A Distal Region in the Interferon-γ Gene Is a Site of Epigenetic Remodeling and Transcriptional Regulation by Interleukin-2∗

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Interferon-γ (IFN-γ) is a multifunctional cytokine that defines the development of Th1 cells and is critical for host defense against intracellular pathogens. IL-2 is another key immunoregulatory cytokine that is involved in T helper differentiation and is known to induce IFN-γ expression in natural killer (NK) and T cells. Despite concerted efforts to identify the one or more transcriptional control mechanisms by which IL-2 induces IFN-γ mRNA expression, no such genomic regulatory regions have been described. We have identified a DNase I hypersensitivity site ~3.5–4.0 kb upstream of the transcriptional start site. Using chromatin immunoprecipitation assays we found constitutive histone H3 acetylation in this distal region in primary human NK cells, which is enhanced by IL-2 treatment. This distal region is also preferentially acetylated on histones H3 and H4 in primary Th1 cells as compared with Th2 cells. Within this distal region we found a Stat5-like motif, and in vitro DNA binding assays as well as in vivo chromosomal immunoprecipitation assays showed IL-2-induced binding of both Stat5a and Stat5b to this distal element in the IFNG gene. We examined the function of this Stat5-binding motif by transfecting human peripheral blood mononuclear cells with ~3.6 kb of IFNG-luciferase constructs and found that phorbol 12-myristate 13-acetate/ionomycin-induced transcription was augmented by IL-2 treatment. The effect of IL-2 was lost when the Stat5 motif was disrupted. These data led us to conclude that this distal region serves as both a target of chromatin remodeling in the IFNG locus as well as an IL-2-induced transcriptional enhancer that binds Stat5 proteins.

IFN-γ1 is a cytokine expressed primarily by, but not limited to, lymphocytes of T and NK cell lineages. NK cells are a major early source of IFN-γ in vivo, and T cell expression of IFN-γ is generally restricted as T helper 1 (Th1) cells (1). These cell types and their predominate cytokine IFN-γ are intimately involved in protection against intracellular pathogens and the development of tumors (2). This is evident in humans with gene mutations that interfere with IFN-γ signaling, including the IFN-γ receptor (3) and Stat1 (4) as well as in IFN-γ-deficient mice (5), which are highly susceptible to infectious agents and prone to certain cancers. In contrast, circumstances in which IFN-γ is overproduced or its effects fail to induce negative feedback loops lead to widespread pathology (6). Not surprisingly, IFN-γ expression patterns are tightly regulated in a tissue-specific manner by precise induction signals.

Much effort has focused on characterizing the proximal IFNG promoter and intervening sequences in an effort to identify potential regulatory regions that are responsible for signal- and cell-specific control of gene expression. This approach has been useful in defining putative regulatory regions near the core IFNG promoter. It is notable, however, that regions proximal to the IFNG gene fail to explain tissue-specific patterns of IFN-γ expression as evidenced by transgenic mice expressing only the proximal IFNG promoter linked to a reporter gene (7, 8). In addition, transgenic mice that contain the full-length human IFNG gene and ~2.7 kb of upstream sequence have similar signal-specific regulatory patterns to the endogenous gene, yet the responses are relatively weak (8). Therefore, it is likely that regions more distal from the core IFNG promoter are involved in determining tissue-specific and optimal signal-specific expression of IFN-γ (9).

In naive T cells, induction signals for activation and IFN-γ expression are provided optimally through T cell receptor (TCR) and co-stimulatory receptor (CD28) engagement (10). Following primary activation, differentiation of effector CD4+ T helper (Th) cell populations occurs over several days and is directed in large part by cytokines such as IL-2, IL-4, and IL-12 (11). Not only do IL-2 and IL-12 induce Th1 cell development, they also directly induce expression of IFN-γ. This is most

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†‡‡ The abbreviations used are: IFNG, interferon-γ; NK, natural killer cells; Th1, T helper 1 cells; Stat1, signal transducers and activators of transcription 1; TCR, T cell receptor; IL-2, interleukin-2; FCγs, fetal calf serum; PBMC, peripheral blood mononuclear cell; Ab, antibody; EMSA, electrophoretic mobility shift assay; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; ChIP, chromatin immunoprecipitation; RPA, RNase protection assay; P/I, PMA/ionomycin.

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apparent in NK cells, because no preceding activation/differentiation signals are required for cytokine responsiveness and transactivation of the IFNG gene (12). Therefore, NK cell models offer an advantage in studying regulation of IFN-γ expression by isolating the effects of autonomous cytokine receptor signaling events.

IL-12 acts via the transcription factor signal transducer and activator of transcription 4 (Stat4) that is thought to mediate trans-activation of the IFNG gene by binding to sequence motifs therein or enhancing the binding of AP-1 to the core IFNG promoter (13). The requirement of Stat4 in regulating IFN-γ is underscored in Stat4−/− T cells and NK cells that are refractory to IL-12-induced IFN-γ expression. In both mice (14) and humans (15), Stat4 has been shown to interact with the IFNG gene in the proximal promoter and/or first intron (16, 17). These elements are based on non-consensus STAT motifs, and their functional role is still not firmly established. Additionally, the human intronic STAT binding region is not present in murine genomic DNA, suggesting its importance in regulating IFNG gene expression may be a recent evolutionary event. Despite concentrated efforts and that fact that IFN-γ is highly regulated by cytokines, the identification of functional STAT binding elements in the IFNG gene has proved to be challenging.

In the case of IL-2, the exact mechanisms by which this cytokine regulates IFN-γ have been surprisingly neglected, despite reports dating to 1983 indicating that IL-2 induces IFN-γ expression (18–20). IL-2 is an important factor in the differentiation of both Th1 and Th2 cells (11). Accordingly, this cytokine is routinely added to culture systems to enhance Th cell development and is thought to act via Stat5 (21). In IFN-γ-secreting cells, induction of IFN-γ mRNA by IL-2 is rapid and occurs at the level of transcription, as demonstrated by nuclear run-on analysis. The induction occurs in the presence of cycloheximide, indicating that protein synthesis is not necessary for the induction of transcription (22). Although IL-2 has long been associated with IFN-γ expression, the molecular mechanisms involved are still unknown.

In this report, we identify and characterize a distal region of the IFNG gene that is a target for cytokine-driven epigenetic modifications, which correlate with competency of tissue-specific IFN-γ expression profiles. Contained within this same region is a distal cis-acting element that binds Stat5 proteins and enhances transcription of the IFNG promoter in an IL-2-dependent fashion. These data indicate that this distal 5′-flanking region of the IFNG gene is a critical part of the functional IFNG gene and provides the first indication of the molecular mechanisms involved in IL-2 regulation of IFN-γ expression.

EXPERIMENTAL PROCEDURES

Antibodies and Cytokines—Antibodies directed against Stat5a, Stat5b, and Stat3 were purchased from R&D Systems and were used in supershift analyses as well as chromatin immunoprecipitation assays. Recombinant human IL-12 was obtained from Hoffmann-La Roche. Recombinant mouse IL-12 was generously provided by Genetics Institute (Cambridge, MA). Recombinant human IL-4 was purchased from R&D Systems. Anti-CD3ε, anti-CD28, anti-IL-4, anti-IL-12, and anti-IFN-γ antibodies were purchased from BD Pharmingen. Anti-acetylated H3, anti-acetylated H4, and normal rabbit IgG were from Upstate Biotechnology (Lake Placid, NY).

Nuclear extracts were prepared from PHA-stimulated human peripheral blood T cells or NK cells, as described previously (23). Briefly, following no stimulation (NS) or treatment with cytokines for 30–60 min, cell pellets were resuspended in lysis buffer (50 mM KCl, 25 mM Hepes, pH 7.8, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM dithiothreitol) and subsequently incubated on ice for 5 min. Cellular suspensions were collected by centrifugation at 2,000 rpm, and the supernatant was harvested as the cytoplasmic protein extract, 1 μg of poly(dI-dC) (Sigma), 4 μl of 5× binding buffer (60 mM Hepes, 7.5 mM MgCl2, 300 mM KCl, 1 mM EDTA, 2.5 mM dithiothreitol, 50% glycerol, and 4-12-aminophenylbenzenesulfonyl fluoride hydrochloride) and 1.5 × 10^6 cpm of [32P]-labeled oligonucleotide were added to each reaction prior to the addition of the probes and incubated in ice for 20 min. In cold oligonucleotide competition experiments, 10- to 100-fold excess of unlabeled oligonucleotide probe was added 10 min prior to adding the radiolabeled oligonucleotide probe.

Immunoprecipitation and Nuclear Extraction—Nuclear extracts were prepared from PHA-stimulated human peripheral blood T cells or NK cells, as described previously (23). Briefly, following no stimulation (NS) or treatment with cytokines for 30–60 min, cell pellets were resuspended in lysis buffer (50 mM KCl, 25 mM Hepes, pH 7.8, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM dithiothreitol) and subsequently incubated on ice for 5 min. Cellular suspensions were collected by centrifugation at 2,000 rpm, and the supernatant was harvested as the cytoplasmic protein fraction. Nuclei were washed in wash buffer (lysis buffer without Nonidet P-40) and harvested by centrifugation at 2,000 rpm. Nuclear pellets
were resuspended in extraction buffer (500 mM KCl, 25 mM Hepes, pH 7.5, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM dithiothreitol), frozen in dry ice, thawed slowly on ice, and finally centrifuged at 14,000 rpm for 10 min. The supernatant was harvested, and nuclear proteins were quantified with the bicinchoninic acid protein assay reagent (Pierce).

**Transfection Assays**—PBMCs were transfected following overnight culture in RPMI 1640 medium containing 10% FCS with or without 10–100 units/ml IL-2 (Chiron Therapeutics, Emeryville, CA). Cells were then washed and resuspended in 250 μl of fresh medium at 2 × 10⁷ cells/ml and electroporated in the presence of 50 μg of reporter construct (600 V, for 9 pulses of 500 μs, with 100 μs between pulses) using 4-mm (gap width) cuvettes in a BTX Electro Square Porator ECM 830 (Biorad, San Diego, CA). A control plasmid containing the β-actin promoter driving a Renilla luciferase (provided by Dr. Christopher Wilson, University of Washington) was co-transfected as an internal standard, and values were normalized to correct for transfection efficiency (generally 30–50%). After electroporation the cells were diluted in fresh medium, allowed to rest for 1 h prior to plating, and then stimulated with 20 ng/ml PMA plus 400 nM calcium ionophore for 4 h. Luminescence was measured using a Promega luciferase assay kit and counted on a six-detector PerkinElmer Life Sciences 1450 Microbeta liquid scintillation counter with coincidence counting deactivated.

**RESULTS**

### IL-2 Induces the Expression of IFN-γ mRNA

The process of T cell differentiation is complicated and involves diverse extracellular signals through multiple classes of receptors, including the T cell receptor (TCR), co-stimulatory molecules, and cytokine receptors (1, 2, 11). This process typically: 1) occurs over an extended period of time (5–10 days), 2) seems to occur with cell division (24), and 3) includes IL-2 in the cultures, either endogenously produced by T cells and/or by exogenous addition. We wanted to isolate the effects of IL-2 on IFNG gene regulation per se from the process of Th cell differentiation. Thus, we have primarily used fresh NK cells and an NK cell line (NK92) as a model to study cytokine-induced epigenetic and transcriptional modifications to the IFNG gene.

We first confirmed that IL-2 treatment of NK92 cells leads to IFN-γ mRNA expression (Fig. 1A). Strong IFN-γ mRNA expression in NK92 was evident at 3 h and was slightly diminished after 6 h of IL-2 treatment. Our group and others have also reported that co-treatment of activated T cells or NK cells with IL-2 and IL-12 leads to a highly synergistic effect on IFN-γ expression (25, 26). A time course experiment was performed on NK92 cells under IL-2, IL-12, and IL-2 plus IL-12 conditions to confirm these data (Fig. 1B). The results confirm that IL-2 and IL-12 induce IFN-γ mRNA expression rapidly and when added together, synergistically induce IFN-γ transcription. These data are similar to what was observed in activated T cells (data not shown). Fig. 1C shows by real-time PCR the response to individual cytokine treatment and the synergistic response to IL-2 and IL-12 stimulation in NK92 cells. Thus, we determined that the NK92 cell line was an appropriate model to study IL-2-regulation of IFN-γ expression.

### Identification of a DNase I Hypersensitivity Site

**Upstream of the IFNG Gene**—DNase I HS mapping strategies have been used to identify functionally identifiable genomic regions that are functionally linked to gene expression. This approach allows for the investigation of the chromatin structure of the native gene in intact nuclei. It has been shown previously that DNase I HSs in the IFNG gene are found only in cells capable of transcribing the gene (27). Furthermore, the appearance or predominance of these sites in T cells is stimulation-sensitive, indicating a dynamic relationship between cell-specific competence for expression and transcription that is activation-dependent (28). Because the proximal 5′-flanking region of the IFNG gene fails to account for normal IFN-γ regulation (8), we sought to determine if more distal regions of the IFNG gene become remodelled upon cytokine stimulation.

To identify potential regulatory regions in the IFNG gene we performed DNase I HS mapping analysis using NK92 cells (Fig. 2). The results show the appearance of two bands by Southern blot analysis. The band of ~12 kb represents the HindIII–HindIII restriction fragment, visible only after phenol-chloroform extraction and complete HindIII digestion. The 3′ HindIII site in the first intron is located at position +669 and the 5′ HindIII site is at position −11,780 relative to the transcriptional start site according to our nomenclature (29). The lower band represents a single DNase I HS that was estimated to be ~4.5 kb upstream of the 3′ HindIII restriction site in the first intron.
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IL-2 Induces Histone Acetylation of the Distal IFNG Site in Primary NK Cells and Is Preferentially Acetylated in Th1 Cells—Alterations in histone acetylation are known to be associated with chromatin remodeling and the ability of a gene to be transcribed. To corroborate our identification of a distal cytokine-regulated site of chromatin remodeling, we assessed histone acetylation of the distal IFNG promoter in primary NK cells using chromatin immunoprecipitation analysis (Fig. 3A). We found that the distal IFNG gene was constitutively acetylated on histone H3 in non-stimulated (NS), freshly isolated NK cells at least to some extent (lane 3). However, when primary NK cells were stimulated with IL-2 alone (lane 6) or in combination with IL-12 (lane 12) we observed a consistent augmentation of histone acetylation. IL-12 treatment alone did not alter the background levels of H3 acetylation at the distal site (lane 9). We confirmed this observation in freshly isolated human NK cells by quantitative ChIP analysis using real-time PCR in which the distal IFN-γ site was amplified (Fig. 3B). Indeed, IL-2 treatment led to quantitative enhancement of histone H3 acetylation of this upstream region. Similar results

Fig. 1. IL-2 induces IFN-γ mRNA, acting synergistically with IL-12. A, ribonuclease protection assay (RPA) analysis of IFN-γ mRNA expression in NK92 cells treated with 100 units/ml of rhIL-2 for 3 or 6 h. B, full time course of IL-2, IL-12, and IL-2 plus IL-12 induction of IFN-γ mRNA expression in NK92 by RPA. C, real-time PCR in NK92 cells to quantify cytokine induction of IFN-γ mRNA levels.

Fig. 2. A cytokine-inducible DNase I hypersensitivity site is present in the distal IFNG gene. NK92 cells were not stimulated (NS) or treated with IL-2, IL-12 or IL-2 plus IL-12, and the nuclei were isolated. The genomic DNA was subjected to DNase I digestion, phenol/chloroform extraction, and digestion with HindIII. The DNA was transferred to a nylon membrane, and Southern analysis was performed using a 32P-labeled human IFN-γ-specific XcmI-HindIII DNA fragment as the probe. A DNase I HS site (large arrows) was identified ~3.5–4 kb 5' to the IFNG transcriptional start site that was responsive to IL-2 and IL-12 stimulation.
were obtained using the NK92 cell line (data not shown).

In T cells, the proximal IFNG promoter has been shown to be hyperacetylated in Th1 cells in comparison to Th2 cells (30).

This region contains a constitutive HS that is not predictive of the selective expression patterns of the native IFNG gene (8).

Thus, we analyzed both the distal and proximal regions of the 5′-flanking region of the IFNG gene for histone modifications in fully differentiated Th1 and Th2 cells (Fig. 3). At the distal site, we found that this region of the IFNG promoter was preferentially acetylated on histones H3 and H4 in Th1 versus Th2 cells. As a control we also analyzed the previously determined site in the proximal IFNG promoter that is a target for histone acetylation. Our results confirm that the proximal IFNG promoter is preferentially acetylated in Th1 cells, however, we found acetylation of the distal IFNG region to be more predictive of IFN-γ secreting Th1 cells (30).

A Novel Stat5 Binding Motif in the Distal 5′-Flanking Region of the IFNG Gene Binds IL-2-induced Stat5 Proteins in Vitro and in Vivo—Stat5 is a key IL-2-activated transcription factor that is critically involved in the regulation of IL-2-inducible genes (31). IL-2 receptor α (CD25) is one of the few IL-2-inducible genes whose promoter has been extensively characterized. This gene contains functional IL-2 response elements and Stat5 binding motifs (31, 32). Upon sequence comparison, however, no similar motifs were identified in the −2.7-kb IFNG promoter, and previous attempts to identify IL-2-responsive elements in the −2.7-kb IFNG promoter were unsuccessful.

Thus, we began to search for IL-2-responsive elements further upstream of the IFNG gene, particularly in regions that are targets for epigenetic modifications. A computer search identified several putative regulatory motifs distal to the IFNG promoter. Of particular interest was a potential STAT binding motif located at position −3.607 (Fig. 4A). The core sequence of the palindrome and the spacing of the internal nucleotides suggested it may be capable of binding Stat5 proteins (31). Interestingly, this motif was also identified at a similar position (−3.554 kb) upstream of the murine IFNG gene. This motif is within the previously identified HS region (Fig. 2) and is contained in the distal region that is acetylated in NK cells and Th1 cells (Fig. 3).

To assess if this motif was capable of binding Stat5 proteins, we made a double-stranded oligonucleotide spanning the site for use in EMSA assays. When activated human peripheral T

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3 J. H. Bream and H. A. Young, unpublished observations.
cells or NK92 cells (data not shown) were stimulated with IL-2, a complex was formed that was not present in non-stimulated cells (Fig. 4B, lane 2). Interestingly, when NK92 cells were treated with IL-15, an inducible complex migrated to the same location to that of the IL-2-induced band, and both Stat5a and Stat5b antibodies shifted the complex (data not shown). Of note, the IL-2-activated complex evident in lane 2 was not inducible by treating cells with IL-12 (lane 3). Cold-competition experiments using excess unlabeled probe reveal the specificity of the complex and requirement for an intact Stat motif for binding (lanes 4–6). Supershift analysis with antibodies against Stat5a or Stat5b displaces the IL-2-induced complex (lanes 7–9). C, ChIP analysis in NK92 cells of the distal IFNG gene using Stat5a or Stat5b antibodies to immunoprecipitate following cytokine stimulation. IL-2 treatment results in the binding of both Stat5a and Stat5b to the upstream IFNG gene (lanes 7–8 and 15–16). One of three representative experiments is shown.

The preceding data indicated that Stat5 isoforms have the ability to bind this distal site in the IFNG gene. However, to establish that IL-2-activated Stat5a and Stat5b interact with the endogenous IFNG promoter in vivo, we again utilized ChIP analysis. We used NK92 cells to test for IL-2-induced Stat5 proteins interacting with the distal IFNG region in vivo. As shown in Fig. 4C, cells that were not stimulated (NS) (lanes 3 and 4) or treated with IL-12 alone (lanes 11 and 12) did not recruit Stat5a or Stat5b to the distal IFNG gene. When cells were stimulated in the presence of IL-2 alone (lanes 7 and 8) or IL-2 plus IL-12 (lanes 15 and 16), however, both Stat5a and Stat5b were immunoprecipitated, bound to the distal IFNG region. To control for nonspecific antibody interactions, normal rabbit IgG was used as an immunoprecipitation control but no evidence of nonspecific binding was observed (lanes 2, 6, 10,
Fig. 5. The distal Stat5 site acts as a transcriptional enhancer element. A, diagram of the IFN-γ reporter constructs used. B, human PBMC were transfected by electroporation with IFN-γ reporter constructs, and the results indicate that maximal P/I induction of the IFN-γ promoter requires IL-2 co-stimulation and the intact distal Stat5 element. One of over three independent, representative experiments is shown.

DISCUSSION

The biochemical and molecular events that control expression of IFN-γ have been intensively studied, yet our understanding of the genomic regions that are required for tissue-specific expression and cytokine-induced transactivation of the IFNG gene is still very superficial. In this study we have identified a distal, 5′-flanking region of the IFNG gene that is a site for cell-specific epigenetic modifications. This region is ~3.5~4 kb upstream of the IFNG gene and contains a cytokine-responsive DNase I HS and is a target of IL-2-activated histone acetylation. This same region includes a Stat5 binding element that is critical to IL-2-induced transcription of the IFNG promoter. Our data show that, in human NK cells, the IFNG locus is constitutively in an “open” conformation yet is responsive to cytokine-induced augmentation of a distal HS site (both IL-2 and IL-12) and to acetylation of the distal promoter within the HS site (IL-2 only). Given that NK cells are capable of expressing IFN-γ in a matter of minutes to hours following stimulation or local infection without prior activation (12, 35), it is logical that the IFNG locus may be regulated differently in NK cells compared with T cells (36). It is interesting that the observed HS region is responsive to both IL-2 and IL-12 treatment, whereas histone acetylation around the Stat5 site responds only to IL-2 stimulation. Because the location of the DNase I HS site is an estimate, and the length of DNA it spans is undetermined, it is possible that other, more IL-12-specific sites may be nearby, possibly further upstream.

Recent studies in T cell subsets have described a CD4+ T cell-specific role for the transcription factor T-bet (37) and a CD8+ T cell-specific role for the related Eomesodermin (38). It is still not clear exactly how these factors exert their influence in the IFNG gene to affect T cell differentiation. In terms of general activation, however, multiple transcription factors appear to play a role in regulating T cell-specific IFN-γ expression. Upon T cell receptor activation signals, AP-1, NF-AT, and
CREB proteins bind to regions close to the transcription start site and act to initiate transcription (39–43). The proximal promoter region, bound by these nuclear proteins, is also a site of DNA methylation, and expression of IFN-γ correlates with the methylation status of this site (44–46). Interestingly, this methylation site is evolutionarily conserved, because it is present in other vertebrates with the exception of avians (47). Regardless, the proximal promoter regions important for TCR-induced activation are not sufficient for normal tissue-specific expression or cytokine-induced IFNG gene induction (8, 9).

In contrast to IFN-γ, regulation of the il4 locus is significantly better understood. Early studies identified sites proximal to or within the il4 gene that are involved in controlling/ enhancing transcriptional events (48–51). During the process of differentiation, the il4 locus in naïve Th2 cells undergoes dramatic changes in the chromatin structure. No fewer than ten Th2-specific HS sites have been identified in this locus (52, 53). Some of these sites provide a physical link between epigenetic modification and transcriptional regulation, including one HS located 3′ of the il4 gene that corresponds to an enhancer element that binds the Th2-specific transcription factor GATA3 as well as NF-AT (54). The use of functional genomics strategies and transgenic approaches has elucidated the contribution of multiple, distal regulatory regions in the il4 locus that encompasses intergenic space between several genes and over 100 kb in distance (53, 55–58).

Like the il4 locus, the chromatin structure around the IFNG gene is regulated in a tissue-specific fashion (56, 59). HS sites have been demonstrated in the proximal promoter and introns of the IFNG gene that correlate with a cell-specific ability to express IFN-γ. Nonetheless, Soutto et al. (8), using a transgenic approach, determined that greater than 2.7 kb of IFNG promoter region was required to mimic endogenous IFN-γ expression profiles (8). Thus, in vivo control of IFN-γ transcription is likely to involve the cooperative effects of regions both proximal and distal to the IFNG gene (8). Accordingly, the 5′-flanking region of the IFNG gene has been suspected of harboring crucial regulatory elements (9), and a PHA/PMA-inducible HS site estimated to be −3 kb upstream was found in Jurkat T cells in 1987 (28). A recent paper by Lee and colleagues (60) describes a distal Th1-specific HS site that was estimated to be −5 kb upstream of the murine IFNG gene. This region has nearby binding sites for T-bet, AP-1, and NF-AT (60), transcription factors previously known to regulate IFN-γ transcription. In addition, this group compared 50 kb of genomic sequence between mouse and human IFNG loci and found a distal region of conservation between species that maps to this HS site. In our study, we have estimated the HS site observed in NK cells to be −3.5–4 kb 5′ to the human IFNG gene. Within this region, we have characterized a Stat5 binding site identified in the IFNG gene that lies 3.6 kb upstream of the transcriptional start site. Interestingly, by sequence comparison, we also identified this Stat5 motif at position −3.554 kb 5′ to the mouse IFNG gene (Fig. 4A). Taken together, this upstream region of the IFNG gene appears to have important functions both in controlling the chromatin structure of the IFNG locus as well as in enhancing transcription.

Originally described as T cell growth factor, IL-2 has been known to up-regulate IFN-γ transcription in both T cells and NK cells. Although IL-2 induction of IFN-γ was reported 20 years ago, little data exists regarding the mechanisms involved. IL-2-induced IFN-γ production has been implicated in several in vivo infectious disease models, including lymphocytic choriomeningitis virus (61) and Toxoplasma gondii (62, 63). In fact, by using IL-2−/− mice, Villegas et al. (64), recently showed that maximal CD3- or PMA and ionomycin-induced IFN-γ expression is dependent on IL-2. We have conducted similar experiments using Stat5a−/− Stat5b−/− mice (65) and found defects in IL-2-regulation of IFN-γ production (data not shown) in CD-3-stimulated splenocytes. It should be noted that these mice have pleiotropic defects in lymphoid development and function. Therefore these data need to be interpreted with some caution. Nevertheless, our PBMC transfection data (Fig. 5B) are consistent with these results and suggest that the distal Stat5 binding element is a functional site for IL-2 enhancement of IFN-γ transcription.

It is important to appreciate, however, that the regulation of IFN-γ transcription is complex, and no single element is altogether responsible for IFN-γ activation. As stated earlier, IL-2 also up-regulates NF-κB proteins that may have both positive and negative regulatory effects on IFN-γ transcription. The mechanisms by which these different transcription factors converge to alter chromatin conformation may well be different depending upon the cell type (66) and extracellular signal. Furthermore, the tremendous synergy observed between IL-2 and IL-12 with respect to IFN-γ expression is likely due, at least in part, to the cooperative activation of multiple transcriptional regulatory regions within the IFNG genomic DNA (such as Stat4 and Stat5 interactions). Although the synergy between IL-2 and IL-12 on IFN-γ expression is dependent on Stat4 and independent of T-bet expression (25), the one or more precise mechanisms are still not clear. Further studies analyzing in vivo interactions of DNA binding proteins with specific regions of the IFNG gene as well as changes in chromatin conformation following stimulation will be required to more precisely define the epigenetic and transcriptional regulatory mechanisms of this gene. Nevertheless, this study supports the notion that the distal 5′-flanking region of the IFNG locus contains specific elements that are involved in tissue-specific and optimal signal-specific expression of IFN-γ.

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