REGULATION OF CLASS I GENES BY INTERFERONS

Thesis by

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This thesis is dedicated to

my shelter and my storm,

James Theiler.

Through the years, a man peoples a space with images of provinces, kingdoms, mountains, bays, ships, islands, fishes, rooms, tools, stars, horses, and people. Shortly before his death, he discovers that the patient labyrinth of lines traces the image of his own face.

Beyond my anxiety, beyond this writing,
the universe waits, inexhaustible, inviting.

-- Jorge Luis Borges

You see, one thing is, I can live with doubt and uncertainty and not knowing. I think it is much more interesting to live not knowing than to have answers which might be wrong. I have approximate answers and possible beliefs and different degrees of certainty about different things...

I don't feel frightened by not knowing things, by being lost in a mysterious universe without having any purpose, which is the way it really is, so far as I can tell...

I don't feel frightened.

-- Richard P. Feynman

Don't be blue, be determined.

-- Leroy Hood

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Abstract

Major histocompatibility (MHC) class I gene expression is increased in response to interferons. In order to identify critical regulatory regions in mouse MHC (H-2) class I genes, the 5' flanking region and the DNA downstream of the transcription initiation site were analyzed separately. The promoters of H-2D^d and H-2L^d were linked to the reporter gene chloramphenicol acetyl transferase (CAT). Conversely, the H-2L^d structural gene was linked to non-interferon regulated promoters. These constructs were transfected into several different cell lines, and their ability to respond to interferons was assessed. Both regions, 5' and 3' of the transcriptional initiation site, were able to independently contribute to the regulation of class I genes by interferons. The basal levels of expression, interferon inducibility, and the relative contributions of the 3' and 5' responses to overall interferon regulation, were cell-type dependent.

Sequence analysis of the 5' flanking region of class I genes led to the identification of multiple DNA motifs that are highly homologous to regulatory elements found in other genes. The H-2D^d promoter contains a TATA box, CAAT elements, enhancer regions, and an interferon consensus sequence that is found in the promoters of many genes that are regulated by interferons. Deletion analysis and expression studies of the H-2D^d promoter revealed several interesting regulatory features of the interferon consensus sequence. It was required for both type I (alpha and beta) and type II interferon (gamma) responses. In some cell types an additional sequence was required for a type I interferon response; this sequence is located 5' and adjacent to the interferon consensus

sequence. Type II interferon action was independent of this upstream sequence in all cell-types tested. Therefore the promoter controlled response to interferons is complex, and the nature of the response depends both on the type of interferon and the cell-type being tested.

We have noted that an interferon consensus sequence homology exists in the promoters of interferon genes. As interferons have a capacity to be auto-regulatory, we propose a model of gene regulation by interferons that incorporates what our studies and others have shown about the regulation of class I genes by interferon, and what is known about the regulation of interferon genes themselves.

CHAPTER ONE

INTRODUCTION

Introduction

This thesis concerns the regulation by interferon of H-2 class I molecules. This is a biologically relevant issue because the interferon induction of class I molecules may have bearing on the success with which an organism mounts an immune response, by enhancing the antigen-specific lytic effect of cytotoxic T cells. This problem is interesting from other perspectives as well. Better understanding of how interferons regulate genes can be fit into the emerging picture of how eukaryotic genes are regulated in general. Also, defining the mechanisms interferons use to regulate immunologically relevant genes may eventually have application for designing therapies that can stimulate or repress different specific aspects of a cellular response to tumors or viral infections.

This introduction describes the structure, function, and expression patterns of major histocompatibility complex (MHC) class I antigens, provides a brief summary of relevant aspects of the role of interferons in the immune response, and describes how the regulatory elements of class I genes can be interpreted in the context of current general models of gene regulation. The ensuing chapters are arranged so that a published paper begins each chapter, followed by an appendix that describes additional experiments that pertain to the major topic of the chapter, and/or a discussion of pertinent data recently published from other laboratories.

Structure and Function of Major Histocompatibility Antigens

H-2 class I molecules are polymorphic membrane-bound glycoproteins

that function as recognition molecules in cell mediated immune reactions (1,2). Their primary physiological role is to present viral or tumor antigens to cytotoxic T lymphocytes. A T cell receptor recognizes antigen in association with a particular MHC class I (or class II, discussed below) molecule (2). This MHC protein mediated restriction of pathogenic antigens is critical for distinguishing self from non-self and for the elimination of diseased cells from their host.

Class I molecules are composed of two polypeptide chains. A heavy chain with a molecular weight of approximately 45,000 daltons spans the membrane bilayer, and is non-covalently associated with a 12,000 dalton light-chain, beta-2 microglobulin (3). Class I heavy chains are encoded within the major histocompatibility complex (MHC) in the H-2K, D, and L loci in mouse, and the HLA-A, B, and C loci in human (4). The structural gene encoding beta-2 microglobulin is located outside the MHC in both species (4). A given individual simultaneously expresses several polymorphic forms of class I proteins from a large pool of alleles in the population. The polymorphic nature of class I molecules presumably provides potential for interaction with a greater range of pathogenic antigens. The particular alleles that an individual expresses are known as its haplotype, and proteins of different haplotypes can be distinguished by specific antibodies. Haplotypes are designated by a lower case superscript letter following the H-2 gene.

Class II molecules are functionally and structurally related to class I molecules. They are also intimately involved in antigen presentation to T lymphocytes, encoded in the MHC, and can be regulated by interferon. A critical difference between class I and class II

molecules is that usually class I molecules present antigen to cytotoxic T cells, and class II molecules present antigen to helper T cells. Whether a T lymphocyte recognizes antigen in the context of a class I or a class II molecule depends on whether the T cell expresses either one or the other of the cell surface molecules CD8 and CD4. (CD8 is also known as Lyt2 in mouse and T8 in human; CD4 is equivalent to L3T4 in mouse and T4 in human.) CD4 is usually expressed in T helper cells and occasionally is found in cytotoxic T cells; the opposite is true of CD8. T lymphocyte recognition is constrained by CD4+ T lymphocytes interacting with targets bearing class II molecules, and CD8+ T lymphocytes interacting with targets bearing class I molecules (5). It has been proposed that the interaction between CD4 or CD8 and their MHC counterparts serves as an intercellular adhesion stabilizing the MHC-antigen/T cell receptor complex (6,7).

Over the past few years, experimental advances have been made that have culminated in a unified structural model explaining MHC restriction and antigen presentation. For both class I and class II restricted cells, a single T cell receptor can be transferred from one T cell to another and impart dual specificity for MHC and antigen (8,9). Viral antigens are not recognized in their native three-dimensional conformation, but are believed to be processed intracellularly to short peptides that associate with MHC molecules (10). This model of MHC restricted antigen recognition is supported by studies of influenza viral specific cytotoxic T lymphocytes that can lyse uninfected histocompatible targets in the presence of exogenously added peptide fragments of the viral protein (11). Also, peptide antigens recognized

by class II restricted T cells bind to isolated class II MHC molecules in vitro, and form complexes that can specifically stimulate T cell responses (12,13).

The recent solution of the crystal structure of a human class I antigen (HLA-A2) by Bjorkman and colleagues (14) is consistent with the existence of a single potential antigen binding site that would accommodate peptides of 8-20 amino acids in length. This remarkable structure revealed that the potential antigen binding site of class I proteins is a deep groove 25 angstroms long and 10 angstroms wide, running along the surface of the molecule facing away from the plane of the membrane (14). The groove is flanked by two alpha helices, and the floor of the groove is formed by a beta-pleated sheet structure (14). Clustered on the walls and floor of this groove are most of the amino acid residues in class I molecules known to influence the recognition of antigen by T lymphocytes (15). The groove contained an intriguing unidentified molecule that co-purified and co-crystallized with HLA-A2, conceivably occupying the site in the same manner as an antigenic peptide primed for presentation to T lymphocytes (14).

Class I molecules are composed of several functional domains. The external outer domains $alpha_1$ and $alpha_2$ form the groove structure that presumably interacts with antigen, are highly polymorphic, and impart the antigen/ T cell receptor specificity (14,16). The third external domain is the membrane proximal domain, and associates with beta-2 microglobulin. It is relatively conserved among class I antigens, and has a structure that identifies it as part of the immunoglobulin super gene family (17). (Members of the immunoglobulin super gene family can

be identified by having homology to a structural unit first described in the constant region domains of immunoglobulins.) Beta-2 microglobulin is structurally similar to a single immunoglobulin-like domain (17). Class I molecules also have a hydrophobic transmembrane region and cytoplasmic tail. The exon/intron pattern in the gene corresponds to the protein domains.

MHC molecules are of interest to immunologists for additional reasons beyond their antigen presentation capacity. MHC proteins serve as targets for immune elimination of transplanted foreign tissue in graft rejections, an effect referred to as alloreactivity. For this reason, the highly immunogenic class I molecules are also known as transplantation antigens. Additional biological functions of MHC molecules are just beginning to be explored. Class I molecules have been found to associate with insulin receptor molecules (18). Antibody studies have indicated that class I molecules may play a role other than antigen presentation in T cell activation (19), and that class II molecules are involved in B cell activation (20). The above observations suggest that MHC class I molecules may function in part as receptors or signal transduction molecules. Class I molecules with similar sequences and genomic exon/intron organization to classical transplantation antigens, known as T1 and Qa antigens, are also encoded in the MHC (21). These molecules are far less polymorphic than H-2K, D, and L, and their function is still a mystery. Qa antigen expression can be enhanced by exposure to interferons (22). It was recently discovered that human cytomegalovirus actually carries a gene similar to class I antigens, also with an unknown function (23).

Expression and Regulation of Class I Antigens

The tissue distribution of transplantation antigens shows a similar pattern of expression in human and in mouse (24). Although class I molecules are expressed in most somatic cells in adults, their basal levels vary. The highest levels of class I antigen expression are generally found in spleen and lymph nodes; moderate in liver and lung; and lowest in heart, kidney, pancreas, and skeletal muscles. Brain, nerves and early embryonic tissues do not detectably express class I antigens. In adult cells, beta-2 microglobulin tends to be coordinately regulated with class I genes (25-27).

Interferons are powerful inducers of transplantation antigens (28), although there are substantial differences in the interferon inducibility of class I antigens in different murine tissues (29). Brain cells show at least a 30-fold increase in expression of class I antigens (30); heart and kidney cells respond with a 13-17 fold increase; and tissues that have the highest basal levels of class I antigens, like spleen, liver, and lung, tend to show the least change in expression (31). Considerable variation in the levels of MHC antigen expression and induction have also been found among cultured lines of normal and tumor cells (32-36). This variation often reflects the tissue of origin. Although early embryos do not express detectable levels of class I antigens, they are inducible by interferons prior to the time that they are normally expressed (37), hence a role for interferon in the developmental onset of class I gene expression has been postulated (37).

Interferons and Their Function in a H-2 Class I Mediated Immune Response

Interferons are a set of proteins that exert anti-viral, cell-growth regulatory, and immunomodulatory effects (for review, 38 and 39). Type I interferons (alpha and beta interferons) are released from cells in response to viral infections. Alpha interferons are a family of immunologically related proteins that are 70-90% homologous in their amino acid sequence, and are expressed mainly in leukocytes (38). Beta interferon is immunologically distinct from and 30-40% homologous to alpha interferons, and is expressed predominately in fibroblasts (38). Type II interferon (gamma interferon) is a lymphokine secreted by activated T lymphocytes and NK cells (38,39). It is immunologically distinct and not related by sequence homology to type I interferons (38). Interferons have a capacity to evoke different phenotypic responses in different cell types, which could be a major reason for the mechanistic complexity of gene regulation by interferons. For example, autocrine beta-related interferons, that are involved in cellular differentiation and activation pathways of hematopoietic cells (40), can stimulate cellular proliferation (41), or cause a loss of proliferative capacity (42), depending on the cell type.

The myriad biological effects of interferons are achieved in part through the transcriptional regulation of gene expression (35,38,39,43-47). RNA stability (44,39) and modifications of the translation apparatus (39) are also involved. Despite many similarities in the biological activities of type I and type II interferons, there are sufficient differences to suggest that the intracellular mechanisms by

which they exert their influence are not identical. Gamma interferon has a distinct cell surface receptor from alpha and beta interferons (48,49), and can elicit immunomodulatory effects that alpha and beta interferons cannot (50,51). Each type of interferon induces the expression of a unique set of genes in addition to the common set they share (43,52-56). For example, in many cell types that normally do not express class II antigens, class II expression can be turned on by gamma interferon, but not by alpha and beta interferons (51,53). (Presumably this class II induction can recruit non-lymphoid cells for antigen presentation to CD4+ cells. This activity may be the epigenetic trigger of some auto-immune diseases (57).) Conversely, the protein encoded by the Mx gene that imparts resistance to influenza virus in mouse, is induced by alpha and beta interferons, but not gamma interferon (43). Some phenotypic responses to interferon, such as its anti-proliferative effect, can be elicited using different mechanisms that depend upon the type of interferon used and the cell type being studied (56). Although both types of interferons can induce class I genes, we have detected differences in regulatory elements required for the response to specific interferons (36).

We have used class I gene regulation as a model system for studying the effects of the two types of interferon on gene regulation in a range of cell types. There are several reasons that the regulation of class I genes are of particular interest. Levels of class I expression may influence the effectiveness of cytotoxic T cell killing of target cells, and also may influence graft rejection. An increase in transplantation antigen expression in response to interferon has been correlated with

enhanced susceptibility of virally infected cells to cytotoxic T cells (58), and a decrease in tumorigenicity of Adenovirus 12 transformed cells (59). Thus interferon regulation of transplantation antigen expression may play an important role in the defense against viral infection and malignant transformation.

Eukaryotic Transcriptional Regulatory Elements

The transcriptional regulatory elements in the promoters of class I genes are a focus of much of this thesis; therefore a brief dicussion of general aspects of eukaryotic transcription regulatory elements follows.

The level of transcriptional activation of a gene is partly determined by multiple sequence-specific DNA-protein interactions that occur in distinct cis-acting regulatory regions (60). Regulatory elements that can influence transcription are composed of basic promoter elements such as CAAT and TATA boxes, enhancer elements, and repressor binding sequences. The combination of sequence-specific DNA-binding proteins that interact with these target sites mediate basal or inducible transcription levels (61). Enhancer elements have the interesting properties of being able to exert positive control on cis-linked promoters in either orientation and often at distances of many kilobases 3' or 5' of the transcription initiation site (62). Although it is not yet clear how enhancer binding proteins exert their influence, direct interactions between DNA binding proteins involving the formation of loop structures are probably important for enhancer function (62-64). Negative regulatory elements can bind repressor molecules that are

capable of preventing transcription from enhancers that would otherwise be constitutively expressed. Derepression is thought to play a key role in the induction of beta interferon transcription upon viral infection (65,66). Therefore, transcription induction by regulatory agents can either occur through activation of positive regulatory elements or derepression of negative elements in a promoter. For many regulatory systems, studies using cycloheximide (which inhibits translation) indicate that gene regulation can be independent of protein synthesis, and presumably a consequence of modification of pre-existing factors (61).

The CAAT and TATA elements are not just simple elements that behave similarly in every context. As transcriptional activator proteins have been purified that bind to the CAAT sequence in different promoters, it has become clear that a multiplicity of DNA binding proteins feature CAAT boxes in their recognition sequences (67). TATA boxes have traditionally been thought of as DNA elements that direct the transcription initiation complex to the appropriate transcriptional start site on the DNA and allow efficient transcription (68). Very recently it was shown that the TATA box of the HSP 70 promoter is the regulatory element for the trans-acting adenovirus protein E1A (69).

Transcriptional Regulatory Elements in Class I Promoters

Deletion analysis of cis-acting elements and DNA/protein binding studies have been used to characterize the regions in class I promoters that direct their transcriptional regulation (35,36,46,47,70-75). These regulatory elements are discussed in detail in Chapters Three and Four

of this thesis. Since the papers included in these chapters were written, several discoveries have been made that have bearing on class I regulation. To discuss these additional findings, I will summarize what is known about regulatory elements in class I promoters.

The regions known to influence transcription of class I genes are, in order from 5' to 3': an enhancer region (70), an interferon response sequence (IRS) (35,36,46,47), and a second enhancer region (70). There are also two CAAT boxes in opposite orientations (76), and a TATA box (76). The IRS contains a consensus sequence found in the 5' flanking region of many genes subject to regulation by interferons (45). We have found that gamma interferon can increase transcription through the IRS alone (35,36), while type I (alpha and beta) interferons require the presence of an additional upstream sequence to influence class I transcription in some cell-types (35,36,46,47). A nuclear factor derived from murine myeloma cells can bind to the IRS (36).

A negative regulatory element located upstream from the IRS may be involved in the developmental switch where class I transcription goes from off to on in F9 cells (73). F9 cells differentiate upon treatment with retinoic acid into parietal endoderm, and upon differentiation, start expressing class I genes. A negative element in the promoter of class I genes may inhibit their transcription in the undifferentiated cells (73).

We have found a murine myeloma cell nuclear factor (or factors) capable of binding to DNA within the two functionally defined enhancer regions (36,76). The purified human transcription activation factor AP-1 also binds to the same sequence of nucleotides in these enhancer

regions (36). Recently it has been shown that the cellular proto-oncogenes c-fos and c-jun compete for the same binding sites as AP-1. (77,78). It is not yet clear whether the protein derived from the myeloma cell extracts that occupies the AP-1 binding sites is a murine analog of AP-1, or a unique protein that shares AP-1's sequence specificity. So far, the best characterized murine analog of a protein in the AP-1 family is the murine transcription factor PEA-1 (79), that was detected by virtue of its binding to the polyomavirus enhancer. There is some indirect evidence that this protein may be a trigger that allows class I expression during development. Undifferentiated F9 cells do not transcribe class I or polyomavirus genes, and PEA-1 activity is not detectable in these cells. Upon differentiation, class I genes are expressed, the cells become permissive to polyomavirus, and the regulatory factor PEA-1 is expressed or activated (79). It is not clear how the proposed positive action of PEA-1 on transcriptional activation of genes during F9 cell differentiation fits into the model based on the negative regulatory element described earlier (73).

The idea that different regulatory proteins share similar DNA binding sequence specificities suggests that complex competitions between nuclear factors for regulatory regions on DNA may ultimately determine the transcriptional activation states of genes. It is interesting to speculate that transcriptional regulatory proteins may be multi-gene families with conserved binding domains that can be linked with different regulatory domains for transcriptional control of sets of genes in different cell types. This notion of shared primordial archetypal domains in regulatory proteins has precedent in homologous

domains found in steroid and thyroid receptors (80). Furthermore, a given transcription factor, depending on its state and the context in which it is acting, may be able to exert either a positive or negative influence on transcription. AP-1 has been observed to act either as an enhancer or negative regulator of transcription (81).

The complexity of the promoter elements required for interferon regulation of class I genes, and the existence of independent 5' and 3' interferon responsive regions, suggests fine-tuning and generally increased class I expression may be vital during an immune response. It is possible that the multiple mechanisms of interferon induction are required to overcome different tissue specific negative regulatory elements that block expression in cells which express low levels of class I antigens and that are subthreshold for eliciting a T cell response. Also, viral infections can sometimes down-regulate class I expression, presumably to evade immune surveillance and destruction of infected cells. Adenovirus-2 is known to decrease class I expression by blocking protein transport to the cell surface (82), while adenovirus-12 can decrease class I expression at the mRNA level (59). If a viral infection or tumor occurs in a tissue that is refractive to cytotoxic T cell killing due to lack of class I antigens, the individual may have a greater chance of eliminating the diseased cells and surviving if the expression of class I genes could be up-regulated by multiple mechanisms.

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CHAPTER TWO

THE REGULATION OF MURINE CLASS I GENES BY INTERFERONS IS CONTROLLED BY REGIONS LOCATED BOTH 5' AND 3' TO THE TRANSCRIPTION START SITE

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Regulation of murine class I genes by interferons is controlled by regions located both 5′ and 3′ to the transcription initiation site

(interferon action/transplantation antigens/gene regulation)

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ABSTRACT **Interferons regulate the expression of a large number of mammalian genes, including the major histocompatibility antigen genes. To investigate the mechanisms involved in interferon action, we have analyzed the ability of murine *H-2L*^{d} and *H-2D*^{d} DNA sequences to control the responses to interferon. The results indicate that interferon regulation of class I gene expression is complex and involves at least two mechanisms that are dependent on class I sequences located upstream and downstream to the transcription initiation site. In transfected mouse L cells, both of these regions are required for full enhancement of class I gene expression, with the major portion of the response controlled by the sequences located 3′ to the transcription initiation site. The fine-mapping analysis of the 5′ region-encoded response also suggests that recombinant α and γ interferons may exert their effects on class I gene expression by using different cis-acting regulatory sequences.**

Transplantation antigens are membrane-bound glycoproteins that function as recognition molecules for cytotoxic T lymphocytes during graft rejection and immune elimination of cells expressing foreign antigens (1, 2). They consist of an ≈45-kDa heavy chain (class I protein) noncovalently associated with a 12-kDa light chain [β_2-microglobulin (β_2m)] (3). Class I proteins are highly polymorphic and are encoded within the major histocompatibility complex by the *H-2K*, *H-2D*, and *H-2L* loci in the mouse (4). Although they are found on most somatic cells of the body, their expression level differs from tissue to tissue (5) and can be modulated by different agents (6). Among the most powerful inducers of class I heavy chains and β_2m are type I (α and β) and type II (γ) interferons (IFNs) (6–9).

Despite many similarities in the biological activities of type I and type II IFNs, there are sufficient differences to imply that the intracellular mechanisms by which they exert their influence may not be identical. They have different cell surface receptors (10, 11), and each induces the expression of a unique set of genes in addition to a common set (12–15). Hence, it is unclear whether the two types of IFNs utilize similar strategies to alter the levels of class I antigens.

Transcriptional as well as posttranscriptional processes were proposed to play a role in gene regulation by IFN (16). Recently, Friedman and Stark (17) identified a conserved sequence that spans ≈30 base pairs (bp) in the promoter regions of several IFN-α-inducible human genes. This sequence is involved in transcriptional regulation of the murine *H-2K*^{b} class I gene by IFN-α/β and IFN-γ in L cells (18). On the other hand, Yoshie *et al.* (19) reported that the expression of a promoterless human class I gene, *HLA-B7*, transfected into L cells is regulated by IFN-β. We show here that sequences upstream and downstream of the transcription initiation site are independently involved in IFN-α and IFN-γ regulation of murine class I genes and that the level of induction controlled by the promoter region constitutes only a minor portion of the response in L cells. In addition, we present results suggesting that the response to IFN-γ and -α may have different sequence requirements in the promoter region.

MATERIALS AND METHODS

DNA Constructs, Enzymes, and Reagents. Class I genes and their derivatives were subcloned from BALB/c cosmid and phage λ clones (20–22). We thank C.-L. Kuo for providing the L^{d}CAT and pBRCAT constructs, T. Wong for FeLVL^d (23), and L. Garfinkel for RSVCAT (24). Sequencing was done by the method of Maxam and Gilbert (25). Recombinant murine IFN-γ (specific activity, 1.3×10^7 units per mg) was supplied by Genentech (South San Francisco, CA), and recombinant human IFN-α A/D [a fusion of the 5′ end of IFN-α gene A and the 3′ end of IFN-α gene D (26); specific activity, 8×10^7 units per mg] was supplied by Hoffmann–La Roche.

Cells and Tissue Culture. All transfections were performed as described by the calcium phosphate precipitation technique (27).

Assays of Chloramphenicol Acetyltransferase (CAT) Activity. CAT protein extracts were prepared and assayed according to Gorman *et al.* (24); protein concentrations were determined by Bio-Rad protein assay.

Quantitative Measurements of Cell-Surface Expression of Transplantation Antigens by RIA. *H-2D*^{d} and *H-2L*^{d} transfectants were treated with IFN-γ (0.2 to 1000 units per ml) for variable times (12 hr to 4 days), and the levels of H-2^d as well as H-2^k antigens were measured by RIA using antigen-specific monoclonal antibodies. To observe a maximal response for both CAT and class I proteins, a 72-hr treatment with 2 units of IFN-γ per ml was sufficient, so a saturating amount of IFN-γ (20–50 units per ml) and a 3-day incubation was chosen for all experiments. Transfected and endogenous transplantation antigens were always induced coordinately in L cells; therefore, it was possible to study factors affecting IFN induction of transfected transplantation antigens quantitatively by standardizing the expression of exogenous H-2^d antigens relative to endogenous H-2^k antigens. Quantitative RIAs were performed as described (27) with saturating concentrations of antibodies [28-14-8 and/or 30-5-7 (anti-H-2L^d), 34-5-8 (anti-H-2D^d), and 11.4 (anti-H-2K^k)] and ^{125}I-labeled protein A.

RNase Protection Assays. The experiments were performed as described by Melton *et al.* (28). Cellular RNA was isolated by the method of Chirgwin *et al.* (29).

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Abbreviations: β_2m, β_2-microglobulin; IFN, interferon; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); RSV, Rous sarcoma virus; EF, enhancement factor; FeLV, feline leukemia virus.

RESULTS

The Region Upstream of the Transcription Initiation Site Is Involved in IFN-γ Regulation. The L cell line selected for the initial study of class I gene regulation expresses high levels of H-2^k class I antigens, comparable to C3H spleen cells (data not shown). When L cells, or class I gene transfectants of L cells, are treated with IFNs, the level of exogenous and endogenous class I antigens is enhanced 2.5- to 8-fold depending on the experiment and the antigen tested. To establish the role of 5′ class I gene sequences in regulation by IFN, the *H-2D*^{d} and *H-2L*^{d} promoter regions were linked to the bacterial gene encoding CAT, transfected into L cells, and assayed for CAT activity. This allows an indirect quantitation of the activity of a eukaryotic promoter (24).

The plasmids D^{d}CAT and L^{d}CAT (Fig. 1*A*) were constructed by ligating 4.8-kilobase (kb) *Hin*dIII-*Bam*HI fragments from the *H-2D*^{d} and *H-2L*^{d} genes to the CAT gene. The 4.8-kb DNA fragments contain sequences homologous to the *H-2K*^{d} class I promoter region for which the transcriptional start site has been mapped (30). This was established by sequencing 0.4 kb of the *H-2D*^{d} flanking region (Fig. 1*B*). The consensus sequence involved in transcription regulation by IFN-α (17) is located in the *H-2D*^{d} promoter at position −165 to −136.

The D^{d}CAT and L^{d}CAT plasmids were stably transfected into fibroblast Ltk$^-$ cells, which lack the gene encoding thymidine kinase. Transfectants were cultured for 3 days in the presence or absence of saturating concentrations of murine IFN-γ. These conditions were chosen on the basis of titration and time course studies. In cells transfected with the L^{d}CAT or D^{d}CAT constructs, an average 1.35 increase in CAT activity was observed in response to IFN-γ (Fig. 2). Although this increase is small, it was highly reproducible; each CAT construct was tested a minimum of seven times, and rigorous analysis of the results showed that the effect is statistically significant (see the legend to Fig. 2). By comparison, H-2D^d and H-2L^d cell-surface protein expression in L cells stably transfected with intact *H-2D*^{d} and *H-2L*^{d} genes was increased by IFN-γ 2.5- to 6-fold (measured by RIA). For controls, the plasmids pBRCAT, which has no eukaryotic promoter, and RSVCAT (Fig. 1), which contains a promoter from RSV (24), were transfected into L cells. The pBRCAT transfectants did not express detectable CAT protein levels, whereas RSVCAT transfectants expressed moderate levels (lower than L^{d}CAT or D^{d}CAT) that were not influenced by exposure to IFN-γ (Fig. 2).

Localization of the IFN-γ-Responsive Sequence in the *H-2D*^{d} Promoter. To map the D^{d}CAT sequence conferring responsiveness to IFN-γ more precisely, a set of promoter deletions was constructed (Fig. 2). The deletion junctions were sequenced and denoted by numbers corresponding to the number of bases remaining in the construct upstream from the transcription start site (Fig. 1*B*). Individual constructs were introduced into L cells, and CAT levels were measured in IFN-γ-treated and untreated cells (Fig. 2).

The deletion constructs Δ-317, Δ-262, Δ-236, and Δ-159 had approximately the same response to IFN-γ as the intact 4.8-kb fragments in the D^{d}CAT and L^{d}CAT plasmids, suggesting that an IFN-responsive site is present in all of these constructs. Inspection of the Δ-159 sequence, from which 6 bp of the IFN-responsive consensus sequence was deleted, revealed that fusion with pBR322 DNA restored almost perfectly the missing nucleotides (see Fig. 3). The expression of the Δ-122, Δ-65, and Δ-56 plasmids was slightly suppressed by IFN-γ, indicating that the integrity of IFN-sensitive site(s) has been destroyed in these constructs.

One of the deletion constructs, Δ-385, was enhanced by IFN-γ approximately twice as much as the D^{d}CAT, L^{d}CAT,

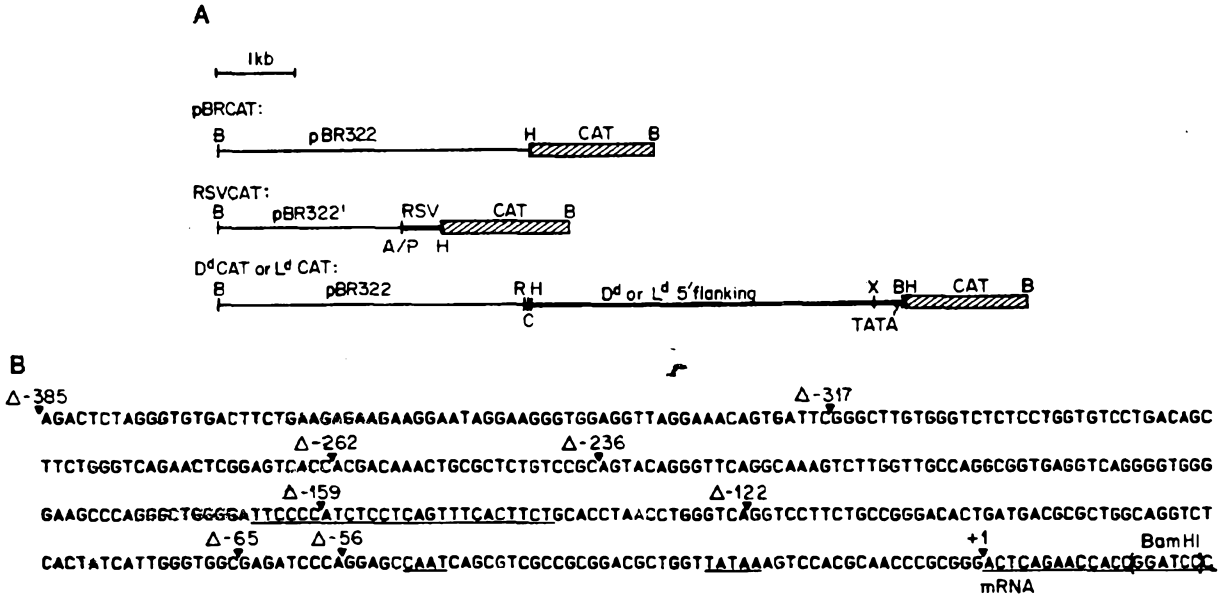

FIG. 1. (*A*) DNA constructs used for 5′ flanking region analyses. The plasmids are shown linearized at the conserved *Bam*HI restriction enzyme site. All constructs contain pBR322 sequences and the bacterial structural gene encoding CAT, followed by a simian virus 40 polyadenylylation signal (indicated by hatched bars). The bold lines indicate regions containing eukaryotic promoters. The plasmid RSVCAT (25) consists of a 2.1-kb fragment of pBR322 (labeled pBR322′), the CAT gene, and a promoter from the Rous sarcoma virus (RSV). The plasmid pBRCAT lacks eukaryotic promoter sequences. To construct the plasmids L^{d}CAT and D^{d}CAT, *Hin*dIII linkers were added to the *Bam*HI sites of the 4.8-kb fragments containing *H-2L*^{d} and *H-2D*^{d} promoter regions, and the fragments were inserted into pBRCAT. Restriction enzyme sites: B, *Bam*HI; A/P, *Acc* I/*Pvu* II junction; H, *Hin*dIII; R, *Eco*RI; X, *Xba* I; and C, *Cla* I. (*B*) DNA sequence of the *H-2D*^{d} promoter region. The nucleotides are numbered relative to the transcription start site (+1). The IFN-responsive consensus sequence, TATA box, and CAAT box are underlined. The positions of deletion end points are indicated by arrows. The *Bam*HI site used to join the *H-2D*^{d} promoter region to the gene for CAT is indicated.

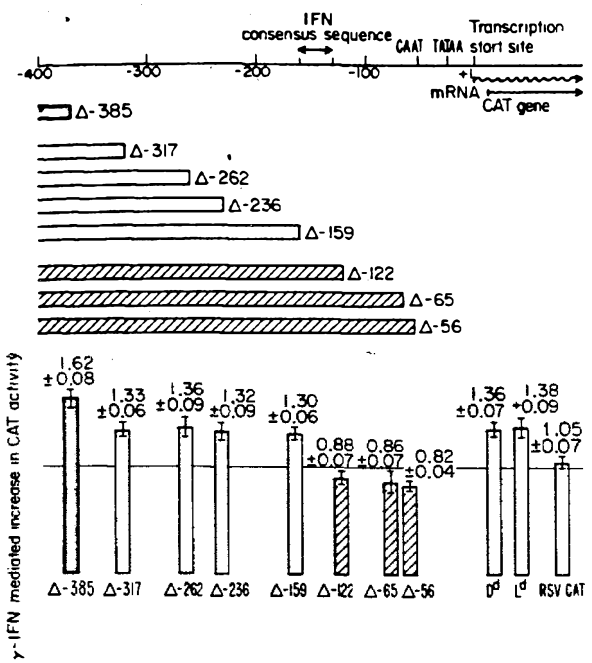

FIG. 2. Localization of the IFN-γ-sensitive site in the 5′ flanking region of class I genes. The horizontal bars indicate the extent of the progressively deleted D^dCAT promoter region. The set of deletion constructs was generated by BAL-31 digestion of D^dCAT plasmid linearized at the *Xba* I site. The DNA was then cleaved at the *Cla* I site in pBR322, end-filled, and ligated to close the plasmid. The deletion constructs are designated by the number of bases remaining relative to the transcription start site. The stippled bar indicates the deletion construct with the highest level of induction, the open bars indicate the deletion constructs that were induced by the same factor as the intact promoter; and the hatched bars indicate deletion constructs that were not induced. The enhancement factor (EF) was calculated as the ratio of the specific CAT activity (CAT activity per protein concentration) in cells treated with IFN to that in untreated cells. The basal levels of CAT gene expression varied between transfectants. Expression of D^dCAT and L^dCAT was ≈5- to 10-fold higher than that of the *H-2D*^{d} deletion constructs; the basal (unstimulated) levels of CAT activity were: ≈130 units per mg of protein for D^dCAT and L^dCAT and ≈14 units for Δ-122, the deletion mutant with the lowest expression (1 unit = 1 nmol of chloramphenicol acetylated per hr at 37°C). Individual transfectants were titrated for CAT activity so that the induction experiments were done with an appropriate amount of protein to fall within the 5–50% acetylated range. The measurements of induced and uninduced levels of CAT activity were made in parallel on the complete set of transfectants. The standard deviation of each estimate is shown. The probability that the EFs are statistically different from the uninduced state (EF = 1) is 99.7% for D^dCAT, L^dCAT, Δ-317, Δ-262, Δ-236, and Δ-159 constructs. The probability that the EF for Δ-385 is different from parental D^dCAT is 99.7%. These calculations were derived by defining the probability of an individual EF as: 1 − confidence level.

Δ-317, Δ-262, Δ-236, or Δ-159 constructs. Sequence analysis of Δ-385 DNA revealed that the fusion of the *H-2D*^{d} DNA and the pBR322 DNA fortuitously created sequences that resemble the IFN-α-responsive consensus sequence (Fig. 3). Therefore, Δ-385 carries several potentially functional IFN-sensitive sequences. Alternatively, the phenotype of Δ-385 could be explained by the existence of an additional sequence that confers IFN responsiveness, between −385 and −317. The activity of such a sequence may be masked in the parental D^dCAT and L^dCAT constructs due to down-regulatory regions upstream of −385 or because of the higher basal level of expression of the parental constructs relative to the deletions.

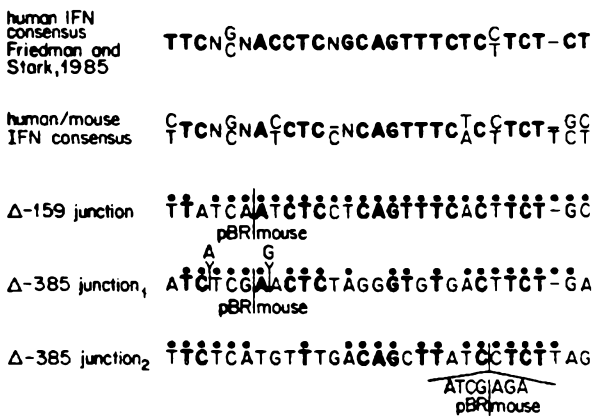

FIG. 3. Comparison of the consensus sequence involved in transcription regulation by IFN with the junctional sequence of deletion constructs. The human consensus sequence (17) is based on the four human sequences previously compared: *HLA-DR*, *HLA-A3*, an unidentified *HLA*, and *MT2*, a metallothionein gene. The human/mouse consensus sequence is based on the sequences above plus five murine class I sequences: *H-2K*^{b}, *H-2L*^{d}, *Q10*, *H-2K*^{d} (31), and *H-2D*^{d}. Bold letters indicate nucleotides conserved in eight of nine genes. The junctions of pBR322 and *H-2D*^{d} promoter DNA in the deletion constructs Δ-385 and Δ-159 are shown, with two alignments of the Δ-385 sequence junction. The dots indicate bases conserved between the junctional sequences and the human/mouse consensus sequence. The 3′-terminal nucleotides of pBR322 are the same in all of the deletion junctions (ATCG, part of the *Cla* I site; see for example Δ-385) with the exception of Δ-159, in which deletion/fusion removed three additional nucleotides, TCG.

To confirm these results by another assay, the experiments were repeated with transiently transfected NIH 3T3 cells and Ltk$^-$ cells. In both cell lines, the IFN-γ response of the entire set of deletion constructs and control plasmids was quantitatively similar to the response in the stably transfected L cells (data not shown).

To address the possibility that the progressive deletion of the *H-2D*^{d} promoter may have resulted in changes leading to incorrect transcription initiation, ribonuclease protection assays were performed (ref. 28; data not shown). RNA isolated from cells transfected stably with D^dCAT, Δ-385, and Δ-159 was initiated properly. Most of the Δ-122 RNA was also initiated correctly, but in addition ≈12% of the Δ-122 RNA used an aberrant transcription initiation site located within the pBR322 DNA. The levels of IFN-γ-induced Δ-385 and Δ-159 CAT RNAs were also measured by quantitative RNase protection experiments. These experiments established that the increase in the number of correctly initiated transcripts correlates with the increase in the CAT protein activity (≈3-fold enhancement for Δ-385 RNA and ≈2-fold for Δ-159; data not shown).

DNA Sequences Located Downstream from the Start Site of Transcription Also Contribute to IFN-γ Regulation. In L cells IFN-γ enhanced the membrane expression of the transfected transplantation antigens from 2.5- to 6-fold, but the analysis of the 5′ encoded response has shown that it accounts for <2-fold increase in expression (<40% of the overall induction effect). Therefore, it is unlikely that the 5′ encoded response plays an important role in overall regulation in L cells, and we reasoned that other mechanisms encoded outside of the promoter must be involved. Therefore, we looked for regulatory sequences located 3′ to the transcriptional start site by studying the regulated expression of the *H-2L*^{d} gene fused to a feline leukemia virus (FeLV) promoter (Fig. 4). The analysis of this construct cannot differentiate between transcriptional and posttranscriptional regulation. The FeLV

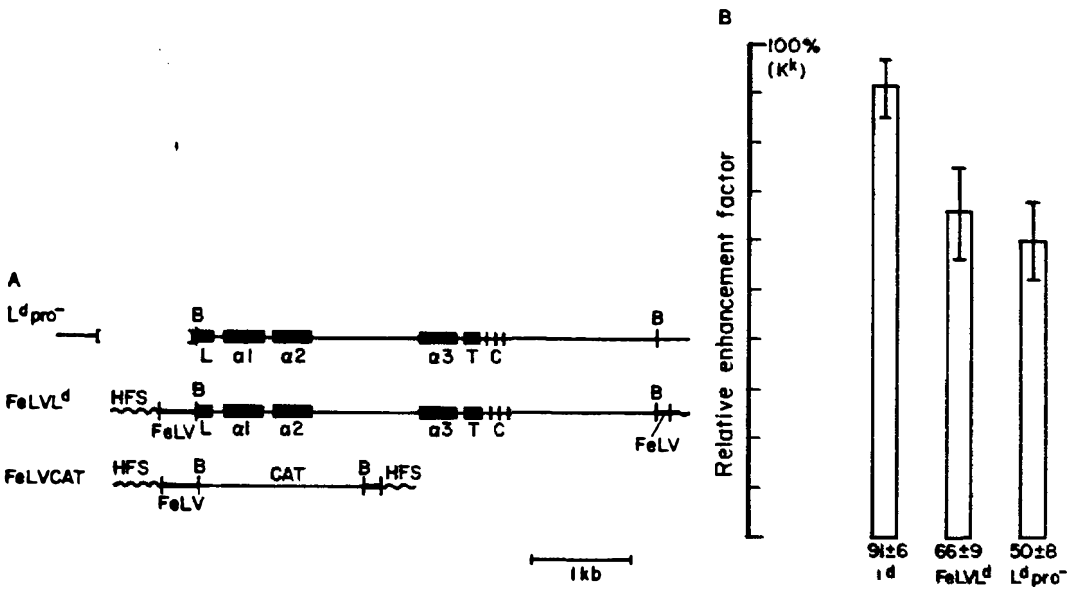

FIG. 4. DNA sequences downstream from the transcription start site contribute to the ability of class I genes to respond to IFN-γ. (*A*) The structure of DNA constructs lacking class I *H-2L*^{d} promoter regions. The coding *H-2L*^{d} region is shown as thin lines (introns) or filled bars (exons). L encodes the leader peptide; α1, α2, α3, the three external domains; T, the transmembrane region; and C, the cytoplasmic tail. The FeLVL^d plasmid has been described (28). It contains a FeLV promoter inserted in front of the *H-2L*^{d} structural gene, in place of parental *H-2L*^{d} 5′ flanking region. The FeLV sequence encodes its own TATA box and transcriptional start site and promotes transcription of the *H-2L*^{d} gene in L cells (24). HFS denotes human flanking sequences present in the plasmid. The FeLVCAT subclone, used to demonstrate that the FeLV promoter is not regulated by IFN, was made by inserting the *Bam*HI fragment carrying the CAT gene (from D^{d}CAT) in place of the *H-2L*^{d} structural gene. The L^{d}pro$^-$ mutant was constructed by excising a 5′ flanking ≈1-kb *Sma* I fragment containing the *H-2L*^{d} TATA, CAAT, and CAP site and the IFN-responsive consensus sequence from the *H-2L*^{d} gene. The downstream *Sma* I site (designated S) is located 12 nucleotides downstream from the transcription initiation site. (*B*) Effect of IFN-γ on the expression of FeLVL^d and L^{d}pro$^-$ mutants. L cells transfected with *H-2L*^{d} or FeLVL^d or L^{d}pro$^-$ mutants were grown in parallel with or without IFN-γ, and the levels of H-2 antigens were quantitated by RIA. The enhancement of the endogenous H-2K^k protein ranged from 3- to 6-fold. The level of transfected H-2L^d protein changed coordinately with the H-2K^k expression. To compare different *H-2L*^{d} transfectants, the data were standardized relative to the internal H-2K^k control (for which the EF was set at 100%). The uninduced level of H-2L^d cell-surface expression was the same in FeLVL^d as in *H-2L*^{d} transfectants, while L^{d}pro$^-$ transfectants expressed <5% of the H-2L^d control. The results are averages of five experiments. The standard errors are shown.

promoter functions efficiently in L cells and can initiate transcription of the *H-2L*^{d} gene (23). The FeLV promoter is not regulated by IFN-γ because, when fused to the CAT gene (Fig. 4*A*) and transfected into L cells, it is expressed at the same level in IFN-treated and untreated cells (Table 1). The cell-surface expression of the FeLVL^d protein in L cells is increased by IFN-γ (2- to 4-fold), but not to the same extent as the parental H-2L^d protein (Fig. 4*B*). Therefore, when the wild-type promoter is replaced with a nonregulated promoter, the ability of class I genes to respond to IFN-γ is diminished but not abolished.

Additional supporting evidence for the existence of IFN-responsive sites located outside the promoter region came from the analysis of an *H-2L*^{d} promoter-minus construct, L^{d}pro$^-$, from which a 1-kb fragment including the IFN-responsive consensus sequence was removed from the 5′ flanking region (Fig. 4*A*). When the L^{d}pro$^-$ construct was transfected into L cells, it was expressed at low levels detectable only by a sensitive RIA procedure (<5% of the wild-type levels; data not shown). It is unlikely that its expression was regulated by a murine promoter located outside of the integrated L^{d}pro$^-$ construct, but rather by a low-efficiency promoter within the plasmid, because 10 different clones of L^{d}pro$^-$ transfectants representing independent transfection events expressed H-2L^d at the same low levels (data not shown). When L^{d}pro$^-$ transfectants were treated with IFN-γ, H-2L^d cell-surface expression was increased by a factor comparable to that seen in FeLVL^d transfected cells (2- to 4-fold; Fig. 4*B*). Thus, it is likely that in both FeLVL^d and L^{d}pro$^-$, the sequences located downstream from the transcriptional start site play a role in responsiveness to IFN.

Table 1. Comparison of the IFN-α and IFN-γ responses of various CAT gene constructs transfected stably into L cells

IFN	EF of selected constructs					
	D^{d}CAT	L^{d}CAT	Δ-385	Δ-159	Δ-122	FeLVCAT
IFN-γ	1.36	1.38	1.62	1.30	0.88	1.05
IFN-α	1.46	1.41	2.17	1.04	0.66	1.07

The EFs are listed for each transfectant. The IFN-γ EFs are from Fig. 2; the IFN-α EFs are based on one series of experiments done in duplicate.

IFN-α Responses Are Also Controlled by the 5′ and 3′ Regions of Class I Genes. Because of the known differences in the two types of IFNs (see Introduction), we asked if the modified class I genes transfected into L cells showed the same pattern of regulation by IFN-α as by IFN-γ. Since murine IFN-α was not available to us, we did a limited number of experiments with recombinant human IFN-α, which is active on murine cells. L cells transfected stably with D^{d}CAT, L^{d}CAT, Δ-385, Δ-122, and FeLVL^d plasmids and with intact *H-2L*^{d} and treated with saturating amounts (800 units per ml) of IFN-α A/D for 3 days showed comparable levels of enhancement of CAT and H-2L^d expression as those treated with IFN-γ (Table 1; the IFN-α EF for FeLVL^d was >50% of the EF for *H-2L*^{d}).

An important difference was observed between responses to IFN-γ and IFN-α. One of the tested plasmids, Δ-159, which is inducible for CAT expression by IFN-γ, did not respond to IFN-α (Table 1). Apparently the presence of the

IFN-γ-responsive site was not sufficient for Δ-159 inducibility by IFN-α.

DISCUSSION

Using deletional analysis of the murine *H-2D*^{d} promoter, we have identified the DNA region necessary for its regulation by IFN-γ. It contains a ≈30-bp sequence located at positions −165 to −136, which is homologous to the human IFN-α-responsive consensus sequence (17) and is important for IFN regulation of the *H-2K*^{b} promoter (18). This region, which is designated "IRS" for IFN-responsive sequence, was proposed to have an effect on transcription initiation by potentiating the action of a functional *H-2K*^{b} enhancer in IFN-treated cells (18). Two restriction enzyme fragments having enhancer properties were previously identified in the *H-2K*^{b} 5′ flanking region (31). The one located between nucleotides −213 to −165 was designated "A," and the other one, located between nucleotides −120 to −61, was designated "B." Israel *et al.* (18) have shown that in L cells the response of the *H-2K*^{b} promoter to type I (α + β) IFN requires the combination of enhancer A and the IRS. Our results with human recombinant IFN-α in L cell transfectants support this conclusion. In contrast the experiments with recombinant murine IFN-γ established that the enhancer A sequence can be deleted from the *H-2D*^{d} promoter without loss of IFN-γ inducibility. It is possible that IFN-γ regulation may act through the IRS independently of enhancer regions or that it may require enhancer B, which is present in all of our IFN-γ-inducible promoter mutants. At present we cannot distinguish between these two possibilities, but it is apparent that in L cells IFN-α and -γ have different sequence requirements for the promoter-dependent response.

We have shown that in L cells the overall induction of the transfected class I antigens is up to 6-fold, whereas the promoter-encoded response to IFN accounts for <2-fold enhancement in transcription initiation. Consistent with this observation is the finding that expression of the *H-2L*^{d} gene transcribed from nonregulated promoters can still be enhanced by 2- to 4-fold. Thus, the two mechanisms, which appear to act independently of each other on the regions located 5′ and 3′ to the transcription initiation site, account together for the full response of class I genes to IFNs.

The location and identity of the downstream regulatory regions have not been established. It may be that these regions are unrelated to the IRS, since no consensus IRS sequence was detected in *H-2K*^{d}, *H-2D*^{d}, and *H-2L*^{d} in a computer search. The 3′ regulatory sequences could be involved in posttranslational events such as an increase in RNA stability (16) or in the rate of the class I mRNA translation. Alternatively, the IFN-mediated increase of FeLVL^d and L^{d}pro$^-$ class I proteins could be explained by the changes in the relative concentrations of β_2m and H-2L^d chains. If β_2m is necessary for the transport of class I proteins to the cell surface and it is present in excess in IFN-treated cells, then the cell-surface expression of transplantation antigens may be more efficient in those cells. We consider this possibility unlikely because FeLVL^d has the same enhancement factor in response to IFN as L^{d}pro$^-$, which expresses basal amounts of H-2L^d protein that are lower by a factor of 20.

The existence of two different regions involved in IFN responses suggests that the mechanisms operating on them may be used for the fine tuning of class I gene expression under different conditions. It would be interesting to define the relative contribution of these two mechanisms in cells and tissues in which the IFN inducibility and the basal level of class I antigen expression varies during development or immune responses. This approach may provide information about the biological significance of each of these mechanisms under physiological conditions.

We thank Kay Blackburn for assistance with tissue culture, and Drs. U. Landegren and S. Hunt for many thoughtful conversations concerning the work. We thank Drs. A. Israel, L. Hoops, N. Lan, P. Lengyel, K. Ozato, S. Rosenberg, E. Rothenberg, and B. Wold for criticism of the manuscript. We are grateful to M. Shepard of Genentech for providing IFN-γ, P. Sorter of Hoffman-La Roche Inc. for IFN-α A/D, and T. Hansen and K. Ozato for monoclonal antibodies. This work was supported by National Research Service Award T32GM7616 and by National Institutes of Health Grant AI 19624.

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Appendix to Chapter Two

The additional data presented here make several points that are relevant to the mechanism acting on the interferon responsive region located 3' relative to the transcription initiation sites of class I genes. First, the 3' (as well as the 5') responsive region was active in several different cell types. Second, the 3' interferon response as measured at the cell surface by RIA was paralleled by an increase in cytoplasmic class I protein tested by two-dimensional protein gels, so the phenomenon was not due merely to interferon triggering increased transport of a steady level of class I antigens to the cell surface. Third, the interferon sensitivity of the transfected genes was tested in cloned cell lines derived from the stably transfected pools of colonies; each cloned line responded to interferon, although to varying degrees, with different basal expression levels.

Summary of Cell Types

Fibroblast-like thymidine kinase deficient L cells (L^{tk-} cells, which will be referred to here simply as L cells) were initially used for these experiments because extensive class I expression studies using these cells had previously been done in our lab and in others (1,2). L cells originated as cultures from an adult C3H mouse and are of the $H\text{-}2^{k}$ haplotype (see ATTC CCL1.1 NCTC clone 929 for a description). L cells normally have high levels of class I expression and are not induced to a great extent by interferons. To better characterize the 5' and 3' responses to interferons, we tested additional cell types. The other three cell lines we tested were two fibroblastoid lines of embryonal

origin, BL5 and BLK SV, and one cell line of neural origin, SDBT. These cell types were chosen because embryonal (3) and neural (4) tissues express low levels of class I antigens that can be induced many-fold by interferons.

These cell types, like the tissues from which they were derived, had dramatic inductions of class I gene expression upon exposure to interferons. Table 1 summarizes the range of interferon inducibility of class I antigens in L^{tk-}, BL5, BLK SV, and SDBT cells.

BL5 cells are derived from primary fibroblast cultures taken from C57BL embryos at 14-16 days of gestation and are of haplotype $H-2^b$ (5). These cells change phenotype while maintained in continuous culture (for example, they become smaller (5)), and with passage begin expressing higher levels of class I antigens (data not shown). Therefore, low passage number cells were used for all experiments described. Cell density, as well as passage number, can drastically influence the basal level of class I expression in these cells. Confluent, heavily populated cultures expressed four- to five-fold more class I antigen per cell than lightly seeded cultures grown in parallel. It is possible that high density in culture causes the cells to differentiate.

SDBT cells were derived from a Rous sarcoma viral induced brain tumor in a CDF-1 mouse and have astrocytoma-like properties (6). CDF-1 mice are the progeny of a DBA x BALB/c cross, and therefore are of $H-2^d$ haplotype. (These cells were kindly provided by Dr. Sussman and Dr. Stohlman at USC). Although these cells are $H-2^d$ haplotype, they do not express endogenous $H-2L^d$ or $H-2D^d$ antigens at detectable levels either before or after interferon treatment. $H-2K^d$, on the other hand, is

expressed in SDBT cells and is induced six- to twelve-fold upon interferon exposure (Table 2.1 and Fig. 2.1). It is fairly common for tumor cell lines to lose either H-2L and H-2D, or H-2K, expression (for review, see 7). Because the endogenous H-$2L^d$ gene is not expressed, the regulation of transfected H-$2L^d$ constructs could be studied.

SDBT cells have several virtues that led us to use them. They are highly responsive to interferon in terms of class I induction, they are stable in culture, easily transfected, and they provided a non-fibroblast line for comparison to the other cell lines used. They also adapt well to suspension culture, and can be used for experiments that require large scale tissue culture preparations.

BLK SV cells are an embryonic fibroblast-like cell line immortalized by SV40 transformation (8), and are unusual in several respects. The class I mRNA levels increased greatly in response to interferon ($\frac{1}{2}$ 15-fold), but the class I antigen levels at the cell surface were barely altered ($\frac{1}{4}$ 2-fold, Table 1). This will be discussed in more detail in Chapter Three. The low level of responsiveness at the cell surface made RIA analysis of interferon induction of class I antigens difficult.

Analysis of the Class I 3' Interferon Responsive Region in Different Cell Types

Both the 5' and the 3' class I response to interferon was studied in L, BL5 and SDBT cells. The 5', promoter region, response will be discussed in the next chapter. The 3' interferon response is illustrated in Fig. 2.1, which compares the induction of expression of

an intact H-2L^d gene with its own promoter, and the FeLVL^d construct with a feline leukemia viral promoter, transfected into BL5, SDBT, and L cells. This figure illustrates that the L^d gene linked to a promoter that is not regulated by interferon can be induced in all three cell types tested, hence the activity of the 3'interferon responsive region is not restricted to L cells alone.

Cytoplasmic Induction of Class I Proteins Encoded by L^d and FeLVL^d

To assess the ability of gamma interferon to induce the total cellular (cytoplasmic as well as cell surface) expression of transfected H-2L^d constructs, versus the cell surface expression measured by RIA that is shown in Fig. 2.1, two-dimensional gel electrophoresis of ^{35}S-methionine labeled total protein was used. The H-2L^d gene products in different stages of glycosylation were identified by comparing the relative positions of H-2L^d and actin protein spots in the total protein gels to H-2L^d and actin in immunoprecipitations of H-2L^d (actin co-precipitates with class I genes, and the actin protein family is readily discernable in total protein gels). The identity of the H-2L^d protein spots was further established by their appearance only in cells transfected with the H-2L^d gene, compared to non-transfected controls (shown in Figs. 2.2 and 2.3). Fig. 2.2 compares the gamma interferon inducibility of H-2L^d expression in BL5 and L cells. This confirms that the low level interferon induction of H-2L^d cell-surface expression observed in L cells is paralleled by a low level cytoplasmic induction, and is not due to limited transport of highly induced protein to the cell surface.

Fig. 2.3 demonstrates that in BL5 cells the interferon induction of the FeLVL^d gene product measured by RIA is not due to increased transport of the antigen to the cell surface, because the cytoplasmic H-2L^d protein encoded by FeLVL^d is also well induced (4.3-fold, see legend to Fig. 2.3). The Proc. Natl. Acad. Sci. paper that makes up the body of this chapter examines only cell surface expression by RIA and does not address this point. We had considered the possibility that interferons may be able to increase the rate of transport of class I antigens to the cell surface through a mechanism such as increased beta-2 microglobulin expression (beta-2 microglobulin association with class I protein has been shown to be important for cell surface expression (9,10)). This study eliminates the possibility that the interferon induction of cell surface expression of the H-2L^d gene linked to a non-regulated promoter is due to intracellular transport, at least in BL5 cells.

Interferon Induction of Cloned Cell Lines of Stable Transfectants

For the studies previously described in this chapter, we used pools of colonies of stably transfected cells in an attempt to obtain unbiased values of the expression levels of transfected genes. To examine the range of expression and induction of the class I transfected genes within these populations, cloned lines were isolated from BL5 transfectants containing L^d, FeLVL^d, and the L^{d}CAT constructs. This set of transfectants was chosen because it enabled us to study the regulation of the entire class I gene, and both the 3' and 5'interferon responsive regions. (The L^{d}CAT construct transfected BL5 clones are

discussed in Chapter Three.)

One of the questions we could address with the sets of clones was whether interferon was causing increased expression of class I gene constructs by causing some portion of the total population to go from off to on -- for example to go from 10% of the population expressing class I genes to 50%, causing a five-fold increase in class I expression in the overall population. All of the clones tested expressed the transfected genes prior to interferon treatment, and all were responsive to interferon (Fig. 2.4). The expression of the transfected H-2L^d constructs and the endogenous H-2K^b gene were both tested simultaneously. There was substantial variation in the initial expression levels and inducibility of the class I constructs among the cloned lines, and the expression and induction of the H-2K^b gene did not tightly correlate with the expression of the transfected genes. The range of induction of the various clones was similar to the range of induction in pools of transfectants in different assays. Quantitative studies of anti-viral effects and mRNA regulation due to interferons have also been shown to vary widely when cloned derivatives of the RD-114 cell line were tested (11).

Fig. 2.1 shows that in BL5 cells the L^d antigen encoded by the FeLVL^d construct is more inducible than L^d transcribed from its own promoter. The L^d promoter is highly responsive to interferon in BL5 cells, and the FeLV promoter was shown to be non-inducible by repeated trials with FeLVCAT construct transfectants (this is shown in Chapter Three). This anomaly is likely to be due to the variability between particular assays. As Fig. 2.4 shows, contrary to the result in Fig.

2.2, in the cloned lines the protein encoded by the L^d gene tended to express lower levels and be more highly induced than its $FeLVL^d$ counterpart. Therefore cloned lines gave more sensible results than their mixed population counterparts, which may arise from the fact that they were tested at very low passage number and therefore are probably more reliable indicators of relative levels of inducibility in BL5 cells.

Current Experiments

Additional studies to determine the nature of the 3' class I interferon responsive region are currently underway. Using a complete H-$2K^d$ cDNA linked to an SV40 promoter, a two-fold increase of H-$2K^d$ in response to interferon was observed in L cells when assayed by RIA (Iwona Stroynowski, personal communication). This indicates that H-$2K^d$, as well as H-$2L^d$, is capable of having a 3' response to interferon, and that the effect is encoded in the exons, as the introns were eliminated from the H-$2K^d$ cDNA. The results that indicate that the 3' interferon response is not due to protein transport are encouraging in terms of studying the effect at the level of mRNA. Studying class I mRNA levels and transcription rates present an extra challenge because of the high degree of homology between class I genes. Probes and hybridization conditions that can distinguish the transfected H-$2L^d$ gene products from the endogenous class I mRNAs are currently being developed for application to this problem (Elly de Pagter and Iwona Stroynowski).

Several plasmids were constructed to look for an interferon responsive enhancer-like activity within the transcribed region of the

H-2L^d gene. The transcribed region of H-2L^d genomic DNA was cut in half at a Bam HI site, and fused in both orientations 5' to a minimal non-regulated promoter linked to the CAT gene (Fig. 2.5). These constructs were transfected into BL5 cells, and expressed extremely low or non-detectable amounts of CAT protein that was not induced by interferon.

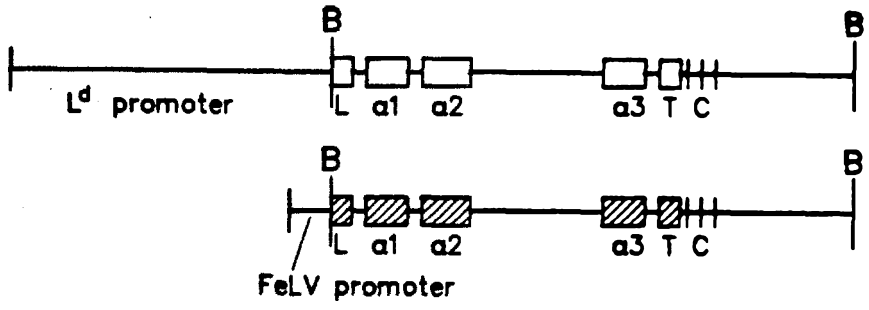
Table 2.1.) A summary comparing different cell types endogenous and transfected class I antigen responses to interferons.

L	BL5	BLK SV	SDBT
2.5-to-8 fold	5-to-30 fold	1.5-to-2.5 fold	6-to-15 fold

The observed range of interferon enhancement factors for both endogenous and transfected intact class I antigens in each cell type is given, assayed by RIA. The enhancement factor is defined as the level of expression after interferon treatment divided by the expression prior to interferon treatment; it indicates the factor by which interferons increase class I expression. For each cell type, optimal interferon concentrations and incubation periods were used. The variability observed depended upon the class I antigen being tested, the particular experiment, and the kind of interferon. BL5 cells tended to have lower enhancement factors using (alpha + beta) interferons versus gamma interferon. Some SDBT transfectants had reduced responses to gamma interferon compared to (alpha + beta) interferons, although others responded equally well to both.

Fig. 2.1.) DNA sequences downstream from the transcriptional start site contribute to the ability of class I genes to respond to interferons in L, BL5, and SDBT cells. (A) Structure of the genes used to study the downstream responsive region. Either the intact genomic sequence of the H-2L^d gene transcribed from its own promoter was transfected into recipient cells, or the Bam HI sites were used to excise the H-2L^d gene and link it to a FeLV promoter. The Bam HI sites are marked with a "B", and the exons are drawn as open or striped boxes to match the graph in part (B). (B) The effect of interferons on the cell surface expression of L^d versus FeLVL^d transfectants as measured by RIA. To compare different H-2L^d transfectants, the data presented here were standardized relative to internal controls of endogenous H-2K gene induction, for which the enhancement factor was set at 100% for a given cell type. The scales of the 100% H-2K standards were drawn to reflect the average relative induction in the different cell types. In these experiments, in L cells the H-2K^k antigen was induced 3-to-6 fold by gamma or (alpha + beta) interferons; in BL5 cells H-2K^b was induced 14-to-21 fold by gamma interferon, and 5-to-8 fold by (alpha + beta) interferons; and in SDBT cells H-2K^d was induced 6-to-12 fold by gamma or (alpha + beta) interferons. The relative induction of L^d is indicated by open bars, and of FeLVL^d by striped bars. These results are the averages of five experiments in L cells, and four each in SDBT and BL5 cells. FeLVCAT transfectants were tested and confirmed that the FeLV promoter is not regulated by interferon in these cell types (see Chapter Three).

A.)


B
B
L^d promoter
L a1 a2
a3 T C
B
B
L a1 a2
a3 T C
FeLV promoter

B.)

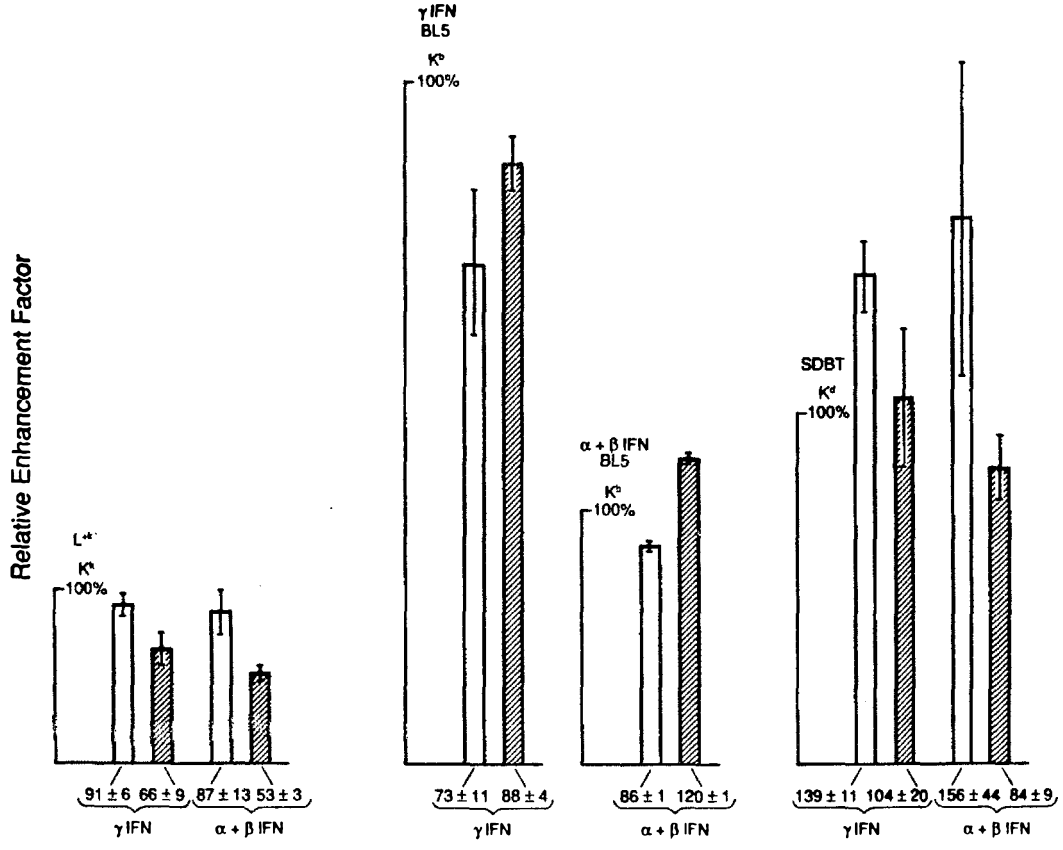

Relative Enhancement Factor
γ IFN
BL5
K^b
100%
α + β IFN
BL5
K^b
100%
SDBT
K^d
100%
91 ± 6 66 ± 9
87 ± 13 53 ± 3
73 ± 11 88 ± 4
86 ± 1 120 ± 1
139 ± 11 104 ± 20
156 ± 44 84 ± 9
γ IFN
α + β IFN
γ IFN
α + β IFN
γ IFN
α + β IFN

Fig. 2.2.) Comparison of gamma interferon regulated expression of L^d transfected into BL5 versus L cells using two-dimensional electrophoresis of ^{35}S methionine labeled total protein extract. The region of the gel where the L^d protein spots are found is circled. Their location was confirmed by comparisons of these gels with immunoprecipitations of L^d antigen; actin co-precipitates and can be used as a marker (data not shown). The actin family is indicated by the arrow. The multiple L^d protein spots are due to different stages of glycosylation -- the lowest most basic spot is the unglycosylated cytoplasmic form, the highest most acidic spot the fully glycosylated membrane-bound form. Parts (a), (b), and (c) were proteins isolated from BL5 cells. Part (a) was from non-transfected cells and serves as a negative control for the presence of L^d. Parts (b) and (c) were isolated from cells that have been stably transfected with the L^d gene, with and without exposure to interferon. All of the different glycosylated forms were heavily induced in this cell type. Parts (d), (e), and (f) were the corresponding studies using L cells. Attempts to quantify these autoradiographs in collaboration with Protein Data Base, Inc., were unsatisfactory, but examination by eye supports the conclusion that the cytoplasmic forms of the L^d antigen are barely induced in L cells and heavily induced in BL5 cells.

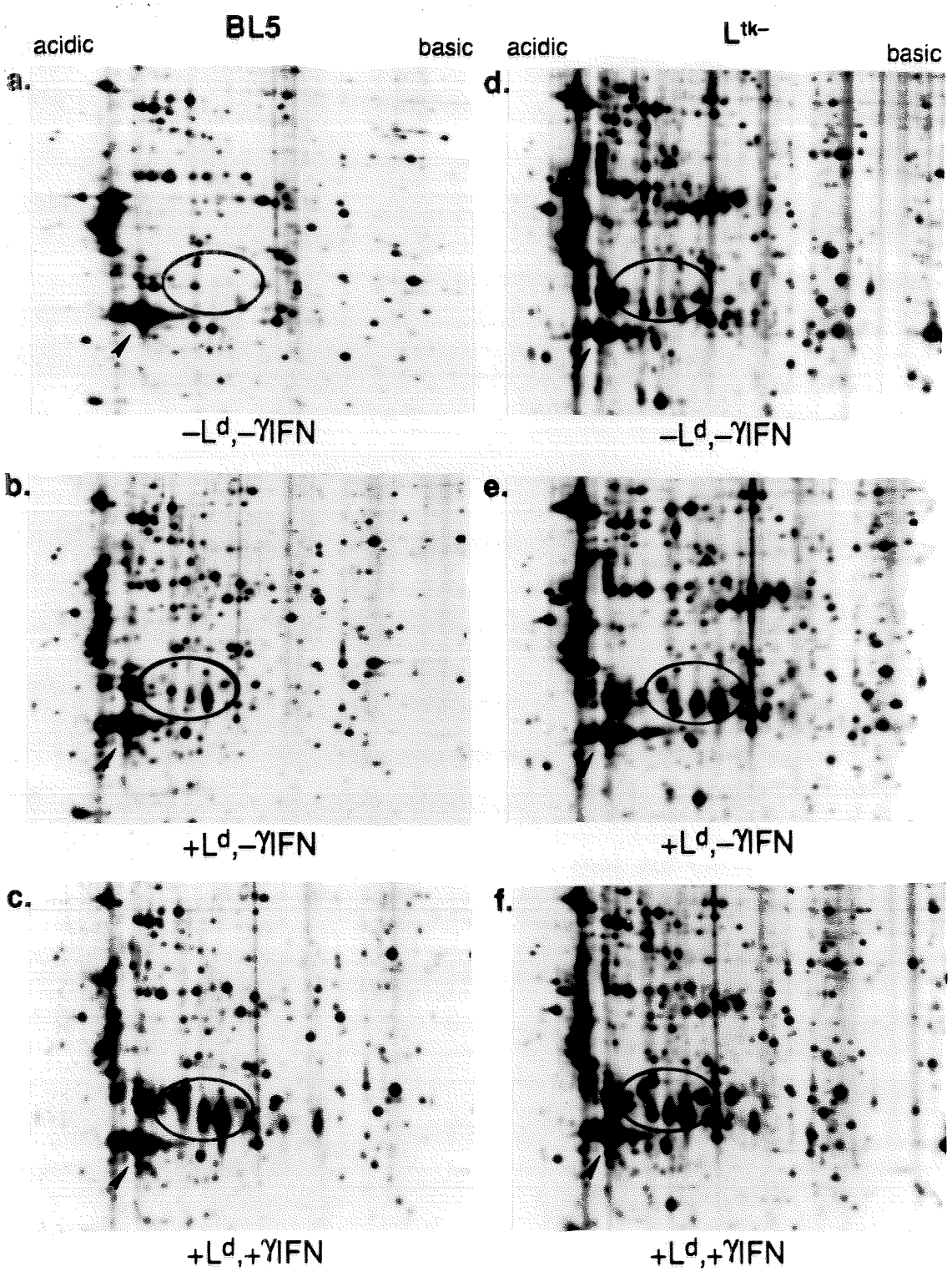

BL5
acidic
basic
a.
−Ld,−γIFN
b.
+Ld,−γIFN
c.
+Ld,+γIFN
Ltk−
acidic
basic
d.
−Ld,−γIFN
e.
+Ld,−γIFN
f.
+Ld,+γIFN

Fig. 2.3.) Comparison of gamma interferon regulation of FeLVL^d versus L^d constructs transfected into BL5 cells. These two-dimensional gels are similar to those in shown in Fig. 2.2; the arrow marks actin and the circled region the location of L^d. Parts (a) and (b) were negative controls for L^d expression in proteins isolated from non-transfected BL5 cells with and without interferon treatment. Parts (c) and (d) show the expression of L^d transfected into BL5 cells, with and without interferon treatment. Scanning densitometry was used to estimate the interferon enhancement factor of the L^d spots in the two-dimensional gels, and a 16.3-fold increase in L^d encoded total protein was measured. RIAs done in parallel with the protein isolation showed an 18.6-fold increase of L^d antigen on the cell surface. (An average of 2,200 cpm of ^{125}I-labeled protein A bound to 2.5 x 10^5 untreated cells/well used for the experiment shown in (c), after incubation with saturating amounts of anti-L^d monoclonal antibody. 41,000 cpm bound to the interferon treated cells used for (d), hence the 18.6-fold increase.) Parts (e) and (f) show the expression of FeLVL^d transfected into BL5 cells with and without interferon treatment. A 4.3-fold increase in FeLVL^d encoded total protein expression in response to interferon was measured by scanning densitometry. The corresponding RIAs done on these cells showed a 2.9 fold increase of cell surface expression. (An average of 14,000 cpm of ^{125}I-protein A bound to untreated cells and 41,000 cpm bound to cells that had been treated with interferon, using 2.5 x 10^5 cells/well taken from the samples used for protein isolation in (e) and (f).)

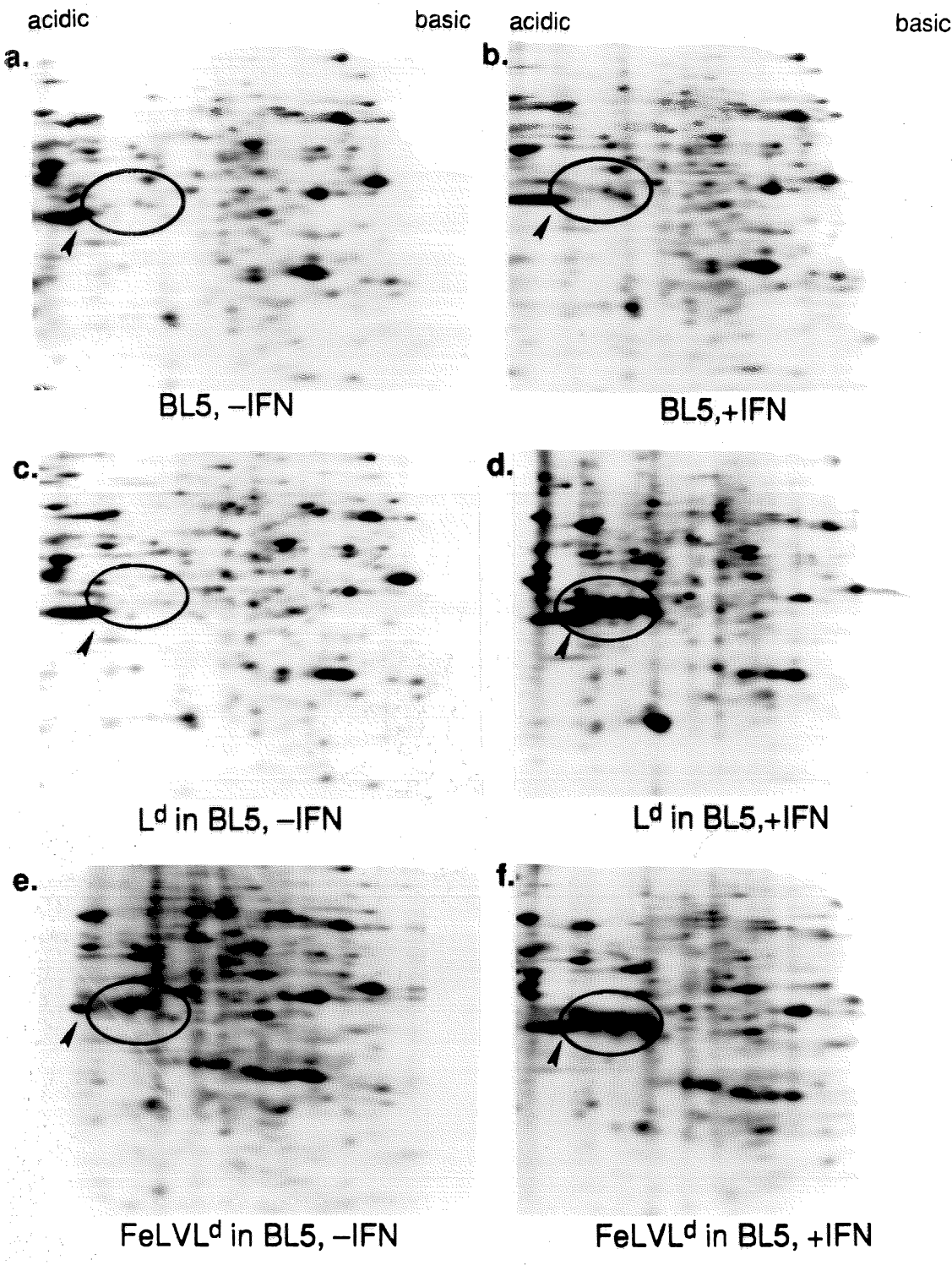
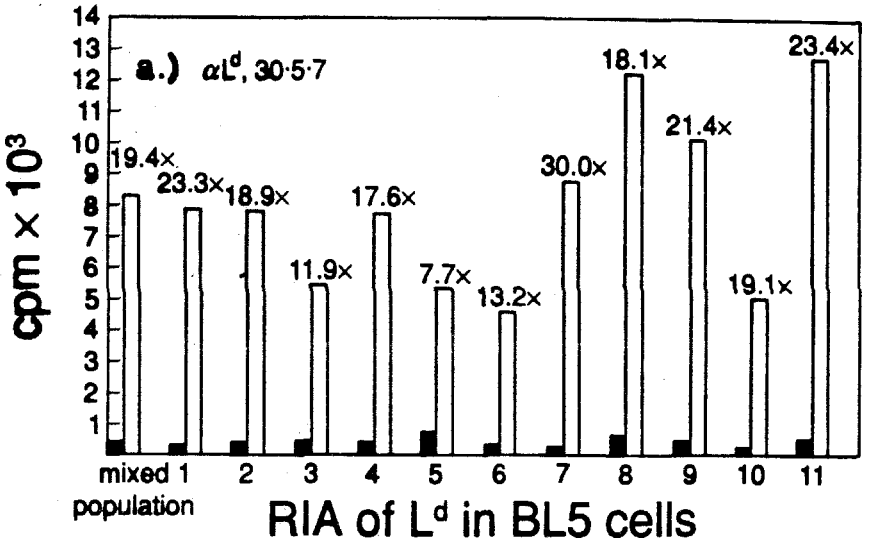
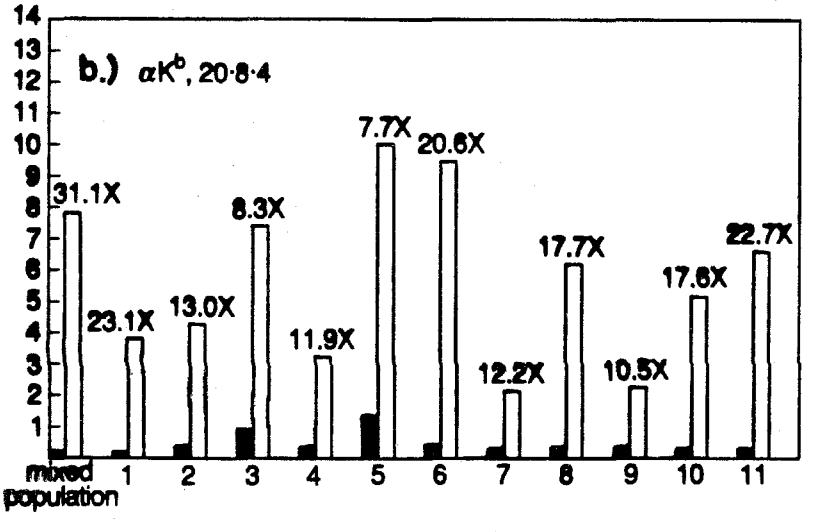
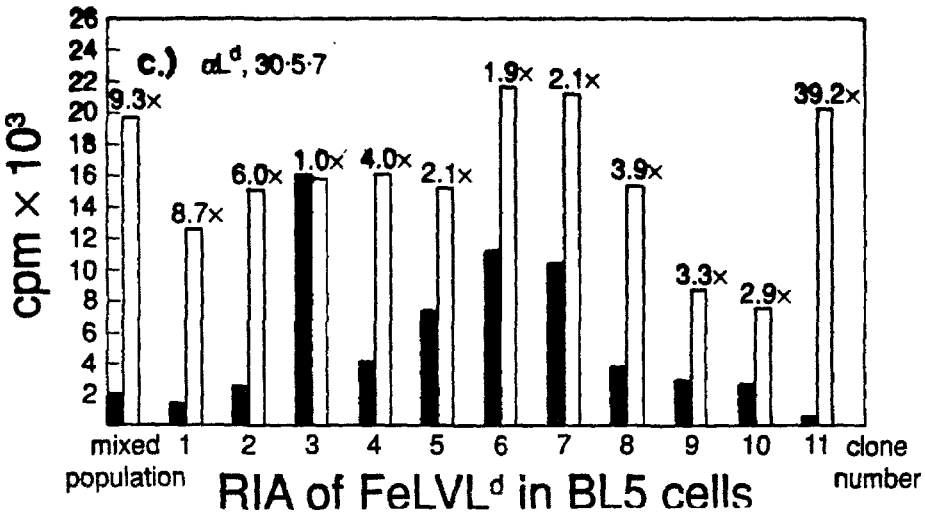

acidic
basic
a.
BL5, –IFN
acidic
basic
b.
BL5,+IFN
c.
Ld in BL5, –IFN
d.
Ld in BL5,+IFN
e.
FeLVLd in BL5, –IFN
f.
FeLVLd in BL5, +IFN

Fig. 2.4.) RIA analysis of expression and interferon induction levels of cloned cell lines isolated from BL5 cell L^d and FeLVL^d stable transfectants. The solid bar indicates expression levels without interferon treatment. The open bar shows expression in cells grown in parallel, but incubated with gamma interferon. RIA analysis was performed simultaneously to measure endogenous H-2K^b and transfected H-2L^d or FeLVL^d expression. The number over the bars is the enhancement factor. The mixed population of cells used to generate the clones was also tested. (A) RIA analysis of L^d transfected clones. Eleven clones were isolated. The clone number is shown on the abscissa; the ordinate indicates how many cpm of ^{125}I protein A bound to 2.5×10^5 cells that had been incubated with saturating amounts of anti-L^d monoclonal antibody. (B) Same as (A) tested with anti-K^b monoclonal antibody. (C) RIA analysis of FeLVL^d transfected clones. Arrangement is similar to (A). Clone 3 does not seem to be induced -- this could reflect saturating expression levels of H-2L^d in the cells prior to interferon treatment, or that the RIA was saturated and did not pick up the interferon induction. The endogenous H-2K^b expression was induced 2.4-fold in clone 3. (D) Same as (C) tested with anti-K^b monoclonal antibody.


a.) αL^d, 30·5·7
cpm x 10^3
19.4×
23.3×
18.9×
11.9×
17.6×
7.7×
13.2×
30.0×
18.1×
21.4×
19.1×
23.4×
mixed population
1 2 3 4 5 6 7 8 9 10 11
RIA of L^d in BL5 cells


b.) αK^b, 20·8·4
31.1X
23.1X
13.0X
8.3X
11.9X
7.7X
20.6X
12.2X
17.7X
10.5X
17.6X
22.7X
mixed population
1 2 3 4 5 6 7 8 9 10 11


c.) αL^d, 30·5·7
cpm x 10^3
9.3×
8.7×
6.0×
1.0×
4.0×
2.1×
1.9×
2.1×
3.9×
3.3×
2.9×
39.2×
mixed population
clone number
RIA of FeLVL^d in BL5 cells

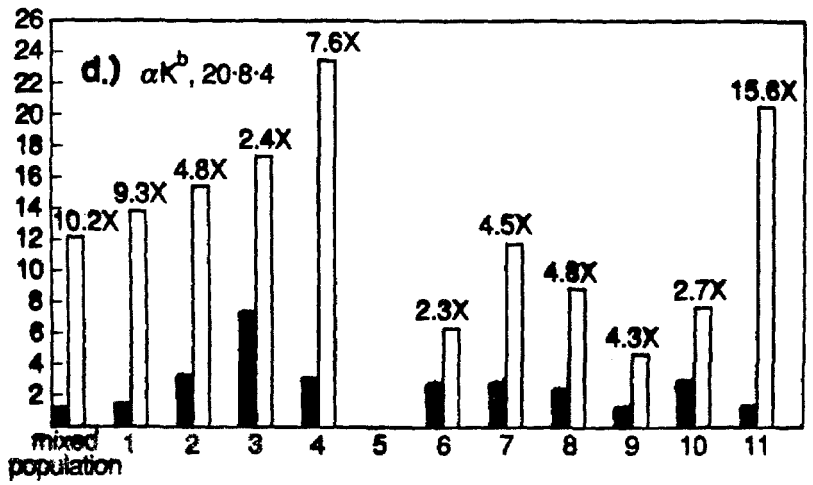
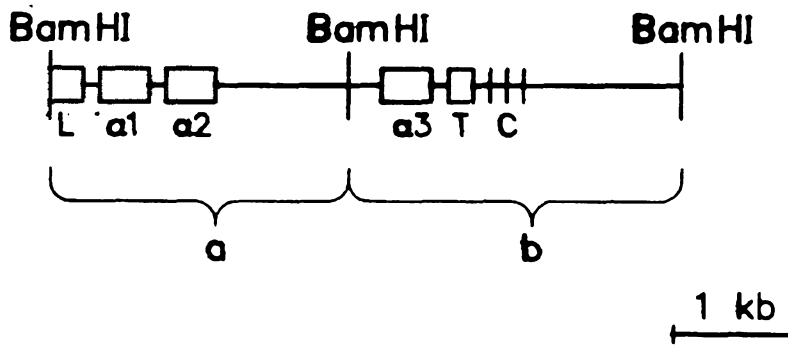
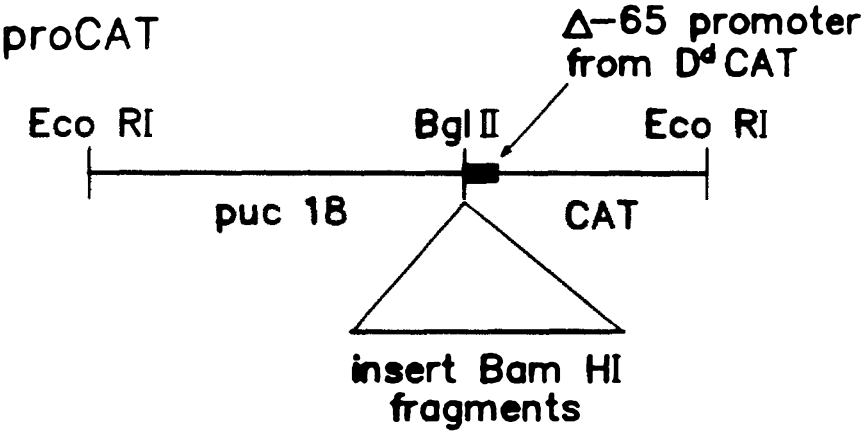

d.) αK^b, 20·8·4
10.2X
9.3X
4.8X
2.4X
7.6X
2.3X
4.5X
4.8X
4.3X
2.7X
15.6X
mixed population

Fig. 2.5.) The plasmids used to test for interferon responsive enhancer elements in the L^d gene. (A) Bam HI fragments were isolated from Ld genomic DNA. The location of the Bam HI sites are marked. (B) The plasmid proCAT was constructed to test for enhancer activity. A minimal promoter with no known enhancer elements, taken from the D^d promoter, was linked to CAT and subcloned next to a polylinker site in puc18. The minimal promoter contains the 65 bases 5' from the estimated start site of transcription in the D^d gene. Fragments a and b from the L^d gene were inserted in both orientations into the site labeled "insert", at a Bgl I site in the polylinker.

A.) L^d genomic DNA



B.) proCAT



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CHAPTER THREE

REGULATION OF GENE EXPRESSION BY INTERFERONS: CONTROL OF H-2 PROMOTER RESPONSES

Regulation of Gene Expression by Interferons: Control of H-2 Promoter Responses

BETTE KORBER, NICOLAS MERMOD, LEROY HOOD, IWONA STROYNOWSKI

The magnitude of the response to interferons and the requirement for individual elements in the promoter of the H-2D^d gene were shown to be cell-specific and dependent on the type of interferon used. Three DNA sequences in the promoter were found to bind murine nuclear factors. Two of these sequences are in functionally defined enhancer regions and also bind to the transcription factor AP-1. The third sequence is part of the region involved in interferon regulation and is homologous to the enhancer element of the interferon β gene. A model for interferon regulation of H-2 promoters is discussed.

INTERFERONS (IFNs) AFFECT SUCH cellular processes as anti-viral responses, cell growth, differentiation, and gene regulation. Type I IFNs (α and β) and type II IFN (γ) are synthesized by different cells, and it is likely that they use different mechanisms to elicit their different cellular responses (*1–3*).

Major histocompatibility (H-2) class I genes are regulated by type I and type II IFNs (*4*). The murine H-2 class I proteins are highly polymorphic, 45-kD polypeptides that function during antigen presentation as cell-surface recognition molecules for cytotoxic T lymphocytes (*5*) and are expressed on most somatic tissues at tissue-specific levels (*6*). Early embryonic tissues do not express H-2 antigens, although expression can be induced by IFNs (*7*). Adult brain cells respond to IFN γ with a 30-fold increase in the expression of H-2 antigens; heart and kidney cells with a 13- to 17-fold increase; and the tissues that have the highest basal levels of H-2 antigens, like the spleen and lymph nodes, show the least change in expression (*8*).

IFN regulation of class I gene expression involves at least two mechanisms, one dependent on sequences upstream and the other on sequences downstream of the H-2 promoter region (*9–12*). IFN induction utilizing the upstream sequences accounts for less than a twofold enhancement of class I gene expression, corresponding to less than 40% of the entire IFN response in L cells (*10*). It is dependent on the presence of a 37-bp region, designated the IRS (IFN response sequence) (*11*) that lies between nucleotides 159 and 122 upstream of the transcription initiation site of the H-2D^d gene (*10*). Induction of class I expression by type I IFNs in L cells requires the concomitant presence of a second sequence located just upstream of the IRS (*10, 11*); this sequence is not required in NIH 3T3 cells (*12*). These conclusions were based on cells that express high endogenous levels of H-2 antigens that are only moderately inducible (2.5- to 8-fold) by IFNs. We have assessed the relative activities of the upstream regulatory sequences in cells in which H-2 antigen expression is highly inducible by IFNs.

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Table 1. Induction of CAT constructs in different cell types by murine type I and type II IFNs. The enhancement factor is a ratio of specific CAT activity (CAT activity per protein concentration) in cells treated with IFN to that in untreated cells. It is independent of the cotransfection efficiency in the individual pools of cells transformed with a mixture of *neo* gene and CAT constructs in all cell lines used. Ten cloned cell lines from a BL5 transfected cell pool were analyzed as controls; all were positive for CAT expression. The basal levels of CAT gene expression and their ability to respond to IFNs varied moderately between different cloned transfectants. Clone data confirmed results observed on pooled transfectants. Levels of class I antigens on different cell lines were measured with different antibodies by radioimmunoassay (32), and compared on a per cell basis with the levels of the class I antigen expression on spleen cells of the same haplotype. L cells expressed 5- to 10-fold higher levels than H-2^k C3H spleen cells, BLK SV cells expressed 3- to 6-fold more than H-2^b C57BL/6 spleen cells, MPC 11 cells expressed 1.5- to 6-fold higher levels than H-2^{d}Balb/c spleen cells, and BL5 cells expressed lower levels of H-2^b antigens than C57BL/6 spleen cells (ranging from 0.25-fold to comparable levels of expression).

Construct	Enhancement factor							
	BL5 + IFN γ		BL5 + IFN (α + β)		BLK SV + IFN γ		BLK SV + IFN (α + β)	
	Range	$\bar{x} \pm$ SD (n)	Range	$\bar{x} \pm$ SD (n)	Range	$\bar{x} \pm$ SD (n)	Range	$\bar{x} \pm$ SD (n)
L^{k}CAT	3.0– 5.2	4.5 ± 0.7 (3)	2.5– 6.6	4.5 ± 1.1 (4)	5.3– 9.9	8.0 ± 0.9 (5)	4.7–8.0	6.6 ± 0.8 (4)
D^{k}CAT	9.7–10.4	10.1 ± 0.2 (3)	9.9–16.7	12.3 ± 1.5 (4)	5.5–11.8	7.9 ± 0.9 (6)	3.8–8.3	5.8 ± 1.0 (4)
Δ-385CAT	5.2– 6.1	5.5 ± 0.2 (3)	4.9– 6.2	5.6 ± 0.4 (3)				
Δ-236CAT	1.9– 4.9	2.9 ± 1.0 (3)	3.4– 5.6	4.5 ± 0.7 (3)				
Δ-159CAT	2.0– 6.4	4.1 ± 0.5 (9)	4.9– 6.3	5.8 ± 0.2 (6)	1.9– 7.0	3.2 ± 0.6 (8)	0.7–1.4	1.1 ± 0.1 (7)
Δ-122CAT*	0.7– 1.1	1.0 ± 0.1 (3)	0.7– 1.2	1.0 ± 0.1 (4)	0.8– 1.6	1.1 ± 0.1 (5)	1.1–1.2	1.2 ± 0.1 (4)

*Endogenous levels of H-2 antigens in Δ-122CAT transfectants were inducible by IFNs to the same degree as in the untransfected cells. The ability of these molecules to respond to IFN served as an internal control for the noninducible phenotype of Δ-122CAT gene.

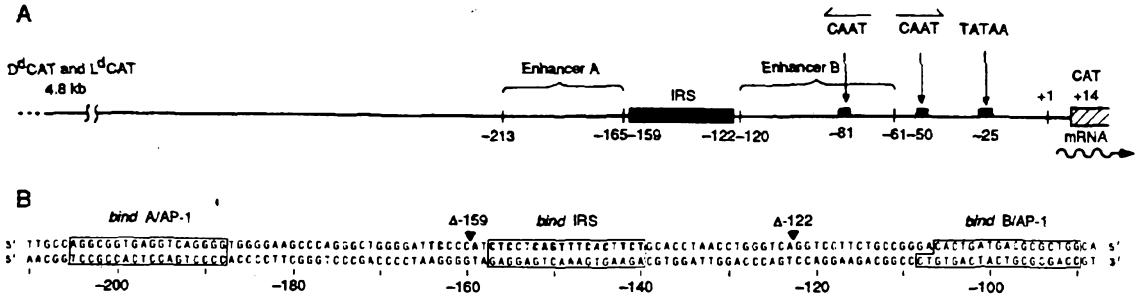

Fig. 1. Regulatory elements in class I promoter regions. (**A**) The D^dCAT and L^dCAT constructs contain 4.8-kb-long restriction fragments derived from the 5′ flanking regions of the H-2L^d or H-2D^d genes, fused 15 nucleotides downstream from their putative transcription start site to the CAT structural gene. The numbering is shown relative to the cap site that is defined by analogy to the mapped H-2K^d gene transcription start site (*33*). The transcription start sites for D^dCAT, Δ-385CAT, and Δ-159CAT have been mapped by ribonuclease I protection in noninduced and induced L cells. In these transfectants transcription initiated at the predicted site; in Δ-122 CAT transfectant ~12% of transcription was initiated at a cryptic pBR322 site. In BL5 cells transcription initiated on Δ-122 CAT template at the predicted site. (**B**) DNase I footprinting. Regions protected by MPC 11 nuclear extracts are boxed. Locations of the homologies between the H-2D^d protected regions and the binding sites of the positive and negative acting proteins of the IRE (*25*) are indicated. The AP-1 core recognition site (*21*) is also shown. Bold letters in the H-2D^d sequence correspond to the homology with the Friedman and Stark consensus sequence (*24*). The locations of the deletion endpoints of Δ-159 and Δ-122 are shown. (**C**) Presence of the enhancer motif of the IFN β gene IRE in the 5′ flanking region of H-2D^d and IFN α and γ genes. The sequence of the IFN α consensus is derived from Ryals *et al.* (*27*), and of the IFN γ gene promoter from Gray and Goeddel (*28*). Homologous bases are indicated by dots. (**D**) Comparison of the DNA sequence homology between the negative regulatory element of the IFN β gene IRE and the H-2D^d IRS. (**E**) Comparison of the DNA sequence homology between the AP-1 core recognition motif and the AP-1/MPC 11 binding sites *bind* A and *bind* B. NC, noncoding; C, coding.

The chloramphenicol acetyltransferase (CAT) gene was fused to H-2 class I promoters and IFN enhancement of CAT activity was studied in transfected cell lines derived from C57BL/6 mouse embryos (H-2^b haplotype). BL5 is a primary fibroblast line derived from 14- to 16-day-old embryos (*13*). Its basal level of endogenous H-2^b antigens is low compared to L cells (Table 1) and it can be induced 15- to 30-fold by IFNs. BLK SV (*14*) is an embryonic fibroblast line immortalized by SV40 transformation. IFN enhances H-2 messenger RNA (mRNA) levels of BLK SV cells at least 15-fold although they express basal levels of endogenous H-2^b antigens similar to H-2 K^k levels on uninduced L cells (*15*).

CAT constructs carrying deletions of the promoter region of the H-2D^d gene were selected from the original deletion set used to transfect L cells (Fig. 1) (*10*). The construct Δ-159CAT contains an IRS sufficient for the induction of the class I promoter by type II IFN in L cells, but the region necessary for a type I IFN response has been deleted; Δ-122CAT lacks the IRS and upstream regions and is not inducible by either type of IFN. Two additional deletion constructs, Δ-385 and Δ-236, which contain all of the known IFN regulatory elements of H-2D^d, were studied in BL5 cells. The deletion constructs and the parental D^dCAT plasmid carrying a 4.8-kb fragment from 5′ flanking region of H-2D^d gene were transfected stably into the two embryonal cell lines. The L^dCAT plasmid derived from 5′ flanking region of H-2L^d gene was also tested (*16*).

In BL5 and BLK SV cells the type II IFN enhancement factor for D^dCAT and L^dCAT was up to tenfold higher than it is in L cells (Table 1). The deletion constructs (except Δ-122CAT) were also inducible although their response was reduced compared to the parental D^dCAT. The reduction may reflect a partial requirement for sequences in the deleted region or may be due to the presence of the pBR322 sequences fused upstream of the deletion junctions.

When type I IFN was used the magnitude of the response in BL5 and BLK SV cells was similarly high (Table 1). In agreement with previous data for L cells (*10, 11*), in BLK SV cells Δ-159CAT was not induced by a mixture of murine α + β IFN or by a purified human recombinant IFN α, while D^dCAT and L^dCAT constructs were inducible. In contrast, in BL5 cells the expression of Δ-159CAT was inducible to an equal degree by both types of IFNs (Table 1). The simplest interpretation of this result is that the IFN regulation of H-2 D^d promoter in BL5 cells is independent of the upstream regulatory element; this may be a consequence of differences between the cell lines, such as the availability of trans-acting factors.

To determine whether trans-acting factors can interact with the functionally defined regulatory regions of the H-2D^d promoter,

deoxyribonuclease I (DNase I) protection analyses (*17*) were done. Since BL5, BLK SV, and L cells did not yield sufficient quantities of active extracts, most studies were carried out with nuclear factors derived from the mouse myeloma cell line MPC 11 (*18*). It expresses high levels of endogenous H-2 antigens (comparable to L cells) and it can be easily grown in suspension to a high density. H-2 antigens on MPC 11 cells are weakly inducible (< twofold) by IFNs. One or more nuclear factors from MPC 11 cells bound to sequences −157 to −140, within the IRS region (Figs. 1 and 2). This site was designated *bind* IRS for the IFN response sequence binding region. In addition, protection was observed at sites designated *bind* A (−205 to −188) and *bind* B (−108 to −90), which are located within the H-2D^d promoter regions homologous to the class I enhancer fragments A and B of the H-2K^b gene (*19*) (Figs. 1 and 2). The DNase I footprint pattern is specific by the following criteria: the protection of all the sites was observed on both strands of the H-2D^d template, and the MPC 11 extracts used in our studies did not footprint randomly chosen fragments of bacterial plasmid DNA.

The presence of IRS-binding activity in untreated extracts from MPC 11 cells suggests that IFN-regulated transcriptional enhancement may involve activation of an IRS-specific factor present in cells in a latent form prior to IFN exposure. Alternatively, MPC 11 cells may constitutively express endogenous IFNs that induce DNA binding factors involved in IFN regulation. The latter hypothesis is plausible because constitutive production of autogenous type I IFNs was reported in several lymphoma cell lines in which H-2 antigen expression was elevated (*20*).

Next, we determined if the mammalian transcription factor AP-1 (*21*) binds to the same regions as the MPC 11 nuclear extracts. Purified AP-1 was incubated with the H-2D^d promoter in DNase I protection experiments (Fig. 2). Two strongly protected regions are apparent: one of them coincides with the *bind* A and the other with the *bind* B site. The footprinted regions appear to be identical as determined with either crude MPC 11 extracts or purified AP-1. Two additional points strengthen this suggestion. First, there is significant homology between *bind* A, *bind* B, and the AP-1 core consensus sequence (Fig. 2). In addition, the *bind* A and *bind* B sites lie within regions with demonstrated enhancer activity, as would be predicted for AP-1 binding sites (*22*). AP-1 also protects four other lower affinity sites (Fig. 2); the significance of this binding remains to be established.

The differences observed in the magnitude of response in different cell lines may reflect availability and inducibility of transacting transcription factors, IFN receptor expression and signal transduction, or the proportion of cells responding to IFN in a population. In both embryonal fibroblastic lines, the magnitude of the promoter-controlled response was up to ten times greater than in L cells. Since the uninduced levels of endogenous H-2 gene expression in BLK SV cells are approximately tenfold higher than in BL5 cells, it is apparent that a low basal level of promoter activity alone does not dictate high IFN inducibility. Consistent with this conclusion is the finding (*12*) that the H-2L^d promoter is inducible in NIH 3T3 cells to the same degree as in BL5 or BLK SV cells in our study.

The cis-acting sequences of the H-2D^d promoter necessary to elicit an IFN response vary among different cell lines. The action of type I IFN in L and BLK SV cells required the presence of a sequence upstream of the IRS region, while in BL5 cells the IRS region was sufficient for induction. The action of type II IFN on H-2 promoters was

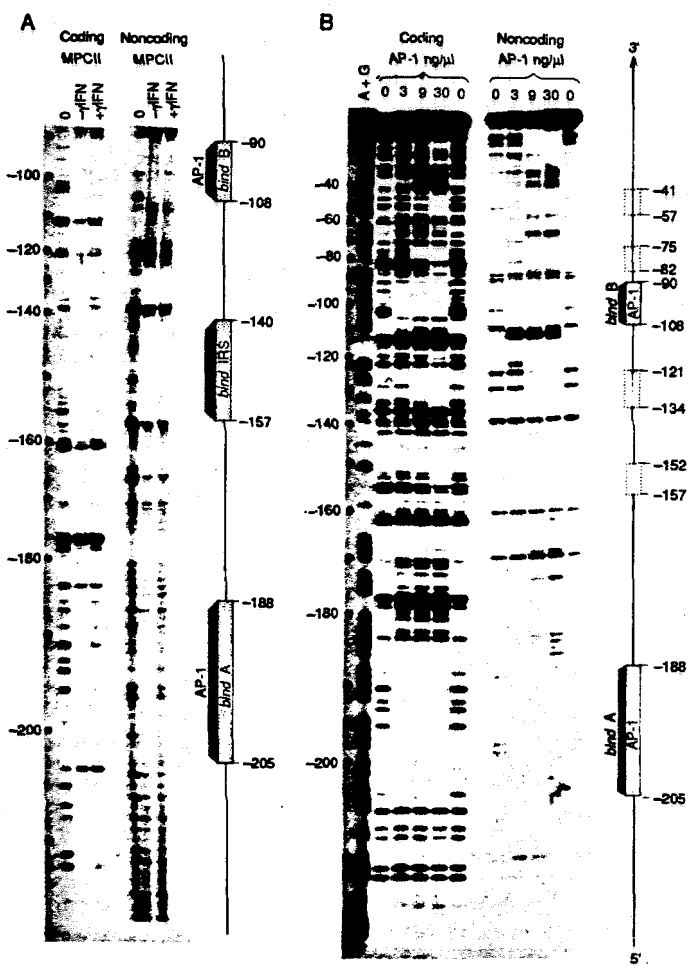

Fig. 2. DNase I protection analysis of the H-2D^d flanking sequences with MPC 11 nuclear extracts (**A**) or AP-1 transcription factor purified from HeLa cells (**B**) (*35*). The coding and noncoding DNA strands were labeled by T4 DNA kinase or by DNA polymerase (Klenow fragment) end-filling at an Eco RI site within the pBR322 vector, ~260 bp upstream from the start point of transcription. For reference the kinase-treated strand was sequenced by means of G+A chemical cleavage reactions. Each labeled strand was digested by DNase I (0 lanes) or incubated with 100 to 150 μg of MPC11 extracts made from uninduced cells (−γIFN) or IFN γ-induced cells (+γIFN). AP-1 concentrations used in footprint analysis in (**B**) are indicated above the gel lanes. The positions of strongly protected sites are indicated by continuous lines, positions of weakly protected sites by dotted lines.

independent of the upstream sequence in all three cell lines tested. These findings lend support to the seemingly conflicting reports on *cis*-acting elements required for H-2 promoters' responses to IFNs (*10–12*).

While the extensive circumstantial evidence points to the *bind* IRS being involved in IFN regulation our data did not demonstrate that the MPC11 factor that binds to this region controls H-2 promoter expression (*23*). The IRS sequence that interacts with the MPC11 binding factor is contained within the 30-bp IFN consensus sequence (*24*) identified by a homology search of the 5′ flanking regions of genes inducible by type I/type II IFN. We have found striking homology between the region protected in our footprinting assay and the inducible response element (IRE) of the human IFN β gene (*25*) (Fig. 1). This element, which is active in either orientation, and controls the induction of the IFN β gene by virus or double-stranded RNA, consists of an enhancer and a repressor motif. Induction of the IRE is thought to involve the release of repressor molecules, thus permitting the binding of a positive-acting protein to the enhancer motif (*25*). The IRS/IRE homology includes the hexamer AAGTGA, present in multiple copies in the 5′ flanking region of the IFN β gene (*26*). The hexamer repeated in tandem was reported to function as a virus-inducible enhancer in the absence of the IRE repressor motif and was proposed to bind a positive regulatory factor (*26*).

The enhancer motif of the IFN β gene IRE is present in the class I IRS on the noncoding strand. The same IRS/IRE homology was found in the promoters of the IFN α (*27*) and γ (*28*) genes (Fig. 1), and in all genes containing Friedman and Stark's IFN consensus sequence (*24*). Thus, IFN enhancement of class I gene expression (and of other IRS/IRE containing genes) may be controlled in part by an IFN-mediated induction of the IRE enhancer-binding transcription factor. This idea is particularly appealing considering that IFN can positively influence its own regulation (*29*).

Another level of control in the IFN regulation of class I genes may be exerted by the dissociation of specific repressors in a situation analogous to IFN β gene IRE induction. The differential response to type I and type II IFNs could then be explained by the use of the IRS enhancer in conjunction with different negative elements responsive to a specific IFN. One interpretation of the requirement for two cis-acting elements in the action of type I IFN on class I genes is that the upstream element (located 5′ of −159) binds to a specific repressor which dissociates after type I IFN induction. There is evidence that a repressor motif exists in this region because the sequence of the H-2L^d promoter from −195 to −159 can act as a negative element in some cell types (*30*). In BL5 cells the repressor-binding factor may be absent and so the induction of class I genes might directly reflect the induction of the IRE-enhancer-binding factor—hence the observed independence of the upstream region (*31*).

Other models may explain the requirement for two cis-acting elements in a response to type I IFN. For example, Israel *et al.* (*11*) proposed that type I IFN regulation of H-2 class I promoters involves a potentiating effect of the IRS on transcription controlled from enhancer A. It is possible that different mechanisms are used to control the IFN response of H-2 promoters in different cells.

Previous and present studies suggest that the transcriptional regulation and IFN induction of H-2 antigen expression is complex. The multiplicity of cis-acting elements which can contribute to the regulation of these genes in a cell-specific fashion suggests that combinatorial effects of trans-acting factors determine the ultimate level of expression. Since quantitative variation in H-2 class I antigen expression influences the acquisition of tolerance, major histocompatibility complex restriction, and the efficiency of cell-mediated immune responses, class I promoters may have evolved multiple cis-acting elements responsive to different regulatory pathways to ensure their specific and sensitive regulation.

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15. IFN enhances endogenous H-2 mRNA levels in BLK SV cells at least 15-fold; the accompanying cell surface class I antigen increase is less than 2-fold (B. Korber, unpublished observation). The discrepancy between the IFN inducibility of the protein on the cell surface and the mRNA may be due to limiting availability of β_2-microglobulin in this cell line. This explanation is based on analogy to SV40-transformed embryonal SVT2 cells [D. Morello, P. Duprey, A. Israel, C. Babinet, *Immunogenetics* **22**, 441 (1985)], for which it was proposed that viral transformation resulted in the 10- to 20-fold induction of H-2 transcripts without concomitant increase in β_2-microglobulin mRNA levels.
16. The 5′ flanking regions of the H-2L^d and H-2D^d genes differ from each other in several positions (*10*) [A. Kimura, A. Israël, O. LeBail, P. Kourilsky, *Cell* **44**, 261 (1986)], but the DNA sequence of the IFN regulatory elements (IRS and upstream region) is identical.
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22. Additional studies are necessary to determine if AP-1 protein functions as a transcription initiation factor for H-2 genes. Since AP-1 expression can be modulated by tumor promoter 12-*O*-tetradecanoylphorbol-13 acetate (TPA) it would be important to test if H-2 promoters are sensitive to TPA.
23. The *bind* IRS is the only sequence within the functionally defined IRS yet shown to interact with nuclear factors. No additional binding sites were detected when extracts from IFN γ–treated MPC 11 cells were used to footprint the H-2D^d promoter (Fig. 1). A region homologous to the *bind* IRS is not always essential for IFN γ responsiveness. For example, the *bind* IRS homology in the 5′ flanking regions of H-2 class II genes can be deleted without loss of the promoter's ability to respond to IFN γ. [J. Boss and J. Strominger, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9139 (1986)]. This may reflect a requirement for different IFN regulatory elements in different genes or cell types.
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29. In some cases induction of IFN gene expression by viral or double-stranded RNA can be augmented by prior exposure of cells to IFN [called IFN priming, T. Fujita and S. Kohno, *Virology* **112**, 62 (1981); T. Enoch, K. Zinn, T. Maniatis, *Mol. Cell. Biol.* **6**, 801 (1986)]. This phenomenon is dependent on protein synthesis and requires an IFN-inducible factor. We speculate that priming may be due to IFN mediated induction of a positive IRS/IRE binding factor.

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31. The functional significance of a partial homology between the IRS region and the repressor region of the IRE (Fig. 1) remains to be evaluated, since dissociation of the repressor from the IFN β IRE is controlled by virus or double-stranded RNA, not by IFN itself.
32. Radioimmunoassays were performed as described (*10*) with the antigen-specific monoclonal antibodies 28-14-8, (αH-2D^b); 20-8-4, (αH-2K^b); or 11-4-1, (αH-2K^k).
33. J. LaLanne, C. Delabre, G. Grachelin, P. Kourilsky, *Nucleic Acids Res.* **11**, 1567 (1983).
34. Time course studies determined the appropriate time lengths for IFN incubations. Endogenous H-2 antigen expression on BL5 and BLK SV cells was fully induced in 48 hours by type I and 60 hours by type II IFN (determined by radioimmunoassay). CAT gene expression driven by the H-2 class I promoter was fully induced in 12 hours by type I IFN, and 36 hours by type II IFN. All inductions were stable for up to 5 days. Therefore 3-day IFN incubation periods were chosen for these experiments. IFN α + β (1000 unit/ml), and IFN γ (20 unit/ml), were fully saturating and used for all experiments described. Murine IFN α + β was purchased from LEE BioMolecular. CAT assays were performed as described (*10*).
35. MPC11 nuclear extracts and footprinting reactions were performed as described in B. Korber, L. Hood, and I. Stroynowski [in *Major Histocompatibility Genes and Their Role in Immune Function*, C. S. David, Ed. (Plenum, New York, in press)]. AP-1 purification and footprinting reactions were performed as described (*21*).
36. We thank K. Blackburn and M. Krempin for superb professional assistance with tissue culture, and D. Bohmann for advice during AP-1 purification. We thank M. Shepard of Genentech for providing the recombinant murine IFN γ, D. Vapnek of AMGen for human α consensus IFN, and T. Hansen for monoclonal antibodies. Supported by a National Research Service Award (T32GM7616) from the National Institute of General Medical Sciences and by an NIH grant AI 19624.

25 August 1987; accepted 31 December 1987

Appendix to Chapter Three

This appendix in part shows data that was referred to but not shown, due to space constraints, in the Science paper that precedes it. It also presents new data concerning the 5' flanking interferon responsive region of class I genes and the nuclear factors that interact with class I promoters. The BLK SV expression studies and RNase protection experiments previously described are presented. Some general data concerning the responsiveness of specific class I genes to interferons in different cell types is discussed. A time course of induction by interferons is shown, illustrating that type I interferon induces class I expression more rapidly than type II interferon. The class I promoter/CAT construct studies were extended to SDBT cells, and clones of L^dCAT transfected BL5 cells were analyzed. Several additional experiments relevant to the DNA/protein binding studies are also discussed.

BLK SV Cells

Using BLK SV cells, we were confronted with the seemingly dichotomous situation of a very low cell surface interferon induction of class I antigens encoded by intact genes (less than two-fold), and a high interferon response of the CAT gene linked to a class I promoter (Fig. 3.1). This was resolved by examining the mRNA levels of class I transcripts and CAT transcripts transcribed from a class I promoter. Class I transcripts are induced fifteen-fold by gamma interferon (Fig. 3.1). The discrepancy between the interferon inducibility of the protein on the cell surface and the mRNA is likely due to limiting

availability of beta-2 microglobulin in a situation analogous to SV40-transformed SVT2 cells (1), for which there is evidence that viral transformation resulted in the 10-20-fold increase of H-2 class I expression without a concomitant increase in beta-2 microglobulin. Beta-2 microglobulin plays an important role in class I transport and expression on the cell surface (2).

RNase Protection Assays

When very short promoter fragments are used to direct the transcription of reporter genes, occasionally aberrant transcriptional start sites occur that originate in the plasmid vector sequences (3). We have used RNase protection studies to ascertain if the expected initiation sites of the CAT mRNAs transcribed from the H-2D^d class I 5' flanking region constructs were used. Class I promoter®CAT constructs' transcription initiation sites were tested in L (Fig. 3.2) and BL5 (Fig. 3.3) cells. The anti-sense RNA probe used for these assays is described in Fig. 3.2. In L cells, the delta-159CAT, delta-385CAT and D^{d}CAT transcripts were correctly initiated. Gamma interferon enhancement values were comparable to, although higher than, the protein induction measured by CAT assays for the two deletion constructs. Transcription from the construct delta-122CAT (this construct does not contain the interferon responsive region and is not inducible) was predominantly initiated correctly, although 10-20% of the RNA originated from an unmapped transcriptional start site located in the pBR322 vector. In BL5 cells, only D^{d}CAT and delta-122CAT were tested, and only correctly initiated mRNA was detected for both constructs. D^{d}CAT mRNA was induced

7-10-fold by interferons, while delta-122CAT mRNA was not induced, as expected.

Interferon Inducibility of Class I Genes and Clonal Analysis of L^dCAT Expression in BL5 Cells

Class I genes have different expression and interferon induction levels depending on the cell type they are expressed in. The relative levels of expression and induction can change over time in continuous culture. Also, there is not some attribute of specific H-2K, D or L molecules that dictate their expression levels relative to other class I molecules. Fig. 3.4 illustrates this point in terms of the interferon inducibility H-2 K, D and L molecules of the d haplotype compared in NIH 3T3 cells (d haplotype), where they are naturally present, and in L cells (k haplotype), where they were introduced by stable transfection. The molecules responded differently in the two cell types — $H\text{-}2D^d$ has the highest interferon enhancement factor in NIH 3T3 cells, and $H\text{-}2^{Ld}$ has the highest enhancement factor in L cells. Because of the variability inherent in this system, we tested the promoters of two class I genes in our studies, $H\text{-}2D^d$ and L^d, to try to get a more general picture of what a typical class I response was like.

In a study similar to the clonal analysis described in the last chapter, L^dCAT transfectants of BL5 cells were cloned and their ability to respond to interferon was measured (Fig. 3.5). There was a wide variation in basal expression levels and in interferon inducibility of the CAT protein. We maintained four of these cloned lines for six months in culture, and reassayed their CAT expression (Fig. 3.5). The

basal level of expression had generally increased, and the relative induction between clones was altered. The increased expression correlates with BL5 cells' tendency to increase the expression of class I genes while maintained in continuous culture, probably due to differentiation of the embryonal fibroblasts (see Chapter Two).

The inherent variability in our system, (as well as similar observations concerning the variability of the interferon induced antiviral and mRNA responses (4)), make it impossible to evaluate quantitatively the absolute value of the enhancement of expression of any given construct. Despite this, whether a construct is inducible or not is indicated qualitatively by the data, and general trends can be followed. For example, the D^dCAT deletion constructs tended to be less highly expressed and have lower interferon enhancement factors than the intact 4.8 kilobase 5'-flanking region of $H\text{-}2D^d$ linked to the CAT gene (with the exception of delta-385CAT in L cells). These observations suggest that other regulatory elements may be present further upstream than our deletion set extends. Also, class I promoter/CAT constructs are generally much more sensitive to interferon induction in BL5, SDBT, and BLK SV cells than in L cells.

Differences in the Kinetics of a Class I Response to Type I and Type II Interferons

In the cell types we tested, the proteins encoded by class I genes and class I promoter/CAT constructs responded to type I (alpha and beta) interferons more rapidly than to type II (gamma) interferon. A similar difference in the timing of the response of HLA mRNAs was observed using

the two types of interferons (5). Fig. 3.6 shows the induction of class I constructs as a function of time after exposure to interferon in L cell and BL5 cell transfectants. A response to type I interferon was detectable in twelve hours, and maximal induction was observed within two days. The response to gamma interferon is not detectable in twelve hours, and requires longer for maximal induction. CAT and class I antigen expression followed the same kinetics of induction. This difference in timing could be a result of a requirement for protein synthesis for some responses to gamma interferon, but not to alpha interferon. Cycloheximide studies support this suggestion (6,7). Therefore alpha interferon may be stimulating modification of pre-existing transcription factors, while gamma interferon may be triggering the de novo synthesis of regulatory factors. This model is compatible with the differences in requirements for cis-acting interferon regulatory sequences between the two types of interferons in L and BLK SV cells.

The 5' Interferon Responsive Region of Class I Promoters in SDBT Cells Compared to L, BL5, and BLK SV cells

The 5'-flanking interferon responsive sequences' capacity to respond to type I and type II interferons was tested in SDBT cells and compared to the data already presented for other cell types (Table 3.1). SDBT cells responded to interferon similarly to BL5 cells in that the interferon responsive sequence remaining in the 5' deletion mutant delta-159CAT was capable of responding to both types of interferon. In contrast, in L cells and BLK SV cells delta-159CAT responded only to

gamma interferon, and a response to alpha interferon required additional upstream sequences. Actual raw data illustrating the differences in magnitude and sequence requirements for a typical response in the four cell types is shown in Fig. 3.7 and Table 3.2. Fig. 3.7A compares the magnitude of the response in L, BL5 and BLK SV cells. Fig. 3.7B shows the difference between the type I interferon response of delta-159CAT in BL5 cells versus BLK SV cells. (A different kind of assay (8) was used to measure CAT activity in SDBT cells than in the other cell types studied (discussed in the Procedures chapter), so this data is presented separately from Fig. 3.7.) Table 3.2 gives typical values of the response in SDBT cells. The requirement for an additional sequence upstream from the IRS for type I interferon regulation was independently observed by Israel et al. (3) in class I promoter deletion constructs transfected into mouse 3T6 cells, so 3T6 cells fall into the same category as L and BLK SV cells, in terms of interferon responsive cis-acting sequence requirements.

Additional DNA/Protein Binding Studies

Improved DNase I protection footprinting data using MPC 11 extracts were obtained after the material was run over a heparin agarose ion-exchange column. The factors that bound to all three regions in a class I promoter (bind A, bind B, and bind IRS) eluted in a 0.6 molar KCl step (Fig. 3.8). This material was then applied to an IRS oligo affinity column, but an earthquake of magnitude 5.9 thwarted its analysis (9).

We used several other types of cells as sources for nuclear extracts and tested for the presence of specific DNA binding activity on

class I promoters. L and BLK SV cell extracts derived from cells that either had or had not been exposed to gamma interferon did not show any binding activity in the IRS, although DNase I protection was observed in the bind A and bind B regions (data not shown). Shirayoshi et al. (10) have also detected binding in the bind A region using gel retardation assays and L cell, F9 cell, or LH8 T cell lines as sources of nuclear extract. Methylation interference studies (10) determined that the protein contact points lie within the AP-1 consensus sequence homology in the class I enhancer A (see Fig. 1, in the Science paper preceding this appendix).

It was reported that nuclear receptors for beta (11) and gamma (12) interferons exist, and that following internalization from the plasma membrane both interferons are translocated to the nucleoplasm (11,12). Therefore, direct binding of interferons with regulatory regions in the DNA seemed plausible. A purified preparation of recombinant gamma interferon was incubated directly with the class I promoter in up to a thousand-fold molar excess and footprinting experiments were attempted. No differences were detectable in the DNase digestion pattern with or without prior incubation of cells with interferon (data not shown). Similar studies were done with either alpha or beta interferon, and again no detectable DNase protection or hypersensitivity was observed. This does not rule out the possibility that interferons in a processed form bind directly to a class I promoter, or that interferons interact with trans-acting regulatory factors which then in turn bind to the DNA, or that detection was beyond the sensitivity of our assay.

Competition experiments were performed using either the unlabeled

fragment of the class I promoter normally used for footprinting, or a non-specific fragment of equivalent size excised from bacterial plasmid sequences. Normally in the footprinting reactions poly (dI-dC) was present in the binding reactions to inhibit non-specific DNA-protein interactions with the labeled class I promoter. Only the factor that bound to site bind A was shown to be specifically competed for by the class I promoter fragment and not by the non-specific fragment at equivalent concentrations (Fig. 3.9). Competition for the factors that bound to the other sites was equally successful using either specific or non-specific competitor DNA fragments. This could be due to a lower affinity of these factors than the bind A factor for their specific binding site in the class I promoter, higher affinity for the DNA in the non-specific competitor DNA, or lower concentrations of these factors in the crude nuclear extract used for these studies. To fully study this effect, purification of the DNA binding proteins must be undertaken.

MPC 11 Cells

We had suggested that the presence of a protein that binds to the IRS in MPC 11 extracts may be due to MPC 11 producing interferon continuously in culture and thus being auto-stimulatory. This could account for the presence of the IRS binding protein, the high basal expression of class I genes, and the low interferon enhancement factor (⅛ 2-fold) in MPC 11 cells. In an attempt to determine if this was the case, filtered supernatant from MPC 11 cultures was applied to other cell types, and class I expression was analyzed.

BL5 cells were incubated in a 50:50 mixture of fresh media and

filtered media that had been collected from MPC 11 cells after three days of incubation. After 48 hours the cells were harvested and endogenous class I gene expression was measured by RIA and compared with cells grown in parallel in fresh media. A total of seven experiments were done that showed slight class I inductions in BL5 cells. On average, H-2D^b was induced 1.9-fold (1.5-2.5) and H-2K^b was induced 1.7 fold (1.3-2.1). Purified (alpha + beta) interferon induction studies done in parallel gave a 6-9-fold induction. Then attempts were made to try to block the MPC 11-media induction by the presence of anti-interferon antibodies, but the previously observed induction was not reproducible. Attempts were made, but no induction was observed. We tried concentrating ten milliliters (mls) of MPC 11 supernatant into one ml and adding it to 10 mls of fresh media in the BL5 cultures, which resulted in BL5 cell death. Experiments using MPC 11 media on SDBT cells were also tried with no detectable induction. It is not clear whether this ambiguous result is due to a change in the MPC 11 cells or the BL5 cells, or is due to some other experimental artifact we could not account for; the experiment was discontinued at this point.

Table 3.1.) Comparison of interferon responses of class I promoter/CAT constructs in BL5, SDBT, L and BLK SV cells. The enhancement factors for various constructs and their standard deviations are given. The enhancement factor is calculated as the ratio of specific CAT activity (CAT activity/protein concentration) from cells treated with interferon to that from untreated cells. D^dCAT and L^dCAT constructs serve as positive controls. Delta-159CAT is induced by type I (alpha and beta) interferons in SDBT and BL5 cells, but not in BLK SV or L cells. It is induced by type II interferon in all four cell types tested. Delta-122CAT and FeLVCAT serve as non-regulated controls, and D^dCAT and L^dCAT as positive interferon regulated controls. The number of assays varied for BL5, BLK SV, and L cells, and are listed in the papers which make up the body of this chapter and Chapter Two. Descriptions of the interferons are also provided in these papers. SDBT cells were tested four times each with each interferon for the D^dCAT and L^dCAT constructs, and six times each for the other three constructs. The basal levels of CAT activity in the different transfectants varied, and titrations were performed to determine the appropriate amount of extract to use in each case. Saturating amounts of interferon were used, and interferon incubations were done for 72 hours except for SDBT cells, which were done for 48 hours. This may explain the limited response of class I promoter/CAT constructs to gamma interferon in this cell type — the cells may not have been fully induced at the time they were assayed.

ENHANCEMENT FACTOR, X ± SD

Cell Type	BL5		SD8T		L^{tk-}		BLK SV		
Interferon	γ	(α+β)	γ	(α+β)	γ	α_D	γ	(α+β)	α_{con}
Construct									
L^dCAT	4.5 ± .7	4.5 ± 1.1	2.4 ± .6	8.1 ± 1.5	1.4 ± .1	1.5	8.0 ± .9	6.6 ± .8	3.3 ± .2
D^dCAT	10.1 ± .2	12.3 ± 1.5	4.3 ± 1.0	9.8 ± 1.3	1.4 ± .1	1.4	7.9 ± .9	5.8 ± 1.0	2.8 ± .1
Δ159CAT	4.1 ± .5	5.8 ± .2	1.9 ± .3	6.0 ± 1.0	1.3 ± .1	1.0	3.2 ± .6	1.1 ± .1	1.0 ± .2
Δ122CAT	1.0 ± .1	1.0 ± .1	1.2 ± .2	.9 ± .1	.9 ± .1	.7	1.1 ± .1	1.2 ± .1	1.1 ± .1
FeLVCAT	.9 ± .1	1.0 ± .2	.9 ± .1	1.1 ± .3	1.1 ± .1	1.1	-	-	-

Table 3.2.) Data from a typical CAT assay experiment performed in SDBT cells. CAT assays were performed using a different assay procedure (8) than previously described. The CAT protein extracts were made in the same way. The reactions were performed at room temperature in the aqueous phase of a 200 microliter reaction layered under two mls of organic scintillation fluid. Rather than using ^{14}C-labeled chloramphenicol, ^{14}C-labeled acetyl co-A was used as the radioactive marker. It is insoluble in the organic scintillation fluid. The chloramphenicol acetylated during the course of the reaction carries the ^{14}C into the organic phase where it can be detected in a scintillation counter. The numbers given are cpm measured after reactions were carried out for a set amount of time (chosen to be well before the assay was saturated) using titrated amounts of extract that varied for each construct.

SDBT CELLS

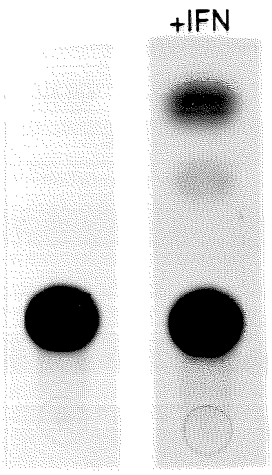
Construct	Ø	α + β	γ	Interferon
L^dCAT	3933	30715 7.8x	6340 1.6x	E.F.
D^dCAT	11712	83844 7.1x	46722 3.9x	E.F.
Δ159CAT	8347	43249 5.2x	14894 1.8x	E.F.
Δ-122	9369	10,544 1.1x	9102 1.0x	E.F.

Fig. 3.1.) Gamma interferon induction of class I versus CAT mRNA and protein levels in BLK SV cells. RIA, CAT, and Northern analysis was performed on the same harvest of D^dCAT transfected BLK SV cells, subdivided for the different assays. Cells were grown in parallel, with or without exposure to gamma interferon. (A) Table showing the endogenous class I protein's response to exposure to interferon by RIA analysis. RIA conditions were the same as described in Fig. 2.3, and the enhancement factor is indicated by EF. (B) CAT assay showing the induction of expression of the D^dCAT protein in response to interferon. (C) Northern analysis of total RNA extracted from uninduced or interferon induced D^dCAT transfected BLK SV cells. Lanes (1) and (2) are probed with CAT coding sequence, and Lanes (3) and (4) are the same filter washed and reprobed with pHII-2a (13), a general class I probe which is taken from the conserved forth exon of class I genes and so hybridizes to all of the endogenous class I genes. Scanning densitometry indicated that class I mRNA was induced fifteen-fold (in contrast to the 1.4- 2.0-fold induction of the antigens at the cell surface), and CAT mRNA was induced four-fold. The positions of the 28S and 18S rRNAs were determined by staining the gel with ethidium bromide; the 1.8 kilobase marker is based on the position of the 18S RNA.

a.

antigen	antibody	cpm, no IFN	cpm, +γIFN	γIFN, EF
D^b	28·14·8	43814	89018	2.0x
K^b	20·8·4	47161	66198	1.4x

b. D^dCAT:



c.

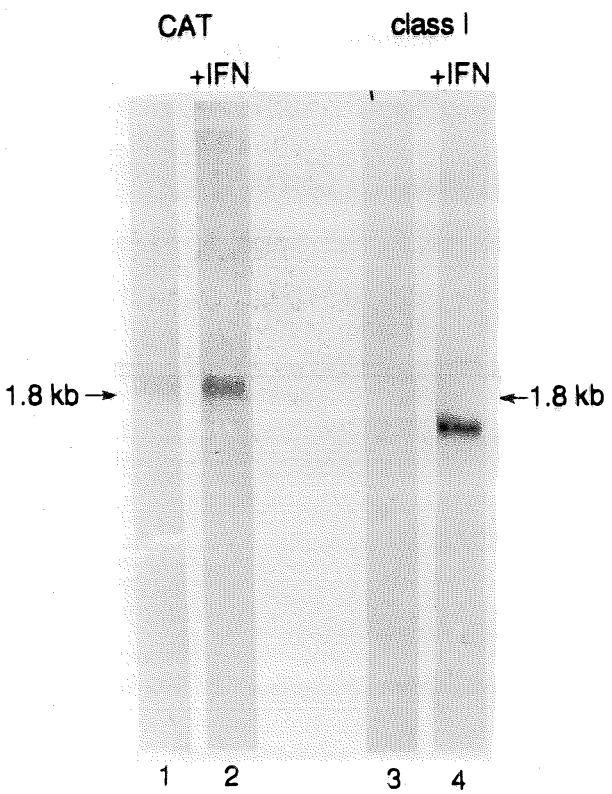
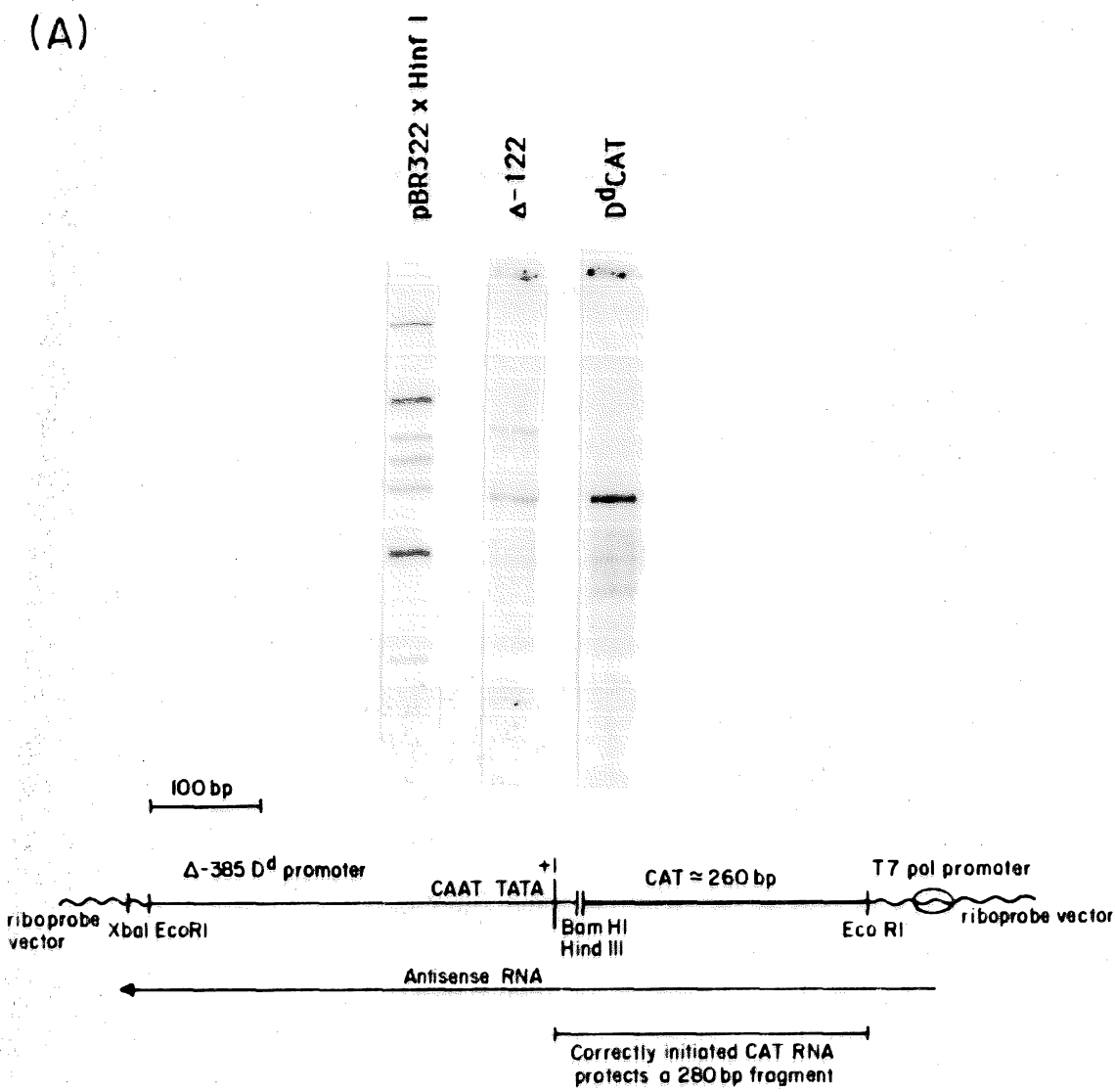
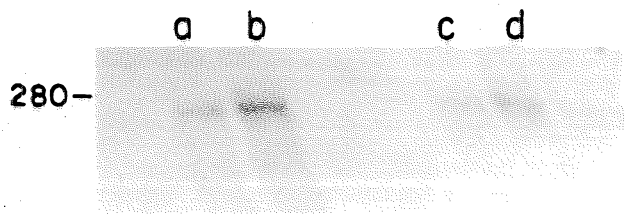


Fig. 3.2.) RNase protection mapping of the transcription initiation site of class I promoter/CAT constructs in L cells. (A) An Eco RI fragment encompassing the first 260 base pairs of the CAT gene linked to 385 base pairs of the D^d promoter was excised from the deletion construct delta-385CAT and subcloned into the vector pGEM-1 (Promega Biotech) to use for generating anti-sense RNA probes for RNase protection experiments. The run-off anti-sense RNA was transcribed from the T7 promoter to the Xba I site in the vector, across the entire insert. Properly initiated CAT mRNA transcribed from a D^d promoter should hybridize with and protect the 280 bases of anti-sense RNA indicated (including CAT coding sequence, a Hind III linker, and the first non-coding transcribed portion of the D^d gene). RNase protection of D^dCAT and delta-122CAT transcripts from uninduced L cells is shown. D^dCAT was properly initiated, and the transcript delta-122CAT was predominantly initiated correctly (80-90%) with an additional transcriptional start site occurring at an unmapped site in the vector. (B) RNase protection studies of delta-159CAT and delta-385CAT. They were properly initiated, before and after interferon induction. Only the protected RNA is shown. Lanes (a,b) show experiments done with RNA isolated from ^{d}elta-385CAT transfectants, lane (a) from untreated cells and lane (b) from cells treated with gamma interferon. Lanes (c,d) show experiments done with RNA isolated from delta-159 transfectants, lane (c) without and lane (d) with interferon treatment. (C) Scanning densitometry to determine the induction levels of the CAT mRNAs. The letters correspond to those described in (B). Delta-385CAT RNA was induced 3.1-fold and delta-159CAT RNA was induced 1.9-fold.

RNase PROTECTION MAPPING OF THE 5' ENDS



(B)


a
b
c
d
280–

(C)

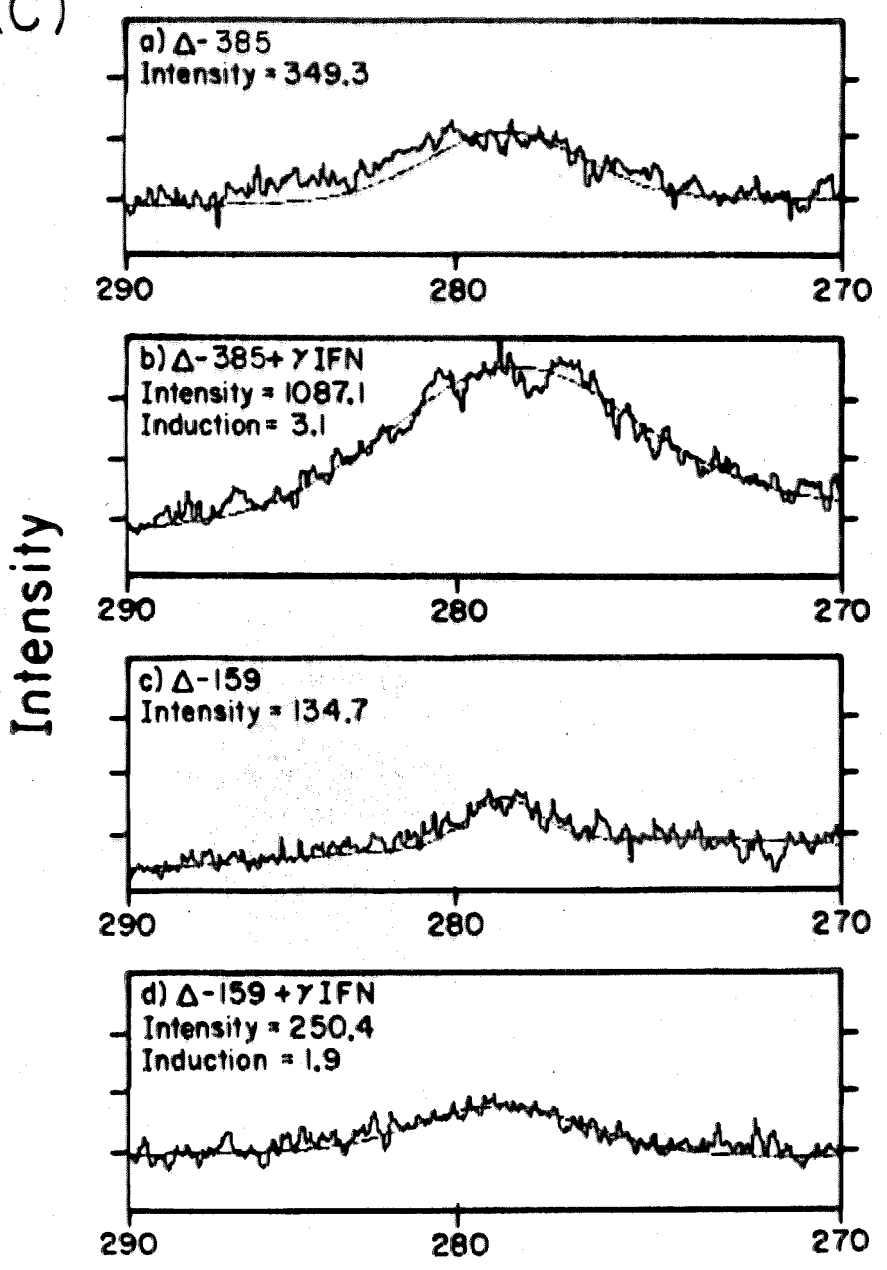
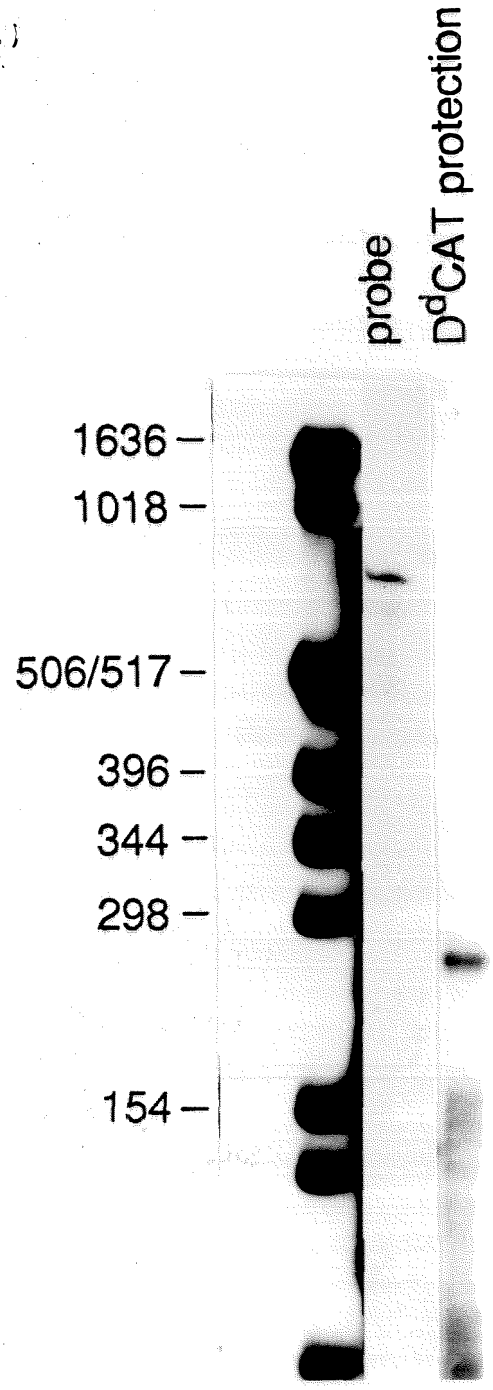

a) Δ-385
Intensity = 349.3
290
280
270
b) Δ-385+γIFN
Intensity = 1087.1
Induction = 3.1
290
280
270
c) Δ-159
Intensity = 134.7
290
280
270
d) Δ-159+γIFN
Intensity = 250.4
Induction = 1.9
290
280
270
Intensity
Size in number of bp

Fig. 3.3.) RNase protection mapping of the transcription initiation sites of class I promoter/CAT constructs in BL5 cells. (A) RNase protection mapping of the D^dCAT transcript in BL5 cells. The same probe was used as described in Fig. 3.2; the intact anti-sense RNA probe is shown. Hybridization with D^dCAT RNA revealed a correctly initiated transcript. (B) Part (a) shows the induction of D^dCAT transcripts by (alpha + beta) (lane I) and gamma (lane II) interferons; (b) shows that delta-122 is properly initiated and uninduced. Scanning densitometry of these lanes revealed D^dCAT gamma interferon induction of 9.4-fold, and an (alpha + beta) interferon induction of 6.8-fold. Delta-122CAT was essentially uninduced with a gamma interferon enhancement factor of 1.3, and (alpha + beta) 1.1.

A.)



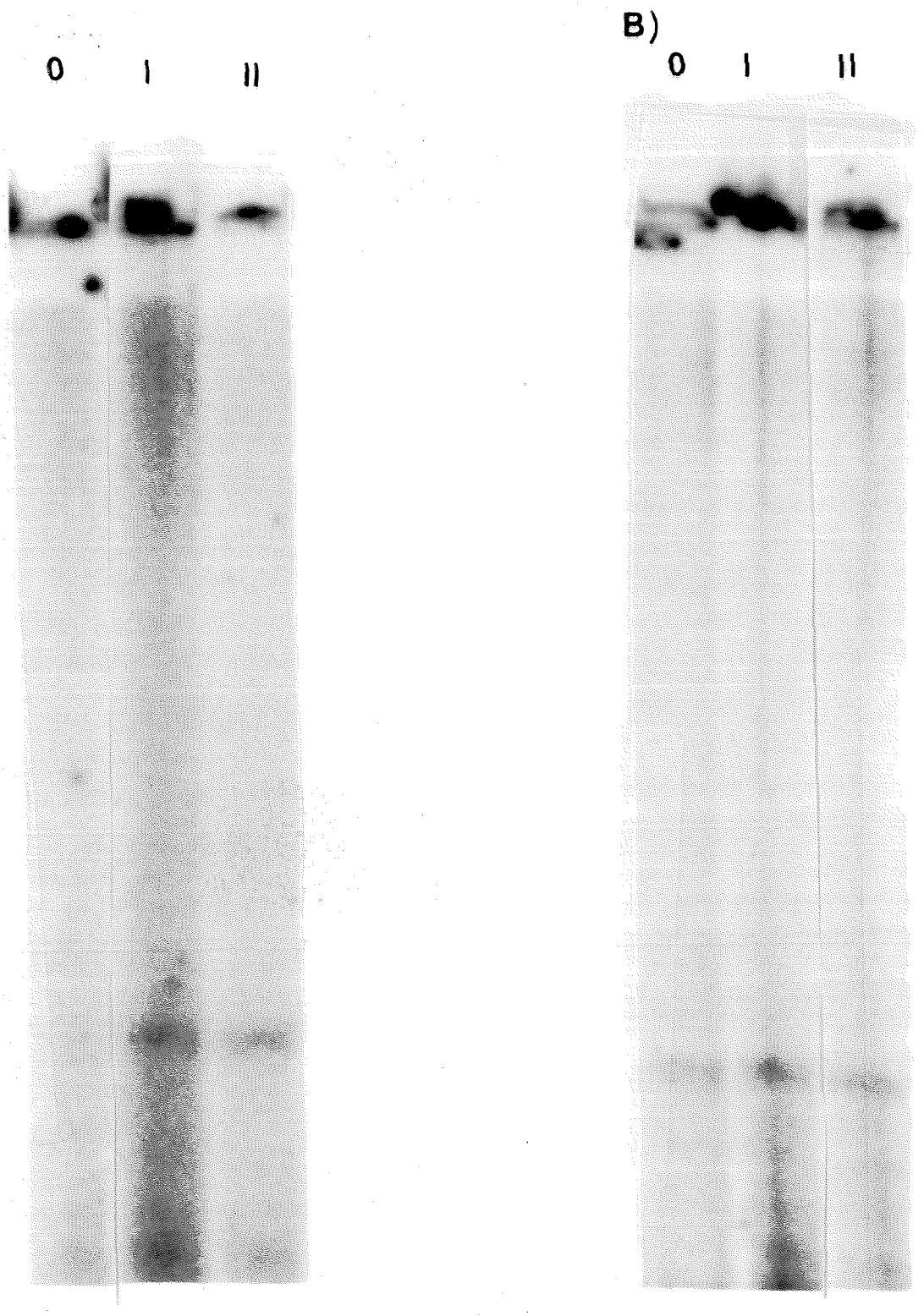
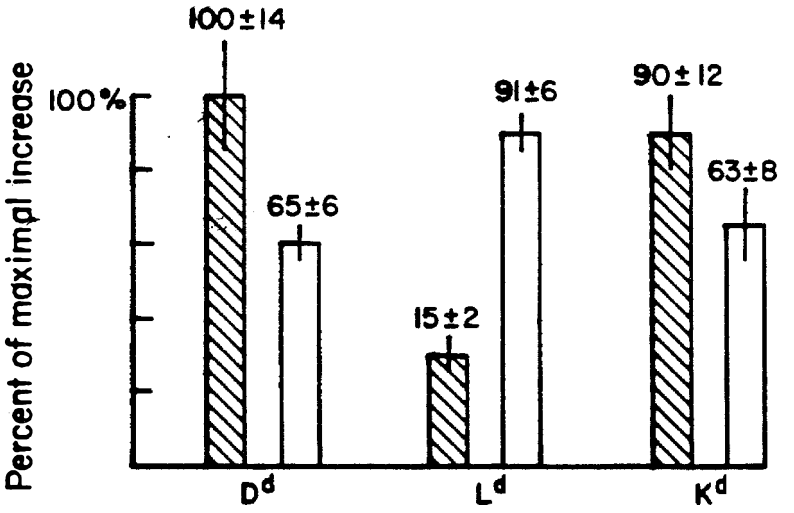

A)
0
I
II
B)
0
I
II

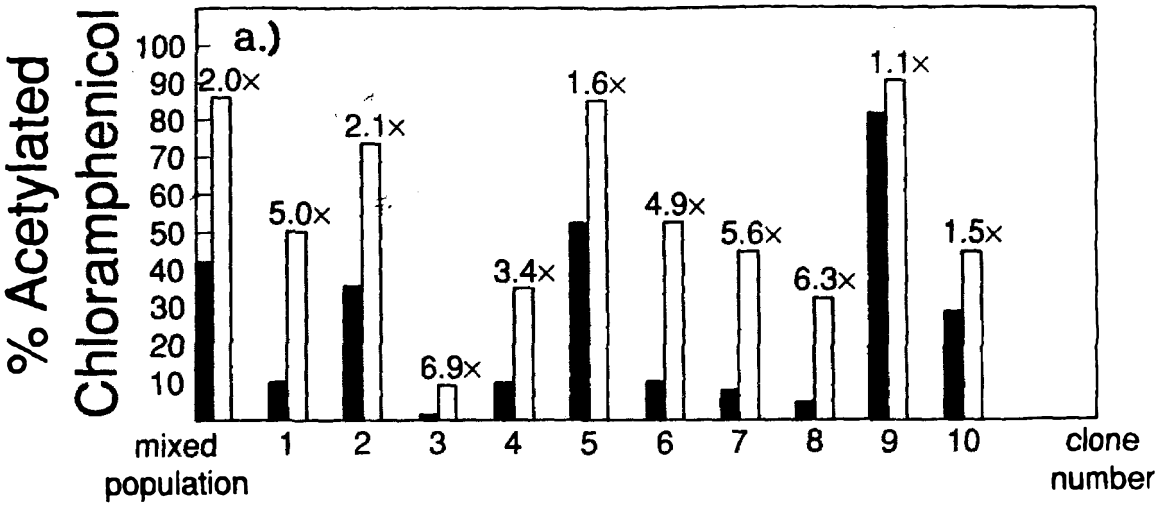
Fig. 3.4.) Comparison between H-2D^d, L^d and K^d induction by interferon in NIH 3T3 versus transfected L cells. The gamma interferon enhancement factors of the three class I genes are expressed as the percentage of the enhancement factor of H-2D^d in NIH 3T3 cells, which was used to define a 100% percent increase on the y-axis because it had the greatest value in these experiments, 3.2-fold. The RIAs for all three class I molecules were performed on the same harvest of NIH 3T3 cells, either grown in the presence or absence of gamma interferon for three days. The L cell studies were performed on independent transfectants. The numbers provided are based on two experiments run in parallel.



Relative levels of increase of Dd, Ld, and Kd expression on 3T3 cells in response to gamma IFN

Relative levels of increase of Dd, Ld, and Kd expression on L cells in response to gamma IFN

Fig. 3.5.) CAT assay analysis of cloned cell lines of L^dCAT transfected into BL5 cells. (A) The ordinate represents the percent of total chloramphenicol acetylated using ten micrograms of protein extract incubated in a half hour reaction. Extracts were derived from cells grown without interferon (solid bar), or with a three day incubation with gamma interferon (open bar). The enhancement factor is written above the bars. The mixed population, and clones 2, 5, 9, and 10, expressed high non-induced levels of CAT protein, so their enhancement factor is a minimal estimate, and titrations would have to be performed to get a quantitative estimate of inducibility. This graph shows that all cloned lines are inducible and have variable basal expression levels. (B) Four of the clones described above were maintained in culture for six months and reassayed for CAT activity and response to interferon. Two micrograms of extract was used in these assays versus ten micrograms in (A). All clones tested increased their basal level of expression, and a general decrease in inducibility was observed.


% Acetylated Chloramphenicol
a.)
100
90
80
70
60
50
40
30
20
10
2.0×
5.0×
2.1×
6.9×
3.4×
1.6×
4.9×
5.6×
6.3×
1.1×
1.5×
mixed population
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2
3
4
5
6
7
8
9
10
clone number

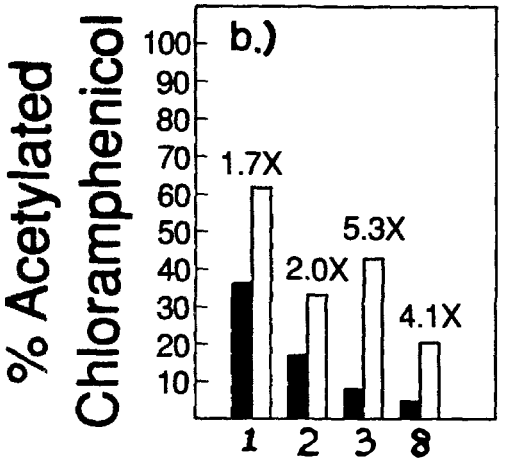
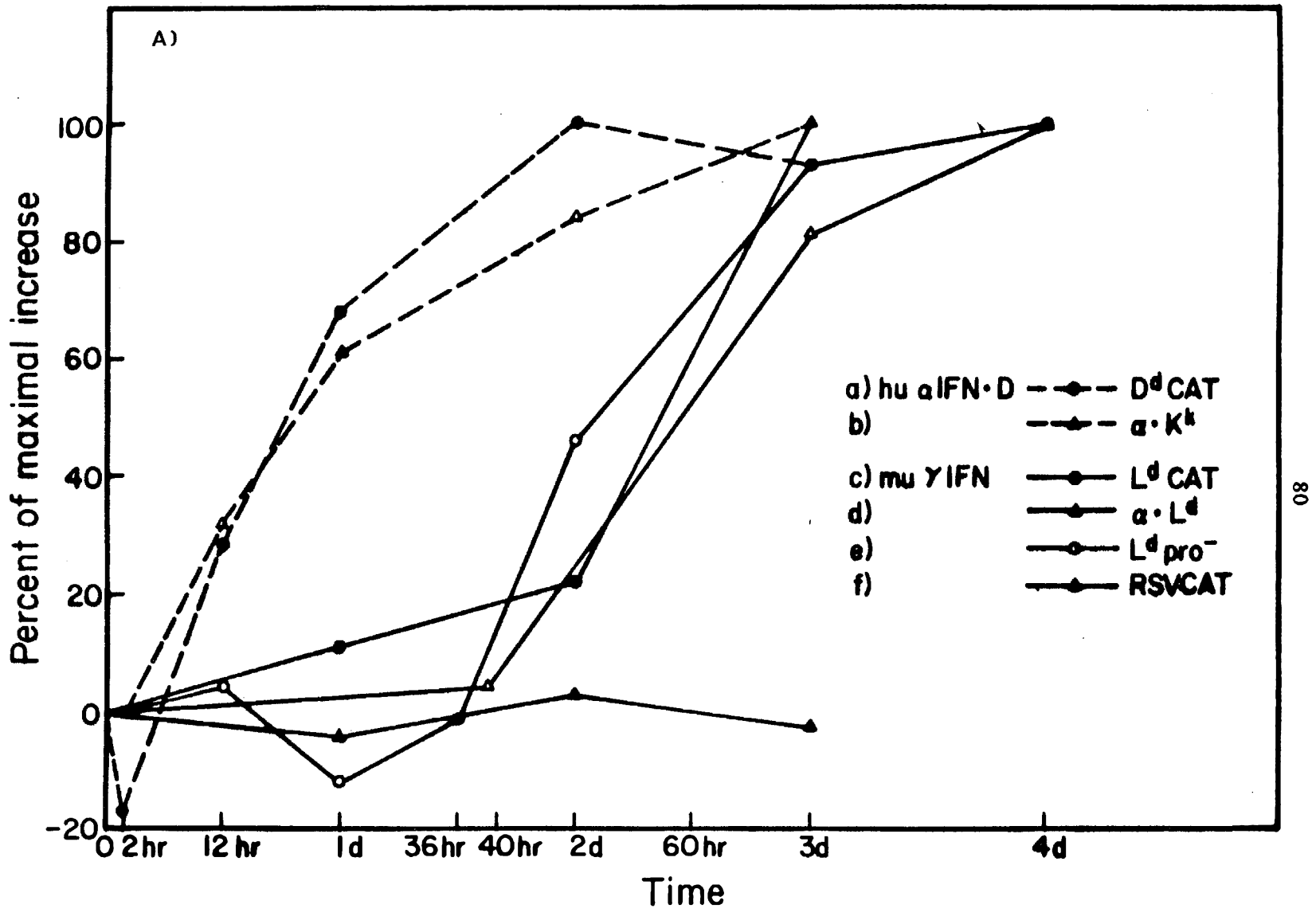

% Acetylated Chloramphenicol
b.)
100
90
80
70
60
50
40
30
20
10
1.7X
2.0X
5.3X
4.1X
1
2
3
8

Fig. 3.6.) Induction of expression of class I genes constructs as a function of time of exposure to interferon. (A) Values are presented as the percent of the maximal enhancement factor observed for a given gene product during a time course in L cells. Parts (a), (c), and (f) were measured by CAT assay and scanning densitometry. Parts (b), (d), and (e) were measured by RIA using monoclonal antibodies to H-2K^k or H-2L^d. Expression of CAT and intact class I genes followed the same time course of induction depending on the interferon (a and b for alpha, and c and d gamma interferon). A class I gene linked to a non-regulated promoter (L^d pro$^-$, described in Chapter Two) paralleled the timing of gamma interferon induction of an intact class I gene (d and e). The maximal enhancement factors for gamma interferon were: H-2L^d, 3.6-fold; L^{d}pro$^-$, 1.8-fold; L^{d}CAT, 1.9-fold; and RSV uninduced so compared with L^{d}CAT. For alpha interferon: H-2K^k, 1.9-fold, and D^{d}CAT, 1.4-fold. (B) CAT analysis of transfected D^{d}CAT, and RIA analysis of the endogenous class I gene H-2D^b, showing the differences in time required for induction by alpha and gamma interferons in BL5 cells. The maximal enhancement factors for gamma interferon were: H-2D^b, 20.7-fold, and D^{d}CAT, 10.8-fold. For (alpha + beta) interferons: H-2D^b, 8.0 fold, and D^{d}CAT, 12.5 fold. The responses in BL5 cells were generally faster than in L cells, and the induction by type I interferon occurs more quickly than induction by gamma interferon.


A)
Percent of maximal increase
100
80
60
40
20
0
-20
0 2hr
12 hr
1d
36hr
40hr
2d
60hr
3d
4d
Time
a) hu αIFN·D
b)
c) mu γIFN
d)
e)
f)
D^dCAT
α·K^k
L^dCAT
α·L^d
$L^d pro^-$
RSVCAT

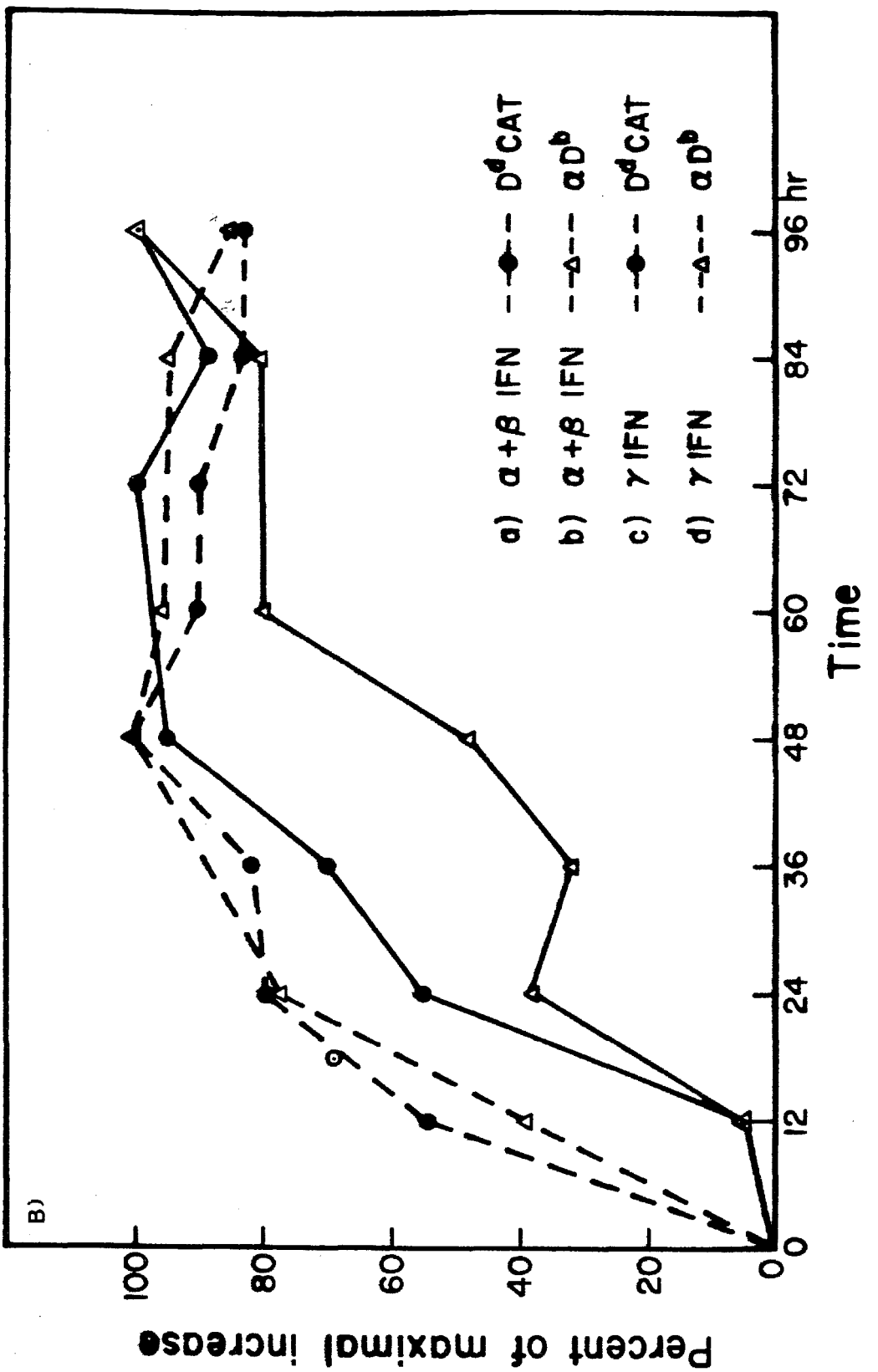
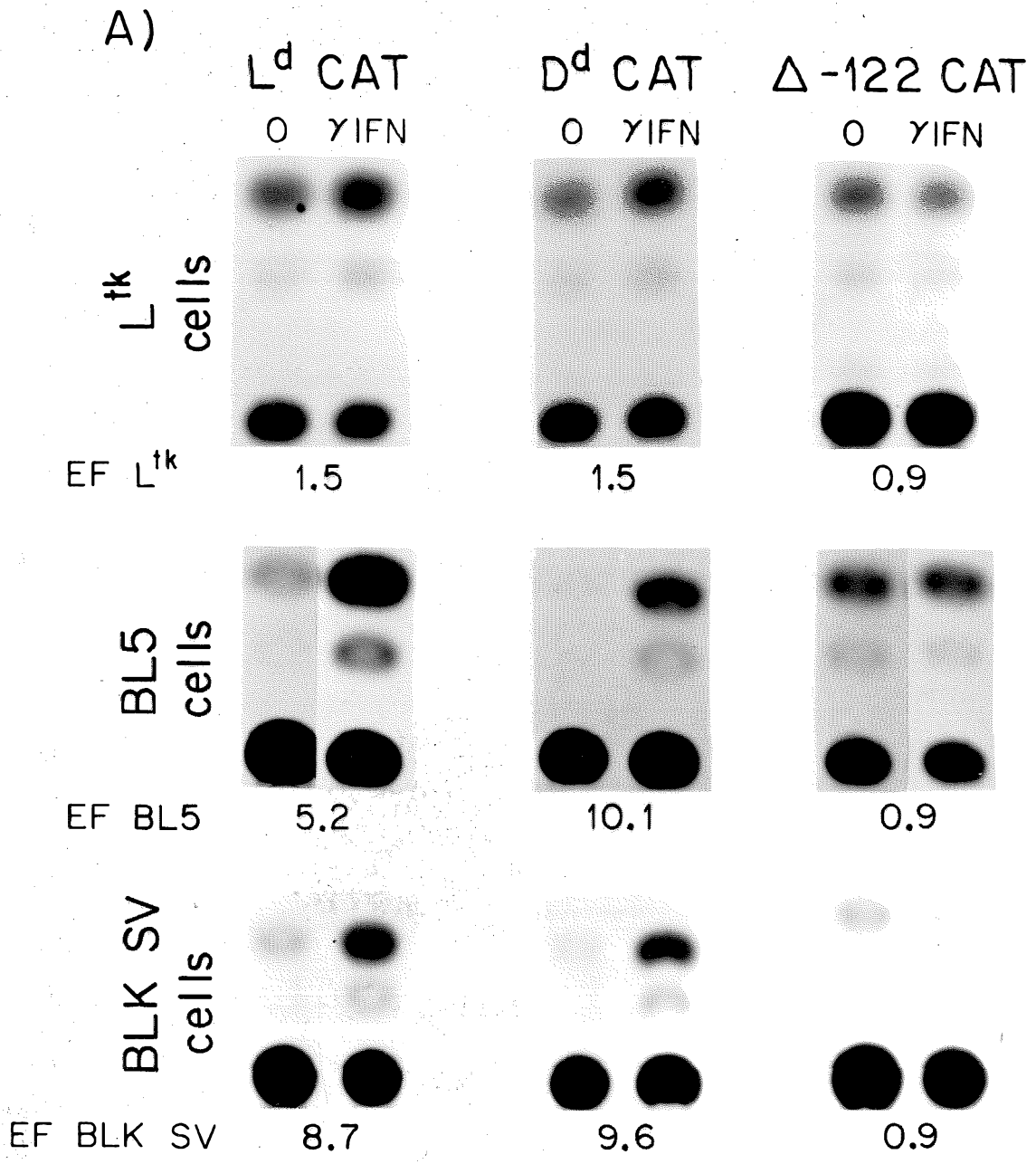

B)
Percent of maximal increase
0
20
40
60
80
100
0
12
24
36
48
60
72
84
96 hr
Time
a) α+β IFN -- D^d CAT
b) α+β IFN -- αD^b
c) γ IFN -- D^d CAT
d) γ IFN -- αD^b

Fig. 3.7.) Representative CAT assays to illustrate class I promoters' responses to interferons. (A) Comparison of class I promoter/CAT constructs response to *gamma* interferon when transfected into different cell types. The enhancement factor for the experiment is shown listed below the individual CAT assay. CAT assays were performed as described in Table 1. (B) Comparison of the response of class I promoter/CAT constructs to gamma versus (alpha + beta) interferons in BLK SV cells versus BL5 cells. Note that delta-159CAT is inducible by (alpha + beta) interferons in BL5 cells but not in BLK SV cells. Delta-159CAT is responsive to gamma interferon in both cell types.


A)
L^d CAT
D^d CAT
Δ-122 CAT
0 γIFN
0 γIFN
0 γIFN
L^{tk} cells
BL5 cells
BLK SV cells
EF L^{tk} 1.5 1.5 0.9
EF BL5 5.2 10.1 0.9
EF BLK SV 8.7 9.6 0.9

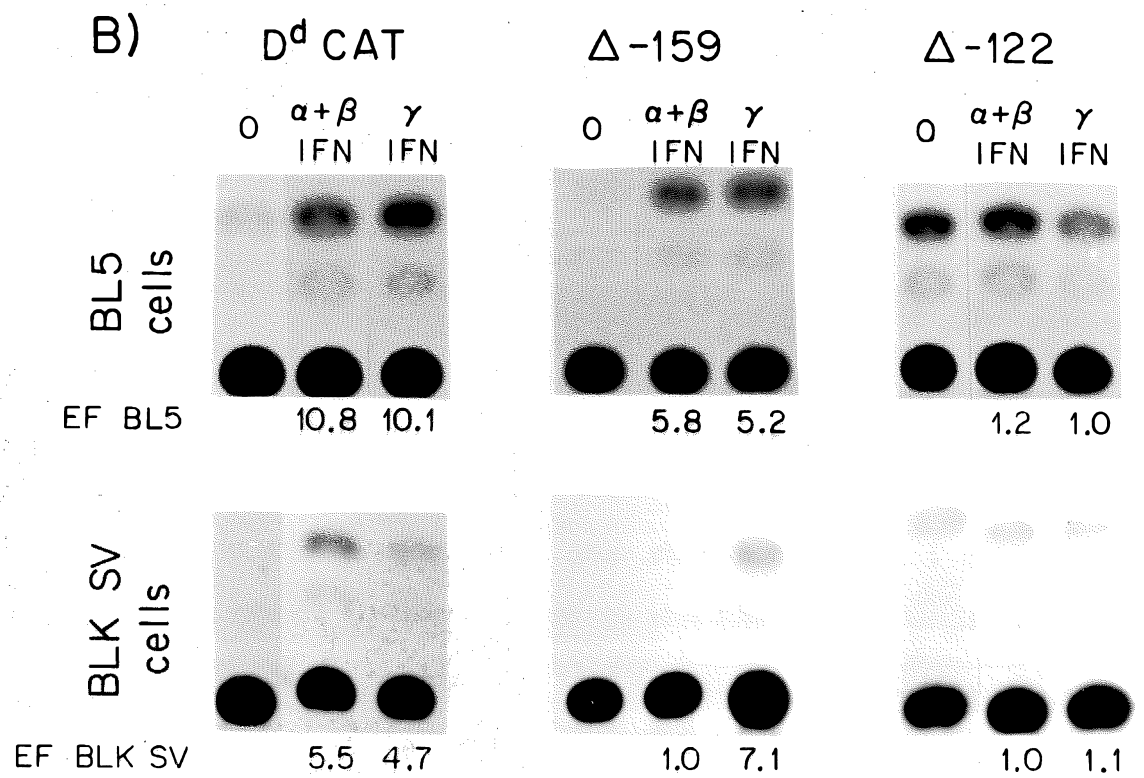
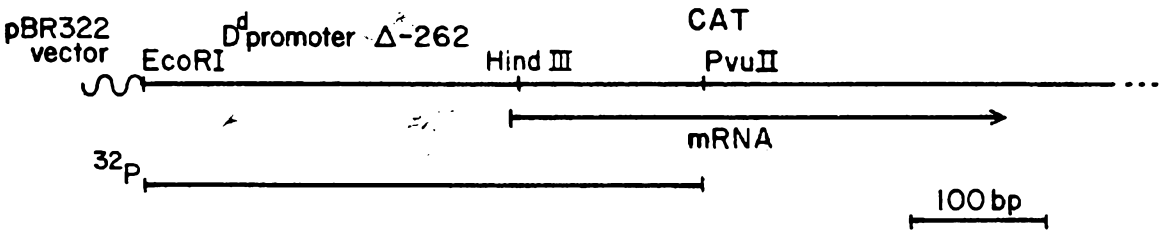
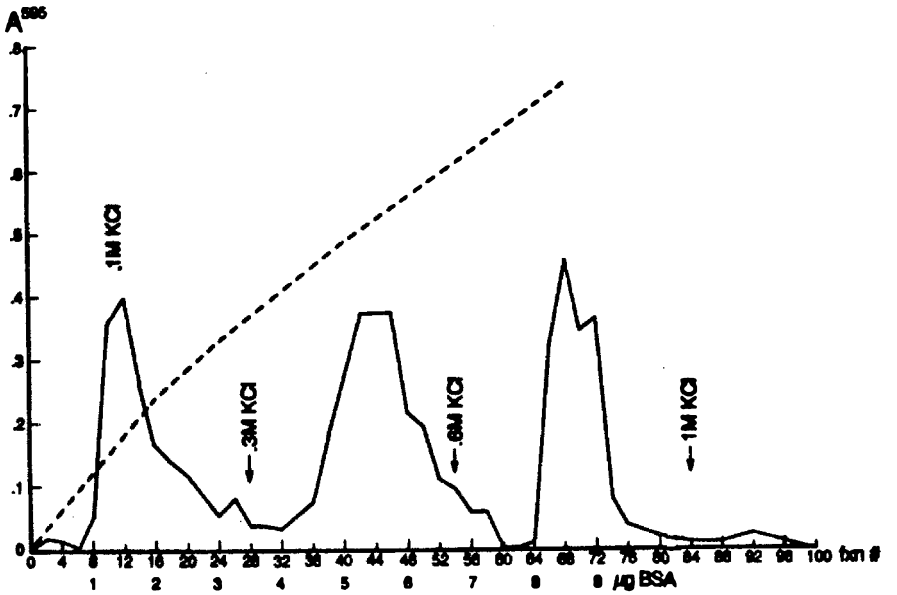

B)
D^d CAT
Δ-159
Δ-122
0
α+β IFN
γ IFN
BL5 cells
EF BL5
10.8 10.1
5.8 5.2
1.2 1.0
BLK SV cells
EF BLK SV
5.5 4.7
1.0 7.1
1.0 1.1

Fig. 3.8.) Elution profile of class I promoter DNA binding proteins from a heparin agarose column. (A) Labeled fragment used for footprinting studies. The coding and non-coding strands were labeled by T4 DNA kinase or by DNA polymerase (Klenow fragment), at the indicated Eco RI site. Secondary restriction digests were done using Hind III. Procedures for making nuclear extracts and footprinting assay are given in detail in the next chapter. (B) Column profile. Protein was loaded in 0.1 M KCl and eluted in 0.3 M, 0.6 M, and 1 M KCl steps. Three mls of MPC 11 nuclear extract was applied to a two ml column, run at two column volumes per hour, and collected in 1/5 column volume fractions in HGKED buffer with PMSF. HGKED is 25 mM Hepes (7.6), 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and KCl added to the appropriate molarity. Protein peaks were determined by testing 1 microliter of the column fraction in a 1 ml BioRad protein assay, and the resulting absorbance at 595 nanometers was measured. The dotted line shows a BSA standard curve relating the protein concentration to the absorbance. (C) Footprinting assay showing that the factors that bind to the sites bind A, bind B, and bind IRS, elute in the 0.6 molar KCl step. Fractions were dialyzed against 0.1 molar HGKED for three hours, and 20 microliters of each fraction was used for the footprinting reactions. The input and the column fractions were incubated with the DNA for 10 minutes on ice. Then DNase I was added for 30 seconds and the reaction was quenched. The input reaction was treated with 100 micrograms/ml DNase I, the column fractions with 10 micrograms/ml DNase, and the no protein control with 1 microgram/ml. The G + A marker lane is a Maxam-Gilbert chemical cleavage reaction (14).

A)


pBR322
vector
EcoRI
D^dpromoter Δ-262
Hind III
CAT
PvuII
mRNA
^{32}P
100 bp

B)


A^{595}
.1M KCl
.3M KCl
.6M KCl
1M KCl
fxn #
μg BSA

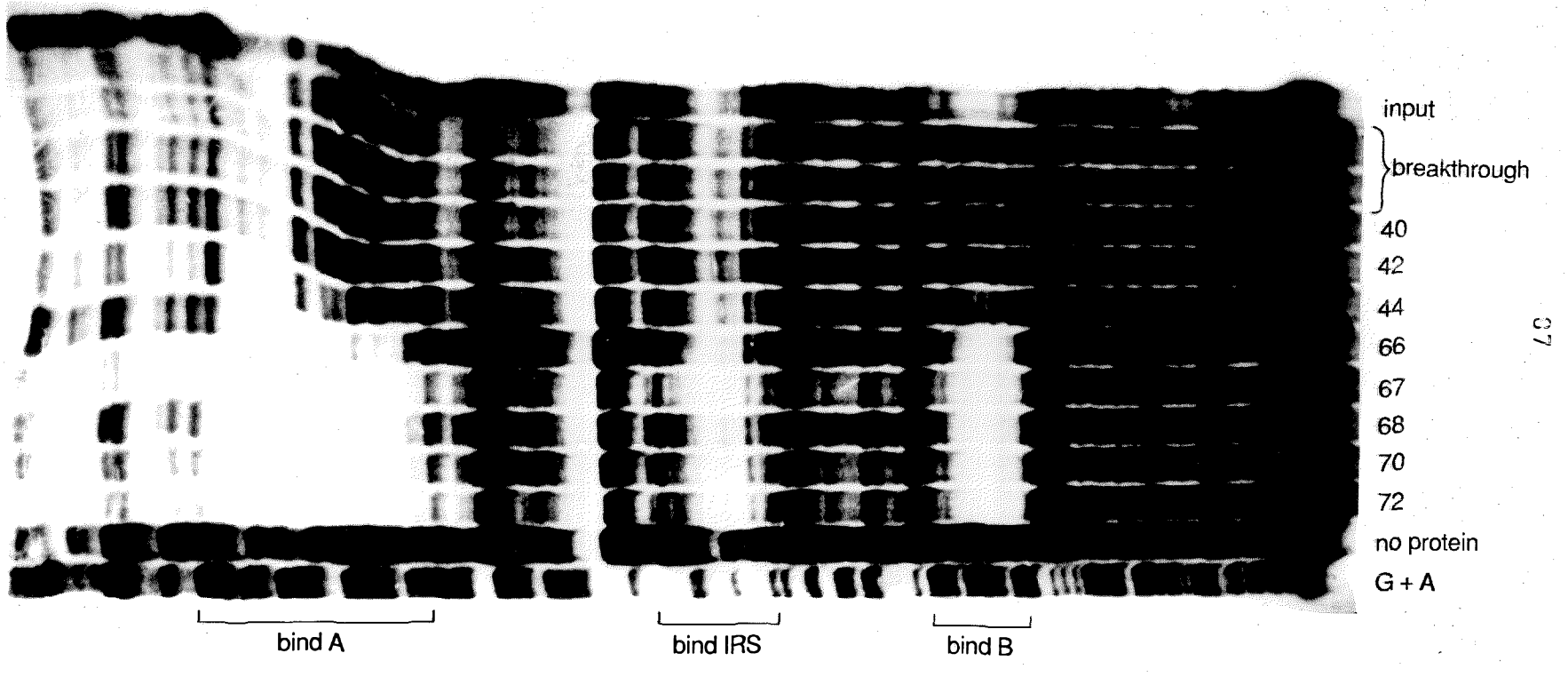
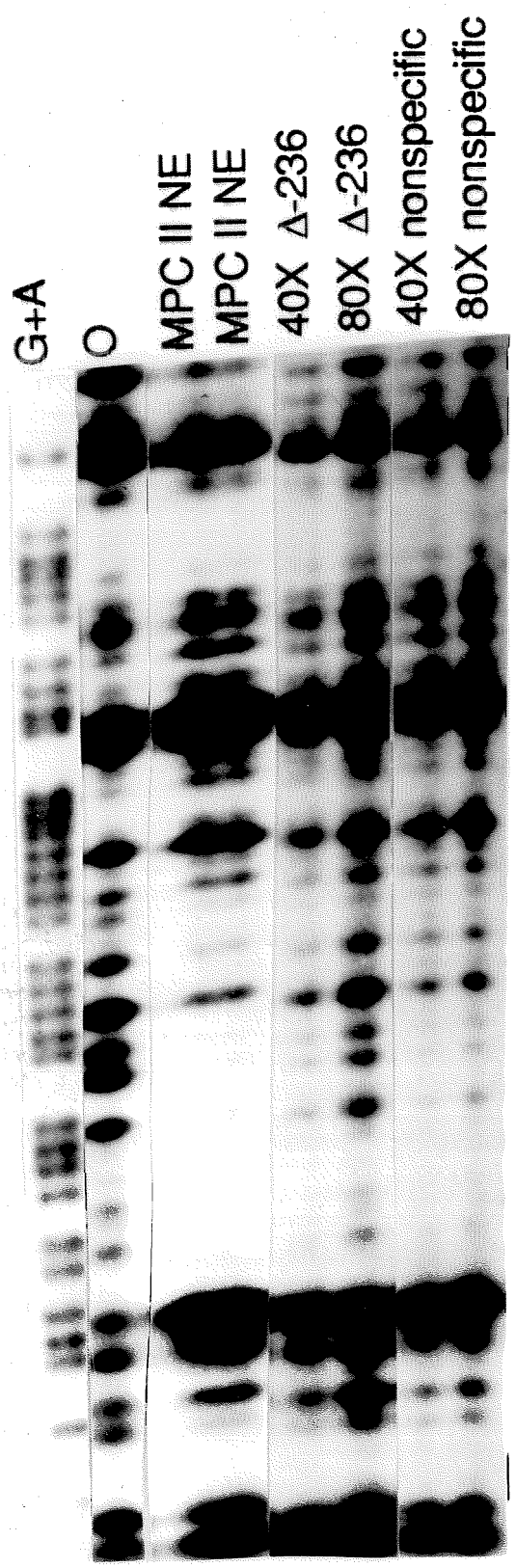

input
breakthrough
40
42
44
66
67
68
70
72
no protein
G + A
bind A
bind IRS
bind B

Fig. 3.9.) Competition study of Enhancer A binding. The first lane is a G + A Maxam-Gilbert sequencing lane (14). Next is a control lane treated with no extract and 2.5 micrograms/ml DNase I. Next are two lanes of DNA incubated with 20 microliters of MPC 11 nuclear extract prior to cleavage by 25 or 100 micrograms/ml DNase I. Delta-236 lanes refer to specific competitor DNA (the same fragment used for footprinting reactions left unlabeled) that was added in a 40- or 80-fold molar excess to the labeled fragment during the binding reaction prior to treatment with 25 or 100 microgrmans/ml DNase I. The nonspecific lanes refer to a DNA fragment cut from pBR322 that was the same size as the specific competitor fragment, but which did not effectively compete for the factor that binds to the bind A site when present in the binding reactions in a 40- or 80-fold molar excess.

Competition Study: Enhancer A binding



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CHAPTER FOUR

REGULATORY ELEMENTS IN THE PROMOTER OF THE H-2D^d CLASS I GENE

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REGULATORY ELEMENTS IN THE PROMOTER OF THE H-2D^d CLASS I GENE

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INTRODUCTION

Murine class I antigen expression undergoes modulation during development and adult life. Early embryonic cells do not transcribe detectable amounts of class I and β_2 microglobulin mRNA and express little, if any, H-2 antigens (1,2). In contrast, adult cells express class I mRNA constitutively and ubiquitously but the relative basal level of class I antigen expression varies on different cells and tissues (reviewed in 3). The quantity of various class I antigens expressed undergoes additional changes upon exposure to agents such as type I and type II interferons and tumor necrosis factor (4-6). Since quantitative variation in class I protein expression can influence the acquisition of tolerance, MHC restriction, and the efficiency of cell-mediated immune responses we were interested in identifying and characterizing the DNA elements and trans-acting factors which are important for the regulation of class I gene expression. This information may be eventually applied for therapeutic purposes to modulate class I protein levels and to affect the immune response in vivo.

Several laboratories have described regulatory elements in the promoters of class I genes (7-11). According to the present view of gene regulation (12) these short DNA sequences bind nuclear transcription factors and control the resulting level of promoter activity. Figure 1 shows a comparison of the published 5' flanking sequences of H-2 class I genes. The locations of cis-acting elements believed to be important for transcriptional regulation are indicated in the figure. Two fragments with enhancer activities (elements which potentiate transcription and act independently of their orientation) were identified upstream of the structural H-2K^b gene (7). A region required for interferon inducibility of the H-2K^b, D^d, and L^d genes, designated the interferon response sequence, or IRS (10), is shown overlapping the class I response element (CRE), which functions as an H-2L^d negative sequence in the embryonal teratocarcinoma F9 cells and as an enhancer in NIH 3T3 cells (8).

Four sequences within these functionally defined regulatory regions were found to bind nuclear factors (13-15). They are located in the enhancer A fragment, in the enhancer B fragment, and in the IRS (Figure 1). Bind A and bind B (designated bind A and bind B because they are regions within enhancer A and B that bind nuclear factors) are protected in a footprinting assay using nuclear extracts made from mouse myeloma MPC 11 cells (15), and are also recognized by the highly purified transcriptional activating protein called AP-1 (15). AP-1 also

interacts with the 12-0-tetradecanoyl-phorbol-13 acetate (TPA) inducible enhancers of the SV40 and metallothionein promoters (16). In these gene systems AP-1 acts as a positive transcription factor. An additional factor binds to a sequence which overlaps with enhancer A (13,14). The promoter of the H-2K^b gene was used to detect this binding activity, so we called the protected region bind H-2K^b (Figure 1). The factor that interacts with the IRS of the H-2 D^d gene may be involved in interferon regulation of class I genes (15). It has been suggested that these DNA binding factors may enhance transcription from H-2 promoters (13-15).

The class I promoter regions encode two CCAAT motifs as well as a TATA motif. These sequences are highly conserved elements found in the promoters of many eukaryotic genes. The TATA box element, in conjunction with the cap sequence, is believed to position the start site of the transcription (reviewed in 17); the CCAAT element is required for the maximum transcriptional activity of many promoters (18). Factors binding to these two elements have recently been partially characterized (19-20). In class I genes the canonical TATA element is located 24 nucleotides upstream of the putative transcription initiation site. Two CCAAT motifs are present 5' of the TATA box (Figure 1). They are arranged in a symmetrical palindrome with the two centers of the motifs spaced 20 nucleotides from each other. We report here that trans-acting factors can bind to both of the H-2D^d CCAAT motifs as well as to the region surrounding the H-2D^d TATA element.

METHODS

Nuclear extracts were prepared by adapting the procedure described in Parker and Topol (21) to MPC11 cells. Cells were seeded at a concentration of 5 x 10^4/ml and grown for three days in roller bottles in suspension. The nuclear extracts were prepared at 4°C by the following procedure. The cells were pelleted at 3,000 rpm for 6 min in a Beckman J6 centrifuge. The cellular pellet was washed in 10 times its volume buffer A: 15 mM KCl, 10 mM Hepes (pH = 7.6, Ultrol, Calbiochem), 2 mM $MgCl_2$, and .1 mM EDTA. The cells were repelleted at 6000 rpm for 6 min in a Sorvall RC-2B centrifuge in a SS34 rotor, and resuspended in 3 times the cellular volume of buffer A and fresh 1 mM DDT. The cells were lysed in a glass Dounce homogenizer. The salt concentration of the hypotonic buffer A was increased by adding 1/10 volume of buffer B: 1.5M KCl, 50 mM Hepes (pH 7.6), 30 mM $MgCl_2$, .1 mM EDTA and 1 mM DTT. The homogenate was centrifuged at 10,000 rpm for 10 min in the Sorvall SS34 rotor, and the crude nuclear pellet was resuspended in 3 times the cell volume of 9:1 buffer A:B. 1/10 of the total volume of 4 M ammonium sulfate (pH 7.9) was added to a final concentration of .36 M ammonium sulfate. The nuclei were lysed by gentle rocking for 30 min. The nuclear lysate was centrifuged at 35,000 rpm for 60 min in a Beckman SW41 rotor. The supernatant was retained, 0.25 g/ml ammonium sulfate was added, and the mixture was rocked for 30 min. The precipitated proteins were pelleted by centrifugation at 35,000 rpm for 15 min in a Beckman SW41 rotor. The supernatant was discarded and the protein pellet was resuspended in 1/2 the volume of the original cell pellet in buffer C: 10% glycerol (Mallinckrodt), 50 mM Hepes (pH 7.6), 20 mM KCl, .1 mM EDTA, and 1 mM DTT. The extract was dialyzed against 250x the extract volume buffer C for 3 hr, divided into aliquots and stored frozen at -80°C. The protein concentration of each extract was determined by BioRad protein assay.

The H-2D^d CAT deletion mutant Δ-262 (10) was used for the footprinting analysis. The DNA was labeled at the Eco RI site (located in pBR322 DNA adjacent to the deletion junction Δ-262) by end-filling with DNA polymerase I (Klenow fragment), or by T4 polynucleotide kinase phosphorylation. After labeling, the DNA was cut with the restriction enzyme Pvu II, at a site which lies in the chloramphenicol acetyl transferase (CAT) coding region, and the 3' or 5' end-labeled Eco RI-Pvu II fragment was gel purified.

The footprinting reactions were done in a 25 µl reaction volume. Twenty µl of nuclear extract or double distilled H_2O was incubated with 2-5 ng of end-labeled fragment, 100 µg/ml of poly(dI-dC) (Pharmacia), 5 mM $MgCl_2$, and 20 mM Hepes (pH 7.6) for 10 min at 4°C. Freshly diluted DNAase I (Worthington) was added to the final concentrations indicated in Figure 2 and allowed to react on ice for 30 sec. The appropriate amount of nuclear extract and DNAse I was determined by titration. The reactions were stopped by the addition of 50 µl of termination buffer (1% Sarkosyl, 100 mM NaCl, 100 mM Tris (pH 8), 10 mM EDTA, 100 µg/ml of proteinase K (Boehringer Mannheim), and 25 µg/ml calf thymus DNA). The tubes were then incubated at 37°C for 15 min, then at 90°C for 2 min. The final mixtures were extracted with 75 µl of phenol, then 75 µl of 50:50 phenol:chloroform. The DNA was precipitated, dried, resuspended in 10 µl of 90% formamide, TBE, and .1% dyes, denatured at 90°C for 5 min, and electrophoresed in a 5% polyacrylamide sequencing gel.

RESULTS

We have previously observed binding of nuclear factors to three DNA sequences in the 5' flanking region of the H-2D^d gene (15). Here we used the same approach of DNA footprinting (22) to identify additional sequences that may interact with nuclear transcription factors. The footprinting assay detects specific DNA/protein interactions by visualizing DNA regions that are protected from DNAase I degradation in the presence of nuclear extracts. The MPC 11 cell line was selected as a source of the nuclear extracts because it expresses high levels of the class I antigens (comparable to those on spleen cells of the parental BALB/c strain). Therefore, we reasoned, it may contain high levels of the class I gene specific transcription factors.

The results of the footprinting experiments on the H-2D^d template are shown in Figure 2. Three DNA regions, that have not been previously reported, are detectably "cleared". They are located at positions -71 to -78, -37 to -50 and -25 to +3 (on the coding strand). These footprinted sequences are also indicated schematically in Figure 1. Two of these regions coincide with the position of the CCAAT elements and the third one overlaps the canonical TATA box and extends into the sequences downstream from the putative transcription initiation site. These DNAse I protected regions are specific by the following criteria: first, they occur on both strands of the H-2D^d DNA template and second, the MPC 11 nuclear extracts used in all of the experiments do not footprint randomly selected fragments of the pBR322-CAT plasmid DNA.

DISCUSSION

We report here binding of nuclear proteins to two elements of the H-2D^d DNA promoter: the CCAAT and the TATA boxes. Based on the conserved nature of these two sequences in many RNA polymerase II promoters, we propose that the observed binding involves transcription factors that are similar to those whose interactions with other promoters have been previously characterized (19-21, 27-28). The following discussion will relate the footprinting patterns observed on the H-2D^d template to the properties of known CCAAT and TATA binding factors.

CTF can contribute to the transcriptional activation of eukaryotic promoters that contain the sequence CCAAT and, in addition, it can act as a cellular DNA binding protein required for the initiation of adenovirus DNA replication (19). CTF factor, purified by sequence-specific DNA affinity chromatography from HeLa cells, consists of a family of polypeptides with molecular weights between 52 and 66 kd. The nature of this heterogeneity in protein size is not presently understood. Purified CTF was shown to recognize CCAAT related sequences with

affinities that vary according to the extent to which the recognition sequence matches the consensus (AGCCAA) and whether or not the consensus is present in a 2-fold rotationally symmetric configuration. The symmetrical sequence TTGGCT (N_3) AGCCAA was proposed to be one of the highest affinity CTF sites (19). The H-2D^d gene encodes a related palindrome: TTGGGT (N_{14}) AGCCAA, which is strongly conserved in all the H-2 genes (Figure 1). Since these two recognition sequences vary only by a single nucleotide and a change in spacing between the putative target CTF sites (one helical turn in the consensus, two helical turns in the H-2 genes) it is possible that CTF can bind strongly to the H-2 promoter region. This is supported by observation that CTF can bind with high affinity even to a single AGCCAA sequence (19). Another important consideration is location of the CCAAT boxes in relation to other regulatory sites. In the H-2 promoters the CCAAT elements are located at positions centered around -50 and -80, upstream of the putative transcription initiation sites. In human ß-globin promoter the functional CCAAT box is located at position -80, while the non-functional CTF binding sites are found further upstream (-125 to -300 region). Hence the location of the H-2 CCAAT boxes approximates sites predicted to be functionally active in promoting transcription.

A second CCAAT binding protein (CBP) has been partially purified from rat liver nuclear extracts (27). It interacts with a sequence closely related to the CTF target site in the HSV thymidine kinase promoter (27,28). CBP is thought to be distinct from CTF because the two proteins have different chromatographic and heat stability properties, and recognize related DNA sequences with different affinities. In particular, a C→G transversion in the first residue of the CCAAT pentanucleotide increases the binding affinity of CBP and substantially reduces the binding affinity at CTF. In addition, the two proteins have reproducibly different DNAase I protection patterns on the HSV-tk promoter (28).

The positions of the footprinted DNA regions in the H-2D^d template are different from the protection patterns predicted for either the CTF or the CBP factors. Whereas in other promoters the CCAAT motif is embedded within the CTF or the CBP binding sites, in the H-2D^d gene the CCAAT boxes are located assymmetrically at the edge of the MPC 11 cleared regions (Figs. 1 and 2). This difference may reflect species or tissue/cell-specific variability of closely related proteins having, otherwise, similar properties. Alternatively, the CTF, the CBP and the H-2D^d CCAAT binding factors may be unrelated to each other except for being able to recognize similar DNA sequences.

Several laboratories have identified factors which bind to the highly conserved TATA motif and are essential components in reconstituted in vitro transcription systems dependent on RNA polymerase II. One such protein, designated the B factor, was partially purified from Drosophila Kc cell nuclear extracts and was shown by DNAase I protection assays to bind to the Drosophila histone H3 and actin 5C promoters (21). Another TATA box binding protein, TFIID, was derived from HeLa cell nuclear extracts and was shown to interact with the adenovirus major late promoter (20). Both the B factor and TFIID protect large regions of their respective promoters' sequences in DNAase I footprinting experiments. The footprinted regions encompass ~30 base pairs upstream of the cap site, including the TATA box, as well as DNA sequences located downstream from the transcription initiation site. The more discriminative method of footprint analysis using methidiumpropyl-EDTA-Fe(II) as a DNA cleavage agent revealed a 10 bp long primary TFIID binding site centered on the TATA motif (23). The MPC 11 extracts also protect large regions of the H-2D^d promoter from DNAse I degradation. The protected regions include the TATAAA consensus as well as sequences downstream from the transcription initiation site. The results of these experiments suggest that the DNAase I protection we have observed using MPC11 extracts may be due to the murine equivalent of the human TFIID or the Drosophila B factor.

Our studies indicate that class I genes may be controlled in part by general transcription factors similar to CTF/CBP and TFIID/B. It should be emphasized, however, that proof of this assertion will require further studies involving DNA mutagenesis of the H-2 TATA and CCAAT sequences and functional analysis of the mutants, as well as assays with purified factors in an in vitro reconstituted transcription system.

Present-day knowledge of the H-2 5' flanking elements suggests a complex view of class I gene transcriptional regulation - the promoter region contains many potential target sequences which may interact with different regulatory proteins. It is possible that regulation during ontogeny, tissue specificity in the relative levels of expression, and adjustment of class I antigen expression during an immune response, bring about the observed complexity in the molecular structure of the H-2 promoters. Future years will likely lead to a better understanding of the mechanisms by which the cooperative protein-protein and protein-DNA interactions cause concerted and controlled expression of these genes.

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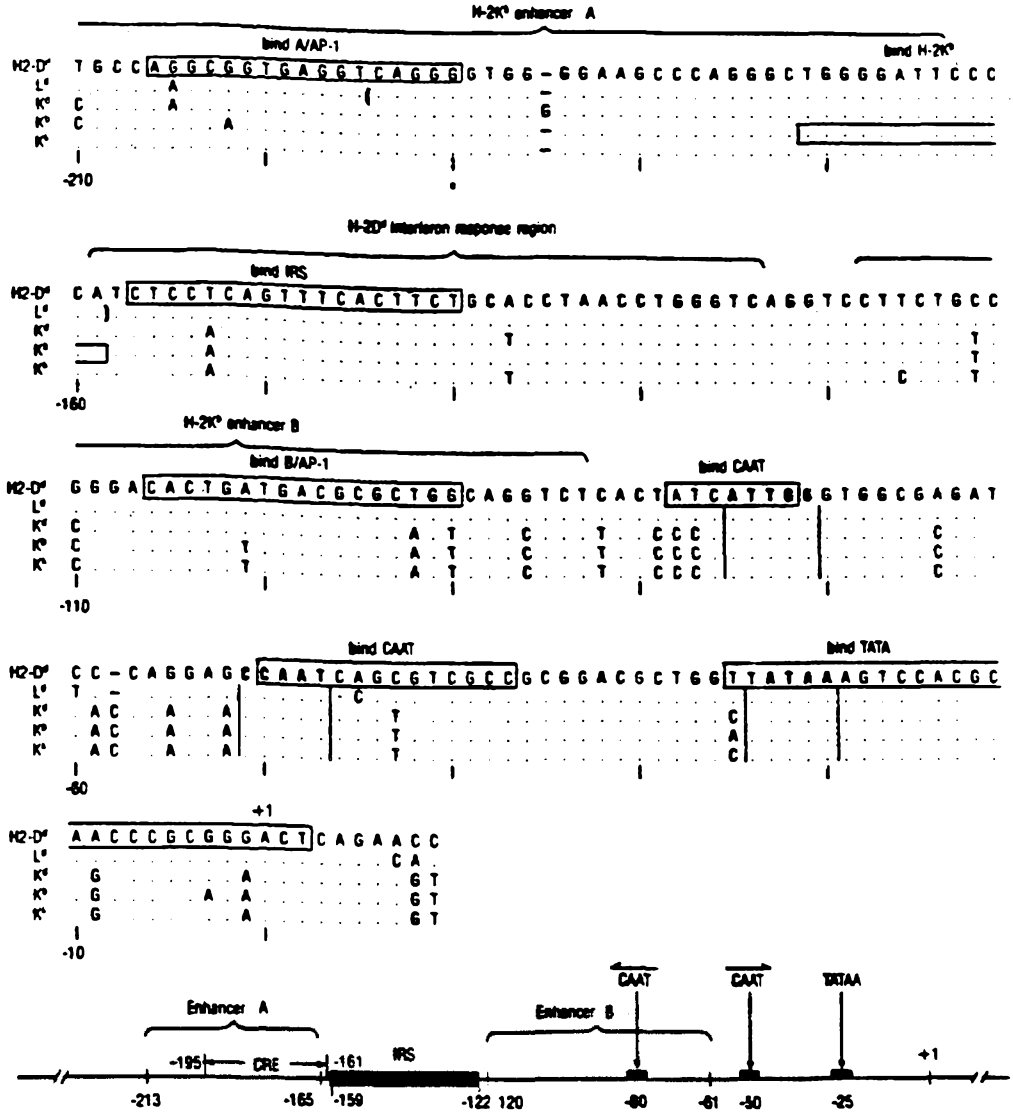

Fig. 1. Regulatory elements in the 5' flanking regions of H-2 class I genes. The sequences are aligned according to the transcription start site mapped for the H-2K^d gene (24). The cap sites for other H-2 genes were not determined experimentally. The locations of the sequences encoding the response to interferons (IRS) in the H-2D^d gene (10), and the enhancers A and B of the H-2K^b gene (7), are indicated. The class I regulatory element (CRE) (8) is enclosed in parentheses in the H-2L^d sequence. The DNA sequences, which bind nuclear factors are boxed. Bind A/AP-1, bind B/AP-1 and bind IRS were determined using the H-2D^d template (15), and bind H-2K^b using the H-2K^b template (13,14). The CCAAT and TATA boxes are indicated in bold letters. Vertical lines highlight the homology of the CCAAT and TATA elements in these H-2 genes. The DNA sequences are derived from references 7, 10, 25 and 26. A schematic representation of an H-2 5' flanking region and known regulatory elements is provided beneath the sequence.

Fig. 2. Footprint analysis of the H-2D^{d} promoter region. The ^{32}P-kinased EcoRI-PVuII restriction fragment, which allows visualization of the coding strand, was either directly digested with DNAase I (the lanes labeled O, using 2.5 or 1 µg/ml of DNAse I) or first incubated with MPC 11 nuclear extract and then digested with DNAase I (the lanes labeled MPC 11, using 250 or 100 µg/ml of DNAase I). The G+A lane was generated by chemical cleavage reactions; the numbering is relative to the proposed cap site. The TATA and CAAT box sequences are indicated, and cleared regions are bracketed.

Appendix to Chapter Four

There are two regions in the 5' enhancer (enhancer A) domain of class I genes that are known to bind specifically to nuclear proteins. One region (bind A) interacts with both AP-1 and our murine MPC 11 cell nuclear factor (1). The other (labeled bind H-2K^b in Fig. 1 in the preceding chapter) is a regulatory sequence that has been shown to interact with two distinct transcription factors (2): H2TF1, (identified by its interaction with the class I H-2K^b promoter) (3,4), and NF-kappaB, a B cell-specific inducible factor (first identified by its ability to interact with an immunoglobulin enhancer) (5). These two factors have sufficiently different properties to classify them as unique factors that share sequence specificity for their DNA recognition site (2). It is worth noting that an additional transcription factor (AP-2) binds to this same sequence in H-2 promoters (6). The binding site for AP-2 in class I genes covers the same region of dyad symmetry as H2TF1 and NF-kappaB:

GGCTGGGGATTCCCCATCT.

There is also an overlapping binding site for AP-2 and NF-kappaB (5) in the SV40 enhancer containing the sequence CTGGGGA. Mutations in the four G residues in SV40 can decrease transcription to 40% of wild-type (7); the G residues have been shown by methylation interference studies to be critical for H2TF1 and NF-kappaB binding to the class I promoter (2). Although AP-2 and H2TF1 have never been directly compared, it is unlikely that they are the same, because AP-2 has a higher affinity for the SV40 enhancer than the class I enhancer (6), and class I DNA competes more effectively for H2TF1 binding activity than

SV40 DNA (3). AP-2 is purified from HeLa cell extracts, and so is also unlikely to be equivalent to NF-kappaB, that is specifically expressed in activated B cells (5). It appears that AP-2, like AP-1, is a member of a family of transcriptional activators with similar DNA-binding sequence specificities.

Phorbol esters are potent tumor promoters that are known to regulate gene expression. AP-1 and AP-2 bind to cis-acting transcriptional regulatory elements that can confer responsiveness to the phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate) (6). Since AP-1 and AP-2 also bind to class I promoters (1,6), we tested if class I genes are able to be expressed at higher levels in cells treated with TPA. Five or fifty nanograms/ml TPA was added to the media of BL5 or BLK SV cells for 12, 24, 48, or 70 hours and class I expression was analyzed by RIA. (TPA and incubation conditions were kindly provided by Ellen Rothenberg and Russel Hill. Although we did not do an internal control to prove the TPA was working, it was an aliquot from an active batch.) No induction was observed in the cell types we tested. Therefore, despite the fact that AP-1 and AP-2 can bind to class I promoters (1,6), that AP-1's activity or abundance can increase in response to phorbol esters (8), and that inducible enhancer elements that bind to AP-1 and AP-2 can confer TPA inducibility on a heterologous promoter (6), class I genes are not inducible by TPA under the conditions tested.

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CHAPTER FIVE

CONCLUSIONS

Conclusions

Although interferons share anti-viral and cell-growth inhibitory effects, they work in a cell-type specific manner, and different interferons can trigger different responses within the same cell type (1,2). The magnitude of a response to interferons varies widely (3). Some genes are able to respond to both types of interferons, while others specifically respond to only one type (1,2). This suggests multiple regulatory pathways may be involved.

Our efforts, along with those of other laboratories (4,5), have begun to reveal the complexity of the interferon regulation of a single type of genes, MHC class I genes. Hopefully, deeper understanding of the mechanisms involved in a cellular response to interferons will begin to explain how divergent regulatory effects can be mediated through similar regulatory elements for cell-type specific responses.

Interferon Regulation of Class I Genes in the Region Downstream from the Transcriptional Start Site

We have found that both type I and type II interferons can stimulate expression of class I genes via a mechanism that acts independently of the promoter region responsive element, through a region downstream from the transcription start site. This response was measured by an assay that detected increased production of protein at the cell surface (RIA), and occurred in each of the three cell types examined. Two dimensional gel electrophoresis established that this response is unlikely to be due to increased transport of protein to the cell surface, so the induction of the protein is likely to be paralleled

by induction of mRNA.

Elly de Pagter and Iwona Stroynowski are currently involved in determining if the mRNA is indeed induced, and if so, if this induction is due to an increase in the transcription rate or due to an increase in mRNA stability. At this point they have successfully distinguished H-$2L^d$ RNA from the endogenous class I RNA in L cells, using probes from the polymorphic alpha 1 and alpha 2 domains of H-$2L^d$. They will use S1 protection experiments to assess the levels of mRNA before and after interferon treatment (6), and nuclear run-on transcription assays to determine the transcription rate in interferon treated and untreated cells (7). Once the stage of expression that the gene is regulated at is determined, deletion and mutation analysis can be applied to distinguish which part of the gene contains the regulatory element, and studies can be instigated to discover how this regulation is implemented.

Interferon Responsive Elements in the Promoters of Class I Genes

The promoters of many (but not all (1,2)) genes that respond to interferons contain a sequence homologous with the interferon responsive sequence (IRS) of class I genes (8). The use of this sequence appears to be cell type specific and quite complex. This sequence is required for class I promoters to respond to both type I and type II interferons, although in some cell types an additional upstream sequence is required as well for a type I interferon response. Class II genes, which also have an IRS homology in their promoters, are not responsive to type I interferon, and surprisingly, this sequence can be deleted from a class

II promoter without loss of type II interferon inducibility (9). Clearly, the IRS does not respond the same in all contexts.

We proposed a model (see Chapter Three) to account for the interferon action on the IRS in class I promoters, incorporating aspects of what is known about the regulatory elements found in the beta interferon gene promoter (10,11), that we discovered had an element homologous to the class I IRS. Beta interferon can be auto-regulatory (12). Our model suggests that the regulation of class I genes by type I interferon in some cell types may rely on derepression of a negative element upstream and proximal to the IRS, which could block enhancer activity of the IRS in the absence of interferon. Additionally, intensification or activation of enhancer activity in response to type II interferon (and type I interferon regulation in some cell types), may explain why an increase in transcription can occur in the absence of the upstream element. Type I interferon would then have to be able to trigger two distinct regulatory pathways in a cell-type dependent manner. All available data concerning class I regulation by interferons can be accounted for within these assumptions.[1]

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[1] The above model also is applicable to the available data concerning regulation of the beta interferon gene. The homologous element in the beta interferon promoter to the class I IRS can serve as a constitutive enhancer when a negative regulatory element is deleted (10). There is evidence that when cells have been stimulated to produce beta-interferon, the negative regulator is inactivated and the enhancer activity dominates the expression. This model is supported by in vivo footprinting experiments where binding patterns in the negative element and the IRS homologous enhancer element change when the gene is induced (11). Alternatively, positive regulation may occur by intensifying enhancer activity, which would explain why a repeated motif from the IRS homology region can stimulate activity in a heterologous promoter in the absence of the negative element (13). Auto-regulation of the beta interferon promoter is due to an effect called priming (12). In some cell types, pre-treatment of the cells with beta-interferon results in

This is not the only model for the role of the IRS in the regulation of class I genes by interferons consistent with the data, but given the homologies between the class I and the beta-interferon promoter elements it is an attractive hypothesis (see footnote 1). Israel et al. (4) have previously presented an alternative model for interferon action on class I promoters by suggesting that the IRS sequence is involved with potentiating an upstream enhancer.

How can these models be tested? Why does the IRS seem to have such different roles in different contexts? These questions can begin to be addressed by purification of and cloning the proteins that bind to the IRS region. Current methods for purification of DNA-binding proteins rely on affinity chromatography using DNA oligo columns specifically constructed to contain the DNA recognition site (14). Antibodies that recognize the purified protein could be generated, and expression libraries could then be screened (15). Alternatively, one could attempt to screen a phage expression library directly with the synthetic oligo that reconstructs the IRS recognition site. This novel technique has recently been used to select a clone that encodes a protein that binds specifically to the site in the class I promoter that is recognized by the factors H2TF1, NF-kappaB, and AP-2 (16).

Once the IRS binding protein(s) is purified and cloned, many

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much greater levels of beta-interferon production when stimulated by virus, hence the cells are primed (12). This effect may be due to the induction of the IRS positive enhancer regulatory factor by interferon pretreatment, which would be free to act only after viral infection caused derepression of the negative element. A comparison of the IRS in the class I promoter and the highly homologous enhancer sequence in the interferon promoter is shown in Chapter Three.

questions could be addressed. Can it serve as a transcriptional activator of promoters that contain the IRS? Is it involved with the interferon regulation of these genes? Does it bind to both the beta interferon and the class I promoter? How is it regulated? Is it transcriptionally regulated or modified in response to interferon?

Additional Regulatory Elements in the Class I promoter

The observation that distinctive transcription factors share a capacity to bind to the same sequence motifs in regulatory regions of class I genes is intriguing (17,18). It is interesting from an evolutionary point of view in terms of the potential for conserved DNA binding domains in regulatory proteins and conserved elements in the promoters of many genes. From the perspective of gene regulation, it provides a new level of possibilities to consider. How are the distinctions made between one tissue and another, when genes that are expressed in a highly tissue specific manner (such as immunoglobulin genes) have regulatory elements that can bind not only tissue specific transcription factors, but transcription factors that are present and active in a broad variety of cell-types? How does the same environmental stimuli (such a as specific type of interferon) trigger one response in one cell-type, and an entirely different response in another? These questions will probably be answered only by a full understanding of what it takes for a promoter to be active — the chromatin structure requirements, the methylation state, and the sum of the specific DNA binding proteins present, how they compete with and interact with each other.

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CHAPTER SIX

PROCEDURES

Procedures

Most techniques have been described in the papers included in the preceding chapters. This section provides more technical detail for some of the methods that were employed, and discusses some additional methods.

Transfections and Interferon Treatment

Long-term transfections of L^{tk-} cells were performed by the calcium phosphate precipitation technique. One microgram of plasmid, cosmid, or phage DNA was cotransfected with ten nanograms of ptk5 (herpes simplex thymidine kinase gene cloned into pBR322) or 50 nanograms of pSV2neo (1) and ten micrograms of L^{tk-} carrier DNA per 5×10^6 cells. A calcium-phosphate-DNA precipitate was incubated with the cells for six hours. The cells were then treated with 8% dimethylsulfoxide (DMSO) for twenty minutes. The cells were refed 16 hours later, and 24 hours later selective HAT or G418 (400 micrograms/ml) supplemented medium was added. For other cell types, only G418 selection was used. Killing curves were carried out with each cell type to determine the appropriate G418 concentration to use: BLK SV cells received 400, SDBT cells 1200, and BL5 cells 800 micrograms/ml G418.

For routine interferon assays $2\text{-}3 \times 10^6$ cells were seeded into 80 mm^2 tissue culture flasks or 100 mM dishes. The next day gamma interferon was added (usually at 20 units/ml) or alpha and/or beta interferon was added (usually at 2000 units/ml), and cells were incubated for 72 hours unless otherwise specified. No cytotoxicity was observed under these conditions.

Assays of CAT Activity

Cells were washed three times in phosphate buffered saline (PBS), harvested from plates by a 5 min incubation in 20 mM EDTA/PBS, pelleted in a microfuge for 20 seconds, and resuspended in 100 microliters 0.25 M Tris (7.8). The cells were then lysed by freezing 2 consecutive times in liquid nitrogen. The cellular debris was pelleted and the protein concentrations of the supernatants were determined by BioRad protein assay. A set amount of protein extract for each construct (determined by titration and ranging from 1 to 100 micrograms per assay) was assayed as described by Gorman et al. (2) using TLC plates, or by using the liquid scintillation-diffusion technique of Neuman et al (3).

Radioimmunoassays

Quantitative cell binding RIAs (4) used monoclonal antibodies and ^{125}I-protein A that were titered to make certain they were used in excess. Cells were harvested, counted, and resuspended in their normal growth media at 10^7 cells/ml (they could be stored at 4 degrees C for several days and reassayed). In 96-well microtiter plates, 20-50 microliters of cells per well were mixed with 50 microliters of antibody diluted in PBS, 0.2% heat-treated fetal calf serum (fcs), and 0.02% NaN_3, and incubated at 4 degrees C. After a 1-4 hour incubation, cells were washed twice in 100 microliters of PBS/fcs/NaN_3. Saturating concentrations of ^{125}I-protein A in PBS/fcs/NaN_3 were added to 50 microliters PBS/fcs/NaN_3. After a 1-12 hour incubation at 4 degrees C, the cells were washed three times and the bound ^{125}I was counted.

The following monoclonal antibodies were used (5,6,7):

28-14-8, anti-H-2L^{d} and H-2D^{b},

20-8-4, anti-H-2K^{b} and H-2K^{d}

30-5-7, anti-H-2L^{d},

34-5-8, anti-H-2D^{d},

11-4, anti-H-2K^{k}.

RNase Protection Experiments

The experiments in L cells were performed according to Melton et al. (8) as recommended by Promega Biotech. ^{32}P-UTP labeled anti-sense RNA was transcribed from the T7 RNA polymerase transcription initiation site. Total cellular RNA was isolated (9), and 30 micrograms was hybridized with excess anti-sense RNA for 12 hours at 50 degrees C. Non-hybridized RNA was digested away with RNase A and T1 RNase in a one hour reaction at 30 degrees C. The length of the protected RNA was determined on denaturing polyacrylamide gels and scanning densitometry was used to quantitate the amount of anti-sense RNA protected by cellular RNA. (All scanning densitometry was performed on a LKB 2202 UltroScan laser densitometer linked to a LKB 2190 GelScan interface and software package.)

The experiments in BL5 cells were done differently than in L cells. RNA was prepared by dissolving cells in GuSCN as before, but RNA hybridization was carried out directly in the non-purified GuSCN solution (10). Cells were trypsonized then washed two times in ten mls PBS, resuspended in two mls of 5M GuSCN, .1 M EDTA, and 10 mM Hepes, and vortexed to dissolve cells. Ten microliters of this solution was ethanol precipitated resuspended in one ml of water so RNA

concentrations were determined. Four micrograms of RNA were hybridized with the anti-sense RNA probe in 20 microliters total volume of GuSCN buffer. After an initial hybridization at 25 degrees C for 16 hours, the reaction volume was increased to 300 microliters of RNase buffer with RNase A and T1 RNase (8), and digested for one hour at 30 degrees C. 20 microliters of 10% SDS were added to terminate the reaction, and 33 microliters of 10 mg/ml proteinase K were added for a 37 degree C, 30 minute reaction. RNA was phenol/chloroform extracted (50:50), ethanol precipitated, and run on a denaturing polyacrylamide gel.

Labeling Cells and 2-dimensional gel electrophoresis

Cells were grown in the presence or absence of gamma interferon for three days. For each T75 flask, media was replaced with two mls of deficient DMEM supplemented with 5% dialyzed fcs, 10 mM Hepes, and interferon (DMEM and fcs were purchased from Irvine Scientific). 500 microCi of ^{35}S-methionine were added, and cells were gently rocked at 37 degrees C for six hours. Cells were either lysed according to instructions provided by Protein Data Base, Incorporated, and 10% acrylamide gels with a pH range of 3-10 were run at their facility, or gels were run here according to protocols adapted by Keith Lewis and Minnie McMillan, based on references 11 and 12.

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...then on to Annapurna.