Distal Regions of the Human *IFNG* Locus Direct Cell Type-Specific Expression

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**Distal Regions of the Human IFNG Locus Direct Cell Type-Specific Expression**

Patrick L. Collins,* Shaojing Chang,† Melodie Henderson,‡ Mohammed Soutto,†,1 Georgia M. Davis,‡ Allyson G. McLoed,† Michael J. Townsend,‡,2 Laurie H. Glimcher,‡ Douglas P. Mortlock,§ and Thomas M. Aune*,†

Genes, such as IFNG, which are expressed in multiple cell lineages of the immune system, may employ a common set of regulatory elements to direct transcription in multi-lineage cells or individual regulatory elements to direct expression in individual cell lineages. By employing a bacterial artificial chromosome transgenic system, we demonstrate that IFNG employs unique regulatory elements to achieve lineage-specific transcriptional control. Specifically, a one 1-kb element 30 kb upstream of IFNG activates transcription in T cells and NKT cells but not in NK cells. This distal regulatory element is a Runx3 binding site in Th1 cells and is needed for RNA polymerase II recruitment to IFNG, but it is not absolutely required for histone acetylation of the IFNG locus. These results support a model whereby IFNG uses cis-regulatory elements with cell type-restricted function. The Journal of Immunology, 2010, 185: 1492–1501.

**C**ell lineage-specific gene expression is a central challenge of multicellular life. Cell type-specific, developmentally regulated genes may be expressed in just one cell type or in multiple cell lineages. Developmentally regulated genes expressed in multiple lineages must be responsive to different arrays of stimuli, transcription factors, and chromatin environments present in the varying cell types in which they are expressed. Conversely, expression must be actively restricted to appropriate cell lineages. In metazoans, lineage-specific expression is thought to be conferred by the use of distal regulatory elements (1, 2). However, mechanisms by which distal regulatory elements direct lineage-specific gene expression are largely unknown. One possibility is that developmentally regulated genes employ a single common set of regulatory elements to direct transcription in all cell types expressing these genes. An alternate possibility is that genes employ unique cell type-specific distal regulatory elements to achieve cell type-specific expression.

IFN-γ is a cytokine produced by selected cells of the innate and adaptive immune systems. IFN-γ is most notably produced by CD4+ Th1 cells, but it is also produced by CD8+ cytotoxic T cells, NK cells, NK T cells, macrophages, and dendritic cells. IFN-γ must not be transcribed in other cell types, such as Th2 cells, developing T cells, and cells outside the immune system. A multitude of transcription factors have been implicated in regulating Ifng, including T-bet, STAT4, Runx3, GATA3, and Hlx (3). The transcription factor T-bet is necessary and sufficient for production of IFN-γ by CD4+ T cells (4). CD8+ T cells and NK cells express a second T-box-containing transcription factor called eomesodermin (5). Because of this second transcription factor, CD8+ T cells do not show the strict reliance on T-bet for Ifng transcription under in vitro conditions as do CD4+ T cells (6, 7). These differences in T-box proteins show that transcription factor regulation of Ifng varies among the different cell types that produce IFN-γ.

Mice carrying an 8.6-kb human IFNG transgene express high levels of human IFN-γ in both Th1 and Th2 cells. Th2 cells should not express IFN-γ. In contrast, a 190-kb bacterial artificial chromosome (BAC) transgene with the human IFNG gene, and 90 kb of both upstream and downstream sequences, does recapitulate Th1/Th2 selective expression (8). This indicates that one or more distal regulatory elements are critical for cell type-specific regulation of IFNG. Regulatory elements within the Ifng locus (9–12), as well as other developmentally regulated loci (1), may exist within distal conserved noncoding sequences (CNSs). For example, a CNS located –22 kb from the mouse Ifng transcription start site (CNS-22) is critical for mouse IFN-γ production in T cells and NK cells (13). Other work has shown that the CNSs within the Ifng locus acquire cell type-specific histone marks that correlate with IFN-γ expression in Th1 cells, as well as histone marks that correlate with repression in Th2 cells (3). However, these studies do not explain how distal regulatory elements within the Ifng locus confer cell type-specific expression. They also do not explain why Ifng possesses a large cis-regulatory region and requires numerous transcription factors for proper regulation. Because the IFNG promotor alone does not confer cell type-specific expression, we sought to determine if a single distal CNS within the IFNG locus

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*Division of Rheumatology, Department of Medicine, †Department of Microbiology and Immunology, ‡Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232; and §Department of Immunology and Infectious Diseases, Harvard School of Public Health and Department of Medicine, Harvard Medical School, Boston, MA 02115

1Current address: Department of Surgery, Vanderbilt University School of Medicine, Nashville, TN.

2Current address: Genentech, South San Francisco, CA.

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dictates cell type-specific expression in all cell types expressing IFNG or if separate CNs are employed to direct IFNG transcription by distinct cell lineages.

To determine the role of distal regulatory elements within the IFNG locus, we created transgenic mice carrying a 190-kb transgene of the human IFNG locus. Activity of the BAC transgene mirrored many aspects of regulation of the endogenous gene, including Th1/Th2 selectivity and reliance upon T-bet and Stat4 for expression of human IFN-γ in CD4+ cells and CD8+ cells. To determine which regions of the 190-kb BAC transgene contain distal regulatory elements, we pursued an unbiased deletion strategy. We created four new BAC transgenic mice with 40-kb deletions of upstream or downstream sequences of the IFNG locus. Using the larger deletions as a guide, we created BAC transgenic mice with 1-kb deletions of individual CNs. We found that a CN located at ~30 kb, relative to the transcription start site, is necessary for human IFN-γ production in T cells and NKT cells, but not in NK cells. This CN binds the transcription factor Runx3 and is necessary to recruit RNA polymerase (RNAP) II to IFNG but not in NK cells. This CNS binds the transcription factor Runx3 and is necessary to recruit RNA polymerase (RNAP) II to IFNG in T cells, but it is not absolutely required for establishment of restricted function.

Results show that distal CNSs of a human gene have cell type-specific function.

Materials and Methods

Mice

C57BL/6 and C57BL/6.Saat4−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME), bred in the Vanderbilt University animal facilities, and used between 4 and 5 wk of age. Thx21 (T-bet)−/− mice have been previously described (4, 8). Research using mice complied with all relevant institutional and federal guidelines and policies.

Cell purification and cultures

CD4+ and CD8+ T cells were purified from splenocytes by negative selection as previously described (8). Purified CD4+ or CD8+ T cells (1 × 10^6 cells/ml) were stimulated with immobilized anti-CD3 (2C11; American Type Culture Collection) and anti-CD28 (CD28.2; BD PharMingen, San Jose, CA), according to the manufacturer’s instructions. Flow cytometry experiments were performed in the Vanderbilt University Medical Center Flow Cytometry Shared Resource. T cell cultures were gated on CD4+ or CD8+ subsets for analysis.

Preparation of transgenic reporter lines

The human IFNG BAC clone RFP11-444B24 (Invitrogen, Carlsbad, CA) was moved into the EL250 Escherichia coli strain to allow controlled recombination and FLP protein expression (14). To mediate deletions with the BAC, 50-nucleotide homology arms were designed to flank segments of the BAC. Long PCR primers containing 5′ homology arms were synthesized (Supplemental Tables 1 and 2) and used to amplify a bacterial tetracycline resistance cassette flanked by FRT sites as previously described (15). This created “deletion cassettes” that were transformed into recombination-competent EL250/BAC cells (14). Tetracycline-resistant colonies were screened by PCR across recombination junctions, and restriction digests were performed to verify predicted recombinations for each BAC deletion. The cassette was deleted by inducing FLP expression, leaving behind only a 34-bp FRT sequences in place of the deleted material. Final verification of correct modification and absence of unwanted alterations occurring during the modification process was confirmed by additional PCR analysis, restriction enzyme digestion and pulsed-field gel electrophoresis, and by direct BAC sequencing.

To prepare BAC clone DNA for microinjection, BACs were purified using a NucleoBond Gigaprep columns (BD Clontech, Palo Alto, CA) followed by treatment with Plasmid-Safe DNase (Epicentre Biotechnologies, Madison, WI) to remove sheared DNA. The remaining circular BAC DNA was desalted using an Amicon 100 centrifugal filtration column (Millipore, Bedford, MA). Transgenic mice were prepared by standard pronuclear injection using circular BAC DNA, which is effective for generating transgenic founders (15). Positive founders were identified by PCR with primers made with flanking tail DNA using appropriate primers for the human IFNG gene. We also used PCR with human-specific primers at various points across the human IFNG BAC sequence to verify that all regions of the BAC transgenes were still present after integration into genomic DNA.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed according to Miltenyi’s online protocol with minor variations: cells were harvested by centrifugation; each protein A agarose-AbChromatin wash was performed two times for 10 min each, and DNA was isolated by phenol-chloroform extraction. Immunoprecipitations were performed using either an anti-acetyl H4 Ab (06-866; Upstate Biotechnology, Lake Placid, NY), anti-H3K9Me2 (ab-1220; Abcam, Cambridge, MA), RNAP II (sc-899X; Santa Cruz Biotechnology, Santa Cruz, CA), Runx3 (39301; Active Motif, Carlsbad, CA), or normal rabbit IgG control. Primers used are presented in Supplemental Table 3. Significance by ANOVA was determined using GraphPad Prism software (GraphPad Software, San Diego, CA). For determining significance at specific genomic locations, a Student t test was performed with Bonferroni’s inequality applied to the calculated probability.

Results

Stat4 and T-bet dependence of reporter gene expression in T cells

To initiate these studies, we examined activity of the 190-kb IFNG-BAC transgene in wild-type (WT), Stat4−/−, and T-bet−/− CD4+ T cells. CD4+ T cells were cultured for 3 d with plate-bound anti-CD3 under Th1 or Th2 conditions (primary stimulation). To measure secondary, effector responses, cells were cultured for 5 d, harvested, and restimulated with plate-bound anti-CD3 alone for an additional 2 d. We found that absence of either Stat4 or T-bet markedly reduced the activity of the IFNG-BAC transgene in Th1 cells during either primary or secondary effector responses (Fig. 1, left panel). Production of human IFN-γ by BAC transgenic CD4+ T cells was reduced by T-bet or Stat4 deficiency to a similar degree as production of murine IFN-γ (Fig. 1, right panel). These results demonstrate that the IFNG-BAC transgene exhibits the same critical T-bet and Stat4 requirements for activity in the CD4+ T cell lineage as does the endogenous Ifng gene.

To determine if the 190-kb IFNG-BAC transgene displays cell type-specific dependence on the transcription factor T-bet, we next examined its expression in CD8+ T cells. We cultured Wt, T-bet−/−, and Stat4−/− BAC transgenic CD8+ T cells with manufacture’s instructions. Flow cytometry experiments were performed in the Vanderbilt University Medical Center Flow Cytometry Shared Resource. T cell cultures were gated on CD4+ or CD8+ subsets for analysis.
anti-CD3 and APCs under Th1 or Th2 polarizing conditions. Similar to CD4+ T cells, absence of Stat4 reduced the ability of CD8+ T cells to produce human IFN-γ. Unlike the CD4+ T cells, transgenic CD8+ cells were able to produce human IFN-γ in a T-bet−/− background (Fig. 2A). These results are consistent with our earlier observations that T-bet does not influence endogenous IFN-γ production in CD8+ T cells that have been stimulated by polyclonal activation with anti-CD3 (5, 16, 17). T-bet−/− CD8+ T cells stimulated in vivo, or in vitro with Ag-specific stimulation, produce significantly reduced amounts of IFN-γ when compared with WT CD8+ T cells (6, 7). IFNG-BAC transgenic mice were backcrossed onto either Wt OT-I TCR or T-bet−/− OT-1 TCR backgrounds. CD8+ T cells were stimulated with the OT-I OVA peptide SIINFEKL, APCs, and IL-2 for 5 d. After 5 d, cultures were harvested and restimulated with plate-bound anti-CD3 only for 2 d (secondary stimulation). Levels of human IFN-γ (left panel) and of murine IFN-γ (right panel) were determined by ELISA.

To determine which 40-kb blocks regulate IFNG-BAC transgene activity in CD8+ T cells, A, CD8+ T cells were purified from IFNG-BAC+ WT, Stat4−/−, or T-bet−/− mice and were cultured under Th1 or Th2 polarizing conditions as in Fig. 1. Cultures were harvested after 3 d (primary responses) or 2 d after restimulation with anti-CD3 (effectors responses) and were analyzed for levels of human or mouse IFN-γ by ELISA. B, CD8+ T cells were also purified from IFNG-BAC+ WT or T-bet−/− OT-I TCR transgene-positive mice and stimulated with peptide Ag, APCs, and IL-2. After 5 d, cultures were harvested and restimulated with peptide and APCs. After an additional 2 d, cultures were harvested and IFN-γ ELISA assays performed.

Forty-kilobase regulatory regions within the 190-kb IFNG-BAC transgene

To identify elements within the 190-kb BAC transgene essential to drive faithful production of human IFN-γ, we first pursued an unbiased deletion strategy. Positions of individual CNSs within the 190-kb IFNG locus are shown in Fig. 3. While the distance between each CNS varies slightly among species, the order of individual CNSs across the locus is absolutely conserved (Fig. 3, bottom). It is unknown how many CNSs across the 190-kb locus participate in regulating IFNG transcription. We divided the transgene into four ∼40-kb blocks for analysis, Δ1–4 (Fig. 3, Supplemental Table 1). We constructed four deletions in the BAC representing each of these 40-kb blocks and prepared transgenic mice. We prepared a fifth 80-kb BAC deletion lacking the Δ3/4 region (Fig. 3). We were able to leave the human homologs to mouse CNS-22 (13), CNS-6 (11, 18), and CNSS+18–20 (9), which have been studied in various reports, in the undeleted region. In this manner, all deletions resulted in removal of novel regulatory elements.

To determine which 40-kb blocks regulate IFNG transcription, CD4+ T cells harboring the different transgenes were cultured under Th1 or Th2 conditions. Cultures were harvested after primary or secondary stimulation, and human and murine IFN-γ levels were determined by ELISA. Removal of Δ1 (+59 to +93 kb relative to the human IFNG transcriptional start site) or Δ2 (+20 to +59 kb) did not affect human IFN-γ production by either primary or effector CD4+ T cell cultures (Fig. 4A). In contrast, the Δ3/4 (∼92 to ∼18 kb) deletion caused a dramatic loss of human IFN-γ production after primary stimulation, as well as a complete loss of human IFN-γ production after secondary stimulation of effector cell cultures (Fig. 4B). Results for Δ3 (∼56 to ∼18 kb) mice were similar to Δ3/4 mice (not shown), and as such Δ3/4 mice (rather than Δ3 mice) were analyzed further to rule out cooperativity effects between the truncated, nonexpressed IL22 and its regulatory elements and the IFNG regulatory elements. Deletion of only Δ4 (∼92 to ∼56 kb) resulted in an increase in human IFN-γ production in both primary and effector cell cultures (Fig. 4B). Levels of murine IFN-γ were unchanged in cultures with the different BAC transgenes (Fig. 4C). We also determined human and mouse IFN-γ levels by ELISA from CD8+ T cells cultured under C11 or C12 conditions. As with CD4+ T cells, deletion of Δ4 resulted in an increase in human IFN-γ in both primary and effector CD8+ cell
culture supernatants (Fig. 4D). The Δ4 deletion removed the IL26 gene, which is a pseudogene in the mouse, and the 3′ end of IL22 contained on the IFNG-BAC. IL26 was not expressed from IFNG-
BAC T cells or NK cells (Supplemental Fig. 1).

To determine the function of these deletions at the transcript level, we verified our results by quantitative PCR of cDNA made from day 3 Th1 cultures (Fig. 4E). Taken together, these results identify two novel 40-kb regions that regulate IFNG transcription in T cells. The −56 to −18 kb region contains elements necessary for human IFN-γ production, while the −92 to −56 kb region represses IFNG transcription. Notably, none of these large deletions resulted in increased production of human IFN-γ in Th2 cells. This phenotype is predicted based on observations that mice carrying only an 8.6-kb IFNG transgene fail to repress human IFN-γ in Th2 cells (8). These results suggest that additional regulatory elements exist within the undeleted regions, −18 kb to +20 kb from the IFNG transcription start site, that govern Th1/Th2 selectivity.

The −92 to −18 kb region is dispensable for IFN-γ production in innate immune cells

To determine if function of regulatory regions within the −92 to −18 kb region is T cell specific, we isolated NK cells from BAC transgenic mice or Δ3/4 BAC transgenic mice and stimulated them with IL-12 and IL-18. To our surprise, in contrast to Δ3/4 BAC transgenic effector Th1 and Tc1 cells, Δ3/4 BAC transgenic NK cells robustly produced human IFN-γ (Fig. 4F). To determine if dependence on the −92 to −18 kb region is T cell intrinsic, regardless of expression of NK cell receptors, we examined human IFN-γ expression in NK cells. NKT cells are cells of the T cell lineage that express a semi-invariant TCR, express NK cell surface markers, and respond to glycolipid Ags (19, 20). NKT cells are the sole cell population stimulated by the NKT cell ligand αGalCer. The Δ3/4 or BAC transgenic splenocytes were stimulated with the NKT cell ligand αGalCer. After 2 d, human IFN-γ levels were determined. BAC transgenic, but not Δ3/4, NKT cells produced human IFN-γ in response to αGalCer (Fig. 4G). Taken together, these results demonstrate that the −92 to −18 kb region is necessary in T cells and NKT cells, but it is not necessary in innate immune cells such as NK cells.

CNS-30 is necessary for human IFN-γ production by T cells

Having identified two large distal regions within the human IFNG-BAC transgene that differentially regulated IFNG transcription, we sought to identify specific CNSs within these regions that recapitulate regulatory properties of these larger genomic regions. To identify the regulatory element responsible for the loss of human IFN-γ production seen in Δ3/4 BAC transgenic T cells, we focused on a conserved NCS located at −30 kb (CNS-30) from the IFNG promoter. The mouse homolog of CNS-30, located in the mouse genome at −34 kb, becomes histone acetylated in T cells, but not in freshly isolated NK cells and cultured NK cells. We examined mouse CNS-34 for conserved binding sites for transcription factors known to regulate Ifng. We identified two conserved AML (also known as Runx) binding sites within the CNS. Runx3 has been shown to be necessary for sufficient Ifng production in T cells (21), but not necessary for Ifng production in NK cells (22).

To verify Runx3 binding to CNS-34, we cultured CD4+ T cells for 3 d under Th1 or Th2 conditions and performed ChIP assays. We observed Runx3 binding predominantly to mouse CNS-34 and to the Ifng promoter in CD4+ Th1 cells (Fig. 5A). When considering these properties, we reasoned that CNS-30 may be the site within the −56 to −18 kb region required for efficient IFNG transcription. We prepared a new BAC construct harboring a 1-kb deletion of a CNS-30 and produced new transgenic mice (∆30, Fig. 3).

Δ30 or IFNG-BAC CD4+ and CD8+ transgenic cells were cultured for primary and secondary stimulation as described above. We observed a dramatic reduction in human IFN-γ production by Δ30 T cells in both the primary and the secondary Th1 cultures (Fig. 5B). We next determined if CNS-30 had an agonist-specific role. We harvested day 5 effector Th1 cells and restimulated them with either plate-bound anti-CD3 or IL-12 and IL-18. Loss of human IFN-γ was evident in effector Th1 cells stimulated with anti-CD3 and cells stimulated with IL-12 and IL-18 (Fig. 5C). Our results were not due to differences in culture conditions, as murine IFN-γ levels were unchanged (Fig. 5B, 5C).

We next sought to distinguish if CNS-30 regulated the percentage of T cells that became IFN-γ producers, and thus by definition Th1 cells, or the amount of IFN-γ produced on a per-cell basis. We restimulated day 4 Δ30 or IFNG-BAC Th1 cells with PMA/ionomycin and measured mouse and human IFN-γ levels by intracellular cytokine staining (Fig. 5D). The Δ30 deletion reduced the overall percentage of CD4+ T cells that became human IFN-γ+. As such, our data indicated that CNS-30 is necessary for human IFN-γ production in Th1 cells in response to both TCR stimulation and IL-12 and IL-18 costimulation.

CNS-30 is dispensable for IFN-γ production in innate immune cells

We next isolated NK cells from BAC transgenic mice or Δ30 BAC transgenic mice and stimulated them with IL-12, IL-18, or a combination of both cytokines. As with transgenic NK cells lacking the Δ3/4 (−92 to −18 kb) region, Δ30 NK cells were able to robustly produce human IFN-γ (Fig. 6A). We also measured IFNG mRNA levels in CD4+ Th1 cells after TCR stimulation and NK cells after IL-12 and IL-18 stimulation. We observed a 10-fold reduction of human IFNG mRNA in Δ30 T cells, relative to IFNG-BAC transgenic T cells, but equivalent production of IFNG mRNA by NK cells from the two transgenic lines (Fig. 6B). These results demonstrate that CNS-30 is a regulatory element necessary for human IFNG transcription in T cells, but it is dispensable for transcription in NK cells.

FIGURE 3. Deletions made in this study. Positions of 40-kb deletions from the IFNG-BAC transgene are shown: red, ∆1; green, ∆2; blue, ∆3; black, ∆4 and small 1-kb CNS deletions; brown, ∆77; and purple, ∆30. Relative positions of human IFNG, IL26, and IL22 in the IFNG-BAC transgene are shown below. Evolutionary mouse-human sequence conservation, locations of CNS (red, >70% sequence conservation between mouse and human spanning ≥200 bp), and locations of transposons (green) within the 190-kb human IFNG-BAC transgene are as identified using the DCODE Web site (www.dcode.org).
NK cells are members of the innate immune system that arise from the lymphoid cell lineage. To determine if CNS-30 was necessary for sufficient IFN-\(\gamma\) production in innate immune cells of the myeloid cell lineage, we examined human IFN-\(\gamma\) expression in macrophages and dendritic cells. For macrophage isolation, we purified CD11b+ cells, and for dendritic cell isolation we purified CD11c+ cells. D\(-30\), D3/4, and BAC transgenic CD11b+ and CD11c+ cells were isolated and stimulated with IL-12 and IL-18. As with NK cells, transgenic D\(-30\) and D3/4 CD11b+ and CD11c+ cells were able to produce human IFN-\(\gamma\) at levels comparable to the complete IFNG-BAC transgenic controls (Fig. 6C). We purified CD11c+ and CD11b+ cells and measured supernatant IFN-\(\gamma\) levels after 48 h of IL-12 and IL-18 stimulation. We did not detect human IFN-\(\gamma\) in these culture conditions. Human IFN-\(\gamma\) expression in our ex vivo culture assay was stimulation dependent, and we did not observe spontaneous IFN-\(\gamma\) production.

To determine which CD11b+ and CD11c+ cell populations produced human IFN-\(\gamma\), we stimulated IFNG-BAC, D3/4, and D\(-30\) CD11b+ and CD11c+ cells with PMA/ionomycin and determined human and mouse IFN-\(\gamma\) expression by intracellular cytokine staining. Among CD11c+ and CD11b+ cells, IFN-\(\gamma\) expression was low in MHC II+ dendritic cell and macrophage populations. However, human IFN-\(\gamma\) was detected among MHC II+ CD11b+ cells.
determined by ELISA. Results are averages of at least three independent experiments.

Thus, unlike T cells and as with NK cells, CD11b+ and CD11c+ populations have been described as an IFN-γ-producing NK cell subset (23). Unlike CD4+ and CD8+ T cells, in which human IFN-γ was almost always coexpressed with mouse IFN-γ (Fig. 5D), human IFN-γ expression in CD11b+ and CD11c+ cells was predominantly in the mouse IFN-γ cell population. Human IFN-γ staining was comparable between IFNG-BAC and Δ3/4 CD11b+ and CD11c+ cells. Thus, unlike T cells and as with NK cells, CD11b+ and CD11c+ cells do not require either CNS-30 or the larger Δ3/4 genomic region for sufficient IFN-γ production.

Copy number variation

We also determined if human IFN-γ production varied with relative transgene copy number. We assessed transgene copy number by standard techniques using quantitative PCR and genomic primer pairs positioned across both the human BAC transgene and the endogenous murine locus in the different transgenic lines. Because we did not have variation in copy number between IFNG-BAC transgenic lines, we selected mice carrying an IFNG-BAC transgenec line. Flow cytometry and intracellular cytokine staining were employed to determine levels of human and murine IFN-γ-producing cells in Δ77 BAC transgenic CD8+ T cells cultured under Th1 polarizing conditions from three separate founder lines. This analysis demonstrated that among the three Δ77 BAC transgenic lines, human IFN-γ production by the Δ77 BAC transgenic lines was proportional to transgene copy number.

CNS-30 is required for RNAP II recruitment, but not for histone acetylation

In eukaryotes, DNA is wrapped around packaging histone proteins. These histones are subject to a series of enzymatically catalyzed chemical modifications that produce a “histone code” of epigenetic information above the genetic code (24). In mice, CNSs within the Ifng locus are marked by histone 4 tail acetylation (H4Ac) during Th1 development by a Stat4- and T-bet-dependent mechanism (3, 25–28). H4Ac is correlated with transcriptionally permissive areas of chromatin (18). To determine if the human IFNG-BAC transgenic locus also acquired H4Ac marks, and to ascertain the role of distal regulatory elements within the Ifng locus in establishing the histone code, we cultured CD4+ T cells from BAC transgenic mice for 3 d under Th1 conditions and performed ChIP assays. Under these conditions the endogenous mouse locus developed extensive, long-range H4Ac marks (Supplemental Fig. 3). We were able to detect H4Ac marks across the human locus in IFNG-BAC transgenic Th1 cells, although at reduced levels than observed at the endogenous mouse locus (Supplemental Fig. 3), consistent with reduced IFNG expression levels (Figs. 1, 2).

We performed H4Ac ChIP assays on day 3 CD4+ Th1 cells from Δ4, Δ3/4, Δ-30, and IFNG-BAC transgenic mice. Deletion of any of the distal regulatory regions examined did not abolish H4Ac at the remaining CNS across the human IFNG locus (Fig. 8A). Whereas a paired ANOVA test demonstrated significant difference in the H4Ac values between the different groups (p < 0.01), a control t test did not identify any significance at any exact genomic position. Furthermore, H4Ac levels between BAC transgenic lines in CD4+ Th1 cells did not absolutely correlate with human IFNG mRNA levels in day 3 Th1 cells. For example, the Δ3/4 deletion did not significantly affect H4Ac at any specific location but completely
abrogated human *IFNG* mRNA production by day 3 Th1 cells. Conversely, the Δ4 deletion resulted in up to a 10-fold increase in human *IFNG* mRNA compared with *IFNG*-BAC transgenic controls. However, H4Ac levels across the *IFNG* locus in Δ4 Th1 cells were not 10-fold above H4Ac levels in BAC transgenic Th1 cells (Fig. 4F).

We next sought to determine if distal regulatory elements functioned to remove a repressive histone mark. The di-methyl-histone 3, lysine 9 (H3K9Me2) histone mark is thought to mark gene repression in mammalian euchromatin (18). CD4+ T cells develop this mark during both Th1 and Th2 cell polarization, but they subsequently lose the mark after 3 d of Th2 polarization (25). We performed ChIP assays from day 3 Δ3/4 or *IFNG*-BAC Th1 cultures. H3K9Me2 levels did not vary significantly (*p* = 0.13 by ANOVA) between Δ3/4 and *IFNG*-BAC T cells (Fig. 8B). Although these negative results do not rule out a role in establishing histone acetylation and methylation throughout the *IFNG* locus, we next considered that the −92 to −18 kb (Δ3/4) region and specifically CNS-30 functioned to recruit components necessary for transcription to *IFNG*. To test this hypothesis, we performed
ChIP assays for RNAP II on day 3 Δ3/4, Δ-30, and IFNG-BAC Th1 and Th2 cells. RNAP II was recruited predominantly to the IFNG-BAC promoter, CNS-16, and CNS-30 in Th1 cells. In contrast, RNAP II recruitment to the IFNG promoter and the CNS-16 sites was significantly reduced in Δ3/4 and Δ-30 Th1 cells, compared with IFNG-BAC Th1 cells (Fig. 8C). Taken together, these results indicate that CNS-30 is necessary for efficient RNAP II recruitment to the IFNG locus in Th1 cells, but it is not absolutely necessary to establish histone marks across the locus.

Discussion

Results presented in this study demonstrate that the IFNG-BAC transgene recapitulates many functions of the endogenous Ifng gene. These include high-level expression, Th1/Th2 and Tc1/Tc2 selective expression, requirement for T-bet by CD4 and CD8 cells to express IFNG, responsiveness to IL-12 and IL-18 stimulation in both T cells and NK cells, and responses to Ag stimulation by effector CD8 T cells via a T-bet-dependent mechanism. Thus, the IFNG-BAC transgene is capable of integrating the multiplicity of signals required for both differentiation and expression of effector function by three distinct cell lineages independent of its position of integration in the genome.

There are two known distal regulatory elements necessary for sufficient IFN-γ production. However, their function in CD4+ T cells is nonredundant. Human CNS-16 (mouse CNS-22) is a major binding site for the transcription factor T-bet (13). Human CNS-30 (mouse CNS-34) binds the transcription factor Runx3. While this paper was in revision, a separate group also identified CNS-34 as a site that binds a Runx family cofactor, CBFβ, and demonstrated that inhibition of Runx family members by a dominant negative approach markedly diminishes IFN-γ production in CD4+ Th1 cells (29). CNS-30 is necessary for efficient RNAP II recruitment to IFNG, but it is not absolutely required for histone acetylation throughout the IFN-γ locus. Thus, a high level of cooperativity is needed among distal regulatory elements, which bind separate transcription factors, to achieve proper transcriptional control. In this model, T-bet binds to CNS-16 and provides initial chromatin remodeling of the Ifng locus to provide locus accessibility to Runx3 and other transcription factors such as Hlx (21). Second, binding of these auxiliary transcription factors to their own distal regulatory elements is necessary to promote...
recruitment of members of the basal transcription complex, such as RNAP II, to the locus.

Removal of distal regulatory elements from −18 to −92 kb of the IFNG transcription start site does not completely eliminate IFNG locus H4Ac, even though removal of these regulatory elements markedly interferes with IFNG transcription. Other groups have proposed that CNS-4 (12), or the mouse homolog CNS-6 (30), responds to an early signal in effector Th1 cell differentiation to promote histone acetylation throughout the IFN-γ locus. Two recent studies have shown CNS located −63, +1.5, and +119 kb from the IFNG transcription start site participate in formation of chromatin loops by binding the chromatin insulator CTCF (31, 32). In this model, CNS-63 and CNS+119 shield the IFNG locus from neighboring chromatin and regulatory elements. However, Δ4 transgenic T cells, which lack both CNS-63 (within Δ4) and CNS+119 (not in the IFNG-BAC transgene), produce robust quantities of human IFN-γ in a Th1/Th2-specific and stimulus-specific manner, demonstrating that these distal CTCF binding sites are not required for cell type- and stimulus-specific gene expression. As such, future studies will focus on determining what role these numerous distal regulatory elements have in determining IFNG chromatin accessibility and expression.

CNS-30 is a distal regulatory element necessary for human IFN-γ production by T cells and NKT cells, but not NK cells. The mouse homolog of human CNS-30, CNS-34, develops histone acetylation upon Th1 differentiation, but it is not acetylated in freshly isolated or cultured NK cells (27). CNS-34 binds the transcription factor Runx3, which is necessary for sufficient Ifng production in Th1 cells (21) but not in NK cells (22). In contrast to the cell type-specific function of CNS-34, previous studies have identified mouse CNS-22 (the homolog to human CNS-16) as a distal regulatory element necessary for IFN-γ production in T cells and NK cells (13). Unlike CNS-34, CNS-22 is acetylated in NK cells (27). As such, CNS-22 is a distal regulatory element necessary for IFN-γ production in cells that participate in both innate and adaptive immunity, whereas CNS-34 (human CNS-30) is a regulatory element only necessary in T cells but is dispensable in innate immune cells (Supplemental Fig. 4). Future work will determine if innate immune cells have their own unique distal regulatory elements, or if cells of the innate immune system have a more permissive array of transcription factors and chromatin modifiers allowing use of fewer regulatory elements to achieve IFNG transcription.

The ability of myeloid cells to express IFN-γ is controversial (33). In the mouse, the identity of which exact dendritic cell subset produces IFN-γ has not been consistent throughout various reports (34–36). Furthermore, mouse CD11c+ and CD11b+ NK cell subsets have made the identification of IFN-γ-producing dendritic cells difficult (23, 37). To our knowledge, human IFN-γ–producing dendritic cells have not been described. Therefore, to understand the possible extent for cell type-specific cis-regulatory elements, we determined which IFNG-BAC CD11c+ and CD11b+ NK cells were capable of producing human IFN-γ. Between IFNG-BAC transgenic CD11b+ and CD11c+ populations, IFN-γ expression is detectable within the MCH II+ NK cell populations. Despite FMA/synergy stimulation, human IFN-γ was close to background in MHC II+ populations. Human IFN-γ expression was very low in CD11c+B220+MHC II+ cells, which contain “IFN-γ-producing killer dendritic cells” (36). We also did not detect human IFN-γ among CD11c+CD8+ cells, which have been reported to produce IFN-γ (34). Therefore, human IFN-γ, in IFNG-BAC mice, is expressed in T cells, NKT cells, and NK cells. Unlike in CD4+ and CD8+ T cells, in MHC II+CD11b+CD11c+ NK populations, human IFN-γ is predominantly expressed in the mouse IFN-γ population. These results further substantiate cell type-specific regulation of IFNG.

Mice carrying IFNG-BAC transgenes without the +20 to +93 kb regions (Δ1 and Δ2 mice) were capable of producing human IFN-γ in CD4+ and CD8+ T cells. Because these areas contain CNSs, which are by definition implicated in fitness, we consider it likely that the +20 to +93 kb region has a role in regulating IFNG. Future work will focus on the +20 to +93 kb region. It is possible that there are elements within this region with cell type-specific roles in NK cells, where the −92 to −18 kb region is dispensable. Alternatively, there may be unique roles for regulatory elements in vivo. For example, CNSs within the IFNG locus could have tissue-specific, or even pathogen-specific, functions. Both spontaneous and Ag-specific human IFN-γ expression in endogenous tissues will be examined in future studies.

These results demonstrate that distal regulatory elements of a human gene function in a cell type-specific manner. This attribute of human gene regulation is recapitulated in the mouse genome. In the mouse Th2 cytokine locus, CNS-1 is necessary for IL-4 production in Th2 cells but not in mast cells (38). Other regulatory elements within the IL-4 locus do not show cell type specificity (39). Thus, different distal regulatory elements have varying cell type-specific functions (IFNG CNS-30 and mouse Th2 CNS-1) or alternatively have a general function (mouse Ifng CNS-22; see Ref. 13). This modular use of distal regulatory elements in the mammalian Th2 cytokine and IFNG loci is similar to what has been found in studies of Drosophila promoters, where modular elements of gene promoters can function independently to direct cell type-specific expression (40, 41). Thus, metazoan developmentally regulated genes, in which expression is restricted to more than one cell type, use modular distal regulatory elements with cell type-specific regulatory elements in possible conjunction with regulatory elements that function in multiple cell types.

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References


