

Two Essential Regulatory Elements in the Human Interferon γ Promoter Confer Activation Specific Expression in T Cells

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Summary

Like interleukin 2 (IL-2), interferon γ (IFN- γ) is an early response gene in T cells and both are prototypical T helper cell type 1 (Th-1) lymphokines. Yet IL-2 and IFN- γ production are independently regulated, as demonstrated by their differential expression in certain T cell subsets, suggesting that the regulatory elements in these two genes must differ. To explore this possibility, the 5' flank of the human IFN- γ gene was analyzed. Expression of IFN- γ promoter-driven β -galactosidase reporter constructs containing 538 bp of 5' flank was similar to that by constructs driven by the IL-2 promoter in activated Jurkat T cells; expression nearly as great was observed with the construct containing only 108 bp of IFN- γ 5' flank. These IFN- γ promoter constructs faithfully mirrored expression of the endogenous gene, in that expression required activation both with ionomycin and PMA, was inhibited by cyclosporin A, and was not observed in U937 or THP-1 cells. The region between -108 and -40 bp in the IFN- γ promoter was required for promoter function and contained two elements that are conserved across species. Deletion of 10 bp within either element reduced promoter function by 70%, whereas deletions in nonconserved portions of this region had little effect on promoter function. The distal conserved element (-96 to -80 bp) contained a consensus GATA motif and a potential regulatory motif found in the promoter regions of the GM-CSF and macrophage inflammatory protein (MIP) genes. Factors binding to this element, including GATA-3, were found in Jurkat nuclear extracts by electromobility shift assays and two of the three complexes observed were altered in response to activation. One or both of these motifs are present in the 5' flank of multiple, other lymphokine genes, including IL-3, IL-4, IL-5, and GM-CSF, but neither is present in the promoter of the IL-2 gene. The proximal conserved element (-73 to -48 bp) shares homology with the NFIL-2A element in the IL-2 promoter; these elements compete for binding of factors in Jurkat nuclear extracts, although the NFIL-2A element but not the IFN- γ element binds Oct-1. Factors binding to this element in the IFN- γ gene were present in extracts from resting and activated Jurkat T cells. However, by *in vivo* footprinting of intact cells, this element was protected from methylation only with activation. The factors in Jurkat extracts that bound to the proximal and distal conserved elements were not detected in U937 or THP-1 extracts. Notably, the critical regions of the IFN- γ promoter do not contain sequences homologous to the NF-AT or AP-1 sites in the IL-2 promoter. The differences in essential *cis* elements in the IFN- γ and IL-2 promoters, and the factors binding thereto, may play an important role in the differential expression of these genes in naive vs. memory T cells and in the relative preservation of IFN- γ as compared to IL-2 gene expression in anergic Th-1 T cell clones.

IFN- γ is an important immunomodulatory molecule produced by activated T cells and NK cells (1, 2). Though named according to its ability to induce cellular resistance to viral infection, IFN- γ has little genetic homology with

the classical interferons, IFN- α and IFN- β (3, 4), and its primary role is modulation of nonspecific and antigen-specific immune responses via widespread receptors (3, 5). The effects of IFN- γ on the afferent and efferent aspects of immunity

are multiple and include: (a) enhanced expression of MHC class I and of MHC-linked proteins involved in antigen processing and transport (6); (b) increased expression of MHC class II on conventional APC and de novo expression on non-professional APCs leading to enhanced antigen presentation to CD4 T cells; (c) regulation of T helper cell differentiation and B cell isotype expression (7, 8); (d) activation of macrophages; and (e) enhanced NK cell cytotoxicity (1).

Like IL-2, IFN- γ is an early response gene expressed by T cells upon activation. Yet the IL-2 and IFN- γ genes are independently regulated as demonstrated by their differential expression in certain subsets of T cells. Production of IL-2 is comparable in naive and memory T cell populations; and in both populations IL-2 is expressed predominately by the CD4⁺ T cell subset (7–10). IFN- γ is produced to a greater extent by the CD8⁺ subset than by the CD4⁺ subset, and in both subsets it is produced almost solely by memory T cells, with very little expression by naive T cells (2, 9–13). Moreover, NK cells express IFN- γ but not IL-2 (1, 14). The differential regulation of the IFN- γ and IL-2 genes in T cells has been shown to occur primarily if not solely at the level of gene transcription (10, 15).

Unlike the relatively well-characterized promoter–enhancer of the IL-2 gene, much less is known about the regulation of IFN- γ transcription. Introduction of an 8.6-kb fragment containing the human IFN- γ gene into transgenic mice produced appropriately regulated tissue-specific expression, indicating the key regulatory elements are located within this fragment containing 2.7 kb of 5' flank and 1.0 kb of 3' flank. These results also suggest that the murine and human genes share evolutionarily-conserved regulatory factors (16). In T cell lines capable of expressing IFN- γ , two constitutive T cell-specific DNase hypersensitivity sites have been identified, one in the 5' flank and another in the first intron; in addition, there is an activation inducible, hypersensitivity site \sim 200 bp 5' from the transcription start site (17, 18). Consistent with the location of the hypersensitivity sites, transient transfection of reporter constructs with human IFN- γ sequences linked to the basal SV40 promoter into a murine T lymphoblastoid cell line suggested that T cell- and activation-specific, inducible regulatory elements were present between –540 and –47 bp (relative to the transcription start site). Enhancer activity also was identified in the first intron, which was inducible in a T cell line but constitutively active in a fibroblast cell line (19). Similarly, analyses in the human Jurkat T cell line and short-term cultured human T cells suggested that elements regulating the IFN- γ promoter are contained within \sim 550 bp of the transcription start site and that the key elements may be located within the proximal 200 bp (20–22).

Such findings offer the possibility that IFN- γ gene expression, like that of the IL-2 gene, is governed primarily by a series of essential regulatory elements (and factors binding thereto) within a relatively compact region of the 5' flanking sequence of the gene. We have sought to provide a higher resolution delineation of the regulatory elements of the 5' flank of the IFN- γ gene and to provide an initial characterization of the factors binding to these elements. In this re-

port, evidence is provided to suggest that two essential elements within 108 bp of the transcription initiation site of the IFN- γ gene are sufficient to confer activation-specific promoter function in T cells. These elements are conserved across species and for the most part differ from those governing IL-2 expression.

Materials and Methods

Cells. A line of Jurkat T cells, that express IFN- γ following activation, was originally obtained from H. Spits, DNAX Institute, Palo Alto, CA. This line was maintained in RPMI 1640 (Bio-Whittaker, Inc., Walkersville, MD) supplemented with 10% FCS (Hyclone Labs., Logan, UT), 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 20 μ g/ml gentamicin (RPMI + 10% FCS). Cells cultured continuously for >6 wk progressively lost the capacity to produce IFN- γ , even though they continued to produce IL-2. Accordingly, aliquots of cells frozen at early passage were recovered from liquid nitrogen monthly, and used for experiments between 1 and 5 wk after thawing. Cells maintained under these conditions produced 200–3,000 pg/ml of IFN- γ , as determined by sandwich ELISA (antibodies provided by Genentech, San Francisco, CA), when activated with 1.5 μ M ionomycin and 50 ng/ml PMA; unstimulated cells produced <20 pg/ml. Two monocyte–macrophage cell lines, U937 and THP-1, were obtained from American Type Culture Collection, Rockville, MD, and maintained in RPMI 1640 supplemented as described previously.

Plasmid Constructs. The IFN- γ plasmids were constructed by subcloning fragments of the IFN- γ gene into a promoterless *LacZ* plasmid, pEQ3, which was derived from PON 1 by mutating the bases flanking the ATG site to create an optimal sequence for mammalian translation initiation (23). With the exception of the –2700 to –538 bp BamHI-XbaI fragment (base pairs numbered relative to the transcription start site), the designated sequences of the IFN- γ gene were generated by PCR amplification using the human genomic BamHI fragment, obtained from P. Gray, Genentech, Inc., San Francisco, CA, as a template (24). Constructs were generated by amplifying the entire fragment (pIFN-29, –39, and –108) or a proximal portion (all the other constructs) with primers that generated a BglII site at a variable 5' terminus and a BamHI site at the common 3' terminus (+64 bp); the TATA box begins at –28 bp. These fragments were cloned into the BglII site of pEQ3, regenerating only the 5' BglII site. For the constructs in which –538 was the 5' end (including those with internal deletions Δ –108/–40, Δ –177/–109, Δ –214/–178, and Δ –258/–109), a distal portion was amplified with a 5' primer containing the XbaI site at –538 and a 3' primer containing a BglII site; these fragments were then cloned into the appropriate promoter construct containing the proximal portion. The pIFN-2.7 construct was created by cloning the BamHI-XbaI genomic fragment into the XbaI site at –538 in the pIFN-538 construct. A partial BclI digestion of the pIFN-538 construct generated the pIFN-339 construct. The 5' truncation constructs, with the exception of pIFN-108, contain a BglII site at –39, while the internal deletion constructs noted above contain a BglII site at the junction of the deletion. The –538 constructs with scanning 10-bp internal deletions from –108 to –29 each contain a BglII site at –177 and were prepared using PCR site-directed mutagenesis and splicing by overlap expression (25). The sequence of PCR generated fragments and junctions was confirmed after subcloning with Vent (exo-) DNA polymerase dideoxynucleotide chain termination sequencing.

The pIL2-568 plasmid contains the HindIII fragment (–568 to +50) of the IL2 promoter (obtained from G. Crabtree, Stanford

University, Stanford, CA) subcloned into the pEQ3 *LacZ* plasmid. The β -actin CAT plasmid was prepared by subcloning the β -actin promoter (obtained from L. Keddes, University of Southern California, Los Angeles, CA) into a promoterless plasmid containing the chloramphenicol acetyl-transferase (CAT)¹ gene. The pEQ 176 plasmid, containing the CMV immediate-early gene promoter driving *LacZ* transcription was obtained from A. Geballe, The Fred Hutchinson Cancer Research Center, Seattle, WA.

Transient Transfection Assays. Cells were transiently transfected by electroporation using a gene pulser set at 0.25 kV and 960 μ f capacitance (Bio-Rad Laboratories, Richmond, CA) with both an IFN- γ *LacZ* plasmid and a control β -actin CAT plasmid in 0.5 ml volume of RPMI 1640. Where indicated, cells were pretreated for 10 min with cyclosporin A (CSA). 1 h after electroporation cells were diluted to 10^6 cells/ml in RPMI + 10% FCS, and a portion of the cells were stimulated with 25 ng PMA and/or 1.5 μ M ionomycin. After incubation for 40 h cell lysates were prepared by three cycles of rapid freezing and thawing. Lysates were assayed for β -galactosidase activity spectrophotometrically using chloro-phenol red as substrate (26) and CAT by ELISA (5 Prime 3 Prime, Inc., West Chester, PA). β -Galactosidase activity was corrected for transfection efficiency by normalizing to the measured CAT value. Results are expressed as a percentage of the stimulated pIFN-538 value in each transfection series. Data from the indicated number of transfections are presented as the mean \pm the standard error of the mean.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from unstimulated and stimulated Jurkat T cells, and both U937 and THP-1 monocytic cells as described (27, 28), in the presence of phosphatase inhibitors (50 mM NaF, 0.2 mM NaVO₄). Cells were stimulated for 2 h unless indicated otherwise, because IFN- γ mRNA accumulation under our conditions was maximal between 2 and 6 h. Recombinant murine GATA-1 was prepared by DEAE-dextran transfection of COS cells with an expression plasmid, pXM, containing murine GATA-1 cDNA (obtained from D. Martin, The Fred Hutchinson Cancer Research Center, Seattle) (29, 30). Whole cell lysates from the GATA-1 transfected and mock transfected cells were prepared as described (30). The protein content of nuclear and COS cell extracts was quantitated using Coomassie blue Plus (Pierce Chem. Co., Rockford, IL). In vitro transcription and translation of recombinant human GATA-3 was performed as previously described (31).

Oligonucleotides were labeled with γ -[³²P]dATP using T4 kinase, annealed, and filled in with the Klenow fragment of DNA polymerase when necessary, then purified by nondenaturing 10% PAGE. Nuclear extract was preincubated at room temperature with buffer and competitors for 5–10 min before addition of the indicated probe. Binding reactions were incubated for 15–30 min at 30°C and, except as noted, contained the following: 12,000 cpm probe, 175 ng polydI-dC (Pharmacia Fine Chemicals, Piscataway, NJ), 2 μ g nuclear extract or COS cell lysate, and designated annealed cold oligonucleotide competitor in a 15- μ l reaction volume. Reaction buffer consisted of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 2 mM PMSF, and 2 μ g/ml pepstatin A. In experiments using Oct-1 anti-peptide antiserum (provided by K. Ullman and G. Crabtree, Stanford University), the peptide and antiserum were preincubated together for 15 min before the addition of protein. Bound complexes were resolved on 4% PAGE gels at room temperature in low ionic buffer (5 mM Tris-HCl, 3 mM sodium acetate, 1 mM EDTA, pH 7.9).

¹ Abbreviations used in this paper: CAT, chloramphenicol acetyl-transferase; CSA, cyclosporin A; EMSA, electromobility shift assay; MIP, macrophage inflammatory protein.

Electromobility shift assay (EMSA) using recombinant GATA-3 (provided by J. Leiden, University of Chicago, Chicago, IL) or a murine monoclonal antibody to GATA-3 (α GATA-3; provided by D. Engel, Northwestern University, Chicago, IL) were resolved in 0.25 \times TBE as described previously (31). Stimulated Jurkat nuclear extract was preincubated with 1 μ g polydI-dC and 2 μ l of the monoclonal antibody for 10 min at 4°C before the addition of probe.

In Vivo Footprinting. In vivo footprinting was performed on Jurkat T cells placed in medium containing 10% FCS and 0.5% dimethyl sulfate (DMS; freshly prepared) for 4 min at room temperature. The cell suspension was diluted 20-fold by adding ice-cold PBS followed by centrifugation. After washing the cell pellet with ice-cold PBS, nuclei were prepared and DNA was purified by the addition of 200 μ g/ml of proteinase K, 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 2.5 mM EDTA, 0.5% SDS for 4 h at 50°C. The DNA was then phenol-chloroform extracted, precipitated in ethanol, and redissolved in 10 mM Tris-HCl, pH 7.8, 0.1 mM EDTA at 50°C overnight. After reprecipitation in 300 mM sodium acetate and ethanol, the DNA was resuspended in 1 M piperidine and allowed to react in sealed tubes at 90°C for 30 min. Piperidine was removed by drying in a Speedvac, redissolving pellets in water and lyophilization, which was repeated twice. Pellets were resuspended in water and precipitated in 3.75 M ammonium acetate and ethanol three times. Ligation-mediated PCR (32, 33) was performed using an IFN- γ specific coding strand primer 1, 5'-CACAGGTGGGCATAATG-3' (-216 to -200 bp relative to the mRNA cap site) annealed to the piperidine-cleaved DNA fragments and extended to blunt ends by using Sequenase (33). The oligonucleotide linker,

5'-GCGGTGACCTAGGAGATCTGAATTC-3'
3'-CTAGACTTAAG-5'

was ligated to the blunt-ended primer-extended molecules according to published procedure (32). After precipitation of the DNA, human IFN- γ -specific fragments were amplified with Taq polymerase by using the longer oligonucleotide of the linker and primer 2, 5'-TAA-TGGTCTGTCTCATCGTCAAAGGAC-3'. 20 amplification cycles were performed. Primer 3, 5'-TAATGGGTCTGTCTCATCGTCAAAGGACCAAGG-3' was labeled with T4 kinase and used for the last extension. The sequence ladders were visualized separately on a standard sequencing gel.

Northern Analysis. Total cellular RNA was isolated and prepared as previously described (34). The full-length human elongation factor 1- α cDNA and a 400-bp fragment of the human IFN- γ cDNA were labeled by the random hexamer primer method and used as described (9).

Results

Important cis Regulatory Elements Are within 108 bp of the Transcription Start Site. To identify the *cis* elements regulating transcription of the human IFN- γ gene we transfected β -galactosidase reporter constructs containing various portions of the 5' flank into Jurkat T cells. For comparison we also transfected constructs containing no promoter, pEQ3, or the well-characterized IL-2 promoter, pIL2 -568 (35). The first series of five IFN- γ promoter constructs (Fig. 1) contained progressive truncations of the IFN- γ 5' flank from 2.7 kb to 39 bp of the transcription start site. Optimal expression was seen with 538 bp of 5' flank (pIFN-538), though expression with constructs containing only 108 bp

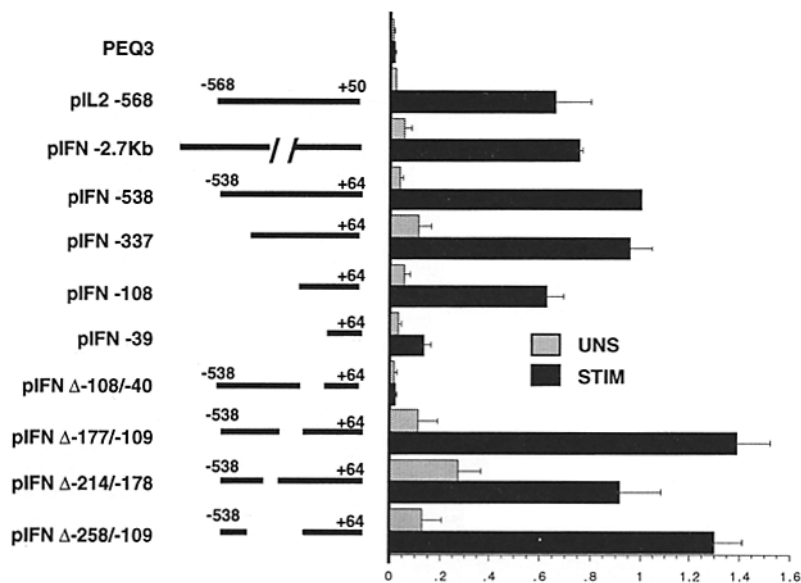


Figure 1. Transfection analysis of the IFN- γ promoter. The promoterless vector plasmid pEQ3 or reporter plasmids containing various portions of the IFN- γ 5' flanking sequence or the IL-2 promoter driving transcription of a β -galactosidase gene (line figures on the left) were transiently transfected into Jurkat T cells with a control β -actin CAT plasmid. A portion of the cells were stimulated with PMA (50 ng/ml) plus ionomycin (1.5 μ M). Cell lysates were assayed for β -galactosidase activity and CAT protein. Results are expressed as a ratio of the stimulated pIFN-538 value after correction for transfection efficiency, and represent means \pm SEM of at least three transfections. β -Galactosidase activity in lysates of the stimulated pIFN-538-transfected cells was 10–25 nmol CPR/ 10^6 cells/min.

of 5' flank (pIFN-108) was nearly as great. The results with these IFN- γ constructs were comparable to those with the IL-2 promoter construct (pIL2 -568), which has been shown by others to contain all the *cis* elements necessary for appropriate expression after transfection in T cell lines, and for proper expression in transgenic mice (35–38). Truncation of the promoter to -39 bp markedly diminished activation-induced expression. In the absence of stimulation there was minimal or no expression with these constructs. The promoterless pEQ3 plasmid gave values with or without activation of <0.25 nmol/ 10^6 cells per min (data not shown).

To examine the importance of regions within the near 5' flank, four constructs containing internal deletions within the pIFN-538 construct were created and similarly transfected into Jurkat T cells. The results are depicted in the bottom portion of Fig. 1. The most striking effect was seen with the deletion of sequences from -108 to -40 (pIFN Δ -108/-40), which reduced expression $>95\%$, comparable to that seen with the promoterless pEQ3 vector. Deletion of sequences between -177 and -109 gave maximum expression (~ 1.5 -fold that of pIFN-538). Comparison of these internal deletion constructs and the 5' truncation constructs suggests that: (a) the elements essential for activation-induced expression are contained within 108 bp of the transcription start site; (b) the region between -177 and -109 has a modest net negative effect on induced expression; (c) the region between -337 and -258 has a modest net positive effect on induced expression; and (d) the region between -214 and -178 may repress constitutive expression since deletion of that region led to increased unstimulated expression.

In Vitro Transfection Assays of IFN- γ Reporter Constructs Parallel Endogenous IFN- γ Gene Activation Requirements, CSA Sensitivity, and Cell Specificity. The induction of IFN- γ in T cells requires interaction between the TCR and antigen presented in association with the MHC on an APC, as well as accessory signals provided by the APC (38–40). Signals delivered through the TCR can be mimicked by PMA, acting

in concert with a calcium ionophore. The immunophilin ligands CSA and FK506 can block induction of IFN- γ , IL-2, and certain other lymphokines (41) by pathways downstream of the increase in intracellular Ca^{++} , most probably through interaction with calcineurin (42, 43). As shown in Fig. 2, the efficient induction of the pIFN-538 or pIFN-108 reporter constructs required both PMA and ionomycin. Further, induction was reduced 70–90% with CSA pretreatment of cells. These results paralleled those of the pIL2-568 construct and the production of IFN- γ protein by Jurkat T cells (Fig. 2).

To determine if these IFN- γ constructs were expressed in hematopoietic cells that do not produce IFN- γ , we transfected them and the pIL2 -568, and pEQ 176 (the pEQ3 vector with the strong cytomegalovirus immediate-early pro-

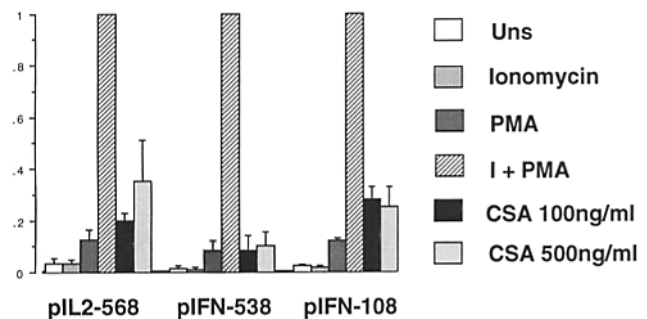


Figure 2. Induction of the 108-bp IFN- γ promoter requires PMA plus ionomycin and is sensitive to suppression by CSA. The pIFN-538 and -108 constructs were transiently transfected into Jurkat T cells that were subsequently incubated in media containing PMA, ionomycin, both, or pretreated with CSA (100 or 500 ng/ml), then incubated with PMA + ionomycin. This figure depicts means \pm SEM of three transfections (except the 100 ng/ml CSA data, which was repeated twice); results of each treatment group are expressed as ratio of the PMA + ionomycin value for each plasmid. Production of endogenous IFN- γ protein measured in supernatants from identically treated Jurkat T cells paralleled the transfection results: unstimulated, <4 pg/ml; ionomycin alone, 23 pg/ml; PMA alone, 70 pg/ml; both, 541 pg/ml; and 500 ng/ml CSA plus PMA and ionomycin, <4 pg/ml as determined by ELISA.

motor driving Lac Z) constructs into two monocytic cell lines, THP-1 and U937 (data not shown). The pEQ 176 plasmid gave expression four- to eightfold higher than the promoterless pEQ3 plasmid in THP-1 and U937 cells and was further induced 120-fold by stimulation with ionomycin and PMA in the U937 cell line. Expression of the pIFN-538, pIFN-108, or pIL2-568 plasmids was equivalent to that of the promoterless pEQ3 in THP-1 cells and in unstimulated U937 cells and not more than two- to fourfold greater than pEQ3 plasmid in stimulated U937 cells. This contrasts markedly with the results in activated Jurkat T cells in which these IFN- γ and IL-2 constructs produced ~60- to 100-fold greater expression than the promoterless pEQ3 (Fig. 1). Thus, the activation signal requirement, CSA inhibition and lack of expression in the monocytic cell lines with these IFN- γ reporter constructs faithfully paralleled expression of the endogenous IFN- γ gene.

Potential Importance of the -108 to -40 Region Based on Sequence Homology to Known Regulatory Elements and Cross-species Conservation. The data from the initial transfection analyses described above indicated that sequences between -108 and -40, within the context of the IFN- γ TATAA box and sequence extending to +64 bp, were both necessary and sufficient for activation-specific and CSA-sensitive induction. In seeking to define the critical elements in this region, we first searched this sequence for homology to known regulatory elements as well as sequences conserved between the human, rat, and murine genes. These searches revealed two regions in which the sequences are highly conserved across species and contain homologies to known regulatory factors (Fig. 3).

Two consensus GATA motifs are present in the human gene, the most 3' of which is conserved in the rodent genes and contained within the distal conserved element (-96 to -80

bp). GATA motifs are found in the promoters-enhancers of the TCR α , β , and δ chain genes, and bind the T lineage-restricted factor GATA-3 (31, 44-46). Abutting the 3' GATA motif in the distal conserved element of the human IFN- γ gene are sequences (-89 to -82 bp) identical to those found in the promoter region of the human and murine GM-CSF genes (-56 to -46 bp and -63 to -53 bp, respectively), as well as members of the macrophage inflammatory protein (MIP) gene family (47). In the GM-CSF promoter this motif is located within an activation responsive DNase I footprint using extracts from MLA 144 cells (gibbon ape T cells) (48) and is part of the conserved lymphokine element 0 (CLEO) (49).

The proximal conserved element (-73 to -48 bp) in the human IFN- γ gene contains a 17- out of 24-bp match to the NFIL-2A site of the human IL-2 gene. In the IL-2 promoter this element is necessary for maximal induction with activation (36), and has been shown to bind both Oct-1 and the activation-specific factor OAP-40 (27).

Thus, excluding the obviously conserved TATAA box and transcription start site, there appear to be two highly conserved, and thus potentially important, regulatory elements within the -108 to -40 bp region. The distal element, -96 to -80, encompasses the 3' GATA homology and the GM-CSF/MIP sequence; the proximal element, -73 to -48, contains the NFIL-2A homology.

Deletions within Either Conserved Element in the pIFN-538 Construct Markedly Reduce Activation-induced Transcription. To map the location of important *cis* regulatory elements within the -108 to -40 region, we created a series of constructs with 10-bp internal deletions scanning across this region within the pIFN-538 construct. Results of transfections with these constructs are demonstrated in Fig. 4. Consistent with their conservation across species, deletions that included the 3' por-

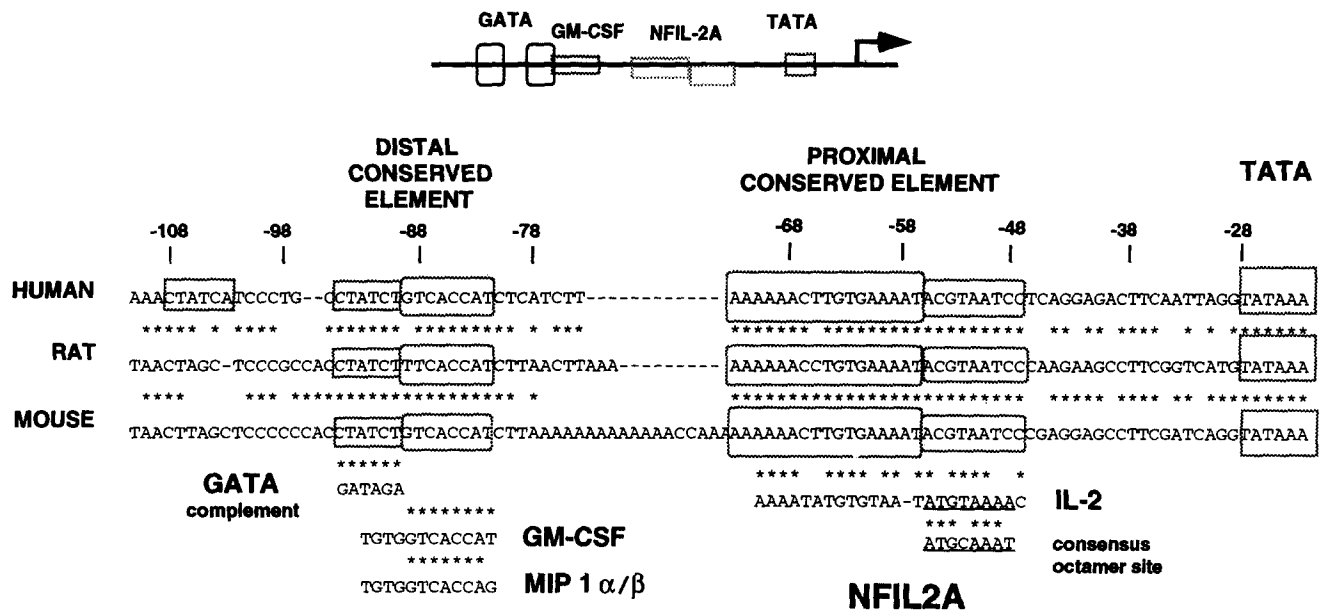


Figure 3. The sequences conserved between the human, rat, and mouse IFN- γ genes in the near 5' flank are shown. Consensus GATA motifs and homologies to elements in the human GM-CSF (-56 to -46 bp), MIP 1 α/β , and human IL-2 (-93 to -69 bp) genes are boxed and shown in detail below. Sequences are numbered relative to the transcription start site of the human gene.

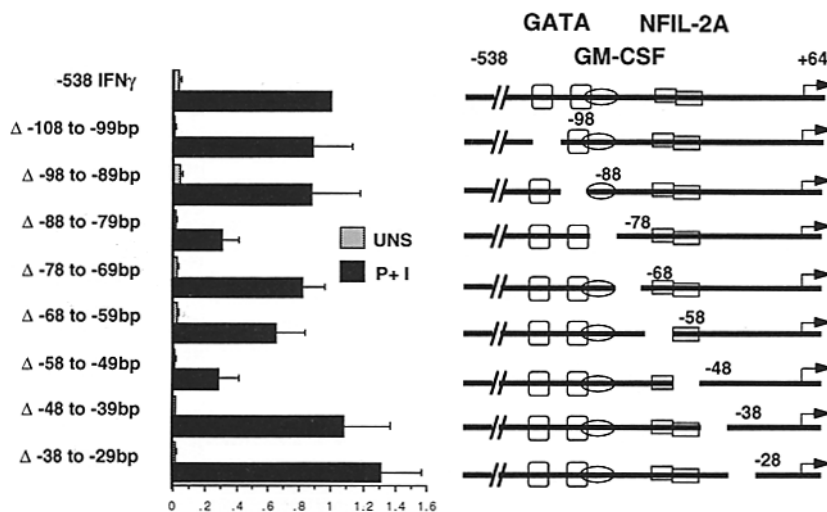


Figure 4. Elements within both conserved regions are essential for activation-induced expression of the IFN- γ promoter. Transient transfection analyses in Jurkat T cells using reporter constructs containing scanning 10 bp internal deletions within the -108 to -40 bp region of the pIFN-538 plasmid. The constructs are shown in line figures on the right. Results were corrected for transfection efficiency and analyzed as described in Fig. 1. Results represent the mean (\pm SEM) of at least four independent transfections.

tions of each of the conserved elements significantly diminished expression. The Δ -88/-79 construct deleted the sequences homologous to elements in the promoter region of GM-CSF and MIP genes within the distal conserved element and reduced expression 69% ($p = 0.001$). Within the conserved proximal element, the Δ -58/-49 construct, which deleted the 3' portion of the NFIL-2A-like element, reduced expression by 72% ($p = 0.005$). The two constructs that contained deletions within the 5' portions of the conserved elements either did not alter expression (Δ -98/-89, which included the GATA motif in the distal element), or reduced expression to a lesser extent (Δ -68/-59, encompassing the homology to the OAP-40 binding site in the proximal element), which was not statistically significant (35%, $p = 0.11$). Deletion of the distal GATA motif (Δ -108/-99) which is not conserved in the rodent genes, had no effect on expression, nor did the Δ -78/-69, Δ -48/-39, or Δ -38/-29 deletions.

EMSA Demonstrate Multiple Factors in Jurkat T Cell Extracts, Including GATA-3, binding to the Distal Conserved Element (-96 to -80 bp), Containing the GATA, and GM-CSF/MIP Motifs. To demonstrate factors binding to the regulatory regions defined by transfection, we performed EMSA with Jurkat nuclear extracts. A probe encompassing the distal conserved element, which contained the GM-CSF/MIP motif and the conserved 3' GATA motif (-98 to -78 bp) produced three specific complexes (designated A_d , B_d , and C_d ; Fig. 5 A, lanes 3-4); the pattern of complexes produced by a probe which extended upstream and also contained the 5' nonconserved GATA motif was identical, though complexes formed with this probe migrated slightly faster overall (WT-L; -115 to -78, Fig. 5 A, lanes 1-2). Additional evidence suggested that the 5' nonconserved GATA motif either did not contribute or was not necessary for the formation of these three complexes: (a) the complexes formed with either probe were competed equally well by oligonucleotides with or without the 5' GATA motif and (b) mutation of the 5' GATA motif had little or no effect on the capacity of an oligonucleotide containing an intact 3' GATA motif to compete for binding to the probes containing one or both GATA motifs (data not

shown). In contrast, mutation of the 3' GATA motif did affect formation of and competition for these complexes (Fig. 5, A and B). EMSA demonstrated a more rapid electrophoretic mobility of the most abundant complex in extracts from the stimulated Jurkat T cells (designated C_d') compared with the unstimulated cells (C_d); in addition, an increased quantity of the middle complex (designated B_d) in the stimulated extracts was noted. Using nuclear extracts prepared in parallel after 2, 4, 6, 12, or 24 h of stimulation, we found that both the qualitative change from the C_d to the C_d' complex and the quantitative change in the B_d complex occurred by 2 h (Fig. 6, the B_d complex is somewhat better seen with the M1 probe, which reduces complexes C_d and C_d'). These changes paralleled temporally the accumulation of IFN- γ mRNA seen upon Northern analysis, which peaked by 2-6 h and declined thereafter (Fig. 6).

Two reciprocal approaches were used to identify the specific bases critical to these complexes: (a) comparison of the ability of probes M1-4 (Fig. 5 C), containing mutations within either the GATA motif (M1, M2) or the GM-CSF/MIP motif (M3, M4), to generate complexes in an EMSA (Fig. 5 A), and (b) comparison of the ability of these unlabeled oligonucleotides to compete for the binding of the complexes by the wild-type IFN- γ distal conserved element probe (Fig. 5 B). Formation of the least abundant, slowest migrating complex A_d , which was present in both unstimulated and stimulated extracts, appeared to require an intact GM-CSF/MIP motif for optimal binding since both M3 and M4 probes failed to generate complex A_d (Fig. 5 A, lanes 7-10). Complex B_d demonstrated the greatest stability or least stringent recognition, since it was formed (Fig. 5 A) and competed (Fig. 5 B) by all the oligonucleotides except M3. Complexes C_d and C_d' were identical in both the ability to be bound by the mutant probes (Fig. 5 A, lanes 5-10) and to compete complexes bound to the wild-type IFN- γ probe (Fig. 5 B, lanes 3-7; competition for complex C_d in unstimulated extracts was identical; data not shown). Binding was reduced by M1 and M2, abolished by M3, and unaffected by M4. The M3 mutation (centered in the GM-CSF motif) was clearly the most disruptive alteration of this distal element, since

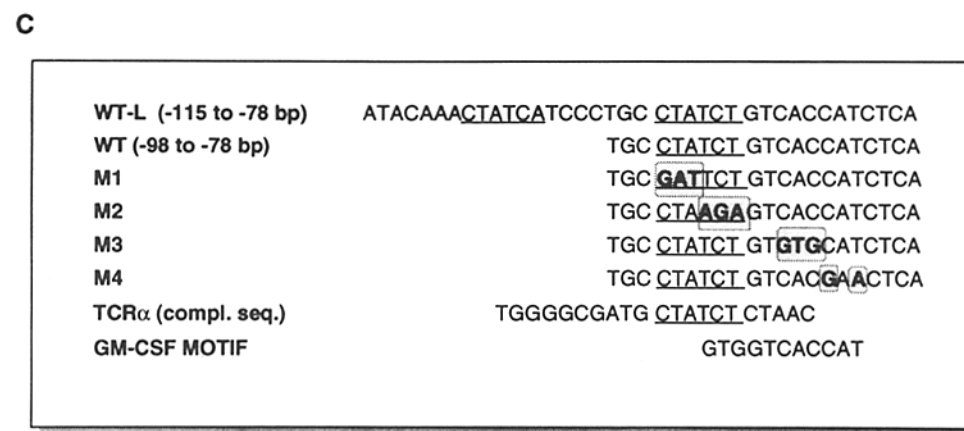
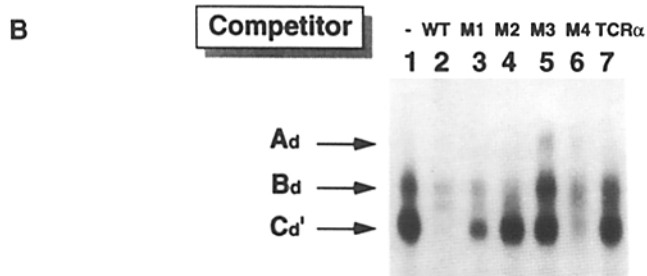
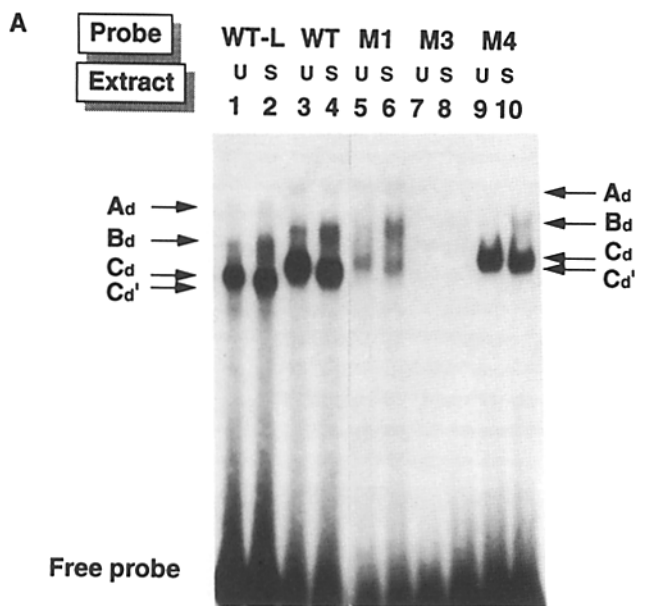


Figure 5. EMSA demonstrating binding of proteins in nuclear extracts to the distal conserved element of the IFN- γ promoter. (A) EMSA with labeled oligonucleotide probes encompassing the distal conserved element in the IFN- γ promoter (WT) and mutations therein (M1, M3, and M4) or extending to include the nonconserved 5' GATA motif (*wild-type long*; WT-L) using nuclear extracts from unstimulated (U) or ionomycin + PMA-stimulated (S) Jurkat T cells. Specific complexes are designated (A_d, B_d, C_d/C_d'). (B) EMSA using the WT probe of the distal conserved element illustrates the reciprocal results obtained by competition for factors bound in stimulated Jurkat nuclear extract by unlabeled WT IFN- γ oligonucleotide, or oligonucleotides containing mutations (M1-M4), or the TCR α GATA element. Reactions contained 5 ng of the indicated competitors. (C) The sequences of oligonucleotides used in these experiments as well as the GM-CSF sequence for comparison. The GATA motifs are underlined and mutations are highlighted.

only a very faint complex B_d was formed with this oligonucleotide as probe (Fig. 5 A, lanes 7-8) and M3 failed to compete for any of the complexes formed by the wild-type probe (Fig. 5 B, lane 5).

We pursued several experimental approaches in an attempt to determine whether GATA-3 was included in the complex observed with the distal conserved element. Initially, we determined that the IFN- γ element bound recombinant human GATA-3 (rGATA-3) (Fig. 7 A). Under these conditions, which were optimal for detection of binding to the TCR α site (Fig. 7 B), the IFN- γ GATA motif competed somewhat less effec-

tively for binding of rGATA-3 to itself or to the TCR α site (data not shown) than did the wild-type TCR α site. In an EMSA with stimulated Jurkat nuclear extract and the IFN- γ wild-type probe, a slower migrating super-shifted complex was observed using a monoclonal antibody specific to GATA-3 (Fig. 7 C, lane 2). This super-shifted band was not observed with three irrelevant monoclonal antibodies that recognized cell surface antigens (Fig. 7 C, lane 3; two additional control antibody supernatants were also tested; data not shown). To demonstrate which bases were critical for binding, more detailed studies were done using recombinant murine GATA-1

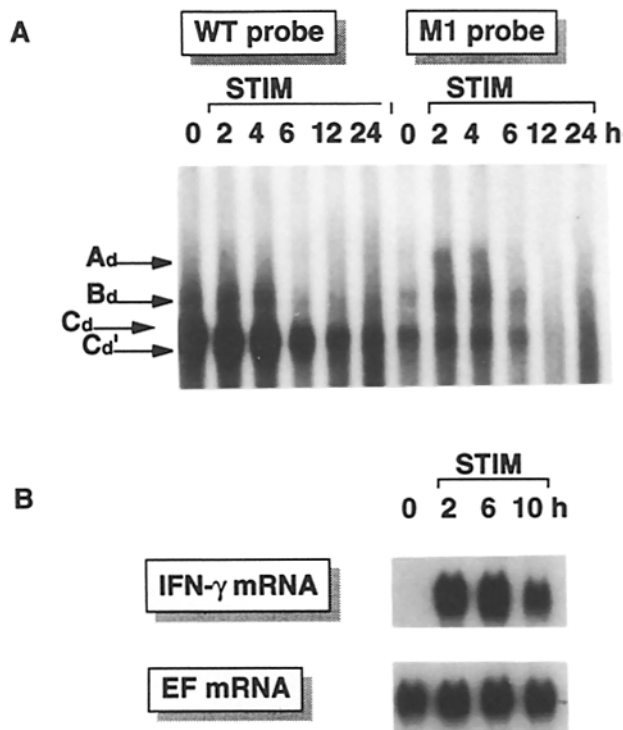


Figure 6. Activation-induced changes in EMSA complexes bound to the distal conserved element of the IFN- γ promoter parallel mRNA accumulation. (A) EMSA demonstration of complexes generated with both the wild-type and M1 probes (sequences shown in Fig. 5 C) incubated with nuclear extracts prepared from Jurkat T cells stimulated with PMA plus ionomycin for the indicated time periods. (B) Northern analysis of 15 μ g total RNA isolated from similarly treated Jurkat T cells probed for IFN- γ mRNA and elongation factor 1- α mRNA, which confirmed equivalent loading of RNA.

(rGATA-1) because it was more readily produced than rGATA-3, had a homologous DNA binding domain, and, like GATA-3, was capable of activating transcription of the TCR δ promoter (46). When conditions optimal for binding of nuclear extracts or rGATA proteins to the IFN- γ probe were used, both the IFN- γ and the TCR α elements bound rGATA-1 equally and demonstrated identical competition with each other and with the mutated IFN- γ probes (Fig. 7 D, lanes 1-6; data for binding to the TCR α site not shown). The complexes formed with the IFN- γ probe and rGATA-1 migrated with a mobility similar to that of the complex C $_d$ '. As would be predicted, competition for binding of rGATA-1 was markedly reduced by mutations in either half of the GATA motif, M1 and M2 (Fig. 7 D, lanes 3 and 4), paralleling, albeit to a greater degree, the effects of these mutations on competition for formation of complexes C $_d$ and C $_d$ ' with Jurkat extract (Fig. 5 B, lanes 3 and 4). In contrast, the M3 mutation in the GM-CSF motif did not affect the binding of rGATA-1, as shown by competition of M3 for binding of rGATA-1 to the IFN- γ probe (Fig. 7 D, lane 5), though this mutation abolished binding of proteins in Jurkat extracts (Fig. 5). Other factors found in the Jurkat extract that bind to the GM-CSF/MIP motif contained in the IFN- γ element must play a pivotal role in formation of each of these complexes because the TCR α probe bound (data not shown) and

competed effectively for rGATA-1 (Fig. 7 D, lane 7) and rGATA-3 (Fig. 7 A), but this oligonucleotide failed to generate a comparable complex with stimulated Jurkat extract (Fig. 7 D, lane 8) or to compete any of the complexes produced by the IFN- γ probe with Jurkat extract in EMSA conditions optimized for the IFN- γ probe (Fig. 5 B, lane 7).

Thus, GATA-3 in Jurkat nuclear extract and rGATA proteins bind the IFN- γ distal conserved element. However, mutations within the GATA motif only inhibited the formation of complex C $_d$ and C $_d$ ' partially and did not affect complex A or B, and deletion of this motif did not significantly alter expression. Thus, GATA-3 does not appear to play a critical role in promoter function under the conditions studied. In contrast, the factor(s) binding to the GM-CSF/MIP motif may play a more pivotal role, since mutations blocked formation of each complex and deletion markedly reduced promoter function.

EMSA Demonstrate Multiple Factors Binding to the Proximal Conserved Element (-71 to -40 bp) Containing the NFIL-2A Homology. In Vivo Footprinting Reveals an Activation-specific Footprint within the Most 3' Portion of the Element. A probe containing sequences in the proximal conserved element (-71 to -40) of the IFN- γ gene, including the NFIL-2A homology, when incubated with unstimulated nuclear extract generated several specific complexes designated A $_p$, B $_p$, C $_p$ 1, and C $_p$ 2 (Fig. 8 A). Competitor oligonucleotides with mutations in either the 5' or 3' portion of the element did not compete any of the complexes as efficiently as wild-type oligonucleotide (Fig. 8 A, compare lane 9 with lanes 10 and 11). To allow a direct comparison, the first lane of this gel shows the complexes produced by the octamer site of the DR α gene (50) and the second lane contains the complexes produced by the NFIL-2A site of the IL-2 gene (27). The IFN- γ and IL-2 probes appear to share affinity for factors giving rise to complexes C $_p$ 1 and C $_p$ 2 since both produced an identical rapidly migrating doublet, for which the NFIL-2A and the IFN- γ oligonucleotide competed equally well (Fig. 8 A, lanes 3-5). The NFIL-2A oligonucleotide competed the factor(s) in the uppermost complex A $_p$, formed with the IFN- γ probe (though only at the higher concentration of competitor), and failed to generate a complex with the same electrophoretic mobility when used as a probe. The complexes generated by the IFN- γ probe with stimulated extracts were identical to those shown with unstimulated extracts (data not shown). These results indicated that the proximal conserved element of the IFN- γ promoter bound multiple factors that appeared to require recognition of specific sequences in both halves of the element and to share partial affinity with the NFIL-2A site of the IL-2 gene.

In contrast to the competition between the NFIL-2A and IFN- γ oligonucleotides, the DR α octamer oligonucleotide failed to consistently alter any of the complexes formed with the IFN- γ probe (Fig. 8 A, lane 6). Furthermore, the complex shown to contain Oct-1 by antisera super-shifting (data not shown), which is shared by the DR α and the NFIL-2A probes (marked as Oct-1 in Fig. 8 A), migrated differently than any of the complexes produced by the IFN- γ probe. Likewise, Oct-1 antisera had no effect upon any of the com-

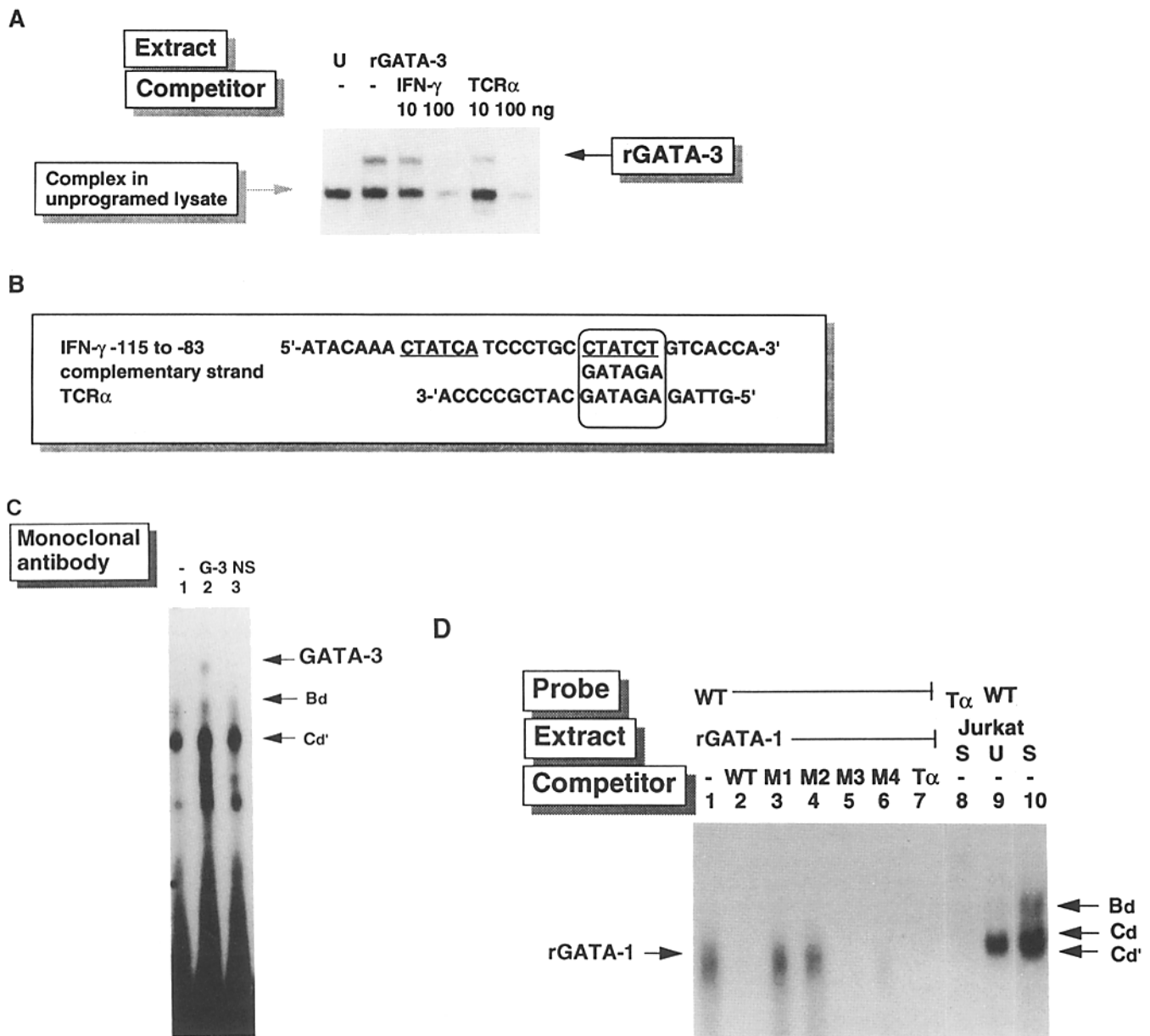


Figure 7. The GATA motif in the distal conserved element of the IFN- γ promoter binds rGATA-3 and rGATA-1. (A) EMSA performed using an IFN- γ probe (exact sequence, B), shows a specific rGATA-3 complex (as well as nonspecific binding in the unprogrammed lysate) that is competed by the TCR α GATA motif. This EMSA was run in $0.25 \times$ TBE buffer, that is optimal for binding of rGATA-3 to the TCR α probe. (B) Sequence of oligonucleotides used in the rGATA-3 EMSA. The GATA motifs are underlined in the IFN- γ sequence. (C) EMSA demonstrating complexes seen with stimulated Jurkat extract and the IFN- γ WT probe in $0.25 \times$ TBE buffer (lane 1). Addition of $2 \mu\text{l}$ of monoclonal antibody to GATA-3 in lane 2 generates a super-shifted band (GATA-3), which is not seen with the control antibody, 24G2, lane 3. (D) EMSA demonstrating the sequences essential for binding of rGATA-1 to the WT IFN- γ probe lanes 1-7, (sequences of these probes and competitors are shown in Fig. 5 C; T α denotes the TCR α oligonucleotide). Reactions contained 5 ng competitor DNA, and EMSA was run in low ionic strength buffer, which is optimal for detection of specific binding to the IFN- γ probe. To allow a direct comparison on the same gel the last three lanes show complexes generated by the TCR α probe with stimulated (lane 8) or the WT IFN- γ probe with unstimulated or stimulated Jurkat nuclear extracts (lanes 9 and 10).

plexes generated by the IFN- γ probe (data not shown). Thus, unlike the NFIL-2A site, this element in the IFN- γ gene does not appear to bind the ubiquitous transcription factor, Oct-1, although competition with the NFIL-2A site suggests other factors may be shared between the two genes.

Though we did not see differences using EMSA, the im-

portance of this region for activation specific expression suggested by transfection analyses was further supported by *in vivo* footprint analysis. As shown in Fig. 9, a footprint was consistently detected over the 3' portion of the proximal conserved element in stimulated but not unstimulated Jurkat T cells. This protected base lies in the NFIL-2A site homology

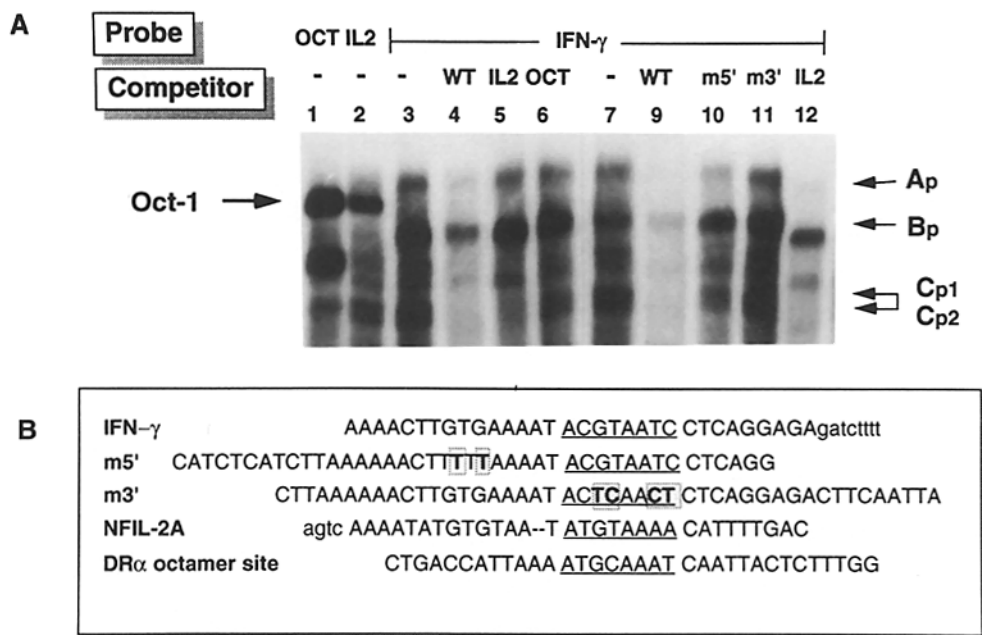


Figure 8. EMSA demonstrating binding to the proximal conserved element of the IFN- γ promoter. (A) EMSA using the designated labeled probes binding factors in unstimulated Jurkat nuclear extracts in the presence of 5 ng of the specified competitor (lanes 4-6) or 15 ng (lanes 9-12). The complexes containing Oct-1, as demonstrated by antiserum super-shifting (not shown) with the DR α octamer probe (OCT) or the NFIL-2A (IL2) probe are labeled, as are the specific complexes generated by the proximal IFN- γ probe. The complexes in lane 12, due to an artifact in the gel, appear to have migrated farther than those in the other lanes. (B) Exact oligonucleotide sequences of the probes or competitors used in the EMSA above are shown; (OCT) the DR α oligonucleotide; (IL2) the NFIL-2A oligonucleotide.

at a CpG dinucleotide, which is not present in the IL-2 element, but is conserved in the IFN- γ genes of human, rat, and mouse.

Factors Binding to Either the Distal or Proximal Conserved Elements of the IFN- γ Gene Are Not Found in U937 or THP-1 Nuclear Extracts. EMSA using nuclear extracts from resting or activated U937 and THP-1 monocytic cells with either the distal or proximal conserved elements of the IFN- γ gene

as probes did not produce complexes (U937) or yielded a poorly defined band (THP-1) that was not similar to those seen with Jurkat extracts (Fig. 10). These THP-1 and U937 extracts contained intact DNA-binding proteins because they did generate discrete complexes with the human immunodeficiency virus long terminal repeat NF- κ B element (Fig. 10). These results paralleled the poor expression of the IFN- γ reporter constructs in these cells in transient transfection assays.

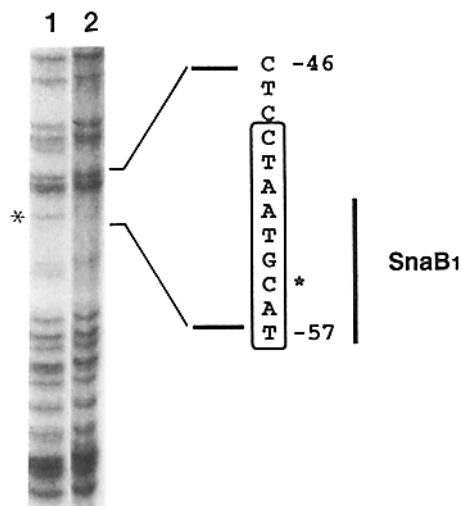


Figure 9. Activation-specific in vivo footprint of the proximal element of the IFN- γ promoter visualized by ligation-mediated PCR amplification. The complementary strand is shown, as revealed using coding strand primers. The sequence contained within the brackets is indicated to the right of the gel. Lanes 1 and 2 contain DNA from unstimulated and PMA plus ionomycin-stimulated Jurkat T cells, respectively. The protected base is marked by an asterisk, the 3' portion of the NFIL-2A homology in the proximal-conserved element of the IFN- γ promoter is boxed and the SnaB1 restriction enzyme site is marked by the bar. Similar results were obtained in two other experiments.

Discussion

The production of IFN- γ , a pleiotropic and potent immunomodulatory cytokine, is closely regulated both by the selectivity of the cells in which it is produced, and by the activation-specific control of expression within those cells. By transient transfection of reporter constructs, we have identified the immediate 108 bp of 5' flanking sequence of the IFN- γ promoter as both necessary and sufficient for activation-induced promoter function in Jurkat T cells, which was comparable to that seen with the well-characterized IL-2 promoter. Regions conferring modest positive (-337 to -258 bp) and negative (-177 to -109 bp) effects upon activation-induced expression, and one that may repress low level constitutive expression (-214 to -178), were also identified. However, they were not required for faithful promoter function. Results with constructs containing 538 or 108 bp of IFN- γ flank appeared to accurately reflect the endogenous gene's requirements for induction, suppression by CSA, and expression in T cells, but not in monocyte-macrophage cell lines. Moreover, two highly conserved *cis* regulatory elements have been mapped within this 108-bp 5' flanking region. Using EMSA, factors binding to these elements have been identified in Jurkat nuclear extracts, but not in extracts from monocyte-macrophage cell lines. The abundance or apparent mobility of the factors binding to the distal element and the

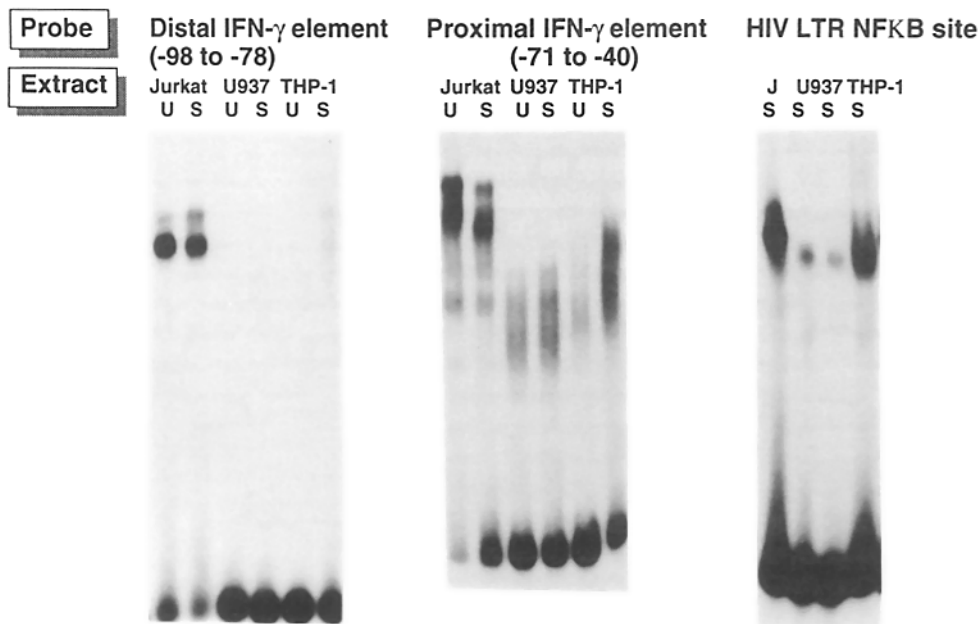


Figure 10. EMSA demonstrating factors binding to either the distal or proximal conserved elements of the IFN- γ gene are not found in monocytic nuclear extracts. The first six lanes of this EMSA contain the distal WT IFN- γ probe with 2.5 μ g of either unstimulated (U) or ionomycin plus PMA-stimulated (S) nuclear extracts from the designated cell types: Jurkat T cells and two monocytic-macrophage cell lines, U937 and THP-1. Similarly the middle six lanes contain the proximal WT IFN- γ probe with these same nuclear extracts. The last four lanes contain the NF κ B binding site of the HIV LTR as probe and the stimulated extracts of these cells. This demonstrates that the U937 and THP-1 nuclear extracts do contain NF κ B proteins.

binding in vivo of factors to the proximal element were altered in response to T cell activation, supporting their role in activation-specific expression of IFN- γ .

The current data are consistent with previously published studies that indicated the importance of the near 5' flank of the IFN- γ gene in its regulation, but more precisely define the region required for proper expression and identify two *cis* elements within this region as critical. Previous studies demonstrated that \sim 500 bp of 5' flank was sufficient for activation-induced expression in a murine T cell line (19) and that the -215 to -53 region conferred expression in primed short-term cultured human T cells (20). Chrivia et al. (20), using 5' truncation promoter constructs, found the -251 to -215 region to contain a strong element repressing both basal and induced transcription, whereas Brown et al. (21), also using 5' truncation constructs, did not. Our internal deletion constructs suggest that the -214 to -178 region attenuates constitutive expression, and the -177 to -109 region has a modest net negative effect on activation-induced promoter activity. In transient transfection assays of Jurkat T cells with Bal-31 5' deletions of the IFN- γ promoter, Brown et al. (21) found that maximal activation-induced expression was observed with 284 bp of 5' flank (\sim 40-fold induction), although near maximal induction (\sim 20-fold) was obtained with 181 bp of 5' flank. In our truncation and internal deletion constructs, sequences between -337 and -258 also had a modest net positive effect on expression. The latter group subsequently reported the presence of a bipartite region in the 5' flank of the IFN- γ gene, in which sequences between -36 and -30 bp and -124 and -114 bp appeared to interact in the formation of complexes with DNA probes and to play a role in activation-induced expression (22). Our data do not support an essential role for the bipartite region identified by Brown et al. (a) The -124 to -114 site was 5' of the flanking sequence sufficient for nearly maximal in-

duction in our assays, though an additional positive role for this site is not excluded by the current findings. (b) Our Δ -38/-29 construct, which removed the proximal portion of the putative bipartite element (Fig. 4), produced expression that was at least as great as the wild-type -538 construct. (c) This putative bipartite element was not as highly conserved in the murine or rat genes (6 of 11 bp and 3 of 7 bp complete identity across species in the proximal and distal portions, respectively), as the critical elements identified in the current studies, suggesting that this bipartite region is less likely to play an essential regulatory role. The differences in findings may reflect technical differences in the reporter analyses in the studies and the complexity of this compact promoter.

Within the -108 to -40 bp region of the human IFN- γ gene, which was necessary and sufficient for faithful expression, two regions with nearly complete cross-species identity were found to contain critical regulatory elements by scanning deletional analysis (Fig. 4). The distal conserved element contains an 8-bp sequence, which is shared with an important regulatory element in the GM-CSF gene, and a consensus GATA motif (Fig. 3). Deletion of the GM-CSF/MIP motif profoundly reduced promoter function. The concept that the IFN- γ and the GM-CSF promoters may share transcription factors is attractive. Both cytokines are expressed well by memory T cells, whereas neonatal, naive T cells express very little IFN- γ and in these cells GM-CSF expression is diminished and delayed relative to adult and memory T cells (8, 11-13, 51). Also, the delayed GM-CSF expression in neonatal T cells appears to reflect IL-2-dependent production rather than expression occurring as a direct result of activation through TCR-related pathways (13). The GM-CSF/MIP motif (47), that is identical in the two genes, overlaps the most 5' portion of the previously identified CLEO element (49). In the GM-CSF promoter, Nimer et al. (52)

found that mutation of two bases in this shared motif markedly reduced promoter activity in transient transfection assays of MLA-144 gibbon T cells and activated primary human T cells. The distal conserved element in the IFN- γ promoter demonstrated three specific complexes by EMSA. The M4 mutant oligonucleotide, which replicates the mutation shown by Nimer et al. (52) to impede the function of the GM-CSF promoter in T cells, inhibited formation of complex A_d only. However, mutation of three bases centered within the GM-CSF/MIP motif (M3, Fig. 5 C) essentially abolished binding of each of the three complexes. The effects of the M3 mutation upon binding of factors by EMSA paralleled results obtained when the GM-CSF/MIP motif was deleted from reported constructs, indicating that this motif is essential for the function of the distal conserved element and plays a critical role in IFN- γ promoter function.

Just 5' of the GM-CSF/MIP motif in the distal conserved element of the IFN- γ promoter is a consensus GATA motif that is capable of binding rGATA-3 and rGATA-1. Deletion of this GATA motif had little effect on reporter gene expression and mutations had less effect on EMSA complexes than did the M3 mutation in the GM-CSF/MIP motif. Yet the formation of the C_d and C_d' complexes was impeded by mutations within the GATA motif and GATA-3 was contained within these complexes. Of note, the promoter regions of several other lymphokine genes (including IL-3, IL-4, IL-5, and GM-CSF genes, but not the IL-2 gene) contain consensus GATA motifs, suggesting that GATA-binding proteins may contribute to regulation of these lymphokines. Also, CD3⁻CD16⁺ NK clones, that would be expected to express IFN- γ and not IL-2, recently have been shown to express GATA-3 mRNA (53). Although a critical role for the GATA motif or GATA-3 was not evident in the current studies, full elucidation of GATA-3's role in the formation and activation-induced alteration of the complexes formed with the distal conserved element and in the regulation of IFN- γ gene expression will require additional investigation.

The second and more proximal conserved element that was critical for expression with activation, shares sequence homology with the NFIL-2A site of the IL-2 gene. This proximal IFN- γ element appeared to bind more than one factor and the binding of each required that both the 5' and 3' portion of the element be intact. Like those binding to the proximal element, factors binding to this proximal element were not detected in monocytic cell line extracts. Although by EMSA proteins binding to this element did not appear to differ between unstimulated and stimulated Jurkat cells, binding to this element appeared to be regulated by activation in intact cells, since an *in vivo* footprint was seen only after activation. Unlike the NFIL-2A element (27), this IFN- γ element did not bind Oct-1, but shared affinity with other factors binding to the NFIL-2A site. OAP-40, which binds to the 5' half of the NFIL-2A element, may be one such factor (27). It is the 3' portion of the NFIL-2A element that binds Oct-1. The failure of the proximal conserved element of the IFN- γ gene to bind Oct-1 may relate to the presence of a CpG rather than a TpG dinucleotide in its 3' portion; it is this CpG dinucleotide that lies within the activation-specific

in vivo footprint. This CpG dinucleotide provides a potential site for methylation of the endogenous IFN- γ gene by methyltransferases, thus allowing an additional putative level of regulatory control by altering the accessibility of critical *cis* elements for the binding of transcriptional regulatory factors. In recent studies the extent of methylation of this site, as measured by the methylation sensitive restriction enzyme Sna B1, was found to correlate inversely with IFN- γ gene expression in several T cell subsets including: (a) murine TH-0, TH-1, and TH-2 T-cell clones (Young, H. A., J. Ye, J. Lederer, A. Lichtman, P. Ghosh, J. R. Gerard, L. A. Penix, C. B. Wilson, A. J. Melvin, D. B. Lewis, et al., manuscript in preparation), (b) freshly isolated human naive, neonatal, and adult memory T cells (A. Melvin, and D. B. Lewis, manuscript in preparation) and (c) human NK cells (H. A. Young, unpublished observations).

In this report we have identified a 108-bp 5' flanking region of the IFN- γ promoter that is necessary and sufficient to direct activation-specific and CSA-inhibitable expression in T cells, and that contains two highly conserved *cis* elements critical for promoter function. In addition to these elements, other regions of the 5' flank appeared to modulate IFN- γ promoter activity. Further, it is likely that in the context of the intact gene, and in response to signals transduced through accessory ligands such as CD28, additional complexity contributes to proper regulation of this gene. Portions of the first intron have enhancer activity that, however, is not T cell specific (1, 19). Elements in the 5' flank that are responsive to estrogen (54) and to the tax protein of HTLV (21) may enhance IFN- γ expression in the appropriate context. This additional complexity notwithstanding, the current data provide insight into potential mechanisms by which the IL-2 and IFN- γ genes may be independently regulated, though both are expressed early after T cell activation, require the same signals for induction and are inhibited by CSA. The distal conserved element contains GATA and GM-CSF-MIP motifs not found in the IL-2 promoter, but present in the promoters of several other lymphokine genes that are preferentially expressed in memory vs. naive T cells. The proximal conserved element is homologous to the NFIL-2A element in the IL-2 promoter. Although sharing affinity for factors in T cell extracts, the NFIL-2A element binds Oct-1, but the IFN- γ proximal element does not. NF-AT and AP-1 elements, which appear to play a critical role in IL-2 promoter function (37, 55), are not present in the IFN- γ promoter sequences that were essential for expression (108 bp of 5' flank) or yielded optimal expression (538 bp of 5' flank). Since impaired function of the proximal AP-1 element in the IL-2 promoter appears to be the critical change leading to loss of IL-2 expression in anergic TH-1 T cell clones, the relative preservation of IFN- γ as compared to IL-2 expression in anergic T cells may be related to the lack of a typical AP-1 element in the IFN- γ promoter (15). Conversely, methylation within the proximal element of the IFN- γ gene, at a CpG dinucleotide not present in the NFIL-2A element of the IL-2 gene, may play a role in the limited expression of IFN- γ in naive T cells. The identification of these two essential regulatory elements in the IFN- γ promoter provides a

focus for future studies to define the molecular nature of the factors and events regulating their activation-induced func-

tion and to determine their role in the control of IFN- γ expression in vivo.

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