein binding domain within the IRE region is the P1 sequence motif which has been described previously as the minimal inducibility core of the enhancer element (26,28); 2) other IFN promoter domains including the P4 domain which contains overlapping positive and negative elements (31) do not compete for the factors interacting with the IRE; 3) the inability of the IFN-α1 promoter, the SV40 72 bp repeats, and the c-fos enhancer elements to compete effectively for IRE binding implies that these factors may be unique to the IFN-ß enhancer core domain.

Protein Binding to the IRE Enhancer Core. When the 16 bp P1 oligonucleotide fragment (-79 to -64) was used to probe the nuclear extracts, similar amounts of protein(s) from the uninduced and induced HeLa S3 extracts

Figure 3. Factor Binding to the IFN-ß IRE. The ^{32}P-labeled IRE probe (0.1 ng) was incubated with 8 µg of nuclear protein from uninduced or virus-induced HeLa S3 cells, 10 µg of poly(dI-dC), and 0-10 ng of unlabeled IRE DNA or 10-20 ng of pAT75 (a 75 bp fragment, derived from plasmid pAT153) in a total volume of 20 µl as described under "Materials and Methods". Three differentially migrating complexes (A,B,C) are indicated by arrows; a noncompeting complex (NS) is also indicated as is the position of non-complexed probe DNA. Samples were electrophoresed through a low ionic strength 6% polyacrylamide gel, dried down, and exposed to autoradiographic film. The type and amount of competing DNA (in nanograms) and type of extract used are indicated above the lanes of the gel.

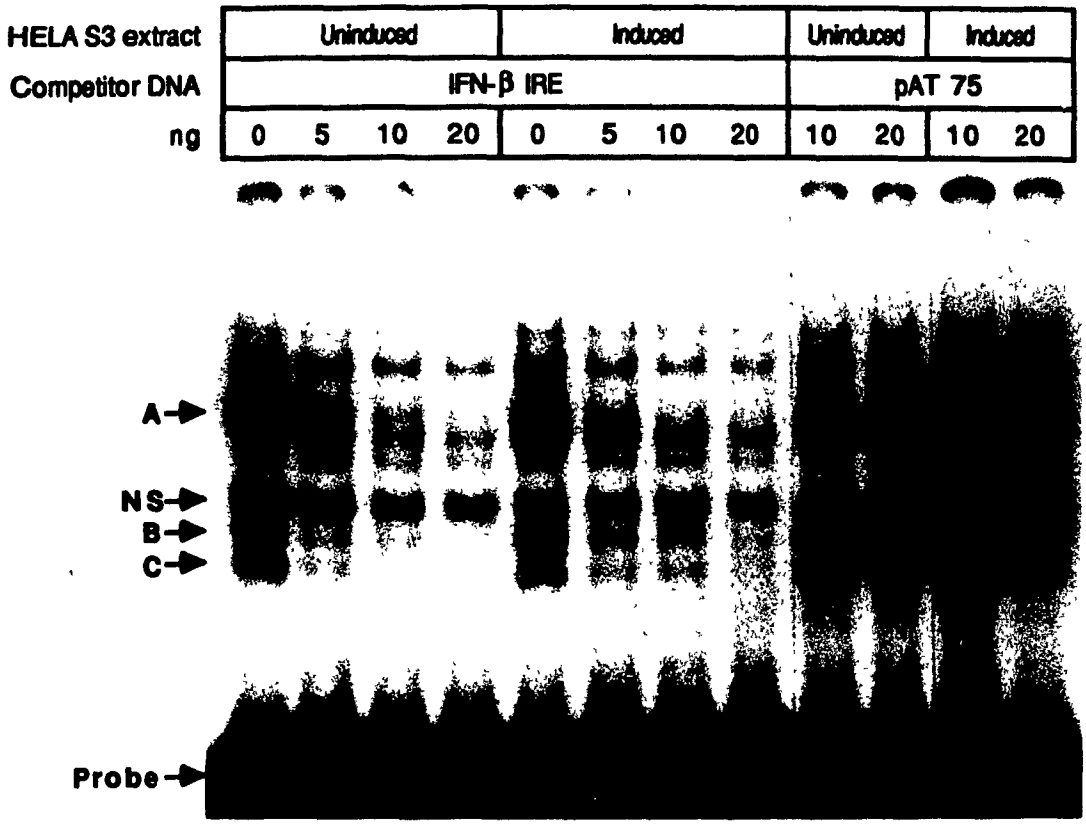
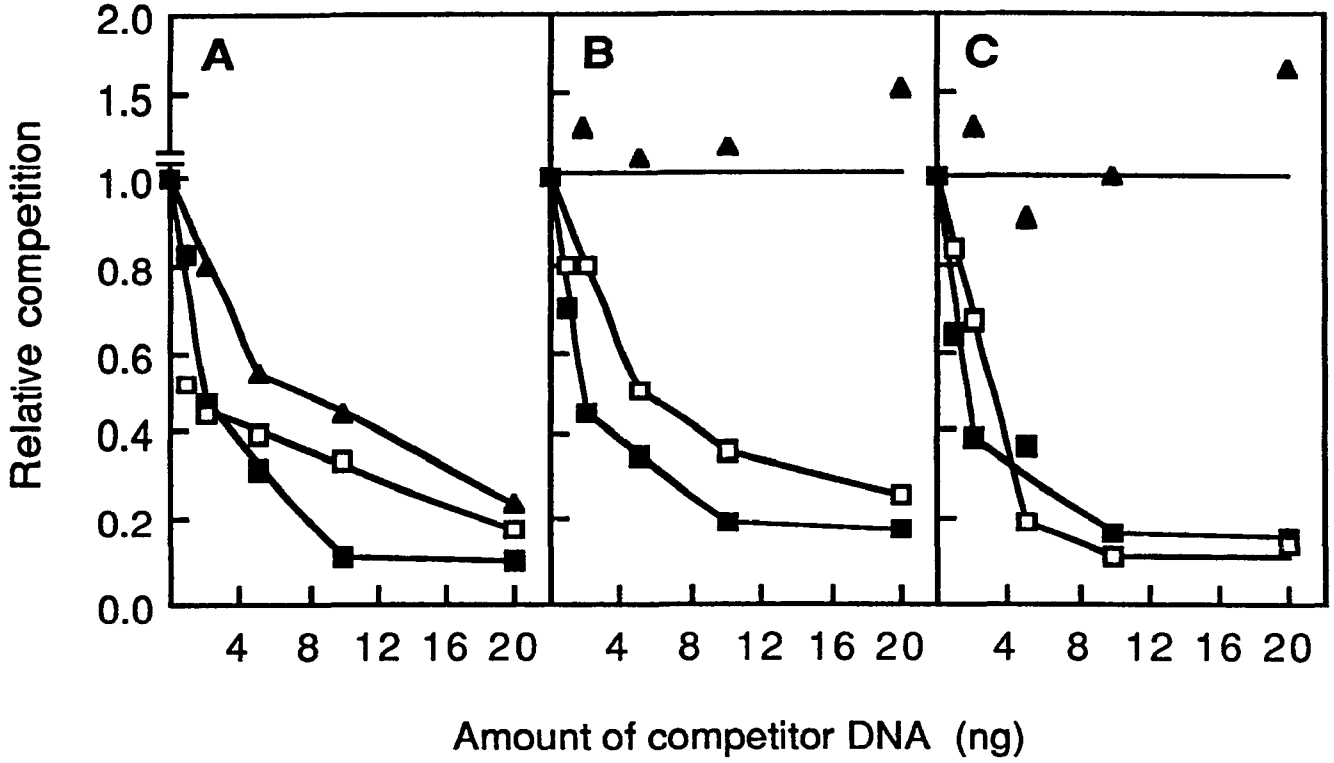

HELA S3 extract
Competitor DNA
ng
Uninduced
Induced
Uninduced
Induced
IFN-β IRE
pAT 75
0 5 10 20 0 5 10 20 10 20 10 20
A
NS
B
C
Probe

Figure 4. Competition for IRE Factor Binding by Different IFN-ß Promoter Domains. The binding reactions were set up as described in Fig. 3, using 0.1 ng of ^{32}P-labeled IRE probe and 0-20 ng of unlabeled DNA fragments corresponding to the IFN-ß domains illustrated in Fig. 2. Protein-DNA complexes generated in the presence of increasing amounts of competitor DNA were analyzed by the gel electrophoresis DNA-binding assay and quantified by densitometric scanning of low exposure autoradiograms. Integration values for complexes A,B, and C were normalized relative to the amount of NS complex in each lane and expressed as a fraction of the complex formed in the absence of competitor (1.0). The values represent the average of three measurements. Competitor DNAs shown are: IRE (■); P1 (□), P4 (▲). P5, P6, α60, the c-fos enhancer, and the SV40 72 bp Sph I fragment also failed to compete for IRE binding activity.

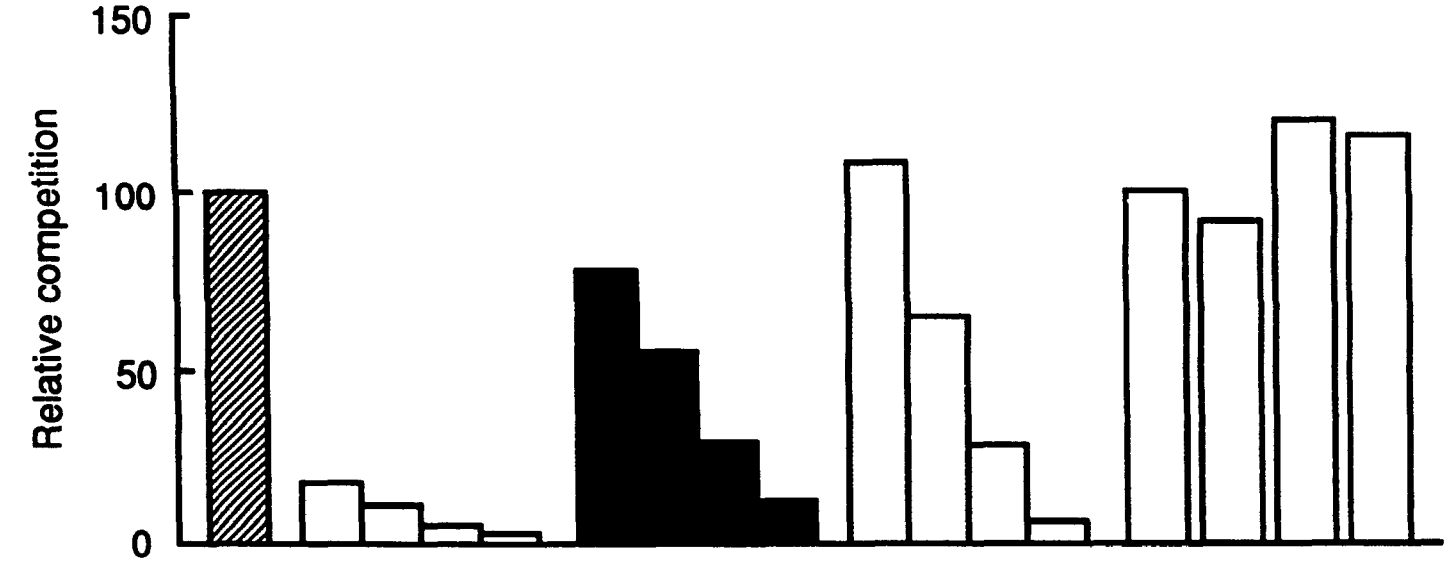

A
B
C
2.0
1.5
1.0
0.8
0.6
0.4
0.2
0.0
Relative competition
4 8 12 16 20
4 8 12 16 20
4 8 12 16 20
Amount of competitor DNA (ng)

bound to P1 (data not shown). A series of competition studies similar to those described in Fig. 4 were performed to determine if other IFN-ß sequence motifs also bound these factors. Fig. 5 represents the results of competition studies using P1, IRE, P4, P5, P6, ß96, and IFN-α1 as competing DNA fragments in greater than 100-fold molar excess over the labeled P1 probe. The P1 complex was resolved into two bands, only one of which could be eliminated by homologous unlabeled P1 DNA. In addition to the homologous P1 competitor and the IRE fragment, sequences between -94 and -78 (P5) located immediately upstream of the P1 probe also competed for factor binding to the IFN-ß enhancer core sequence. Both P5 and the IRE were about 10-fold less efficient than P1 in the competition reactions. The -132 to -92 upstream sequences (P6) and the sequences -65 to -30 (P4) downstream of the P1 probe did not compete for P1 binding. The 60 bp IFN-α1 fragment which encompasses sequences between -131 and -71 relative to the IFN-α1 mRNA start site also failed to bind protein, indicating that the interaction examined was unique to the IFN-ß promoter.

Initial fractionation of the nuclear extracts has revealed several characteristics of the P1 DNA-binding activity. The P1 DNA-binding activity was enriched about 20-fold by heparin-Sepharose chromatography and eluted in the 0.2 M KCl step. This chromatography step separated the majority of P1 from the more rapidly migrating nonspecific activity (Fig. 6, A and B). Size fractionation on Sephacryl S-300 suggests that P1 activity elutes as two distinct peaks (data not shown).

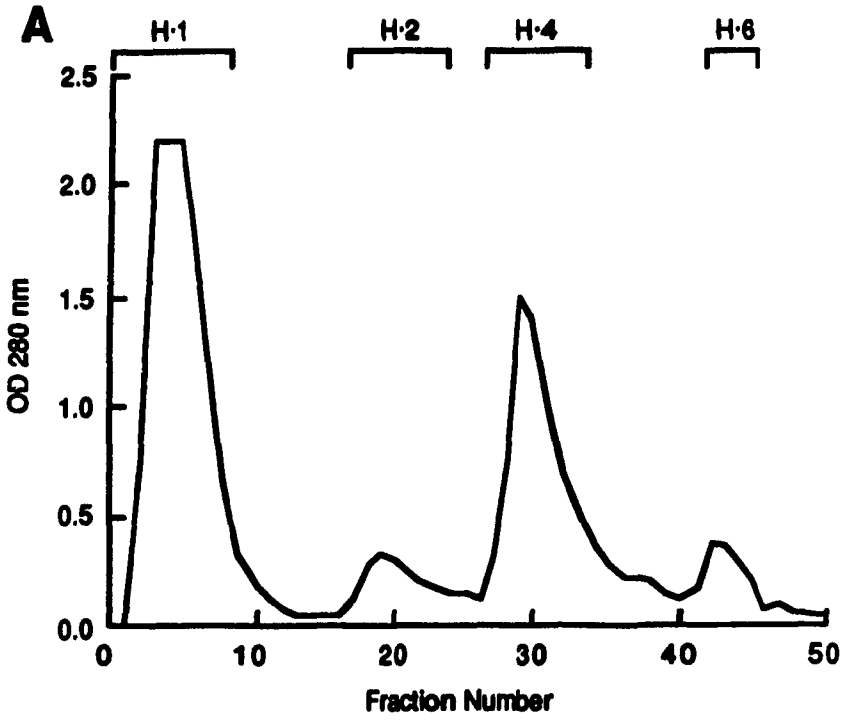
The P1 domain contains two adjacent permutations of the hexameric sequence AAGTGA which is present seven times throughout the IFN-ß promoter between the region -110 and -65 (27); the tetrahexamer of AAGTGA was shown to confer virus inducibility to a heterologous promoter (29). To

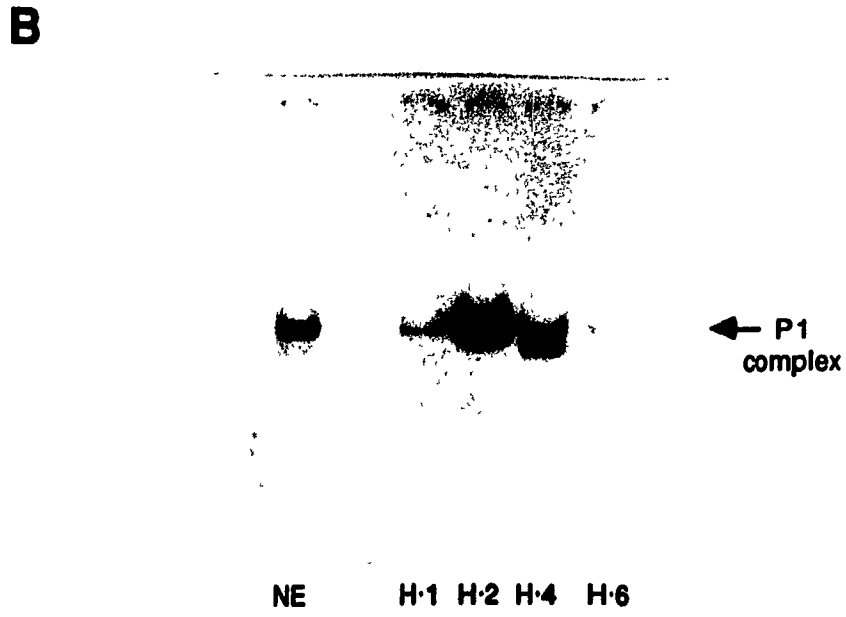
Figure 5. Competition for Binding to the P1 Element. Various amounts of different IFN regulatory domains were used to compete for protein binding to the radiolabeled 16 bp P1 oligonucleotide; the type, amount, and molar ratio (relative to P1 probe) of the DNA fragments used in the competition are shown below the graph. The relative ability of the different DNA domains to compete for P1 factor binding was determined by densitometric scanning of the radioactivity present in P1 complexes following the band retention assay and autoradiography. The calculated values are expressed relative to the amount of P1 complex formation in the absence of competitor DNA (100%) and represent the average of duplicate measurements. The autoradiograph illustrates the P1 complexes generated in the presence or absence of the competitor DNA described below the bar graph.



Comp DNA	–	P1				IRE				P5				P6	P4	β96	α1
ng	0	1	2	5	10	10	20	40	80	10	20	40	80	80	80	80	20
Molar ratio	–	10	20	50	100	38	76	151	302	93	187	373	747	364	373	140	227

Figure 6. Fractionation of P1 DNA-Binding Activity and Competition with Hexameric Oligonucleotides. Crude nuclear protein (20 mg) was applied to a 7 ml bed of heparin-Sepharose in NDB containing 100 mM KCl. The column was eluted stepwise with 14 ml of NDB containing 100, 200, 400, 600, and 800 mM KCl, and the protein profile ($A_{280\ nm}$) was determined (A). The peak protein fractions were pooled, dialyzed against NDB containing 100 mM KCl, and an aliquot from each pool containing 2 μg of protein was assayed for P1 DNA-binding activity (B). The capacity of different synthetic hexameric oligonucleotides corresponding to AAGTGA (lanes 1-5), AAAGGA (lanes 6-9), and ACGCGA (lanes 10-13) to compete for P1 DNA-binding was determined by gel retardation (C). Lane 1 represents the amount of binding to the P1 probe in the absence of competitor. P1 binding was competed with 2,5,10, and 15 ng of the respective oligonucleotides. The results were quantified by scanning densitometry and plotted as a fraction of the binding to P1 in the absence of competitor.


A
H·1
H·2
H·4
H·6
2.5
2.0
1.5
1.0
0.5
0.0
OD 280 nm
0
10
20
30
40
50
Fraction Number


B
P1
complex
NE
H·1
H·2
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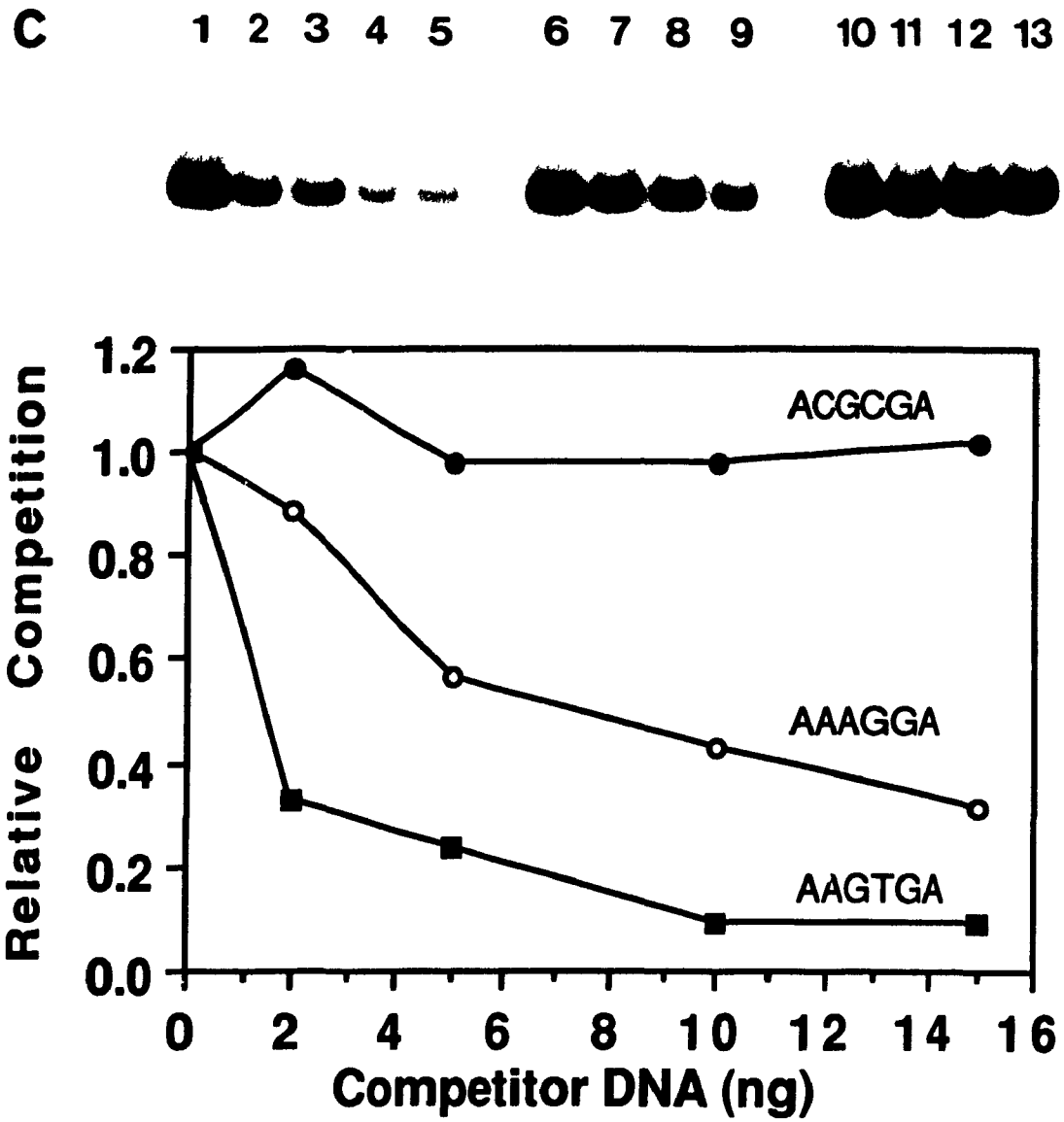
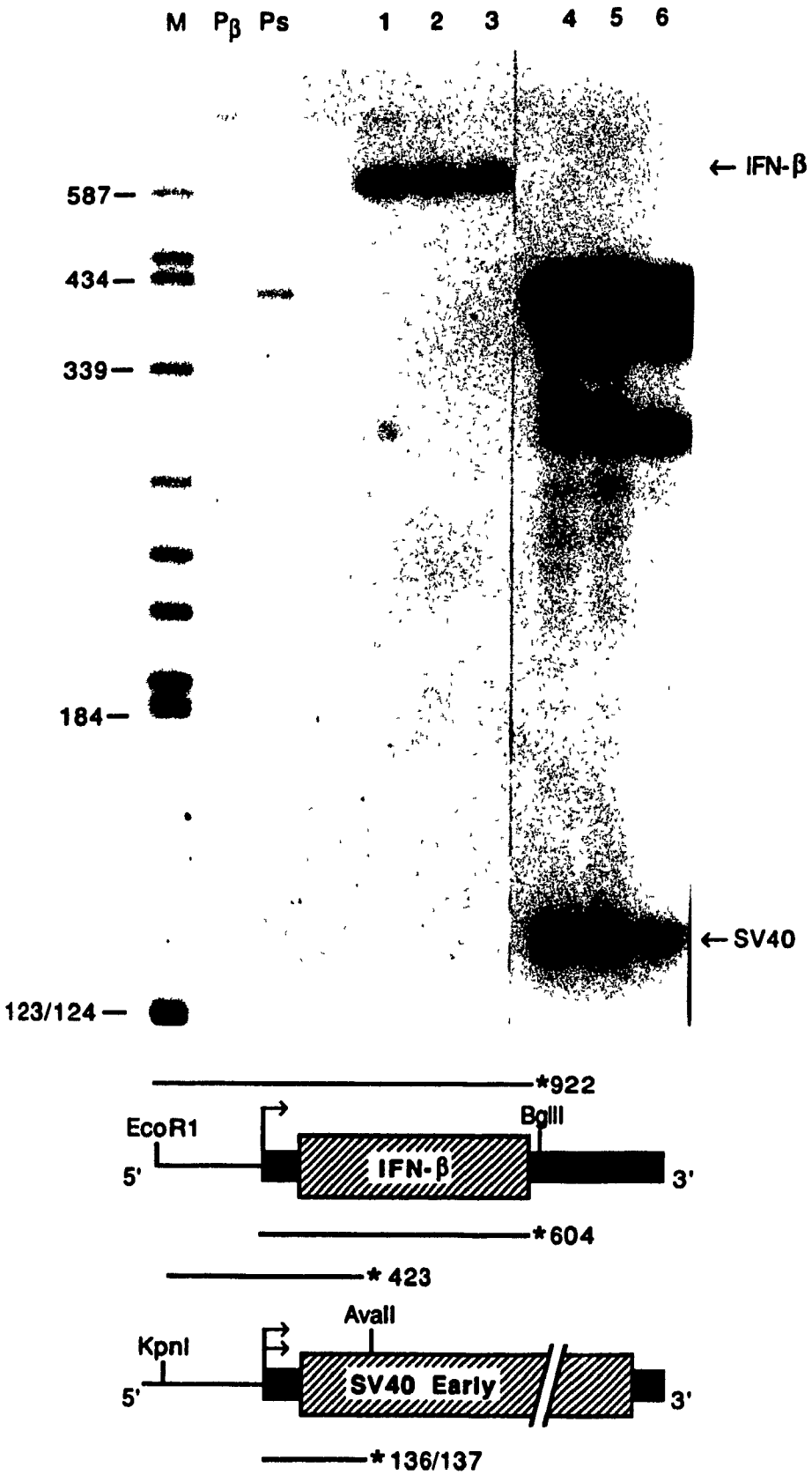
H·6


C
1 2 3 4 5 6 7 8 9 10 11 12 13
Relative Competition
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ACGCGA
AAAGGA
AAGTGA
0 2 4 6 8 10 12 14 16
Competitor DNA (ng)

localize more precisely the factor binding sites in the P1 domain, different hexameric permutations were used to compete P1 binding (Fig. 6C). The AAGTGA tetrahexamer competed for P1 DNA-binding activity at molar ratios of 13 to 99 (2-15 ng) relative to P1 probe (Fig. 6C). Mutation to AAAGGA (a noninducible hexameric permutation) (29) decreased the relative competitive capacity of the oligonucleotide about 5-fold, while a mutation to ACGCGA generated an oligonucleotide that was unable to compete for P1 binding activity (Fig. 6C). These results suggest that the permutations to AAGTGA within the IRE function to bind a factor(s) involved in IFN inducibility.

***In Vitro* Transcription of the IFN-ß DNA Template.** To test the functional significance of the IFN-ß promoter binding proteins, *in vitro* transcription was performed using supercoiled pUCß (41) plasmid (250 ng) and nuclear extracts from uninduced HeLa S3 cells. Preliminary experiments demonstrated that preincubation of the extracts with poly (dI-dC) decreased the amount of nonspecific initiation (detected as protection of the full-length probe) and increased the amount of correctly initiated IFN-ß mRNA by about 10-fold (data not shown). The amount of correctly initiated IFN-ß mRNA synthesized *in vitro* was measured by an indirect S1 mapping procedure using the IFN-ß BglII-EcoRI 922-bp probe fragment (Fig. 7, lane 1). Addition of 20 and 60 ng of 44-bp IRE DNA decreased the IFN-ß transcript level about 5-fold (Fig. 7, lanes 2 and 3), but did not change the relative levels of SV40 early mRNA transcribed from a similar amount of supercoiled pwtSV plasmid containing the SV40 early region (Fig. 7, lanes 4-6). It has not yet been possible to demonstrate inducible expression of the IFN-ß template *in vitro*. Nonetheless, these experiments indicate that cis-acting domains within the

Figure 7. *In Vitro* Transcription of the IFN-ß Template. Transcription reactions (20-25 µl) contained 8 mM $MgCl_2$, 200 µM ribonucleoside triphosphates, 5 mM creatine phosphate, 2 ng of creatine phosphokinase, 25 units of RNase inhibitor (Boehringer Mannheim), 1 µg of poly(dI-dC), and 250 ng of supercoiled pUCß (41) or pwtSV plasmid. After 1 h at 30°C, the reactions were terminated and RNA isolated by the guanidinium isothiocyanate procedure (38). Correctly initiated transcripts (indicated by arrows) were measured by S1 mapping using the 922 bp BglII-EcoRI IFN-ß probe or the 423 bp AvaII-KpnI SV40 early region probe. Competition reactions with the 44 bp IRE fragment were performed as described under "Materials and Methods". M, pAT153 digested with HaeIII; $P_ß$, IFN-ß probe; Ps, SV40 early region probe; lanes 1-3, pUCß DNA template transcribed in the presence of 0,20, and 60 ng of IRE fragment; lanes 4-6, pwtSV transcribed in the presence of 0,20, and 60 ng of IRE fragment.


M
Pβ
Ps
1
2
3
4
5
6
← IFN-β
587
434
339
184
123/124
← SV40
*922
EcoR1
BglII
5'
IFN-β
3'
*604
*423
AvaII
KpnI
5'
SV40 Early
3'
*136/137

IRE interact with transcription regulatory proteins that are capable of mediating IFN-ß transcription *in vitro*.

DISCUSSION

The virus-regulated expression of the interferon-ß promoter provides a model of inducible cell-specific gene expression. To investigate the protein-DNA interactions that contribute to IFN-ß regulation, we have used synthetic oligonucleotides and the gel retardation assay to probe for the presence of IFN-ß transcription factors in uninduced and IFN-primed Sendai virus-induced HeLa cell nuclear extracts. A number of proteins capable of forming stable complexes with the IRE (-79 to -36), and more specifically with the P1 domain (-79 to -64), were detected in both uninduced and induced extracts. Since the P1 element comprises one of two sequence domains defined by deletion analysis as the minimal inducibility element (26,28,31), proteins interacting with this region may represent important components in the control of IFN-ß transcriptional initiation.

The IRE and P1 complexes generated with uninduced and induced extracts are quantitatively very similar. Our findings are consistent with previous reports demonstrating that inhibitors of protein synthesis do not preclude IFN-ß gene activation (40), suggesting that induction does not require de novo synthesis of proteins, but instead may involve biochemical modification of transcription factors present in uninduced cells. The DNA-binding and transcriptional domains may occupy distinct sites on the protein; biochemical modification of the functional domain during induction may therefore not affect the binding affinity of the protein nor the stability of its interaction with the template *in vitro*. For example, alterations in the functional domain of the yeast GCN4 activator protein abolish transcription without affecting DNA-binding activity (42). Sorger et al. propose that phosphorylation of the yeast heat shock element binding protein is required for activation of heat shock gene transcription, but not for factor binding to

DNA (43). Alternatively, the activity of positive factors could be modulated by a non-DNA-binding protein via direct protein-protein interactions, either before or after such factors have associated with the template, as observed with S-300 II and COUP in the chick ovalbumin promoter (13). It is also possible that trans-acting factors may be present but sequestered from the promoter or a modifying protein in uninduced cells. An induction signal would cause release of control by compartmentalization, thereby permitting the factor(s) to interact with appropriate targets. A soluble system and short oligonucleotides would relieve the constraints imposed on protein-DNA interactions by chromatin structure.

Support for these possibilities comes from mutational analysis and genomic footprinting of the IFN-ß promoter in nuclei isolated from induced and uninduced MG63 cells (35). These studies demonstrated that factors in uninduced cell extracts interact with IFN-ß sequences between -68 and -38, as well as with sequences -94 to -167. Following virus induction these factors dissociated and were replaced by proteins binding between -77 and -64, precisely within the P1 domain. More recently, band retention and footprinting analysis has demonstrated factor binding to the positive regulatory domains PRDI (-77 to -64) and PRDII (-64 to -55) of the IFN-ß promoter (36). Although binding to PRDII was similar in uninduced and poly(I)-poly(C)-induced MG63 extracts, two DNA-binding activities interacted with PRDI; one complex was present in both uninduced and induced extracts, while a lower molecular weight complex was present only in induced extracts. Our results differ in two respects from those of Maniatis' group: first similar binding was obtained in both control and virus-infected nuclear extracts; and second, binding was restricted to the P1 element and was not reduced by oligonucleotides containing either the downstream (P4, -65 to

-30) or upstream (P6, -132 to -92) regulatory sequences. Differences in factor concentration, protein stability, or preparation of the HeLa and MG63 extracts may account for the observed variability. Proteolytic cleavage of the factor binding to PRDI may be a requisite modification for inducibility (36) or could be the result of alterations of DNA-binding factors which occur during extract preparation, as described recently for extracts prepared from B lymphoid cells (44).

The IFN-ß DNA sequences between -77 and -37 have been characterized in murine C127 cells as a negatively regulated inducible enhancer composed of multiple domains that contribute to positive and negative control (26,28,31). In other cell lines, however, a more extensive 5' boundary (to -115) appears to be required for virus inducibility (27). Fujita et al. (29) defined the borders of the element important for induction more precisely by demonstrating that synthetic tandemly duplicated copies of the hexameric sequence -AAGTGA- (present as different permutations seven times throughout the IFN-ß promoter between sequences -110 and -65) incrementally restored viral inducibility to homologous and heterologous promoters in mouse L929 cells. Mono- and dihexamers were not sufficient for inducibility, but trihexamers and tetrahexamers displayed 4- and 20-fold inducibility, respectively. In the present study, we show that the tetrahexamer of AAGTGA competed effectively for P1 binding activity, while the non-inducible tetrahexamer of the type AAAGGA competed about 5-fold less efficiently for P1 binding. The P1 domain contains two copies of this reiterated hexamer; interestingly, duplication of Fujita's hexamer (i.e. AAGTGAAAGTGA) regenerates an element that resembles part of the TC-II factor binding motif (underlined) of the SV40 enhancer (45). Kuhl et al. showed that chimeric plasmids containing multimers of the hexanucleotide -GAAAGT- silenced the SV40

and cytomegalovirus enhancers and rendered a truncated ß-globin promoter inducible by virus (30). This sequence bears strong homology to factor binding domains found within several regulated viral and cellular enhancers (28).

The P5 domain (-94 to -78) located immediately adjacent to P1 also competed for P1 binding activity (Fig. 5). We have observed that multimers of P5 confer inducibility upon a truncated SV40 promoter (data not shown) when assayed in a transient CAT expression system (41). The synergistic effect upon induction observed when identical synthetic DNA elements are arranged in tandem (29) may thus mimic the effect produced *in vivo* by the juxtaposition of the P5 and P1 domains, and may be due to a cooperative interaction generated by multiple binding of the same factor to adjacent repeats.

In support of this idea, Porter et al. (46) suggested that the motif -GGAAA- plays an important role in response to environmental signals based on 1) studies with the IFN-responsive 6-16 gene, 2) the conservation of this pentamer within the 5'-flanking region of many inducible genes, and 3) its proximity within inducible promoters, to other DNA sequences implicated in protein binding (46). This element is present at multiple positions in the IRE between sequences -91 and -59. Regardless of whether they are viewed as distinct binding domains recognizing different proteins or overlapping sites for the same protein, it appears that these reiterated sequence motifs bind multiple factors.

The P2 (PRDII) domain was not an effective competitor for IRE factor binding in HeLa extracts, even though a dimer of PRDII was able to bind specific proteins from uninduced and induced HeLa extracts (data not shown). These results are in marked contrast to results with myeloid cell extracts; in these extracts P2 competes for inducible IRE factor binding and interestingly,

the human immunodeficiency virus-1 enhancer (19) also competes for the IRE factors (Hiscott et al. manuscript in preparation). These experiments suggest that IFN-ß gene regulation may be partly controlled by distinct NF-κB-like proteins in epithelioid and myeloid cells.

The functional role of IRE sequences in the activation of IFN-ß transcription has been demonstrated *in vivo* in many studies (26-29, 31). In the present study an *in vitro* transcription competition assay (47) was used to monitor the involvement of IRE binding proteins in the transcription of an IFN-ß template. Increasing amounts of IRE fragment in the transcription reaction resulted in a significant decrease in the amount of IFN-ß mRNA produced *in vitro*. In control reactions pUC8 DNA did not affect IFN-ß transcription, nor did the IRE inhibit transcription from the SV40 early promoter. These experiments suggest that it may be possible to modulate IFN-ß gene expression *in vitro* by addition of either cis-acting DNA fragments or purified transcription regulatory proteins.

CHAPTER 6

ANALYSIS OF PROTEIN-DNA INTERACTIONS WITHIN THE PRDII DOMAIN OF THE INTERFERON-ß PROMOTER REVEALS DISTINCT CONSTITUTIVE AND INDUCIBLE COMPLEXES

PREFACE

The interferon regulatory element (IRE) contains two positive cis-acting domains, PRDI and PRDII (Goodbourn, S. et al. 1988. Proc. Natl. Acad. Sci. USA 85: 1447-1451). We have identified a constitutive DNA-binding protein(s) in Hela nuclear extracts that interacts specifically with the PRDI domain. This protein(s) also appears to interact with sequences present immediately upstream of PRDI and may be related to transcription factors that have been shown to bind different hexameric motifs present within this region (Harada, H. et al. 1989. Cell 58: 729-739). Interestingly, the studies in chapter 5 failed to detect binding to the PRDII domain. However, subsequent experiments conducted by myself and others in the lab demonstrated that PRDII does indeed bind inducible trans-acting factors (Hiscott, J. et al. 1989. J. Virol. 63: 2557-2566). Extensive nucleotide homology between the PRDII domain and the NF-κB recognition site suggests that NF-κB may be involved in the regulation of the IFN-ß promoter. To investigate the possibility that in addition to NF-κB, multiple factors with related sequence specificity bind to PRDII, we examined binding in various cell types after treatment with different inducers. Protein-DNA interactions were monitored by EMSA and UV cross-linking analysis. The latter procedure permits resolution of covalently linked protein-DNA complexes on denaturing SDS-polyacrylamide gels.

ABSTRACT

The human interferon-ß gene (IFN-ß) is transcriptionally activated in a variety of cell types by synthetic double-stranded RNA (poly I:C) and viruses. Inducibility of the IFN-ß promoter is in part mediated by a short decameric element, designated the PRDII domain (-55 to -64), which is the target for the transcription factor NF-κB, and potentially a number of related DNA-binding proteins with similar sequence specificity. In the present study, we have examined factor binding to the PRDII domain in three distinct cell lines (HeLa, U937, Jurkat) following treatment with known inducers of IFN-ß transcription and NF-κB binding activity. UV cross-linking analysis has revealed that all 3 cell types express two inducible DNA-binding proteins of 58 kD and 69 kD and one constitutive protein of 48 kD. Two distinct proteins of 35 kD and 40 kD were found to bind constitutively to PRDII in U937 and Jurkat cells. In HeLa cells, binding of the 35 kD was inducible, while binding of the 40 kD protein was not detected. Competition experiments indicate that all proteins detected in virus-induced HeLa extracts are specific to PRDII, and suggest that multiple factors may interact with this element to regulate IFN-ß gene transcription.

INTRODUCTION

The human interferon-ß regulatory element (IRE) is comprised of two positive cis-acting elements, PRDI and PRDII that mediate inducibility of the structural gene (1,2,3). A single copy of either element is insufficient to confer activity to a linked promoter, however, each element can function individually when multimerized in tandem (1,2,4-6). Several characteristics are unique to PRDI, including the ability to silence the SV40 enhancer when positioned between a basal promoter and the 72 bp repeats (1,2). Furthermore, PRDI is inducible by type I and II interferons (1), a property that is likely attributable to the high degree of sequence homology between PRDI and the interferon-stimulated response element (ISRE) found upstream of several IFN-stimulated genes (7-10).

The PRDII region is unrelated to PRDI, but shares 80% nucleotide homology with the binding site for the transcription factor NF-κB (11, reviewed in ref. 12). Inducible binding of an NF-κB-like factor to PRDII has been independently reported by several groups (5,6,13,14). Binding can be stimulated in a variety of cell types by PMA (4,14) as well as potent inducers of interferon-ß transcription such as Sendai virus (4,13,14) and synthetic double-stranded RNA (polyI:C) (4,5,14). Reagents such as deoxycholate and GTP which stimulate NF-κB binding to other promoters *in vitro* (15,16), have an analogous effect on binding to PRDII (4,5,14). In addition, the NF-κB and PRDII motifs are functionally interchangeable *in vivo* (4,13,14). The activity of hybrid CAT plasmids containing multimerized HIV-κB and PRDII elements linked to an enhancerless SV40 promoter is 3-4 fold inducible by virus in HeLa cells and 10-20 fold inducible by PMA in Jurkat cells (4). CAT activity of similar constructs containing one or two copies of the IRE can also be induced by PMA in Jurkat cells (13).

The activity of the interferon-ß promoter *in vivo* depends on the cell line and/or the type of inducer (17,18). Multimers of PRDI or PRDII are highly virus inducible in murine L929 cells, but mainly constitutive and only weakly or moderately inducible in murine C127 and human 293 and HeLa cells (1, 4-6). At the cellular level, this variability in promoter activity presumably reflects quantitative or qualitative differences in the transcription factors present. Multiple factors may differentially modulate PRDII activity in different cells or in response to different inducers, given that related motifs identified in other viral and cellular genes (19-30) bind distinct proteins (23,28,29,31-34). For example, H2TF and NF-κB bind with high affinity to the same sequence in the MHC class I ($H2K^b$) promoter (29).

To test the possibility that the PRDII domain binds distinct regulatory proteins, extracts from three different cell lines (HeLa, U937, Jurkat) treated with various inducers (PMA, virus, polyI:C) were examined by gel retardation analysis and UV cross-linking. Analysis of protein-DNA complexes formed with a BUdR substituted probe indicates that a number of distinct proteins can interact with the PRDII domain. In all cell extracts two proteins of 69 kD and 58 kD were bound to the P2 oligonucleotide only after induction, while a third protein of 48 kD was bound in uninduced and induced extracts; the concentration of this 48 kD protein was diminished in polyI:C-treated samples. Cell-specific differences were also noted in the UV cross-linking profiles obtained with HeLa versus U937 and Jurkat extracts. In addition, we show that an NF-κB-like binding activity detected in PMA-induced Jurkat cell extracts can recognize the PRDII domain in the context of the natural IFN-ß promoter.

MATERIALS AND METHODS

Cells and Cell Culture:

HeLa, U937 and Jurkat cells were grown in suspension to a final density of 5×10^5 cells/ml in RPMI supplemented with 10% fetal calf serum and antibiotics. Inductions were carried out for 4-6 hours at 37°C using various treatments: PMA (50 ng/ml), Sendai virus (400 HAU/ml) and poly-I:poly-C (100 μg/ml). Interferon primed cultures were treated for 8 hours with 500 IU/ml of Intron A (rIFN-α2; Schering) prior to induction. After induction cells were washed twice with >50 volumes of ice cold PBS, pelleted and used immediately to make nuclear extracts, or frozen in liquid N_2 and stored at -70°C before preparation of whole cell extracts.

Cell Extracts and Fractionation:

Nuclear and whole cell extracts (WCE) were prepared as previously described (35,36). DNA-binding factors from PMA-induced Jurkat cells were partially fractionated by DEAE-Sephacel and heparin-Sepharose chromatography. Thirty-five milligrams of total cell protein was loaded onto a 5 ml DEAE-Sephacel column and eluted in a stepwise manner with two column volumes of WCE buffer (20 mM Hepes, pH 7.9, 0.2 mM EGTA, 0.2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 10% glycerol, 10 mM sodium molybdate, 1 mM DTT, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM PMSF) containing 0.1 M KCl and 0.3 M KCl. The 0.3 M KCl fraction was collected and dialyzed against two changes of 0.1 M KCl WCE buffer (500 ml). The dialysate (15 mg) was then applied to a 3 ml heparin-Sepharose column and eluted stepwise with two column volumes of WCE buffer containing 0.1 M KCl, 0.3 M KCl or 0.5 M KCl. The fractions collected were desalted on NAP-25 columns (P.L. Biochemicals) equilibrated

with WCE buffer (0.1 M KCl) and analyzed for DNA-binding activity by the gel retardation assay (37). All protein concentrations were estimated using the Bio Rad Protein assay. Extracts and fractions were prepared at 4°C, frozen in liquid N_2 and stored in aliquots at -70°C.

Preparation of Labeled Fragments:

The sequences of the human interferon-ß and HIV-1 enhancer oligonucleotides used in this study are outlined below. Preparation of the ß96 interferon-ß promoter restriction fragment has been described elsewhere (38). Oligonucleotides were synthesized on a Pharmacia Gene Assembler and purified by electrophoresis on denaturing polyacrylamide gels. BUdR(5-bromo-deoxyuridine) incorporated oligonucleotides were generated by substituting selected thymidine residues in the nucleotide sequence (shown below) with 5-bromo-deoxyuridine CED phosphoramidite (P.L. Biochemicals):

1)	P2(BUdR=B)	5'-GGGAAABBCCGGGAAABBCC-3'
		3'-CCCTBTAAGGCCCTBTAAGG-5'
2)	P2	5'-GGGAAATTCCGGGAAATTCC-3'
		3'-CCCTTTAAGGCCCTTTAAGG-5'
3)	HIV	5'-AGGGACTTTCCGCTGGGACTTTCC-3'
		3'-TCCCTGAAAGGCGACCCTGAAAGG-5'
4)	HIV(mut)	5'-ACATGGTTTCCGCTGCATGGTTTCC-3'
		3'-TGTACCAAAGGCGACGTACCAAAGG-5'
5)	TH	5'-GATCCAAGTGAAAGTGAAAGTGAAAGTGAG-3'
		3'-GTTCACTTTCACTTTCACTTTCACTCCTAG-5'

6) TH(mut) 5'-GATCCAAAGGAAAAGGAAAAGGAAAAGGAG-3'

3'-GTTTCCTTTTCCTTTTCCTTTTCCTCCTAG-5'

7) IRE 5'-GGGAGAAGTGAAAGTGGGAAATTCCTCTGAATAGAGAGAGGACC-3'

3'-CCCTCTTCACTTTCACCCTTTAAGGAGACTTATCTCTCTCCTGG-5'

Complementary oligonucleotide strands were heated at 70°C for 5 minutes in 20 µl of TE and then annealed by slowly cooling to room temperature. Double-stranded oligonucleotides were purified by gel electrophoresis on native polyacrylamide gels. Probes were generated by 5' end-labeling DNA fragments with [γ-^{32}P]ATP (ICN, 3888 Ci/mmole) and polynucleotide kinase (P.L. Biotechnology) (39). Unincorporated nucleoside triphosphates were removed by Sephadex G-50 gel filtration (39).

Gel Retardation and UV Cross-Linking Analysis:

Gel retardation assays were carried out as previously described (40). For UV cross-linking analysis gel retardation assays were performed using a double-stranded BUdR-substituted γ^{32}P-P2 oligonucleotide probe. Protein-DNA complexes were resolved by electrophoresis through a 4% native polyacrylamide gel. The complexes were cross-linked *in situ* by resting the wet gel directly on a Spectroline transilluminator (model TR-302, 302 nm) and exposing it to UV light for varying lengths of time (as indicated in the figure legends) at 4°C. The region corresponding to the shifted complexes was localized by autoradiography, excised with a scalpel and soaked in 30 ml SDS sample buffer (2% v/v 2-mercaptoethanol, 0.1% SDS, 20% glycerol, 10 µg/ml bromophenol blue, 62 mM Tris-HCl, pH 6.8) for 5 minutes at 68°C. The SDS sample buffer was aspirated and the gel was rinsed twice in 20 ml of stacking buffer (0.1% SDS, 125 mM Tris-HCl, pH 6.8). The gel slice was sandwiched

between two electrophoresis glass plates and anchored to the top of the plates using a 1% agarose solution (prepared in 0.1% SDS, 125 mM Tris-HCl, pH 6.8). A 9% SDS running gel with a 4% SDS stacking gel was cast to within one centimeter of the sandwiched gel slice. The remaining 1% agarose solution was poured around the gel slice to complete the cast. Single tooth combs were inserted into the agarose to form wells into which prestained molecular weight protein standards (Sigma) were loaded. The gel was run at 45 mA until the tracking dye entered the stacking matrix and then electrophoresed at 100-120 V for an additional 12-14 hours. All gels were blotted to Whatman 3MM paper, dried under a vacuum at 60°C and autoradiographed for 1-4 days.

Densitometry:

Relative DNA-binding activities were measured by scanning autoradiograms with an LKB laser densitometer (Ultroscan model 2202); scans were integrated using the LKB 2202 Gelscan software package.

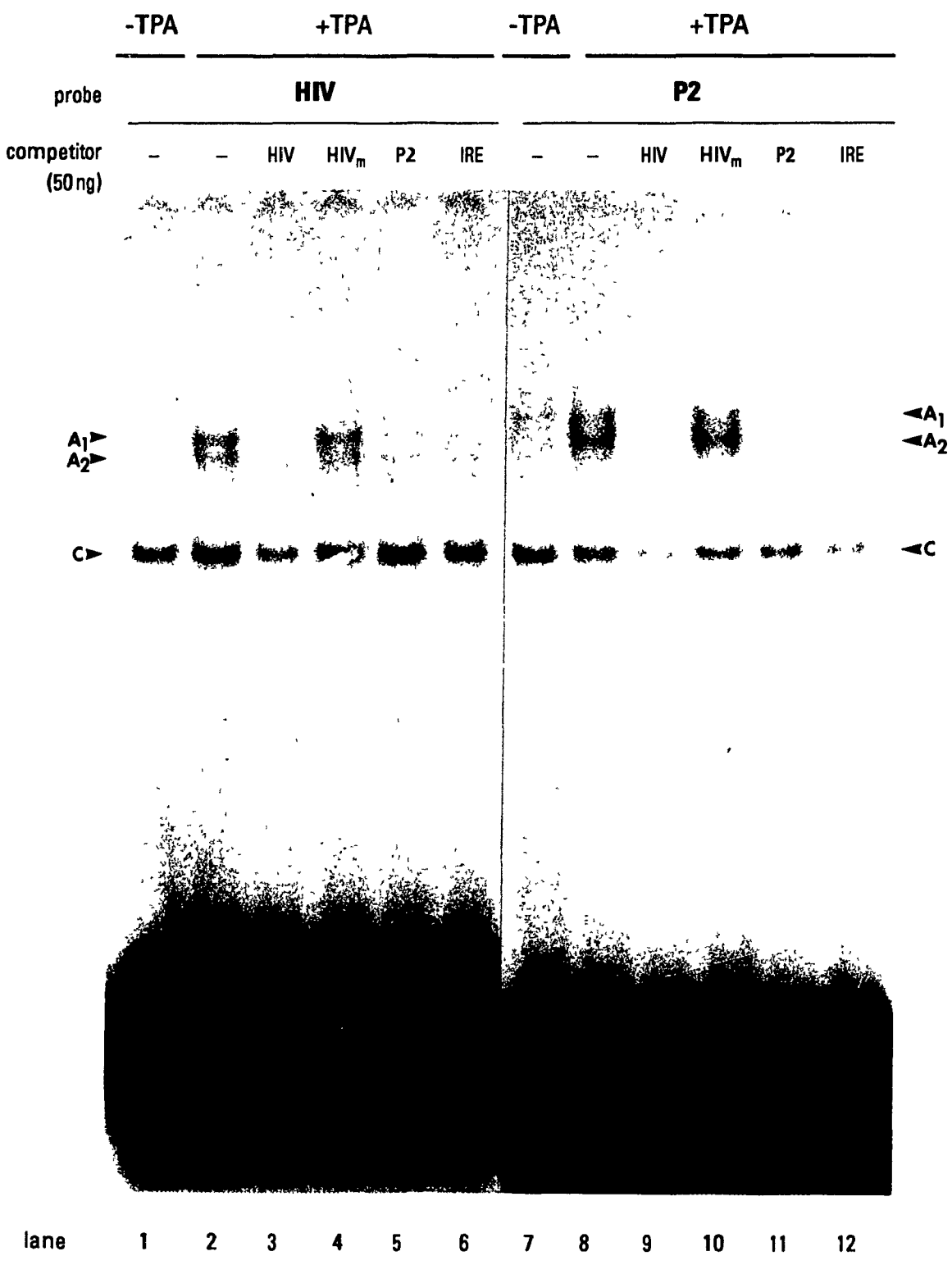
RESULTS

The IFN-ß Promoter Interacts with NF-κB Like Proteins.

Mutational analysis of the IFN-ß promoter has previously shown that the PRDII domain is important for inducible transcription *in vivo* (3). This short motif shares significant homology with the NF-κB recognition sequence (11,12). Inducible binding of an NF-κB-like factor to PRDII domain has been recently reported (5,6,13,14). In the present study several distinct complexes were identified in extracts from PMA(TPA)-treated monocytic U937 cells. Probing with a tandem oligonucleotide dimer of the PRDII domain (P2 oligonucleotide) or an HIV enhancer oligonucleotide, we detected two inducible complexes (A1/A2) which migrated at a discrete doublet in the mobility shift assay (Fig. 1, compare lanes 1 and 2, 7 and 8). A greater amount of complex A1 relative to A2 was detected with the P2 probe in the basal extract (Fig. 1, lane 7). An excess of cold P2, HIV or IRE oligonucleotide, but not a mutated form of the HIV enhancer efficiently inhibited the formation of these complexes (Fig. 1, lanes 3-6,9-12). A third, but faster migrating complex (C) was also identified, although its level in uninduced versus PMA-induced extracts remained unchanged. Complex (C) was not competed by any of the aforementioned competitor templates, and may be the result of non-specific protein-DNA interactions.

The IFN-ß PRDI regulatory domain is juxtaposed to the 5' end of the PRDII domain and extends to the distal boundary of the IRE. Several studies have characterized nuclear factors which bind to PRDI (4,36,40-43). cDNAs encoding these factors (IRF-1, IRF-2) have been recently isolated from λgt11 expression libraries and the corresponding gene products were shown to specifically interact with a tetrahexameric oligonucleotide probe (TH) (42,43). When factor binding to the IRE was examined in Sendai virus-infected U937 nuclear

Figure 1. Competition of PMA(TPA)-inducible Factor Binding in U937 Monocytic Cells. The ^{32}P end-labeled probe (0.5 ng) was incubated with 5 μg of uninduced (lane 1 and 7) or PMA-induced (lanes 2-6 and 8-12) U937 whole cell extract, in the presence of 5 μg of polydI:dC as non-specific competitor DNA and 50 ng of specific competitor oligonucleotide as indicated. The reactions in lanes 1, 2, 7 and 8 were performed in the absence of specific competitor DNA. The sequences of the competitor oligonucleotides are outlined in Materials and Methods and described in the legend to Figure 2. Protein-DNA complexes were analyzed by the gel retardation assay and the positions of the different complexes are indicated by an arrow head.


-TPA
+TPA
-TPA
+TPA
probe
HIV
P2
competitor (50 ng)
–
–
HIV
HIV$_m$
P2
IRE
–
–
HIV
HIV$_m$
P2
IRE
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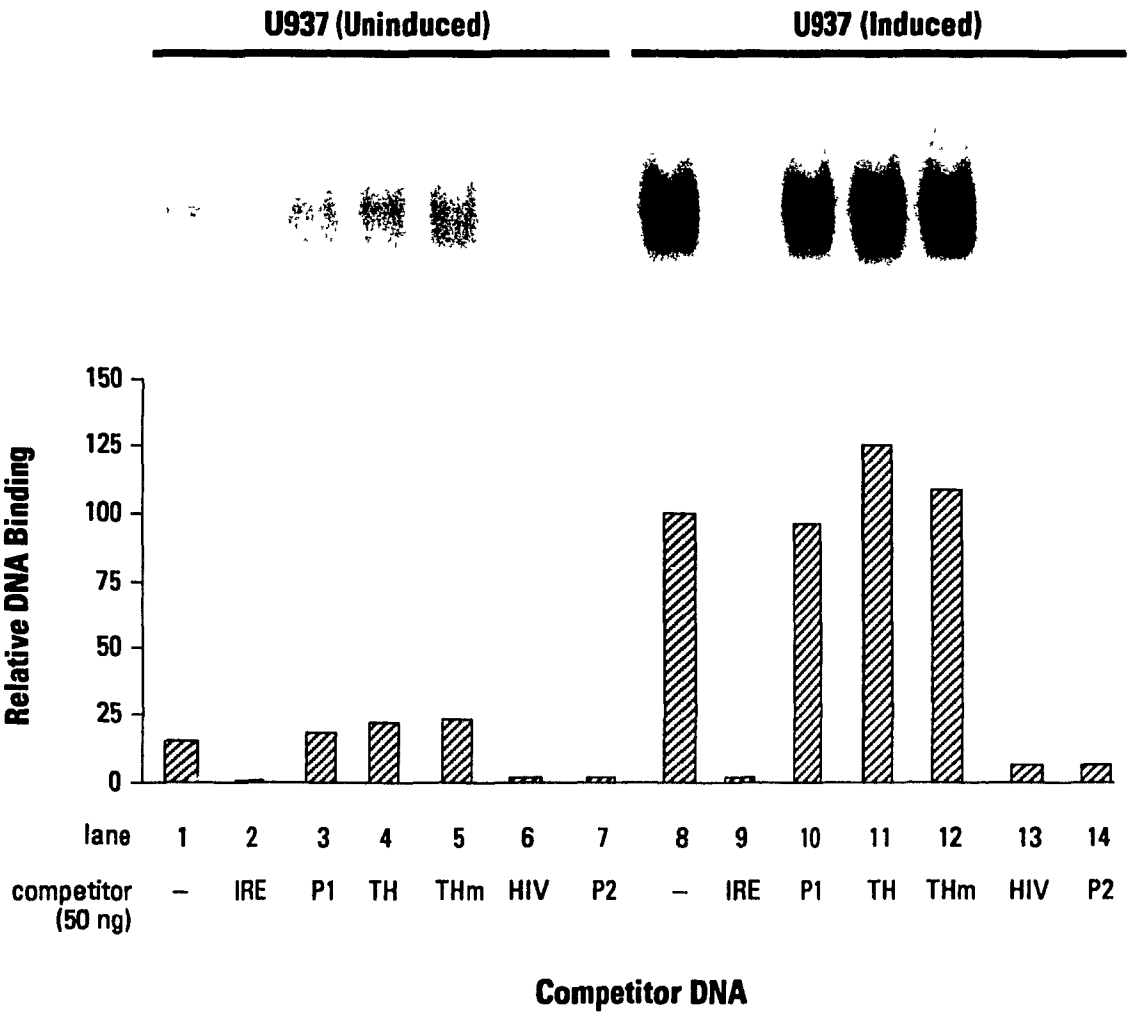
12

extracts, a six fold increase in protein-DNA complex formation was detected as compared to uninduced extracts (Fig. 2, lanes 1 and 8). Competition experiments were performed to further localize the site of interaction. The P1, TH and THm (mutant tetrahexamer) oligonucleotides could reduce binding to the IRE (Fig. 2, lanes 3-5 and 10-12). However, a large excess of cold IRE, P2 or HIV competitor DNA in the reaction mixture inhibited complex formation (Fig. 2, lanes 2, 6, 7, 9, 13 and 14). This clearly indicates that binding occurred within the PRDII domain and could not be attributed to an interaction with IRF-1/IRF-2.

Factor Binding to the Natural IFN-ß Promoter.

PMA-induced whole cell Jurkat extracts were fractionated to enrich for NF-κB-like factors capable of recognizing the PRDII domain in the context of the natural IFN-ß promoter. A combination of DEAE-Sephacel and heparin-Sepharose chromatography permitted detection of specific binding to both the IRE and a longer restriction fragment containing promoter sequences between -40 and -135 (ß96) (Fig. 3, panel A and B). After the initial application to DEAE-Sephacel in low salt buffer (0.1 M KCl), the majority of P2 binding activity eluted within the 0.3 M KCl wash (D.3), however, some activity could be detected in the flow through fraction (Fig. 3A, lanes 2 and 4). The 0.3 M KCl fraction from DEAE-Sephacel was dialysed without loss of binding activity and then applied to a heparin-Sepharose column (Fig. 3A, lanes 5 and 6). Examination of the heparin-Sepharose flow-through and 0.1 M KCl wash revealed that the majority of the activity remained bound to the column (Fig. 3A, lanes 7 and 8). P2 binding activity could be detected in both the 0.5 M KCl (H.5) and 0.3 M KCl (H.3) heparin-Sepharose fractions, although the H.3 fraction was contaminated by proteins associated with complex C (Fig. 3A,

Figure 2. Virus-inducible Factor Binding to the Interferon-ß Regulatory Element. The ^{32}P end-labeled IRE probe (0.1 ng) was mixed with 5 µg of polydI:dC and 10 µg of uninduced (lanes 1-7) or rIFN-α2 primed/Sendai virus-induced U937 nuclear extract (lanes 8-14), in the absence (lanes 1 and 8) or presence (lanes 2-7 and 9-14) of competitor DNA. The type of competitor DNA used is indicated below each lane: IRE, IFN-ß promoter -79 to -35; P1, IFN-ß promoter -79 to -64; TH, a tetramer of the hexameric sequence AAGTGA (2); THm, a permutation of the tetrahexamer, AAAGGA; HIV, an oligonucleotide representing the HIV-1 enhancer element from -105 to -80; P2, a direct duplication of the PRDII sequence in the IFN-ß promoter from -64 to -55. The relative DNA-binding activity (expressed as a pecentage of binding obtained in the induced sample without competitor, lane 7) was quantified by scanning densitometry of the protein-DNA complexes which are indicated by an arrow head).


U937 (Uninduced)
U937 (Induced)
Relative DNA Binding
150
125
100
75
50
25
0
lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
competitor (50 ng) – IRE P1 TH THm HIV P2 – IRE P1 TH THm HIV P2
Competitor DNA

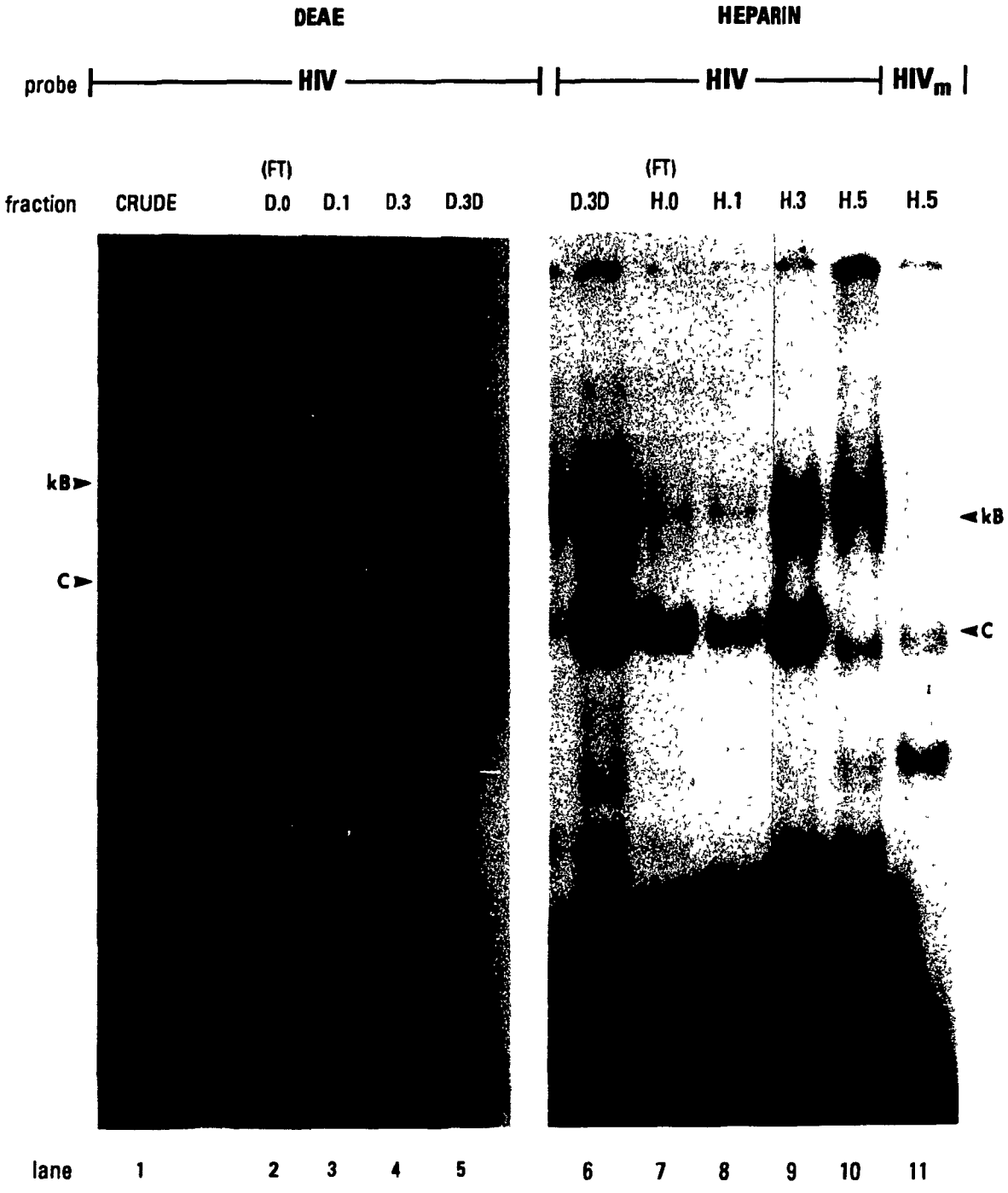
lanes 9 and 10). When the H.5 fraction was assayed for binding, specific complex formation was detected with the HIV, IRE and ß96 probes, but not an HIV mutant probe; in each case the complex migrated at the same position (Fig. 3B, lanes 1,6,9 and Fig. 3A, lane 11). Furthermore, binding was inhibited by either homologous or heterologous cold HIV or IFN-ß competitor template (Fig. 3B). As in previous experiments binding to the HIV enhancer probe could not be inhibited by the HIV mutant oligonucleotide (Fig. 3B, lane 2). Curiously, the P2 protein-DNA complex detected in nuclear or partially fractionated extracts was more diffuse than that detected with more crude whole cell extracts. This is also seen with DNA-affinity purified preparations (4; L. Cohen and J. Hiscott, unpublished observation). Thus with more enriched protein fractions the protein-DNA complex may assume an altered conformation which causes it to migrate differently.

Analysis of PRDII Binding in Different Cell Types.

The IFN-ß gene is transcriptionally activated following treatment with an appropriate inducer. However, the magnitude of the response varies according to the cell line examined and also the type of inducing stimulus (17,18). This variation may reflect quantitative or qualitative differences in the transcription factors that are present in a given cell type. A number of related factors all recognize a common κB consensus motif which is also present in the IFN-ß promoter (11,23,28,29,31-34). Thus, depending on the cellular environment, the inducer, or both, one or more factors may be involved in regulating IFN-ß transcription. To examine this possibility, experiments were performed to compare P2 binding activity in HeLa, U937 and Jurkat extracts (Fig. 4). Cells were induced with either PMA, polyI:C, Sendai virus or a combination thereof. The mobility shift profile was

Figure 3. Fractionation of an NF-κB-like Binding Activity in PMA-induced Jurkat Extracts. (A) Thirty-five milligrams of PMA-induced Jurkat whole cell extract was fractionated by DEAE-Sephacel and heparin-Sepharose chromatography as outlined in Materials and Methods. Bound proteins were eluted from each column in a stepwise manner with two column volumes of WCE buffer containing increasing concentrations of KCl. Left, DEAE-Sephacel fractions: crude extract, lane 1; flow-through (D.0), lane 2; 0.1 M KCl wash (D.1), lane 3; 0.3 M KCl wash (D.3), lane 4; D.3 fraction dialyzed to 0.1 M KCl (D.3D), lanes 5 and 6. Right, heparin-Sepharose fractions: flow-through (H.0), lane 7; 0.1 M KCl wash (H.1), lane 8; 0.3 M KCl wash (H.3), lane 9; 0.5 M KCl wash (H.5), lanes 10 and 11. DNA- binding activity was monitored by the gel retardation assay (see below) using a ^{32}P end-labeled HIV oligonucleotide probe (lanes 1-10), or a ^{32}P end-labeled mutant HIV (HIVm) oligonucleotide probe (lane 11). The positions of the protein-DNA complexes are indicated by an arrow head. (B) Competition of factor binding to the natural IFN-ß promoter. The ^{32}P end-labeled HIV, IRE and B96 probe (0.5 ng) was incubated with 3 μg of protein from the 0.5 M KCl heparin-Sepharose fraction, in the presence of 5 μg of polydI:dC (lanes 1, 6 and 9) and competed with 50 ng of cold competitor oligonucleotide as indicated below each lane. The relative DNA-binding activity (expressed as a percentage of binding in the absence of competitor DNA) was quantified as described in Figure 2.

A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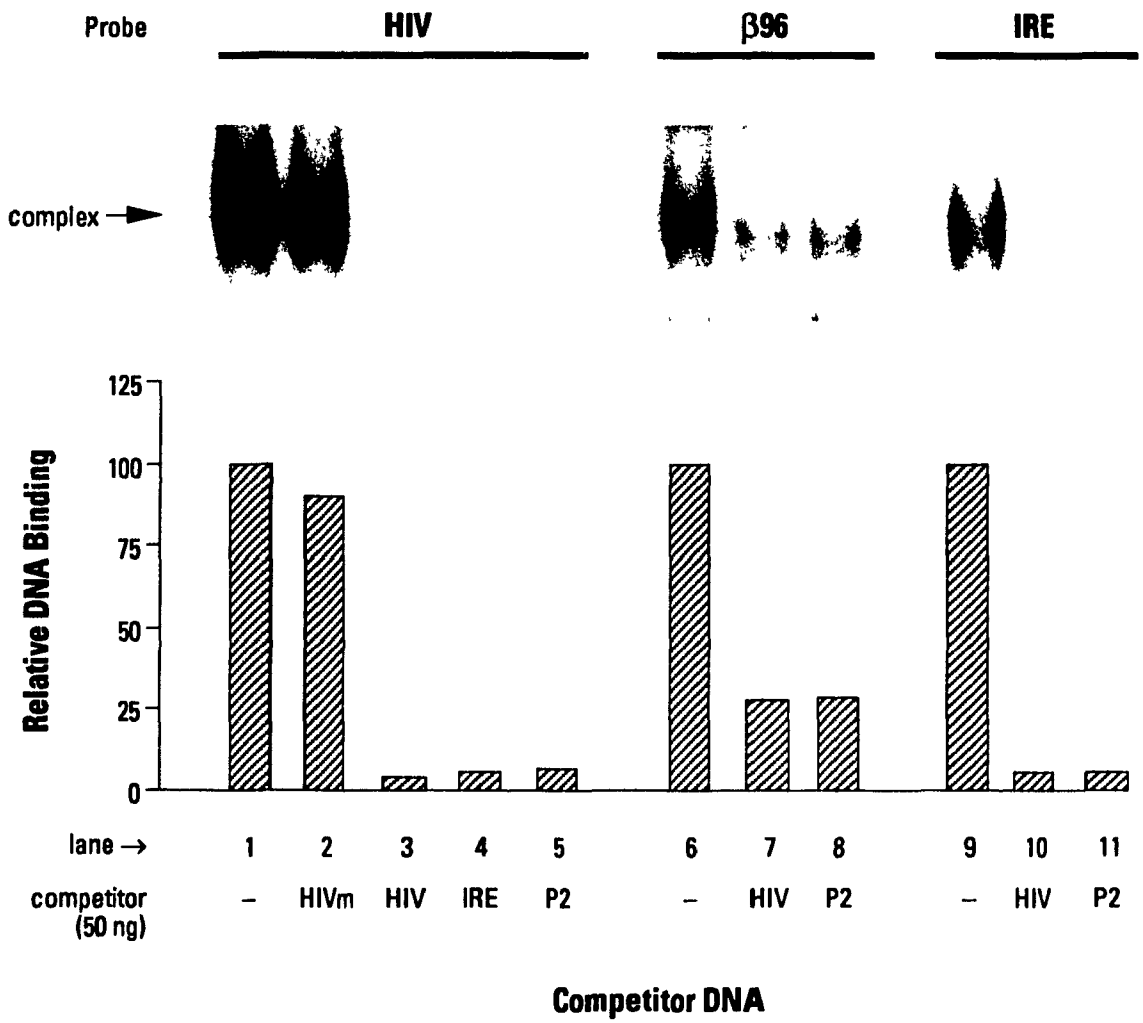
Probe
HIV
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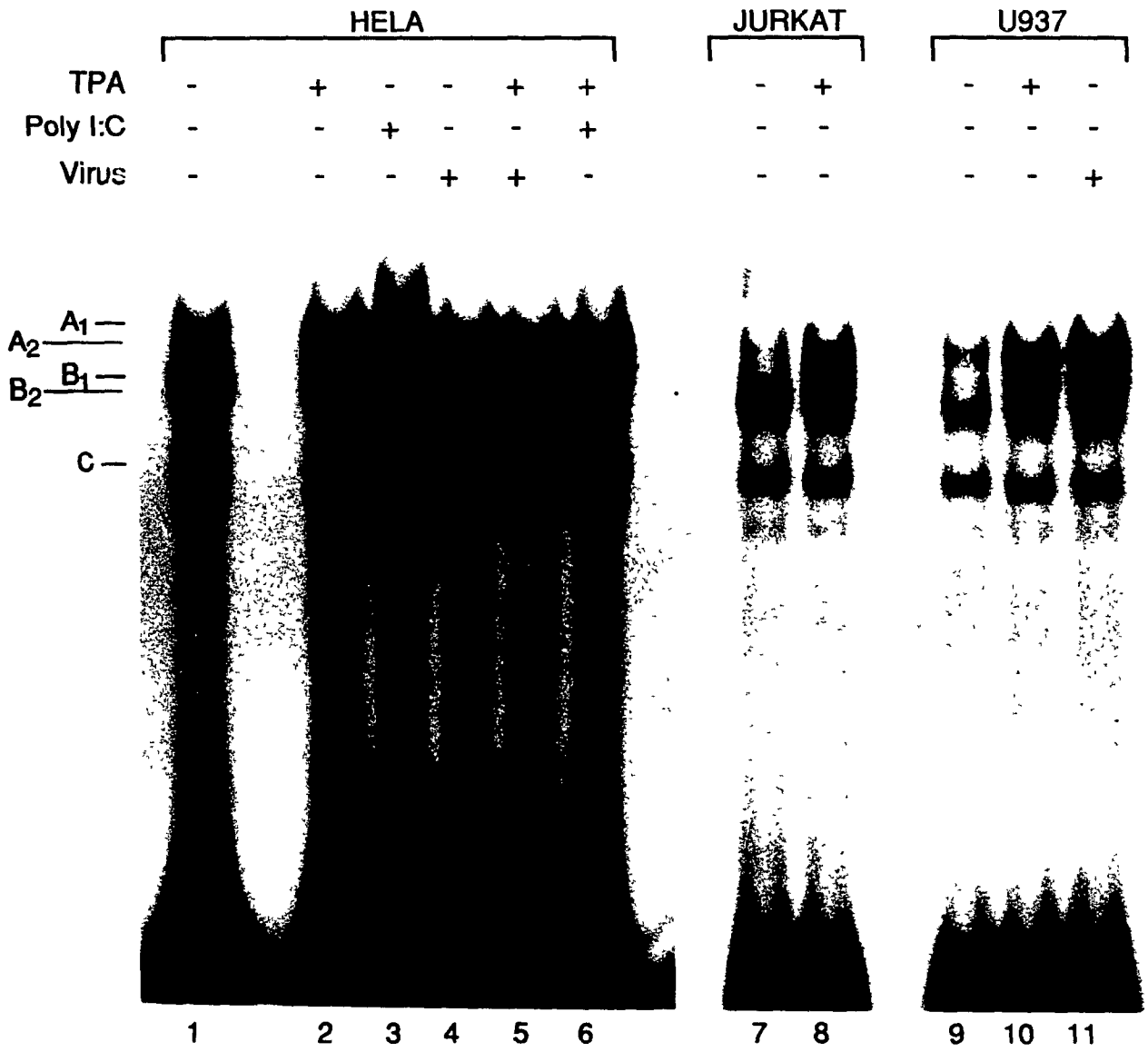
0
lane →
1 2 3 4 5 6 7 8 9 10 11
competitor (50 ng)
– HIVm HIV IRE P2 – HIV P2 – HIV P2
Competitor DNA

essentially the same in all cases, regardless of cell type or treatment. Virus, polyI:C and PMA all induced the formation of complex A1 and A2. Co-induction of cells with PMA/polyI:C or PMA/virus had no significant effect on the level of A1 or A2 binding, indicating that these treatments do not act synergistically (Fig. 4, lanes 5 and 6). The only notable difference between this and earlier experiments was the appearance of a faint doublet (B1/B2) which migrated immediately below the induced doublet (A1/A2) (compare Figures, 1 and 4). It is unclear why the B1/B2 complex was not previously detected, but may be due to differences in extract preparation, gel resolution or specific activity of the probe. Complex B1/B2 was present in all uninduced and PMA or virus-induced cells, but was less prominent in the polyI:C-treated extracts (Fig. 4, lanes 3 and 6). In addition, the decrease in the amount of complex B1/B2 was accompanied by the induction of a band that co-migrated with complex C. Taken together, these experiments conclusively demonstrate that some of the factors which bind to the PRDII domain are not cell-specific and can be induced by a repertoire of biologically and biochemically active agents.

UV Cross-Linking to the PRDII Domain.

To further characterize the factors interacting with PRDII, extracts were examined by UV cross-linking (44). Protein-DNA complexes detected in the gel retardation assay with a BUdR substituted P2 probe were cross-linked *in situ*, and analyzed on SDS-polyacrylamide gels (see Methods and Materials). In calculating and discussing the sizes of the proteins identified, we have subtracted the molecular weight of the P2-BUdR probe (13,200 kD) since samples could not be treated with DNAase prior to electrophoresis. To ensure that cross-linking occurred in a linear fashion, the amount of

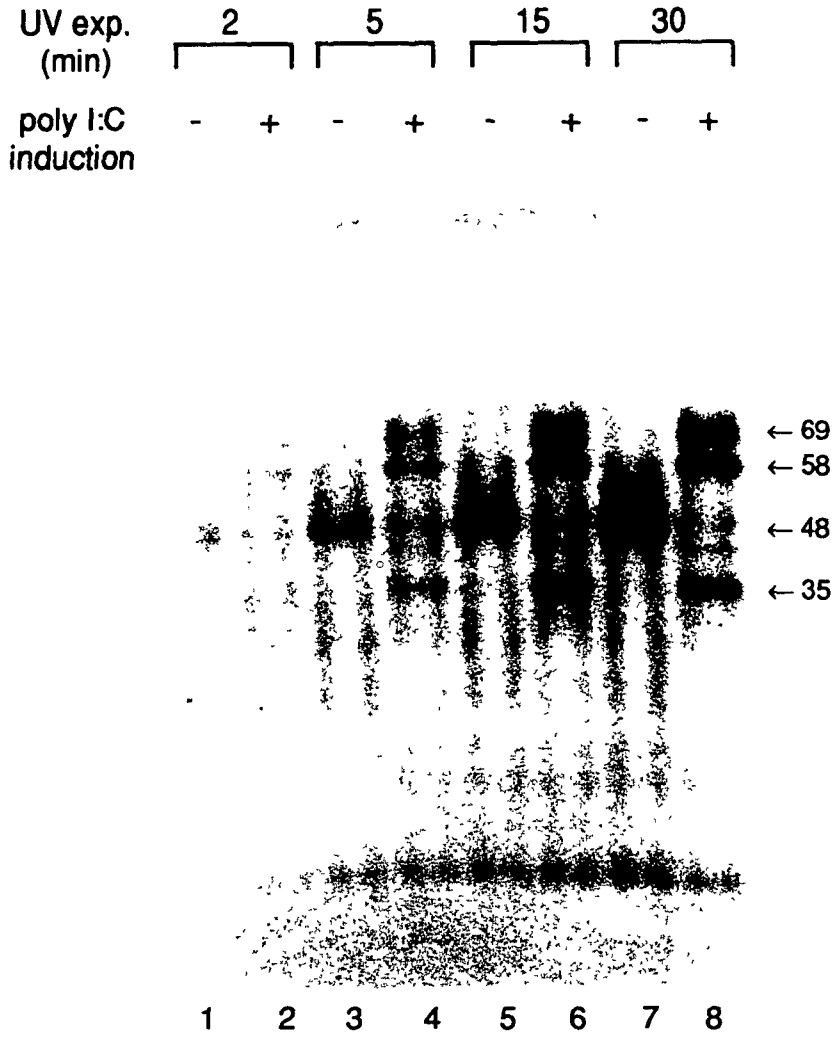
Figure 4. A Comparison of PRDII Factor Binding in a Variety of Cell Types Following Different Treatments. The ^{32}P end-labeled P2 probe (0.5 ng) was incubated with 10 μg of whole cell extract and 3 μg of polydI:dC as non-specific competitor DNA. The cell type and induction protocol is indicated above the gel; a (-) and (+) symbol denotes the absence and presence of inducer, respectively. The specific protein-DNA complexes detected in the gel retardation assay are indicated by a line.


HELA
JURKAT
U937
TPA - + - - + +
Poly I:C - - + - - +
Virus - - - + + -
- + - - - -
- - - - - -
- - - - - +
A1
A2
B1
B2
C
1 2 3 4 5 6 7 8 9 10 11

covalently linked product formed in uninduced and polyI:C-induced HeLa extracts was measured as a function of UV irradiation time. Figure 5 shows that over a 30 min period, the amount of cross-linking was proportional to the length of UV irradiation. Based on this result, an exposure of 25 minutes was chosen for all subsequent studies. This experiment also demonstrates a distinct difference between proteins bound to the P2 probe in HeLa extracts before and after polyI:C induction. Inducible binding of 4 proteins with molecular weights ranging between 35 kD and 70 kD was detected only after treatment with polyI:C (Fig. 5, even lanes). Following induction, however, binding of a single constitutive protein with a molecular weight of 48 kD was decreased relative to the amount detected in uninduced HeLa extracts (Fig. 5, compare lanes 5 and 6).

To determine if the proteins that bound to the P2 probe were cell type or inducer specific, cross-linking was compared in HeLa, Jurkat and U937 extracts before and after induction with either PMA, polyI:C or virus (Fig. 6). Jurkat cells were only treated with PMA in this study because polyI:C and virus do not induce detectable levels of interferon-ß mRNA synthesis in these cells. For a similar reason, the effect of polyI:C treatment on P2 binding was not examined in U937 cells. As shown in Figure 6 the UV cross-linking profiles generated with all three treatments and in all cell types were for the most part similar, although some important differences were detected. Binding of the 58 kD protein and also a larger protein of 69 kD was inducible by PMA in Jurkat cells (Fig. 6, lanes 7 and 8), by PMA and virus in U937 cells (Fig. 6, lanes 9-11), and by PMA, polyI:C and virus in HeLa cells (Fig. 6, lanes 1-6). However, in contrast to what was observed with polyI:C treatment, the amount of 48 kD protein associated with the P2 probe in HeLa cells remained constant and was not diminished by PMA or virus-induction (Fig. 6, compare lane 3 with 2 and

Figure 5. UV Cross-linking of HeLa DNA-binding Factors to PRDII as a Function of Time. The BUdR-substituted ^{32}P end-labeled P2 probe (0.5 ng) was incubated with 10 μg of uninduced (-, odd numbered lanes) or polyI:C-induced (+, even numbered lanes) HeLa whole cell extract, in the presence of 3 μg of poly dI:dC. Protein-DNA complexes were resolved on a 4% native polyacrylamide gel and subjected to UV irradiation for different lengths of time: 2 min., lanes 1 and 2; 5 min., lanes 3 and 4; 15 min, lanes 5 and 6; 30 min., lanes 7 and 8. The complexes were localized by autoradiographing the wet gel and analyzed on a 9% denaturing SDS-polyacrylamide gel as outlined in Materials and Methods. The cross-linked products and their corresponding molecular weights (kD) are indicated by an arrow.

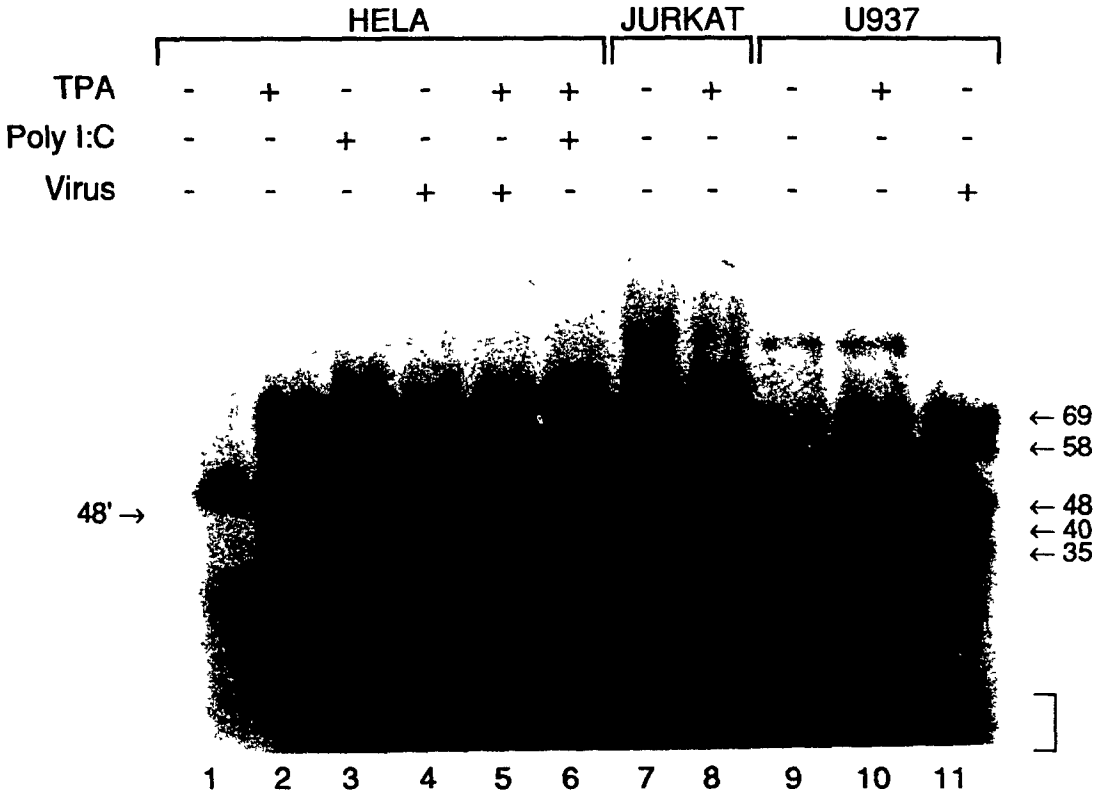

UV exp. (min)
2
5
15
30
poly I:C induction
- + - + - + - +
← 69
← 58
← 48
← 35
1 2 3 4 5 6 7 8

4). In addition, as with the gel retardation assay, the polyI:C-specific decrease in binding of the 48 kD protein coincided with the appearance of a slightly lower molecular weight protein which we designate 48' kD (Fig. 6, lane 3). Both virus and PMA failed to induce binding of the 48' kD protein in any of the cells examined (Fig. 6, lanes 2 and 4). The relationship between the 48 kD and the 48' kD proteins is currently not understood, but these proteins may represent biochemically modified states of the same factor. Alternatively, the decrease in the level of one protein and the corresponding induction of the other could be due to unrelated events and as such the proteins may be distinct.

Some specific differences were also noted among the various cell lines analyzed. Firstly, a protein of 35 kD was inducible in HeLa cells, but bound constitutively to P2 in U937 and Jurkat extracts, regardless of treatment. Secondly, binding of a distinct protein of 40 kD was constitutively identified in U937 and Jurkat cells, but not HeLa cells, even after induction. Thirdly, the concentration of the 48 kD and 35 kD proteins was detectably higher in Jurkat cells than in U937 and HeLa cells. As illustrated in Figure 6, a number of additional proteins of lower molecular weight were also detected in all samples examined (Fig. 6, outlined in brackets). These proteins are more prominent in Jurkat and U937 cells as compared to HeLa cells. However, throughout the course of several experiments, consistency in the binding pattern or level of these proteins was not observed. In light of this, we cannot ascertain their significance and conclude them to be artifactual.

The pattern of protein-DNA complex formation in the mobility shift assay correlated to some extent with the observed cross-linking profiles (compare Fig. 4 and 6). On the basis of this comparison, the induced doublet (complex A1/A2) and the constitutive doublet (complex B1/B2), correspond to the

Figure 6. UV Cross-linking Comparisons of PRDII Binding in HeLa, Jurkat and U937 Cells Following Different Treatments. The BUdR-substituted ^{32}P end-labeled P2 probe (0.5 ng) was incubated with 10 μg of whole cell extract and 3 μg of polydI:dC. Protein-DNA complexes were resolved on a 4% native polyacrylamide gel and cross-linked *in situ* for 25 minutes at 4°C. The profile of cross-linked products was analyzed on a 10% denaturing SDS-polyacrylamide gel as outlined in Materials and Methods. The cell type and induction protocol is indicated above the gel; a (-) and (+) symbol denotes the absence and presence of inducer, respectively. The arrows indicate the different cross-linked products detected and their molecular weight (kD).


HELA
JURKAT
U937
TPA - + - - + + - + - + -
Poly I:C - - + - - + - - - - -
Virus - - - + + - - - - - +
48' →
← 69
← 58
← 48
← 40
← 35
1 2 3 4 5 6 7 8 9 10 11

58/69 kD and 48 kD cross-linked adducts, respectively. To examine this more directly, the two protein-DNA doublets (A1/A2 and B1/B2) formed with virus-induced HeLa extracts were first resolved on native gels and each complex was then independently excised and examined after UV cross-linking. As predicted, analysis of these bands on denaturing gels indicated that complexes B1 and B2 both correspond to the 48 kD protein (Fig. 7, lanes 3 and 4). On the other hand, the pattern obtained with complexes A1 and A2 was more complicated than expected; each complex gave rise to the 35 kD, 48 kD, 58 kD and 69 kD proteins (Fig. 7, lanes 2 and 5). Similar observations have been described by others and would support the notion that a single gel retardation complex can comprise multiple protein-DNA conjugates (45).

In order to assess the specificity of the proteins identified, competition experiments were performed. The 35 kD, 48 kD, 58 kD and 69 kD proteins detected in virus-induced HeLa extracts could be specifically reduced by a cold excess of either the P2 or HIV enhancer oligonucleotides (Fig. 8B, lanes 2 and 3). The HIV mutant oligonucleotide was unable to reduce binding of the 35 kD, 58 kD or 69 kD proteins, but could partially compete for the 48 kD protein (Fig. 8B, lane 4). Also, in the gel retardation assay, the mutant DNA competed for the formation of complexes B1/B2, both of which correspond to the 48 kD protein (Fig. 8A, lane 4). Interestingly, the behavior of the 48 kD protein in this experiment is reminiscent of a group of purine binding proteins that have recently been purified and shown to interact with the sequence GAAANN (46). This short hexanucleotide consensus is conserved in the P2, HIV and HIV mutant oligonucleotide and may account for the ability of all three DNAs to compete in the assay.

Figure 7. UV Cross-linking Analysis of Individual Protein-DNA Complexes in Virus-induced HeLa Cells. The BUdR-substituted ^{32}P end-labeled P2 probe (0.5 ng) was incubated with 10 μg of whole cell extract from Sendai virus infected HeLa cells and 3 μg of polydI:dC. Protein-DNA complexes were resolved on a 4% native polyacrylamide gel and cross-linked *in situ* for 25 minutes at 4°C. Individual complexes were localized by autoradiography and excised from the wet gel prior to analysis on a 10% denaturing SDS-polyacrylamide gel (see Materials and Methods). The complexes detected in the gel retardation assay are shown in a single vertical lane above the gel. The solid lines connect each complex with the lane containing the corresponding cross-linking profile. Lane 1 represents the pattern of cross-linked products obtained when all four gel retardation complexes are simultaneously analyzed. The arrows indicate the different cross-linked products in each complex and their molecular weight (kD).

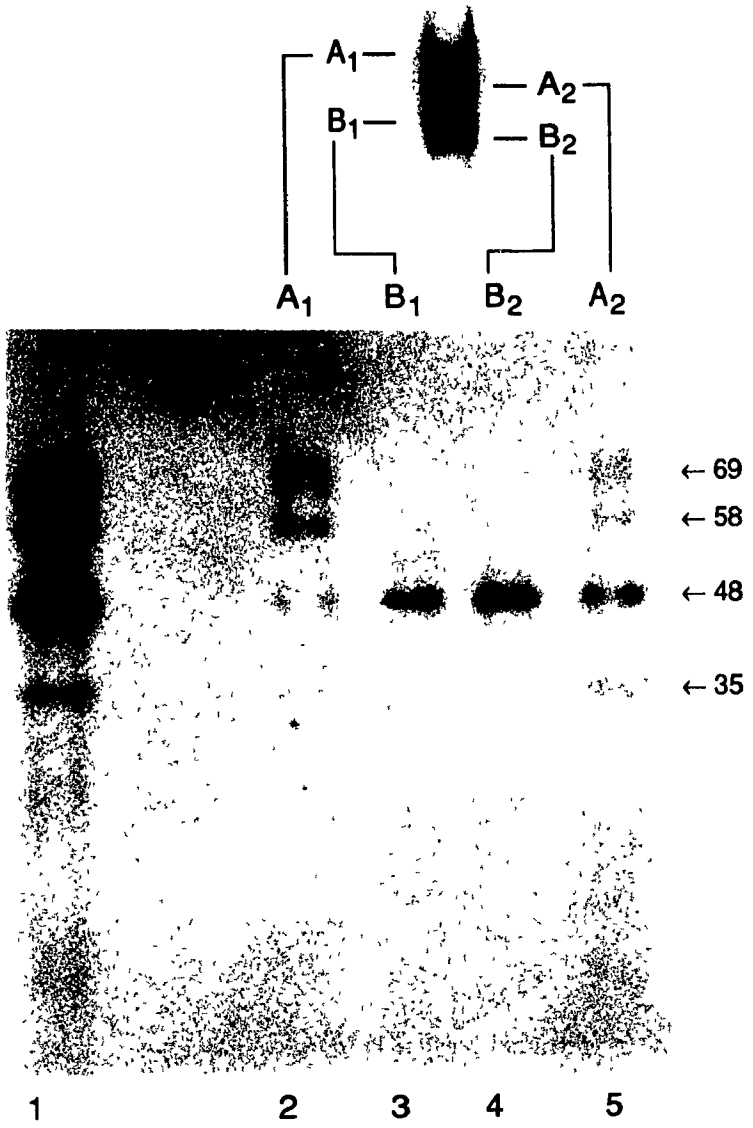
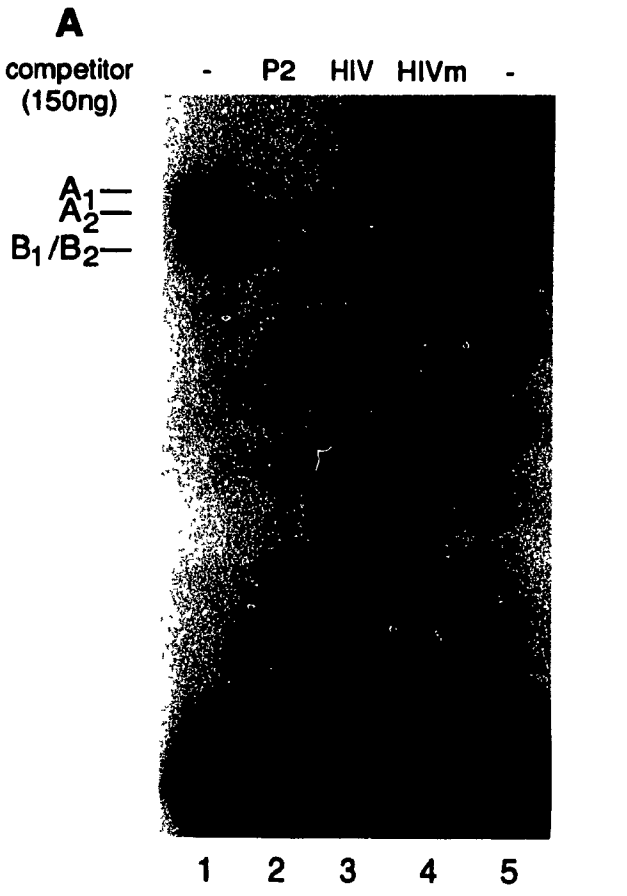

A1
B1
A2
B2
A1
B1
B2
A2
← 69
← 58
← 48
← 35
1
2
3
4
5

Figure 8. Competition of PRDII Binding in Virus-induced HeLa Cells. (A) The BUdR-substituted ^{32}P end-labeled P2 probe (0.5 ng) was incubated with 10 μg of whole cell extract from Sendai virus-infected HeLa cells, in the presence of 3 μg of polydI:dC and 150 ng cold competitor oligonucleotide as indicated above each lane. Protein-DNA complexes were resolved on a 4% native polyacrylamide gel and localized by exposing the wet gel to X-ray film at 4°C for 60 minutes. The individual complexes are indicated by a line. (B) UV cross-linking analysis of the competition assay. The native gel shown in Figure 8A was cross-linked *in situ* at 4°C for 25 minutes and analyzed as described in Materials and Methods. Cross-linked products were resolved on a 9% denaturing SDS-polyacrylamide gel. The competitor oligonucleotides used are shown above each lane. The position and molecular weight (kD) of each cross-linked product is indicated by an arrow.


A
competitor
(150ng)
-
P2
HIV
HIVm
-
A1
A2
B1/B2
1
2
3
4
5
B
competitor
(150ng)
-
P2
HIV
HIVm
-
69
58
48
35
1
2
3
4
5

DISCUSSION

The interferon-ß promoter is composed of at least two regulatory domains, PRDI and PRDII, which bind distinct factors and are essential for maximal induction by virus or synthetic double-stranded RNA (polyI:C) (4-6, 13,14,40,41). Recent work in our laboratory indicates that these domains interact cooperatively; partially purified chromatographic fractions containing PRDI and PRDII binding activity synergistically stimulate transcription of the interferon-ß gene *in vitro* (4). This is consistent with previous genetic studies that demonstrate an interdependence of the two elements *in vivo (1).* The protein-DNA interactions associated with PRDII have been investigated in some detail. Mutations in PRDII which abolish or impair expression of the IFN-ß gene *in vivo,* have the corresponding effect on factor binding to this element *in vitro* (5,14,33,41).

This study confirms previous reports which showed that an NF-κB-like binding activity present in PMA-stimulated Jurkat extracts is capable of recognizing the PRDII domain in the context of the natural IFN-ß promoter (14). The complexes detected using an HIV, IRE or B96 probe were qualitatively similar and migrated with equal mobility in the gel retardation assay. In contrast, a mutated HIV enhancer oligonucleotide was unable to bind the NF-κB-like factor(s) in these extracts. A six fold stimulation of NF-κB-like binding to the IRE was also observed in virus-induced U937 nuclear extracts. Comparable binding to a PRDII dimer oligonucleotide (P2 probe) was seen in U937 cells following treatment with PMA. In these experiments, binding to the P2 probe was efficiently competed by a cold excess of the P2 and HIV oligonucleotides, but not the mutated form of the HIV enhancer.

A number of distinct DNA-binding proteins with NF-κB related sequence specificity have been recently identified. (23,28,39,31-34). Differences in cellular distribution or in promoter utilization of PRDII binding factors, might account for corresponding variations in IFN-ß transcriptional activity among different cell populations or in response to different inducers. In order to assess the possible contribution of multiple PRDII binding proteins to IFN-ß transcription, we have examined PRDII binding in a range of cell types before and after induction. A series of constitutive (B1/B2) and inducible (A1/A2) proteins were found to bind specifically to the PRDII domain as determined by gel retardation analysis. Since significant differences in the pattern of binding obtained with HeLa, U937, and Jurkat cells were not observed, we conclude that similar factors interact with the PRDII domain in a variety of cell types. Furthermore, similarities in the band shifts generated by virus, polyI:C and PMA treatment indicate that these inducers can act via common signal transduction pathways to stimulate binding of several distinct proteins. Protein-DNA cross-linking demonstrated that at least two major inducible proteins of 58 kD and 69 kD interact with PRDII in HeLa, Jurkat and U937 cells. A single protein of 48 kD was found to bind constitutively in all cells examined, although the amount of 48 kD protein that bound to PRDII was slightly decreased in HeLa cells, but only with polyI:C treatment. Induction of the 48' kD protein in HeLa cells was also polyI:C-specific and could not be stimulated by virus or PMA. The 48 kD and 48' kD proteins may be related through modification or they may represent distinct proteins. Further analysis is required to distinguish between these possibilities. In Jurkat and U937 cells two proteins of lower molecular weight, 35 kD and 40 kD, bound constitutively to PRDII, while in HeLa cells only the 35 kD protein was detected and binding was inducible. The appearance of the

35 kD and 40 kD proteins did not consistently coincide with the presence of either the 48 kD, 58 kD or 69 kD proteins in the different cell types examined. Thus, these proteins were probably not generated through modification or proteolytic cleavage of the three higher molecular weight species and may therefore represent distinct factors. Cell specific differences in the uninduced levels of the 35 kD and 40 kD proteins may have some *in vivo* significance. The endogenous IFN-ß gene is transcriptionally more active in U937 cells than in HeLa S3 cells (S.X., data not shown). It is tempting to speculate that a constitutive level of these proteins in U937 cells renders the IFN-ß gene more responsive to induction.

Analysis of the individual gel retardation complexes identified in virus-induced HeLa extracts revealed that the 48 kD protein was present in complexes B1 and B2, while the 35 kD, 48 kD, 58 kD and 69 kD proteins were present in complexes A1 and A2. A similar finding by Lowenthal et al has been previously reported (45). They noted that a single inducible complex from PMA-treated Jurkat cells, identified using a κB element probe from the IL-2Rα gene, resolved into four distinct bands after UV cross-linking. Competition assays in the present study indicate that all four proteins detected interact specifically with PRDII. Interestingly, the P2, HIV and HIV mutant oligonucleotides all contain the sequence GAAANN and were able to compete for binding of the 48 kD protein. It remains to be seen whether this protein is member of a group of purine binding proteins recently shown to interact with similar GA-rich motifs in the regulatory regions of the HSV-1 early genes as well as several IFN- and virus-inducible genes (46). These proteins, designated IEF_{ga}, bind DNA constitutively and purify as a heterogeneous set of bands in the 43 kD to 63 kD range (46). The 48 kD protein identified in this report shares both of these characteristics.

As already mentioned, a family of related DNA-binding proteins has been shown to interact with a range of promoter elements that share nucleotide homology with PRDII (11,19-34). A subset of these NF-κB-like factors is ubiquitous and possesses constitutive DNA-binding activity (23,28,29,31-33). For example, EBP-1 interacts with κB element in the HIV-1 and SV40 enhancers (31,32). DNAase I protection over the HIV enhancer has been obtained with lymphoid and non-lymphoid extracts (31,47). More recently, binding of EBP-1 to the human interferon-ß regulatory element was demonstrated by DNAase I and OP/Cu^+ nuclease footprinting (33). EBP-1 makes similar base specific contacts across one complete turn of the double helix in both the SV40 enhancer κB site and the PRDII domain. Mutations in PRDII which affect basal and inducible transcription *in vivo*, have a parallel effect on EBP-1 binding *in vitro* (33). Purified EBP-1 cross-links as a heterogeneous set of bands with molecular weights between 58 kD and 63 kD (31). In this study we did not detect a constitutive PRDII binding activity within this size range. However, we have used crude extracts in our cross-linking assays in order to minimize the loss of individual binding activities that can occur through fractionation. Thus, the failure to identify EBP-1 in these experiments may simply be a reflection of extract purity. Regardless, this study does suggest that multiple proteins may bind to PRDII to regulate IFN-ß gene transcription. Some of the PRDII bindir ~ activities reported herein are constitutive, while others are clearly inducible. Ultimately, complete functional characterization of these factors in the context of the IFN-ß promoter necessitates their purification and cloning.

CHAPTER 7

GENERAL DISCUSSION

7.1 Transcription Regulation in Eukaryotes

Eukaryotic RNA polymerase II transcription is regulated by cis-acting DNA sequences, promoters and enhancers, present in the 5' flanking regions of the gene. Promoters are typically located within 100-125 nucleotides of the mRNA start site and are required for maintaining accurate and efficient transcription initiation. Enhancers regulate the activity of promoters by either augmenting or suppressing the level of transcription. In general, promoter elements must be positioned within reasonable proximity of each other as well as the mRNA start site in order to operate efficiently, whereas enhancers are capable of modulating gene expression in either orientation when placed several hundred nucleotides upstream of a homologous or heterologous promoter or downstream of the linked gene (1-3).

Structurally, both promoters and enhancers are organized as discrete modules which bind soluble trans-acting factors (4). Promoter modules include the TATA box and upstream elements (UPE's) such as the CAAT box and G-C motifs. The TATA box acts to ensure accurate positioning of the RNA polymerase initiation complex, while the UPEs act to increase the rate of transcription initiation (3, 4). Much of our understanding about the modularity of enhancers stems from studies on viral regulatory elements. Genetic analysis of the prototypical SV40 early enhancer has revealed a hierarchy of enhancer organization (5-7). SV40 viruses that display reduced transcriptional activity because of mutations in specific enhancer motifs, are able to compensate for such mutations by duplicating the remaining functional modules (6). Each module within the SV40 enhancer constitutes an enhanson which is capable of interacting with DNA-binding proteins, but as a single entity lacks activity of its own (6, 7). Three classes of enhansons,

usually 8-12 bp each, have been characterized: class 'A' enhansons exhibit enhancer activity if tandem repeats of the motif are oligomerized; class 'C' enhansons are active following multimerization of the monomer motif; and class 'B' enhansons only generate enhancer activity when juxtaposed to a class 'A' or 'C' motif and then oligomerized. Certain regulatory elements (eg. steroid responsive elements) constitute a fourth class of enhansons which are rather unique in that a single copy of the motif possesses full enhancer activity (7).

It is the spatial and temporal interaction between cis-acting regulatory elements and trans-acting factors that determines the pattern of gene expression within a given cell type (3, 8, 9). Numerous sequence-specific promoter/enhancer binding factors have been purified to homogeneity, and in some cases the corresponding cDNAs have been cloned from bacteriophage expression libraries. A subset of these factors are constitutively active, while others are synthesized de novo or activated from a latent state in response to specific environmental signals or developmental programs (3, 9). Mutational dissection of recombinant transcription factors has delineated the requirements for DNA-binding and transcriptional activation. Defined domains mediating these activities can often be physically dissociated. This has permitted the construction of inter- and intraspecies chimeric transcription factors with DNA-binding and transcriptional activation properties that are characteristic of the parental proteins. DNA-binding moieties identified include the Zn finger motifs, leucine zippers, homeodomains and CTF binding domains. Transcriptional activating moieties include amphipathic α-helices (acid-blobs), glutamine rich domains and proline rich domains (9-13).

Recent attempts to elucidate the mechanisms of transcriptional activation have focused on protein-protein interactions between transcription factors. Accurate RNA polymerase II initiation is dependent on the sequential assembly of multiple protein complexes consisting of at least five general transcription factors (TFIIA, B, C, D, E) and RNA polymerase II (14-17). Interactions between these components and factors binding to upstream regulatory elements may stabilize the formation of active initiation complexes (17-21). The interaction of distantly bound regulatory proteins with the general transcriptional machinery could itself be facilitated through conformational changes in the DNA that permit close juxtaposition of the factors involved. Alternatively, regulatory proteins that bind in close proximity to the transcription apparatus may exert their effects through direct contact or by means of a signal relay system involving proteins bound to adjacent sites (10, 22).

The exact nature of the interaction between transactivating proteins and the initiation complex is still not clear. One possible target of transcriptional regulatory proteins is the TATA factor (TFIID) (18-21, 23-26), although other potential canditates include RNA polymerase II itself (perhaps the COOH-terminus of the large subunit), TFIIA, TFIIB or RNA polymerase II-associated proteins (26). The gene encoding the yeast TATA factor has recently been cloned and efforts to isolate DNAs specifying the mammalian counterpart, as well as other components of the transcription apparatus are underway (27, 28). Once these clones are available, fine point structure-function studies should help define the molecular mechanisms controlling eukaryotic RNA polymerase II transcription.

7.2 Expression of the IFN-ß Promoter

The IFN-ß promoter is appropriately regulated when introduced into heterologous cells on recombinant vectors that are stably or transiently maintained (29-37). We have developed a transient expression system to analyze the transcriptional activation of the IFN-α and IFN-ß promoters in homologous human cells. In this system the intact IFN-ß promoter confers virus inducibility to a CAT reporter gene in a manner that is characteristic of the endogenous promoter. These experiments further demonstrate that in the uninduced state the IFN-α1 and IFN-ß promoters silence the constitutive activity of the SV40 enhancer. Following induction the SV40 enhancer-IFN-ß promoter combination appears to synergistically augment the expression of the linked CAT gene. Suprisingly, in 293 cells the SV40 enhancer also confers a low level of virus inducible transcription to an otherwise inactive IFN-α promoter. This suggests that the factors required for expression of the IFN-α1 gene may exist in 293 cells, but are present at too low a concentration to induce transcription. In this case the SV40 enhancer may act to cooperatively potentiate the interaction of such factors with the IFN-α promoter. Alternatively, the α-factors may be absent in 293 cells and induction may be solely mediated by transcription proteins that interact with the SV40 enhancer.

Using the transient expression system experiments were designed to characterize the nature of the trans-acting proteins interacting with the IFN-ß promoter. These studies identified a negative factor(s) that associates with upstream sequences (-135 to -202) previously found to be involved in negative regulation (38). In contrast, the sequences downstream of -135 were shown to interact with a positive trans-acting factor(s). Depletion of this

factor(s) precluded induction of the IFN-ß promoter and partially inhibited activity of the SV40 early promoter, implying that similar proteins may recognize both templates.

The sequences to which we have localized the interaction of a positive factor(s), encompass the interferon regulatory element (IRE) (39). Site-directed point mutagenesis and deletion analysis revealed that the IRE is composed of two genetically distinct positive domains, PRDI and PRDII, and one negative domain, NRDI (40, 41) (figure 1). The combined activity of these domains confers properties of an inducible enhancer to the IRE (39).

7.3 Functional Analysis of the PRDI Domain

The IFN-ß promoter contains a modular arrangement of sequence motifs. A 13 bp degenerate sequence is reiterated 5 times within 115 nucleotides of the mRNA start site. An equivalent number of related motifs are also present in the 5' flanking region of the IFN-α2 gene (39). One of the 13mer motifs localizes to PRDI (-76 and -64) (39-41) and shares homology with the regulatory elements of several eukaryotic viruses and cellular genes, including: adenovirus 5, 7 E1A (42) , polyoma virus (43), SV40 (7), heat-shock protein 70 (44), and a number of IFN-stimulated genes (45-52). Three 13mer elements are located between -110 and -64 and overlap significantly with 7 copies of a permutated hexameric motif identified by Fujita and co-workers (figure 1) (53).

One of these hexameric motifs, AAGTGA (-69 to -74) is present in the PRDI domain (ie. 13mer IV). In L929 cells four tandem copies of the sequence AAGTGA (refered to as the tetrahexamer from here on) are able to confer an appreciable level of inducibility to a truncated IFN-ß promoter fused to a

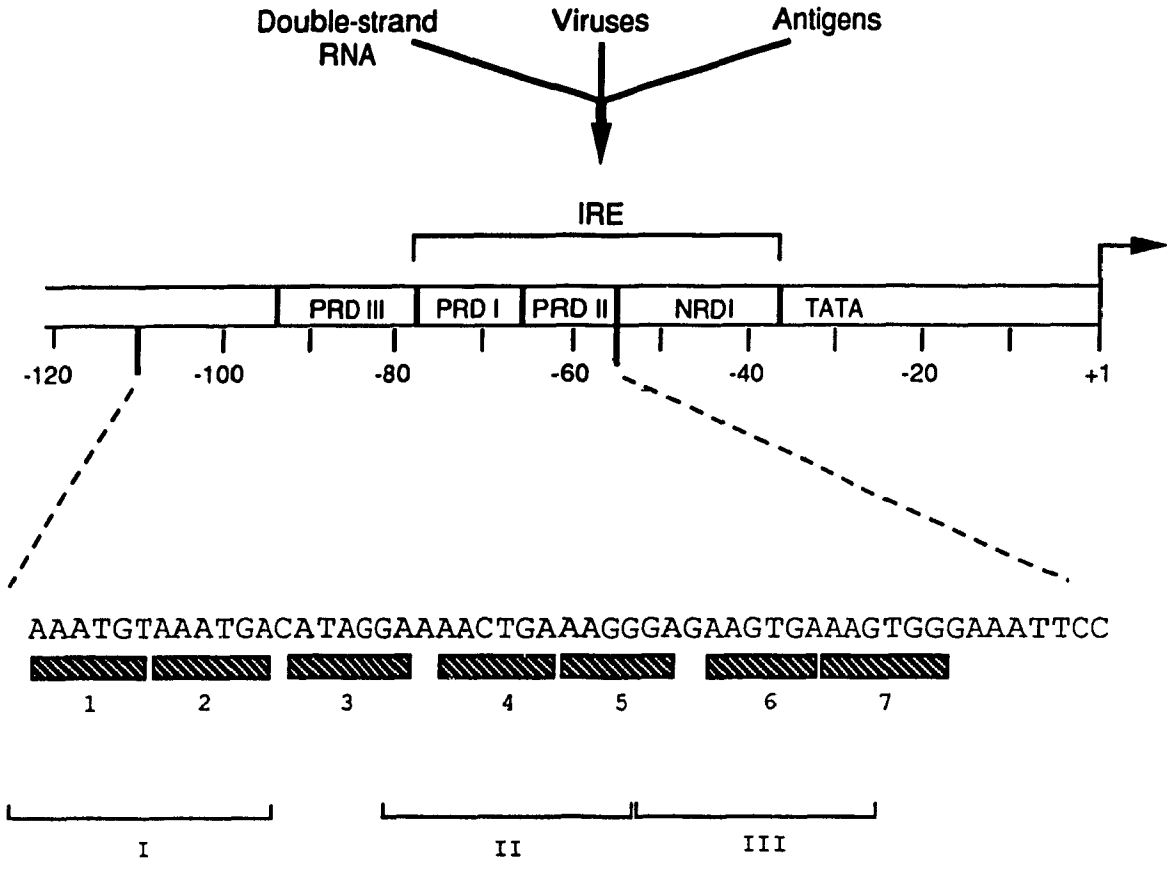


Figure 1. Schematic of the IFN-ß Regulatory Domains. The positive (PRD) and negative (NRD) promoter domains are indicated within the open boxes. The sequences between -55 and -107 are expanded below the gene. The hatched boxes represent the hexameric motifs (53). Copies I, II, and III of the 13 mer motifs are drawn beneath the hexamers (39). The negative upstream regulatory region is not illustrated in this diagram.

chloramphenicol acetyltransferase (CAT) reporter gene (54, 55). However, four copies of a two different hexamers, AAATGA and AAGGGA, are only 7.5 and 2.4 fold inducible, respectively. Constructs containing four repeats of a mutated hexamer, AAAGGA, which does not appear in the natural promoter, fail to respond to induction (54). Multimerization of the most efficient hexamer, AAGTGA, has a cooperative effect on induction; two copies are inactive on a -53 truncated promoter, whereas three, four and eight copies confer 2, 20, and 53 fold inducibility, respectively (54). In addition, both the tetrahexamer or an octahexamer renders the -53 deletion construct responsive to induction in an orientation independent manner, and confers inducible activity to a heterologous IL-2 or ß-globin promoter (54, 55).

Interestingly, related hexameric motifs also appear to regulate expression of the IFN-α1 promoter (56). The IFN-α1 VRE contains two direct imperfect 19 bp repeats, repA and repB. Multiple copies of repA render a truncated or intact ß-globin promoter virus inducible. Two different permutations of the hexamer occur in the IFN-α1 VRE (GAAAGC in repA and GAAAGT in repB). Duplication of the IFN-ß hexamer motif, AAGTGA, generates one copy of the sequence GAAAGT (AAGTGAAAGTGA), while tetramerization of AAGTGA yields three tandem copies of the repB permutation. Four tandem copies of the GAAAGT motif mediate virus inducibility when placed in front of a ß-globin promoter. Tetrameric repeats of repA and of the GAAAGC or GAAAGT motifs silence the SV40 enhancer if interposed between the 72 bp repeats and the ß-globin TATA box. The silencing effect is relieved by induction and transcription is synergistically augmented by the combination of both elements (56). A similar silencing effect has also been noted with the IFN-ß tetrahexamer motif (55, 57).

The capacity of the tetrahexamer to confer inducibility in L929 cells suggested that contrary to previous proposals, PRDI might not comprise a constitutive element (40). However, it was later demonstrated that the tetrahexamer possesses constitutive activity in C127 cells (55). This finding implied differences in the basal levels of PRDI binding factors in C127 versus L929 cells. Such variability may account for conflicting reports about the 5' sequence requirement in these systems (39, 53). In C127 cells high level induction is maintained in the absence of distal copies I and II of the 13mer motif (or copies 1-4 of the hexamer motif) (39), while in L929 cells a full complement of these elements is required (53). Since the basal activity and perhaps the absolute levels of PRDI factors may be lower in L929 cells as compared to C127 cells, it is possible that additional copies of the 13mer (or hexamers) are required in L929 cells to cooperatively maximize inducible transcription.

It is important to consider that none of the studies described have tested the activity of the intact PRDI domain per se. We have recently found that the tetrahexamer is weakly inducible in 293 cells and that multiple copies of PRDI do not confer inducible activity to a truncated SV40 promoter, although a low level of constitutive PRDI activity is detectable (Leblanc, J. F. et al., submitted). However, multiple copies of a domain which we designate, PRDIII (-78 to -94), renders the SV40 basal promoter virus inducible. PRDIII is juxtaposed to PRDI and contains two complete hexamers. The enhanced activity of PRDIII relative to PRDI is consistent with the notion that additional 5' sequences constituting a distinct positive domain are required for activation in some cell systems (53, 58).

7.4 Protein-DNA Interactions within PRDI

DNA-binding proteins which interact with the PRDI domain have been identified in a number of human and murine fibroblastic cell lines (57, 59-61). We have identified a constitutive factor in uninduced and IFN-primed virus-infected Hela cells which interacts specifically with PRDI and can be competed by synthetic oligonucleotides corresponding to the IRE, PRDI, PRDIII and the tetrahexamer $(AAGTGA)_4$. A related factor with constitutive binding activity (PRDI-BFc) and also polyI:C inducible factor (PRDI-BFi) were characterized in nuclear extracts from MG63 cells (60). Similar to the PRDI factor that we detect in Hela cells, binding of PRDI-BFc and PRDI-BFi is competed by the tetrahexamer and preliminary data indicates that both proteins are capable of interacting with PRDIII. The induction of PRDI-BFi binding does not require de novo protein synthesis, suggesting that the factor pre-exists in uninduced cells and is post-translationally regulated in response to induction. Mutations in PRDI which impair IFN-ß transcription *in vivo*, concomitantly prevent binding of PRDI-BFc and PRDI-BFi *in vitro*. DNA-protein complexes formed with PRDI-BFi migrate more rapidly in native gels than those formed with PRDI-BFc, but the proteins display similar DNAase I footprints and make identical contacts within PRDI (60). Hence, it is possible that the inducible factor arises from the constitutive factor through non-specific proteolytic cleavage resulting during preparation of the extract. Assuming, however, that PRDI-BFc and PRDI-BFi constitute distinct proteins then binding in uninduced and induced cells, respectively, may account for the constitutive and inducible activity of templates which lack part of the negative domain (NRDI) (40). Similarly, elevated levels of PRDI-BFc in C127 versus L929 cells,

may explain differences in the basal activity of the tetrahexamer in these systems (55).

Our data is consistent with a model in which constitutive factors play a role in IFN-ß regulation. We have found that proteins required for specific transcription of an IFN-ß template *in vitro* are present in uninduced Hela extracts, and can be depleted by adding an excess of IRE oligonucleotide to the reaction mixture. It is therefore possible that functionally active factors pre-exist in uninduced cells, but are sequestered from the promoter because of constraints imposed by negative proteins or chromatin structure. Support for the latter possibility comes from *in vitro* studies which demonstrate that nucleosome formation impedes the interaction of transcription factors with the adenovirus major late promoter (62). Partial relief of this constraint in uninduced cells may account for the low level of basal activity sometimes associated with recombinant IFN-ß plasmids *in vivo* (38, 39, 58).

The interaction of soluble trans-acting factors with the tetrahexamer has been decribed both *in vivo* (54) and *in vitro* (57, 60, 63, 64). Transcription proteins required for the expression of a recombinant IFN-ß test gene *in vivo* can be depleted by co-transfecting a competitor plasmid containing multiple copies of the tetrahexamer motif (54). At least one DNA-binding protein identified in L929 extacts requires sequences upstream of -66 for protein-DNA complex formation *in vitro*. Binding to the natural promoter is inhibited by the tetrahexamer and different permutations thereof, and to a lesser extent by the IFN-α VRE. The efficiency with which a given permutation is able to compete for binding correlates with its functional activity *in vivo*. A factor designated IRF-1 was initially shown to interact with the tetrahexamer motif in L929 cells and thought to mediate both silencing and activation of the promoter (57). More recent evidence suggests that IRF-1 may only mediate

activation and that a functionally distinct factor, IRF-2, mediates the silencing activity (64). Cell extracts from L929 cells contain a mixture of IRF-1/IRF-2 and the proteins share identical sequence specificity. Both are detectable in uninduced cells, but a 2-3 fold stimulation in binding is observed following virus induction. DNAase I footprinting assays demonstrate that these proteins protect the hexamer motifs in the natural IFN-ß promoter in a cooperative and concentration-dependent manner. The two most proximal hexamer motifs which localize within PRDI bind IRF proteins with the highest affinity. Furthermore, the tetrahexamer exhibits little functional activity in R66 monkey cells which lack IRF binding activity (57). At the moment we do not know if the PRDI binding factor which we detect in Hela cells corresponds to IRF-1/IRF-2 or a distinct protein with similar sequence specificity. Recent experiments in our lab indicate that constitutive and inducible proteins interact with the tetrahexamer in Hela cell extracts. Binding of these proteins is partially competed by PRDI oligonucleotides, but only the constitutive protein is detectable with a radiolabeled PRDI probe (Cohen, L., unpublished observation).

DNA templates encoding IRF-1 and IRF-2 were isolated from a λgt11 L929-cDNA expression library (63, 64). The DNA-binding properties of the cell-derived and cloned IRF proteins are biochemically indistinguishable. Mutations which decrease transcription and prevent binding of cell-derived IRF, also inhibit the binding of cloned IRF-1 (63). Methylation interference footprinting assays demonstrate that IRF-1 and IRF-2 make identical contacts within the IFN-ß and IFN-α1 promoters, recognizing concatamers of a hexameric sequence rather than individual motifs. Interestingly, the affinity of IRF-2 is approximately 5 times greater for the tetrahexamer than IRF-1;

however, the proteins share approximately equal affinity for the natural promoter (64).

The functional activity of IRF-1 and IRF-2 was examined by introducing cloned IRF genes into mammalian cells and monitoring the effect on transcription. Overexpression of IRF-1 in monkey cells minimally induces endogenous IFN-α and IFN-ß gene expression (65). On the other hand, IRF-1 fails to stimulate the activity of a -123 truncated IFN-ß promoter in L929 cells. In this system, IRF-1 can only activate transcription of a template containing the tetrahexamer linked to a -53 promoter deletion. A stimulatory effect does not materialize if both IRF-1 and IRF-2 are cotransfected, suggesting that IRF-2 antagonizes activation by IRF-1 (64). The enhanced activity of IRF-1 on the tetrahexamer relative to the natural promoter is not currently understood, but may partly reflect differences in DNA-binding affinity for these templates (64). However, a more likely possibility is that induction of the natural promoter is dependent on additional factors that recognize PRDII.

Expression studies using chimeric IRF fusion genes indicate that the DNA binding and transcriptional regulatory domains of these proteins are specified by the amino- and carboxy-terminal halves of the molecule, respectively (64, 65). IRF-2 shares considerable amino acid homology (65%) with IRF-1 at the N-terminus, but the proteins diverge significantly within their C-terminal domain (63, 64). The DNA-binding domain of IRF-1 and IRF-2 can be exchanged without affecting functional activity (as specified by the C-terminus); fusion of the IRF-1 binding domain to the carboxyl portion of IRF-2 generates a molecule (IRF1/2) with silencing activity, while the reciprocal fusion (IRF-2/1) is capable of mediating transactivation (64). Structurally, the DNA-binding domain of IRF-1 lacks any of the characteristic Zn finger, helix-turn-helix, or homeo-binding motifs previously identified in

the corresponding regions of other eukaryotic transcription factors. The activation domain of IRF-1 contains several serine-threonine clusters which represent potential phosphorylation sites, as well as an arrangement of amino acids that are known target sites for casein kinase II (63).

The IFN-ß gene is not inducible by interferon, but induction can be augmented or 'primed' by pretreating cells with interferon. Several pieces of data implicate IRF-1 as the priming factor. IRF-1-mediated activation appears to require new protein synthesis (63), as does the development of the primed state in some cell systems (66-69). More importantly, IRF gene expression is stimulated in response to interferon treatment (64). This is consistent with reports showing that the activity of the tetrahexamer is inducible not only by virus, but also by IFN-ß and IFN-γ (55). Physiologically, interferon secreted from a virus-infected cell may serve to amplify the induction effect in neighbouring cells after challenge by the invading virus. According to this model IFN-priming would act to increase the intracellular concentration of IRF-1 in the absence of induction. Delivery of the inducing signal would activate other proteins which in combination with IRF-1 could stimulate the promoter. Synthesis of IRF-1 prior to induction would also reduce the lag phase of the activation response and increase the magnitude of induction, both of which have been shown to occur as a result of priming (66, 69, 70).

Perhaps the most interesting finding from the IRF/PRDI studies relates to the overlap between the regulation of the IFN-ß promoter and the promoters of several IFN-stimulated genes. The interferon-stimulated response element (ISRE) shares extensive nucleotide homology with PRDI and the tetrahexamer motif (45-52). A number of genes known to be activated by interferon can also be stimulated by virus and polyI:C (71, 72). Synthetic ISRE oligonucleotides confer virus inducibility and reversible silencing activity to a

heterologous promoter *in vivo*. Anti-IFN antibodies do not abrogate inducibility, suggesting that the effect is directly mediated (55). IRF-1/-2 binding to PRDI and the MHC class I (H-2D^d) gene ISRE is indistinguishable as determined by gel retardation and methylation interference footprinting analysis (64). The basis for these similarities has recently become evident. DNA clones encoding IRF-1 and ISGF2, the putative negative regulator of ISRE-mediated transcription, were isolated by independent laboratories, but it is now apparent that the genes are identical and specify the same protein (63, 73). Furthermore, IRF-1/ISGF2 may also be related to an IFN-γ inducible ISRE/PRDI binding factor (IBP-1) detected in Hela extracts (61). As with the bovine papilloma virus E2 (74) and adenovirus E1a protein (75, 76), two distinct regulatory properties impinge on a single transcription factor. IRF-1/ISGF2 plays a dual role, acting as a repressor of IFN-stimulated genes and an activator of IFN-ß and perhaps IFN-α transcription. There is now evidence to suggest that IRF-1/ISGF2 may exist in multiple phosphorylation states (Pine, R., personal communication). It is therefore tempting to speculate that differences in post-translation modification confer distinct regulatory activities to a single IRF-1/ISGF2 molecule.

7.5 Functional Analysis of the PRDII Domain.

A number of experiments analogous to those performed with the tetrahexamer motif were used to functionally characterize the activity of PRDII. Synthetic oligonucleotides containing multiple copies of PRDII have been assayed *in vivo* when positioned upstream or downstream of truncated/intact promoters, at varying distances, and in different orientations (55, 77-79). Single copies of PRDII positioned 5' of an HSV TATA box fused to

a luciferase gene confer a small increase in basal transcription to the hybrid template, but fail to further stimulate transcription after induction by polyI:C (79). As with PRDI the activity of PRDII varies between cell lines; PRDII is mainly constitutive in C127 (55, 79), Hela and human 293 cells (79; Leblanc, J. F. et al., submitted), but is predominantly inducible in MG63 cells (79). In most cases, constitutive PRDII activity can be marginally augmented by induction. In L929 cells this element can act in either a constitutive or inducible manner depending on the substrain (55, 77, 79).

Two copies of the PRDI-like tetrahexamer are able to stimulate expression of homologous or heterologous promoters when positioned 5' of a linked gene, although as many as eight copies have no effect if placed 3' to the gene (54, 55). Two or more copies of PRDII can similarly confer activity when positioned in front of an intact ß-globin promoter or a -77 IFN-ß promoter deletion (55). However, four or more copies of PRDII are able to confer activity when positioned downstream of these transcription units. Multiple PRDII elements can also stimulate transcription when positioned upstream of a truncated β-globin promoter, but in a distance-dependent manner. The ability of PRDII to function as a dimer at various positions on intact promoters, suggests that this element possesses greater intrinsic activity than PRDI. However, unlike PRDI, PRDII is unable to reversibly silence the activity of a viral enhancer when interposed between the enhancer and a heterologous promoter (55).

7.6 Protein-DNA Interactions within PRDII

The induction of immunoglobulin kappa gene transcription during B cell development correlates with the binding of the transcription factor NF-κB to the kappa enhancer (80-82). NF-κB is constitutively active in mature B cells (82), but its activity can be induced in pre-B cells with lipopolysaccharide (LPS) (82, 83), and non-B cells by a variety of reagents such as phorbol esters (ie. phorbol myristate acetate) (82), the HTLV-I tax protein (84, 85), the hepatitis B virus X gene product (86), inhibitors of protein synthesis (82), and TNF-α (87, 88). The activity of NF-κB is regulated by a post-translational mechanism. In the cytoplasm, NF-κB exists as a heterodimer coupled to an inhibitor protein, termed IκB. Phosphorylation of IκB is believed to mediate dissociation of the complex. NF-κB is then translocated to the nucleus where it binds to a decanucleotide recognition sequence (GGGACTTTCC) and exerts its transcriptional effect (89, 90).

The PRDII domain (GGGAAATTCC) shares 80% nucleotide homology with the NF-κB recognition site. Related sequence motifs have been identified in the regulatory regions of several eukaryotic viruses and cellular genes including SV40 (91), cytomegalovirus (92), human immunodeficiency virus type-1 (93, 94), ß2-microglobulin (95, 96), serum amyloid A (97), interleukin-2 receptor-α (98, 99) and the class I (95, 100, 101) and II major histocompatibility complex genes (102). A number of experiments have recently shown that the PRDII and κB motifs are functionally interchangeable *in vivo* and *in vitro* (70, 78, 79). Multiple copies of the κB motif confer virus inducibility on a truncated c-fos promoter in L929 cells (78). Similarly, hybrid CAT plasmids containing reiterated HIV-κB and PRDII elements linked to a basal SV40 promoter are 3-4 fold inducible by virus in Hela cells and 10-20

fold inducible by PMA in Jurkat cells (Leblanc, J. F. et al., submitted). Constitutive PRDII activity has also been demonstrated in mature B cells which contain high levels of active NF-κB (78).

Using an electrophoretic mobility shift assay (EMSA) we identified an inducible NF-κB-like complex which forms specifically with the PRDII domain. Inducible PRDII binding is detectable in a variety of cells following treatment with physiological inducers of IFN-ß transcription and NF-κB binding activity. Related studies showing similar binding have demonstrated that mutations in PRDII which preclude IFN-ß transcription *in vivo*, concomitantly prevent NF-κB-like complex formation *in vitro* (78, 79). In addition, PRDII binding factors have biochemical properties that are characteristic of NF-κB (78, 79) and generate similar methylation interference footprinting patterns (78).

Cell-specific differences in PRDII activity suggest that aside from NF-κB, distinct proteins with related sequence specificity may interact with the PRDII domain. Several factors with similar binding specificity have been extensively characterized. Three members of this family, H2TF, KBF-1, and EBP-1 are ubiquitous and constitutively active (95, 101, 103-105), while a fourth protein designated HIVEN86 is constitutively active in chronically infected monocytes and inducible by PMA in leukemic Jurkat T cells (106). Thus, it is possible that the high basal activity of PRDII in some cell systems results from interactions with constitutive transcription factors. In addition to EBP-1 (105), at least one other constitutive PRDII binding activity has been identified in nuclear extracts (60). A cDNA clone encoding a PRDII binding factor (PRDII-BF1) has also been described (107). PRDII-BF1 is distinct from NF-κB and H2TF and is both virus and serum inducible. The functional significance of this protein is not clear given that the kinetics of PRDII-BF1

expression do not correlate with either activation or shutoff of IFN-ß transcription. We have recently used comparative EMSA/UVcross-linking analysis to identify a number of constitutive and inducible protein-DNA interactions within the PRDII domain. These experiments demonstrate that similar proteins are capable of binding to PRDII in a variety of cell types and in response to different inducers. At the moment we cannot make definite conclusions about the functional activity of these proteins. Additional characterization is required to distinguish between those proteins that fortuitously interact with PRDII and those which have a bona fide role in IFN-ß transcription.

7.7 Negative Regulation of the IFN-ß promoter

The IFN-ß promoter contains two distinct negative regulatory elements. One of these is located in the upstream regulatory region from -107 to -210 (38), while the second, designated NRDI, extensively overlaps PRDII and extends to the 3' border of the IRE (40, 41). We have identified a factor, KGß, in unstimulated KG-1 nuclear extracts that binds to a 67 bp restriction fragment (-202 to -135) within the negative upstream domain. A portion of the binding site for KGß may extend 3' of -135 given that sequences downstream were able to partially compete for KGß complex formation. Even though we have no direct evidence that KGß is involved in negative regulation, this possibility is suggested by *in vivo* competition assays which demonstrate that the 67 bp fragment interacts with a negative factor(s) in 293 cells. Curiously, the IFN-α promoter was also able to compete a negative activity *in vivo*, but failed to inhibit KGß binding *in vitro*. The reason for this discrepancy is not understood, but may reflect differences in the assay or cell

type used. Analogous interactions with the upstream promoter sequences have been reported by other groups. Differentiated EC cells and murine fibroblasts contain factors which bind IFN-ß promoter sequences between -70 and -210 in the uninduced state (108). *In vivo* footprinting studies demonstrate that the negative regulatory sequences between -94 to -168 and -38 to -55 (NRDI) bind factors in uninduced MG63 cells; within 1-2 hrs following polyI:C induction the putative negative factors are displaced from the promoter (59).

Negative protein-DNA interactions with NRDI have yet to be described *in vitro*, but the negative activity of this domain is implied from mutational studies of the IRE in C127 cells (40, 41). Several point mutations spanning NRDI have been shown to increase the basal activity of the promoter (41). These mutations might impede binding of a negative factor(s) and thereby facilitate the interaction of constitutive factors present in uninduced cells. A negative factor with NF-κB-like sequence specificity could conceivably interact with the NRDI sequences overlapping PRDII. However, additional negative regulation may be imposed by other factors which recognize PRDI. One possible candidate for PRDI-mediated repression is IRF-2 (64), and more recent studies indicate that a distinct factor, designated PRDI-BF1, might also be involved (109). Overexpression of cloned PRDI-BF1 inhibits virus inducibility of a recombinant reporter plasmid containing tandem copies of PRDI (109). Moreover, the regulatory activity of IRF-2 and PRDI-BF1 is antagonized by inhibitors of protein synthesis (64, 109). These findings support the idea that superinduction of the IFN-ß gene is in part mediated by a transcriptional mechanism.

Preliminary studies in our lab indicate that PRDIII also imparts negative regulation to the IRE (Leblanc, J. F. et al., submitted). We have found that an

oligonucleotide corresponding to the PRDI/II domains is weakly constitutive and fails to respond to induction in 293 cells when linked to a basal SV40 early promoter. The basal activity of this construct is diminished and inducibility is restored only when the PRDIII domain is fused to the PRDI/II sequences. Thus in this system, the presence of PRDIII is critical for maintaining both positive and negative promoter activity. The negative activity confered by PRDIII may be due to IRF-2 or PRDI-BF1, since PRDIII and PRDI interact with similar factors.

7.8 Summary and Future Prospects

The information available to date suggests that IFN-ß gene induction is mediated by temporal interactions of positive and negative factors with the promoter. Maximal derepression of the positive cis-acting domains (PRDI/II/III) requires a secondary signal provided by the inducing stimulus. Positive factors which bind only in response to induction could displace negative factors, and act alone or in concert with constitutive factors to maximally stimulate transcription. Although multiple factors are likely to be involved in activation, as few as two of these , IRF-1 and NF-κB, seem able to moderately induce IFN-ß transcription *in vivo*. We have recently found that co-transfection of an IRF-1 expressing plasmid and induction of NF-κB by phorbol ester or tax protein, is sufficient to induce the natural promoter in cells that normally do not express IFN-ß (Leblanc, J. F. et al., submitted). Attempts to purify these and other proteins are currently underway. By assaying purified factors in an *in vitro* transcription system we can begin to examine how different components in the signal transduction pathway interact to stimulate or repress transcription.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The following novel findings have been demonstrated in the thesis and are listed according to chapter:

Chapter 2

A transient expression assay was developed that permits cell specific regulated expression of the human IFN-α and IFN-ß promoters in homologous cells. The induction kinetics of the transfected IFN-ß template closely mimic those of the endogenous gene. Analysis of the IFN-α promoter in 293 cells suggests that the trans-acting factors which mediate IFN-α expression are either lacking or present in insufficient quantities in this cell system. In the course of these experiments it was also demonstrated that under basal conditions, the IFN-ß promoter exerts a dominant negative influence over the constitutive SV40 enhancer

Chapter 3

The transient expression assay provides a convenient means by which to examine protein-DNA interactions required for IFN-ß induction. *In vivo* competition experiments detected positive and negative factors involved transcriptional activation and repression, respectively. The DNA sequences inplicated in this control correlate well with previous genetic delineation of the promoter. These experiments further demonstrate that positive trans-acting factors recognizing IFN-ß regulatory sequences may be common to the SV40 early promoter, and that E1a-mediated repression of the SV40 enhancer in 293 cells is reversible.

Chapter 4

In vitro analysis of protein-DNA interactions identified a nuclear protein(s), KGß, in uninduced myeloid cells that binds to a negative upstream region of the IFN-ß promoter. KGß fails to recognize sequences from other eukaryotic promoters including IFN-α1. Based on the functional activity of the KGß binding sequences *in vivo*, we suggest that this protein(s) could represent a transcriptional repressor.

Chapter 5

EMSA analysis identified multiple protein-DNA interactions within the IFN-ß regulatory element (IRE). Factor binding to the IRE was localized to the PRDI domain. The PRDI binding activity characterized is constitutive and shares sequence specificity with IRF transcription factors.

In vitro transcription of the IFN-ß gene has not been previously demonstrated. We have established an *in vitro* transcription system that supports accurate initiation of the IFN-ß promoter in crude nuclear extracts. Competition experiments adapted to this assay indicate that IRE binding factors pre-exist in uninduced Hela cells and are important for IFN-ß expression *in vitro*.

Chapter 6

These studies have demonstrated inducible binding of an NF-κB-like activity to the PRDII domain. PRDII is functionally interchangeable with the

HIV-1 enhancer κB motifs in the EMSA assay. Additional characterization of PRDII binding factors was achieved using a modified UV cross-linking procedure developed by the candidate. These studies reveal that a number of distinct proteins can recognize the PRDII domain. In general, proteins which interact with PRDII bind constitutively or can be induced to bind in a variety of cell lineages (epithelioid, myeloid, and lymphoid) with reagents that stimulate IFN-ß transcription or NF-κB binding activity.

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CHAPTERS 1-7

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