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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^dCAT and L^dCAT (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^dCAT and L^dCAT 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^dCAT or D^dCAT 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^dCAT or D^dCAT) 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^dCAT 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^dCAT and L^dCAT 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^dCAT, L^dCAT,

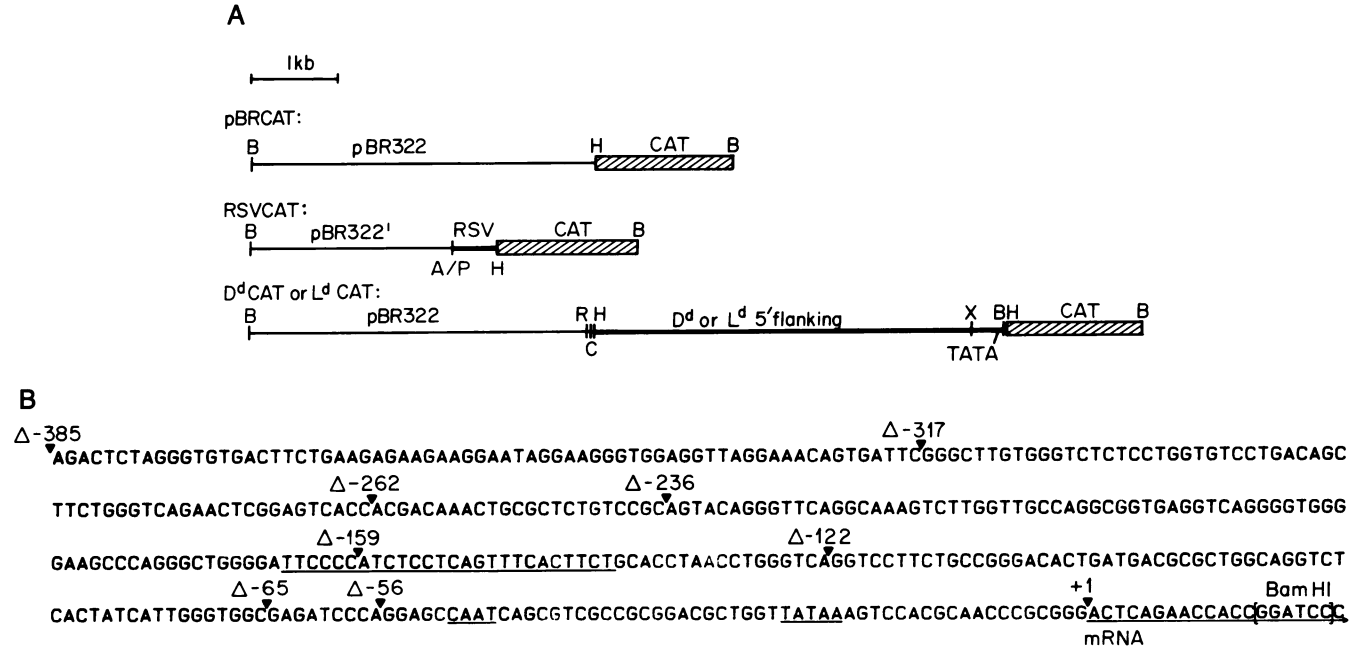

FIG. 1. (*A*) DNA constructs used for 5′ flanking region analyses. The plasmids are shown linearized at the conserved *Bam*HI restriction enzyme site. All constructs contain pBR322 sequences and the bacterial structural gene encoding CAT, followed by a simian virus 40 polyadenylylation signal (indicated 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^dCAT and D^dCAT, *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.

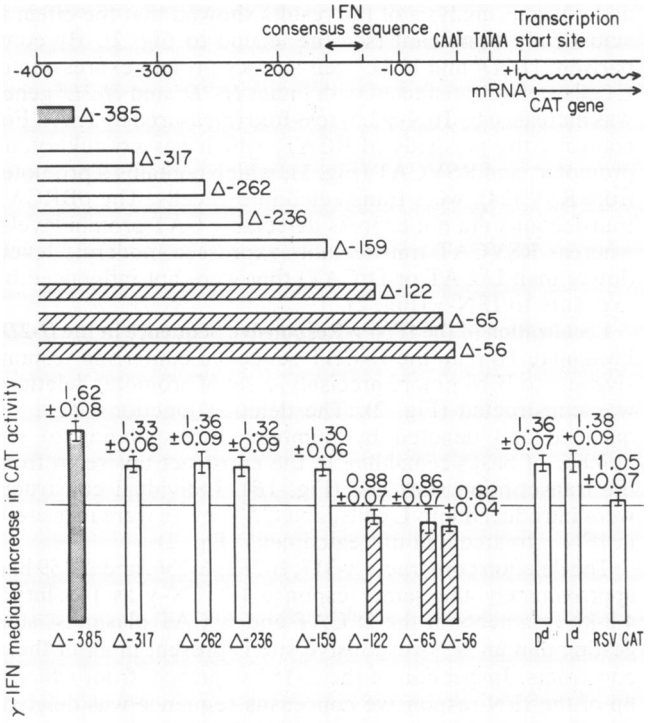

FIG. 2. Localization of the IFN-γ-sensitive site in the 5′ flanking region of class I genes. The horizontal bars indicate the extent of the progressively deleted D^{d}CAT promoter region. The set of deletion constructs was generated by BAL-31 digestion of D^{d}CAT plasmid linearized at the *Xba* I site. 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^{d}CAT and L^{d}CAT 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^{d}CAT and L^{d}CAT 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^{d}CAT, L^{d}CAT, Δ-317, Δ-262, Δ-236, and Δ-159 constructs. The probability that the EF for Δ-385 is different from parental D^{d}CAT 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^{d}CAT and L^{d}CAT 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.

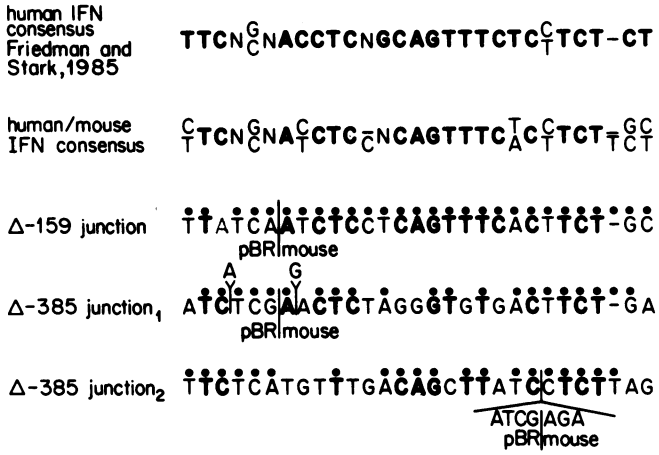

FIG. 3. Comparison of the consensus sequence involved in transcription regulation by IFN with the junctional sequence of deletion constructs. The human consensus sequence (17) is based on the four human sequences previously compared: *HLA-DR*, *HLA-A3*, an unidentified *HLA*, and *MT2*, a metallothionein 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^{d}CAT, 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

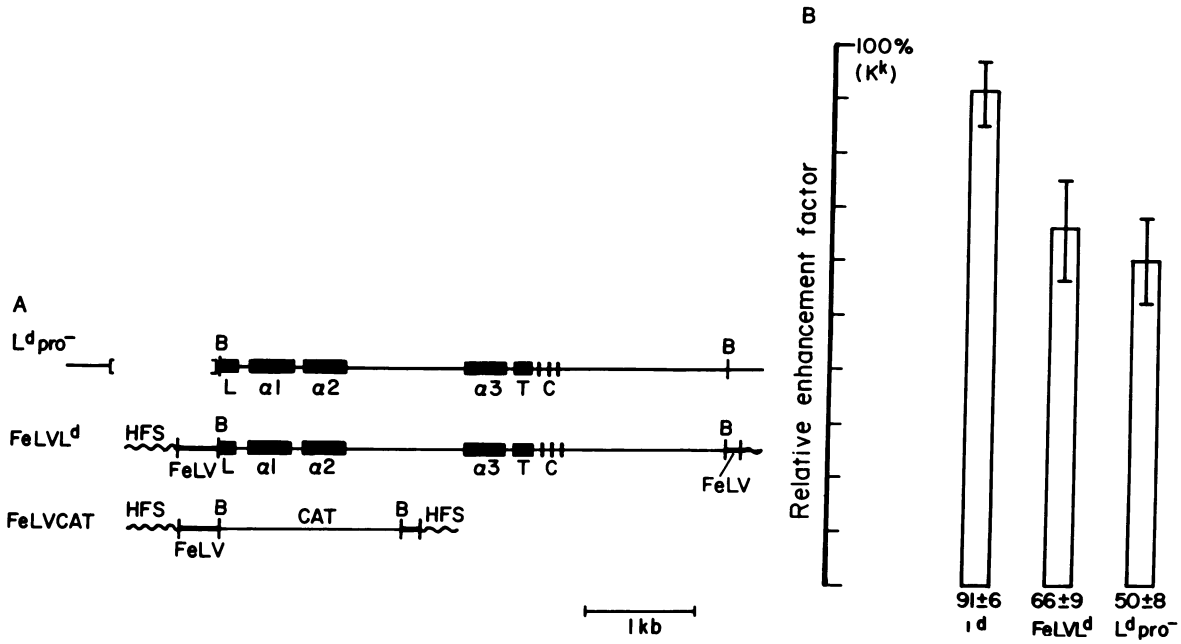

FIG. 4. DNA sequences downstream from the transcription start site contribute to the ability of class I genes to respond to IFN-γ. (*A*) The structure of DNA constructs lacking class I *H-2L*^{d} promoter regions. The coding *H-2L*^{d} region is shown as thin lines (introns) or filled bars (exons). L 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.

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