Diverse functions of distal regulatory elements at the *IFNG* locus

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Abstract

Previous studies have identified multiple conserved noncoding sequences (CNS) at the mouse *Ifng* locus sufficient for enhancer activity in cell-based assays. These studies do not directly address biology of the human *IFNG* locus in a genomic setting. *IFNG* enhancers may be functionally redundant or each may be functionally unique. We test the hypothesis that each *IFNG* enhancer has a unique necessary function using a bacterial artificial chromosome transgenic model. We find that CNS-30, CNS-4 and CNS+20 are required at distinct stages of Th1 differentiation while CNS-16 has a repressive role in Th1 and Th2 cells. CNS+20 is required for IFN-γ expression by memory Th1 cells and by NKT cells. CNS-4 is required for IFN-γ expression by effector Th1 cells. In contrast, CNS-16, CNS-4 and CNS+20 are each partially required for human IFN-γ expression by NK cells. Thus, *IFNG* CNS enhancers have redundant necessary functions in NK cells, but unique necessary functions in T helper cells. These results also demonstrate that distinct CNSs are required to transcribe *IFNG* at each stage of the Th1 differentiation pathway.

Introduction

A primary signal in the defense against intracellular infections is the cytokine interferon gamma (IFN-γ) (1). IFN-γ is expressed by NK and NKT cells, CD8+ cytotoxic T cells and CD4+ T helper (Th) 1 cell subsets. During the initial CD4+ T cell maturation stage, a naïve CD4+ T cell can polarize into various T helper cell subsets including Th1 and Th2 subsets (2). Th2 cells must repress *IFNG*. This *IFNG* repression in Th2 cells is dependent upon the noncoding segment of the genome. Mice carrying an 8.6 kb transgene of the human *IFNG* gene fail to repress *IFNG* in Th2 cells (3). In contrast, mice carrying a 190 kb bacterial artificial chromosome (BAC) transgene with *IFNG* and the surrounding noncoding region both correctly express human IFN-γ in Th1 cells and repress human IFN-γ production in Th2 cells (3). As such, cell-type selective expression of human IFN-γ depends upon the noncoding segment of the genome.

The majority of the conserved portion of the human genome is noncoding. Further, the majority of the human common single nucleotide polymorphisms associated with disease traits are noncoding (4). As such, understanding the noncoding segments of the genome will be important to understanding human health. The noncoding portion of the genome includes various types of functional elements, including enhancers. Enhancers are thought to be necessary for driving tissue-specific, as well as species-specific gene expression (5, 6). Some genes are regulated by multiple, redundant enhancers, which some models propose are necessary to allow for expression under sub-optimal signaling conditions (7). As enhancers

drive tissue-specific expression, and tissue-specific expression of *IFNG* is critical for protection from intracellular infections in humans (8), a relevant question is the mechanism of how *IFNG* enhancers drive tissue-specific expression of *IFNG*.

Transcription factor binding to mouse *Ifng* distal regulatory elements is cell type and stimulus type-specific. The transcription factors T-bet (9, 10), STAT4 (10), STAT5 (11, 12), NF-κB family members (13), and Runx3 (14) positively regulate *IFNG* expression and directly bind to distinct conserved noncoding sequences (CNS) of the mouse *Ifng* locus in a Th1 and stimulus dependent manner. Transcription factor binding is accompanied by Th1-specific covalent histone modifications at conserved noncoding sequences (13, 15, 16). These observations have led to the hypothesis that proper regulation of interferon gamma is conferred by transcription factor interactions with CNSs. In transgenic model systems, a mouse CNS −16 kb from the *Ifng* start site (mCNS-16) is needed for Thy1.1 reporter expression from a mouse *Ifng* BAC (9). In addition to mCNS-16, additional mCNSs display enhancer activity in reporter assays (15, 17, 18) and also function with other *Ifng* CNS to synergistically stimulate transcriptional activity (18).

Our understanding of human *IFNG* distal regulation in the setting of an intact genome is incomplete. We considered two non-exclusive hypotheses. First, CNSs may have redundant function where each CNS is necessary for a fraction of *IFNG* expression in all responder cell types in response to diverse stimuli. Second, CNSs may possess unique functions such that each individual CNS provides a unique contribution to developmental decisions and stimulus-specificity to achieve proper *IFNG* transcriptional regulation. To test these hypotheses, we employed an *IFNG*-BAC transgenic system (3, 14). In this model, mice are created with a transgene that contains *IFNG* and surrounding regulatory regions with or without specific CNSs. Normal production of mouse IFN-γ is not affected and serves as an internal control. We have previously characterized a conserved noncoding sequence −30 kb from the *IFNG* start site (CNS-30) necessary for transgenic *IFNG* expression in T cells but not NK cells (14). Here, we extend those studies to comprehensively characterize *IFNG* CNS, and detail the necessary function of CNS-16, CNS-4, CNS+20 and CNS+120. We find an essential role for CNS-4 in effector/memory Th1 cells. CNS+20 is required for human IFN-γ production by *in vivo* generated memory Th1 cells, as well as NKT cells. Lastly, we find that CNS-16 has a repressive role in both Th1 and Th2 cells, opposite to what was found in the mouse BAC transgenic system (9). In contrast, each of these CNSs, CNS-16, CNS-4, and CNS+20 contribute a fraction to *IFNG* transcription in NK cells. These results demonstrate that the necessary functions of distal regulatory elements are dependent upon developmental context, as well as species, cell type and stimuli.

Materials and Methods

Mice and preparation of transgenic reporter lines

C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME), housed in the Vanderbilt University animal facilities, and used between 4-5 weeks of age. Preparation of human *IFNG*-BAC transgenic lines was preformed as described previously (14). Briefly, human *IFNG*-BAC CTD-3002C24 was used to make 210 kb *IFNG*-BAC mice. Alternatively, CTD-3002C24 was moved into EL250 *E. coli* and CNS deletion was achieved using homologous recombination followed by FRT-mediated removal of the selection marker. Targeting primers are described in supplemental table 1. We used human-specific PCR primers to verify insertion integrity of the different *IFNG*-BAC transgenes. Lines without full-length insertions were excluded from analysis. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Cell purification and cultures

CD4+ and CD8+ T cells were purified from splenocytes by positive selection per manufactures instructions (Miltenyi Biotec). T cells were cultured with plate bound 2 μg/ml anti-CD3 (hybridoma 2C11, American Type Culture Collection, Manassas, VA) and anti-CD28 (BD Biosciences). For Th1/Tc1 cultures, cells were cultured with 10 ng/ml IL-12 and 10 μg/ml anti-IL4 (11B11 hybridoma, ATCC). For Th2 cultures, CD4+ T cells were cultured with 20 ng/ml IL-4 and 10 μg/ml anti-IFN-γ (hybridoma, ATCC). In addition, cultures were harvested after three days and re-cultured with IL-2 for two additional days. Effector Th1/Tc1 cells were re-stimulated with either plate bound anti-CD3, 10 ng/ml IL-12 and 10 ng/ml IL-18, 10 ng/ml IL-12 and 10 ng/ml IL-2, or 50 ng/ml PMA and 1 μM ionomycin. NK cells were purified from spleen by negative selection (Miltenyi Biotech).

Immunization

Five-week old transgenic mice were immunized with 50 μg soluble ovalbumin in complete Freund's adjuvant (Sigma Chemical Co.) by intraperitoneal injection. At day ten, mice received an additional boost of Ova in incomplete Freund's adjuvant (IFA). Splenocytes were harvested at day 35 for analysis. Splenocytes were either re-stimulated with 0.1 mg/ml Ova, 0.1 mg/ml hen egg lysozyme, or 10 μg/ml Ova 257-264 peptide. After two days, human and mouse IFN-γ was determined in culture supernatants by ELISA. Alternatively, splenocytes were restimulated overnight with 10 ng/ml IL-12 and 10 ng/ml IL-18. The next day, BD GolgiPlug was added, and cells were cultured for an additional six hours and analyzed by flow cytometry.

Results

Creation of 210 kb BAC ΔCNS-16, ΔCNS-4 and ΔCNS+20 transgenic mice

Previous reports have detailed that mouse CNS-22, mCNS-6, mCNS+22 and mCNS+67 contribute to *Ifng* transcriptional regulation (19). We used UCSC genome alignments to identify respective orthologs at human CNS-16, CNS-4, CNS+20 and CNS+120. CNS-4, CNS+20 and CNS+120 showed strong conservation in placental and non-placental mammals, but we were unable to find a non-placental ortholog to CNS-16 (Fig. 1). We verified correct enhancer ortholog location by referencing Th1 and Th2 DNase I hypersensitivity tracks. We next designed new *IFNG*-BAC transgenes with or without these conserved noncoding sequences. While a 190 kb *IFNG*-BAC transgene correctly recapitulates developmental and signaling-dependent expression of *IFNG* including dependence upon the transcription factors T-bet and STAT4 (14), the 190 kb IFNG-BAC transgene does not contain CNS+120. We identified a new 210 kb *IFNG*-BAC transgene which differed mainly by the inclusion of CNS+120 (Fig. 1). To assay functional roles of CNS-16, CNS-4 and CNS+20 we created new 210 kb *IFNG*-BACs with 1 kb deletions of CNS-16 and CNS-4 or a 3 kb deletion of CNS+20. A 3 kb region was chosen for CNS+20 to include all Th1 specific DNase I hypersensitivity sites as well as all regions homologous to mouse CNS+22. The exact location of deletions, transgene locations, and homologous mouse locations are found in supplemental table 1. We next created new transgenic mice with the full 210 kb IFNG-BAC transgene, or with the ΔCNS-16, ΔCNS-4 or ΔCNS+20 transgenes.

Transgenic mice displayed no overt abnormalities, were born at Mendelian ratios and gained weight at appropriate rates. Mouse IFN-γ production was consistent among different transgenic lines and was not affected by the presence of the transgene. The large size of BAC transgenes is thought to help protect against position integration effects, but lead to potential partial integration effects (20). We verified full integration of all transgenes by human-specific PCR of the BAC transgene from transgenic mouse genomic DNA (Fig. 1).

Transgenic mice without full integration were not analyzed further. We have previously observed that IFN-γ expression from the 190 kb *IFNG*-BAC is dependent upon copy number (14). We next analyzed relative copy number by quantitative PCR. Copy number of all new lines of BAC transgenic mice was consistent, equivalent to two copies in a 190 kb *IFNG*-BAC control. We next assayed for human IFN-γ expression in the 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4 and ΔCNS+20 BAC transgenic mice.

CNS-4 is necessary for IFN-γ production by effector Th1 cells

We began our assays by using an *in vitro* tissue culture system. To test for expression in Th1 cells and repression in Th2 cells, CD4+ T cells were isolated and cultured under Th1 polarizing conditions (IL-12 and anti-IL4) or Th2 conditions (IL-4) with plate bound anti-CD3 and soluble anti-CD28 for three days. To test for expression in CD8 cells, CD8+ T cells were isolated and cultured under Th1 polarizing conditions (IL-12). At day three, human and mouse IFN-γ levels in cultures were assayed by ELISA. Alternatively, Th1 cells were cultured for two additional days in IL-2. On day five, Th1 cells were re-stimulated with either anti-CD3, IL-12 and IL-18, or IL-2 and IL-12 to test for defects specific to either T cell receptor stimulation or coordinated cytokine stimulation. IFN-γ was measured in cultures two days later.

We first assayed for human IFN-γ expression from T cells with the 210 kb *IFNG*-BAC, the ΔCNS-4 and the ΔCNS+20 *IFNG*-BAC transgenes. For comparison, we assayed for human IFN-γ expression from previously created mice with the 190 kb IFNG-BAC transgene, or 190 kb *IFNG*-BAC transgenes lacking CNS-30 or CNS-77, which have been previously described (14). In day three Th1 cultures, human IFN-γ culture supernatant concentrations did not significantly differ between 190 kb and 210 kb *IFNG*-BAC cultures. Further, presence or absence of CNS-77, CNS-4 or CNS+20 did not significantly change human IFN-γ concentrations in Th1 or Th2 cultures (Fig. 2*A*: Left). Control mouse IFN-γ concentrations did not vary among transgenes (Fig. 2*A*: Right). As previously described (14), removal of CNS-30 led to a loss of detectable human IFN-γ in cultures.

We continued our *in vitro* protocol and rested day three Th1 cultures in IL-2 for two days. CNS-4 has been proposed as an IL-2 responsive element (11). Effector Th1 cells were re-stimulated with plate-bound anti-CD3, IL-12 and IL-18, or IL-12 and IL-2. Human IFN-γ was measured in cultures two days later by ELISA. Human IFN-γ levels did not significantly differ between Th1 cells with the 190 kb *IFNG*-BAC transgene or the 210 *IFNG*-BAC transgene (Fig. 2*B*). Presence or absence of CNS-77 or CNS+20 also did not significantly alter concentrations of human IFN-γ in cultures. As previously reported, and as seen in primary cultures, removal of CNS-30 resulted in loss of detectable human IFN-γ in re-stimulated Th1 cell cultures. However, unlike in primary cultures, removal of CNS-4 resulted in a significant decrease in the concentration human IFN-γ in culture supernatants of re-stimulated effector Th1 cells. The CNS-4 defect was observed in both T cell receptor re-stimulated and IL-12 and IL-18 re-stimulated Th1 cells. As such, removal of CNS-4 resulted in a loss of human IFN-γ production by differentiated effector Th1 cells.

Lack of CNS-16 leads to increased human IFN-γ expression

We next investigated the role of CNS-16 in the regulation of IFN-γ in the human *IFNG*-BAC transgenic system. Using UCSC genome alignments, we identified CNS-16 as the ortholog of mouse CNS-22. mCNS-22 is necessary for Thy1.1 reporter expression from a murine BAC (9). mCNS-22 is also a T-bet responsive enhancer in two independent cell-based reporter assays (9, 15). As such, we hypothesized that CNS-16 would be required for human IFN-γ production in the *IFNG*-BAC transgenic system. We began by assaying human IFN-γ expression from cultures of 210 kb *IFNG*-BAC or ΔCNS-16 *IFNG*-BAC

transgenic Th1 and Th2 cells. In direct contrast to our hypothesis, removal of CNS-16 led to relatively higher human IFN-γ in Th1 cultures, compared to controls (Fig. 3*A*). In addition, removal of CNS-16 led to increased human IFN-γ in Th2 cells. Control mouse IFN-γ was appropriately repressed in Th2 cultures (Fig. 3*A*: Right). In this study, and in previous studies of *IFNG*-BAC transgenic mice (14), measurable human IFN-γ above background levels was a phenotype unique to ΔCNS-16 Th2 cultures. These results indicate that ΔCNS-16 plays a key role repressing IFN-γ expression in both Th1 and Th2 cell cultures.

We next assayed for human IFN-γ in cultures of CD8+ T cells. We cultured 190 kb *IFNG*-BAC, ΔCNS-77, ΔCNS-30, 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4 and ΔCNS+20 transgenic CD8+ T cells for three days and measured human IFN-γ by ELISA. As previously reported (14), removal of CNS-30 resulted in concentrations of human IFN-γ below levels of detection (Fig. 3*B*). Similar to Th1 cell cultures, removal of CNS-16 resulted in relatively high concentrations of human IFN-γ in CD8+ T cell cultures (Fig. 3*B*). Choice of 190 kb *IFNG*- or 210 kb *IFNG*-BAC or presence or absence of CNS-4 or CNS+20 did not significantly affect human IFN-γ production by CD8+ T cells under these culture conditions. To verify our results at the transcript level we cultured transgenic CD4+ T cells for three days under Th1 polarizing conditions. After cDNA synthesis, we determined human *IFNG* and mouse *Ifng* transcript levels by quantitative PCR. We observed relatively high human *IFNG* transcripts in ΔCNS-16 Th1 cultures compared to 210 kb IFNG-BAC cultures. Mouse *Ifng* transcripts were not different among cultures with the different transgenes (Fig. 3*C*). To verify that results were not due to contaminating NK cells, we assayed human and mouse IFN-γ expression by intracellular cytokine staining. 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4 and ΔCNS+20 transgenic CD4+ T cells were cultured under Th1 conditions for 5 days, rested for 2 days in IL-2 containing media, re-stimulated with PMA/ionomycin, and analyzed for IFN-γ by flow cytometry. Similar to measures of levels of human IFN-γ in cultures of effector cells, we observed a high percentage of human IFN-γ positive ΔCNS-16 Th1 cells and a low percentage of human IFN-γ positive ΔCNS-4 Th1 cells, relative to controls. As such, in our *in vitro* cultures of CD4+ and CD8+ T cells, production of human IFN-γ was dependent upon CNS-4 and CNS-30 with CNS-16 having a repressive role.

STAT4 and T-bet bind to the transgenic IFNG promoter

We next determined if any deletion was associated with altered T-bet and STAT4 binding to the *IFNG*-BAC promoter. We cultured 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4 and ΔCNS+20 transgenic CD4+ T cells for three days under Th1 polarizing conditions and processed cells for chromatin immunoprecipitation assays using antibodies against T-bet, STAT4 or an IgG isotype control. We observed specific T-bet and STAT4 binding to the human *IFNG* and mouse *Ifng* promoters in BAC transgenic Th1 cells (Supplemental Fig. 1). Levels of STAT4 binding to the transgenic *IFNG* and mouse *Ifng* promoters were essentially equivalent. Similarly, levels of T-bet binding to the transgenic *IFNG* and mouse *Ifng* promoters were essentially equivalent Further, deletion of CNS-16, CNS-4, or CNS+20 did affect binding of STAT4 or T-bet to the *IFNG* promoter. As such, these deletions were not associated with changes T-bet or STAT4 binding to the *IFNG* promoter.

The CNS-16 ortholog, mCNS-22, is required for Thy1.1 reporter expression from a mouse *Ifng* BAC transgene (9). As mCNS-22 and human CNS-16 have opposing functions, we analyzed the mouse and human interferon gamma loci for obvious differences. We first compared conservation of the human *IFNG* locus (Supplemental Fig. 2*A*). A small region of the human CNS-16 was not conserved in the mouse and rat genomes, but was conserved in other rodents and mammals outside Muridae. We also observed a poorly conserved site within this region marked by Th1-specific DNase I hypersensitivity. As the ΔCNS-16 deletion covered the murine-conserved portion of CNS-16, we reanalyzed conserved transcription factor binding sites across CNS-16. Analysis of transcription factor binding

sites in the segment of CNS-16 not conserved in mice identified a cluster of T-bet and Runx3 binding sites (Supplemental Fig. 2*B*). To confirm the ability of the non-conserved segment of CNS-16 and the poorly conserved CNS-21 to bind transcription factors, we analyzed publically available chromatin immunoprecipitation followed by sequencing (ChIP-seq) data for binding of STAT4, STAT5A/B and YY1 (21, 22) in Th1 cultures because of their known ability to regulate *IFNG* transcription. This analysis showed that STAT4 and STAT5 bound to both CNS-21 and the portion of CNS-16 not conserved in mice. These data identify CNS-21 and the portion of CNS-16 not conserved in mice as likely regulatory elements that may contribute to their observed functional differences.

CNS+20 is necessary for IFNG expression by memory Th1 cells

Our previous experiments relied upon *in vitro* maturation and polarization of T helper cells using experimental procedures optimized for murine IFN-γ production. Because previous reports have described differences in long-range chromatin modifications of the mouse *Ifng* locus during *in vivo* and *in vitro* Th1 polarization (15), we addressed the necessary functional requirements of human *IFNG* distal regulatory elements, *in vivo*. Mice were immunized by i.p. injection with OVA in CFA and boosted at day ten with OVA in IFA. At day thirty-five splenocytes were isolated and re-stimulated with irrelevant protein, HEL, OVA protein, or OVA 257-264 SIINFEKL peptide, which is recognized by CD8+ T cells on the C57BL/6 background (23). After two days, human and mouse IFN-γ concentrations in cultures were determined by ELISA (Fig. 4). Human and murine IFN-γ production were antigen-specific and not observed in HEL stimulated cultures. Concentrations of human IFN-γ were higher in ΔCNS-16 cultures when re-stimulated with either OVA or OVA (257-264) peptide relative to controls. Consistent with results from *in vitro* generated Th1 cells, removal of CNS-4 resulted in a significant decrease in human IFN-γ production in the antigen-specific memory responses. Further, while human IFN-γ production by *in vitro* generated ΔCNS+20 Th1 cells did not significantly differ from controls, human IFN-γ concentrations in the cultures of ΔCNS+20 T cells were significantly lower in response to antigen-specific re-stimulation of *in vivo* generated memory cells. These results demonstrate a necessary role for CNS+20 in memory responses *in vivo*.

Among T cells, IFN-γ expression in response to IL-12 and IL-18 stimulation is restricted to effector/memory Th1/Tc1 lineages. Therefore, we analyzed human IFN-γ expression by freshly isolated CD4+ and CD8+ T cells after stimulation with IL-12 and IL-18 overnight. Eighteen hours later single cells producing human and mouse IFN-γ were quantified by flow cytometry (Fig. 5*A & B*). Cells were gated on murine IFN-γ positive populations to restrict analysis to effector/memory populations. Under these stimulation conditions, up to 45% of mouse IFN-γ positive BAC-transgenic CD4+ T cells were also human IFN-γ+. Percentages of human IFN-γ+ positive cells did not differ between 190 kb *IFNG*-BAC and 210 kb *IFNG*-BAC transgenic mice. Consistent with antigen-specific re-stimulation of *in vivo* generated antigen-specific Th1 cell responses, removal of CNS-4 or removal of CNS+20 resulted in a significant decrease in the percentages of human IFN-γ+ cells compared to 210 kb *IFNG*-BAC T cells. Further, stimulation of ΔCNS-16 T cells resulted in a relatively higher percentage of human IFN-γ+ cells compared to controls. Results were similar for CD8+ T cells. Unlike changes in the percentages of human IFN-γ producing cells, mean fluorescence intensities of human IFN-γ staining did not differ among transgenes. As such, CNS-4 and CNS+20 determine numbers of IFN-γ+ cells rather the amount of human IFN-γ produced per cell.

Natural killer T cells require CNS+20 for human IFN-γ production

Natural killer T (NKT) cells represent an innate-like T cell subset arising from the T cell lineage but which express NK cell markers. Upon recognition of glycolipid antigens, NKT

cells rapidly express IFN-γ and other cytokines characteristic of multiple T cell lineages (24). Therefore, we determined which distal regulatory elements were required for *IFNG* expression by NKT cells. We first assayed NKT cell expression after antigen-specific stimulation. Splenocytes from 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4 and ΔCNS+20 transgenic mice were isolated and stimulated with the glycolipid α-galactosylceramide (α-GalCer), or a mock stimulus. After two days, human and mouse IFN-γ concentrations in cultures were determined by ELISA (Fig. 6*A*). Concentrations of human IFN-γ did not significantly differ among 210 kb *IFNG*-BAC, ΔCNS-16, or ΔCNS-4 cultures. In contrast, removal of CNS+20 resulted in a marked decrease in production of human IFN-γ.

To directly compare NKT cell regulation with T and NK cell regulation of the *IFNG*-BAC transgene, we examined NKT cell expression of human and mouse IFN-γ after stimulation with IL-12 and IL-18. Isolated splenocytes were stimulated with IL-12 and IL-18 for 16 hours and analyzed by intracellular cytokine staining (Fig. 6*B*). To identify NKT cell populations, we used a tetramer specific for the NKT cell receptor CD1d. Cells were gated on MHC class II-negative populations to remove non-specific tetramer staining. Percentages of human IFN-γ positive cells did not vary among 210 kb *IFNG*-BAC, ΔCNS-16 and ΔCNS-4 transgenic NKT cells. By contrast, we did not detect ΔCNS+20 transgenic NKT cells positive for human IFN-γ. These results are consistent with a necessary role for CNS +20, but not other CNS, for NKT cells to express human IFN-γ from the *IFNG*-BAC transgene.

Natural killer cells partially require CNS-16, CNS-4 and CNS+20

In previous work, we demonstrated that CNS-30 is not required for human IFN-γ expression by NK cells (14). Human CNS-4 is acetylated in natural killer cells, and is an enhancer in reporter assays using human peripheral blood mononuclear cells (11). Further, NK cells immigrate to the periphery fully able to rapidly produce IFN-γ, much like effector Th1 cells, which required CNS-4 for human IFN-γ expression. As such, we hypothesized that CNS-4 was required for human IFN-γ expression by NK cells. To determine distal regulatory usage by NK cells, purified 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4, and ΔCNS+20 transgenic NK cells were stimulated with IL-12 and IL-18. After two days, we measured human and mouse IFN-γ in cultures (Fig. 7*A*). In contrast to T cells, removal of CNS-16 resulted in a significant decrease in human IFN-γ concentrations in NK cell cultures. In addition, removal of CNS-4 or CNS+20 resulted in a significant decrease in human IFN-γ concentrations in culture supernatants, compared to controls. Concentrations of human IFN-γ in NK cell culture supernatants were noticeably higher than concentrations of human IFN-γ in T cell cultures under identical stimulation conditions (Fig. 2*B*). We verified that cultures were 95% pure DX5+, NK1.1+ (Fig. 7*B*). The high concentrations of human IFN-γ in the cultures and functional requirements for CNS-16 suggests that NK cell regulation of the BAC transgene differed from T cell regulation.

We directly compared distal regulatory element usage in T cells and NK cells by intracellular cytokine staining of IL-12 and IL-18 stimulated splenocytes from immunized transgenic mice. After gating on mouse IFN-γ+, CD8− populations, we directly compared T cells and NK cells by comparing CD4+ T cells to CD4−, DX5+ cells (Fig. 7*C*). In 210 kb *IFNG* BAC NK cells, 40% of cells were human IFN-γ+ (Fig. 7*D*), similar to expression by T cells. Consistent with measurements of protein concentrations, each of the CNS-16, CNS-4 and CNS+20 deletions individually resulted in a slight decrease in percentages of human IFN-γ+ NK cells (Fig. 7*D*). Taken together, CNS-16, CNS-4 and CNS+20 all contribute to a fraction of the total human IFN-γ+ expression by NK cells but have markedly different functions in CD4+ and CD8+ T cells, and NKT cells.

Discussion

Here we determined the necessary functional roles of *IFNG* distal regulatory elements CNS-16, CNS-4, CNS+20 and CNS+120, using a BAC transgenic system. In T cells, these distal regulatory elements all have discrete functions. CNS-16 is a repressor of IFN-γ expression in both Th1 and Th2 cells. CNS-4 is necessary for IFN-γ expression by effector Th1 cells in response to secondary TCR stimulation. CNS+20 is necessary for memory responses *in vivo*. In contrast, only CNS+20 is required by NKT cells to produce human IFN-γ in response to antigen stimulation. Unlike T cells and NKT cells, CNS-16, CNS-4 and CNS+20 each are partially required for *IFNG* expression in NK cells, and removal of no individual distal regulatory element completely abolishes human IFN-γ expression. As such, NK, NKT and Th1 cells employ very different usage of distal regulatory elements to achieve lineage-specific *IFNG* transcription.

Conserved noncoding sequences at −30 kb, −4 kb and +20 kb from the *IFNG* start site are necessary for expression of human IFN-γ. In an earlier report, we used 40 and 80 kb deletions in the *IFNG*-BAC transgenic model to identify the −30 to +20 kb region as necessary for *IFNG* expression while regions outside this core regulatory element, −80kb to −40 kb and +20 kb to +100 kb, were dispensable (14). CNS-30, CNS-4, and CNS+20 show clear non-placental orthologs in our conservation analysis. In addition, CNS-30, CNS-4, and CNS+20 possess Th1-specific DNase I HS. Distal regulatory regions which are either not conserved in non-placental mammals or do not show Th1-specific DNase I hypersensitivity were not required for *IFNG* expression. Thus, in our model system the combination of Th1-specific DNase I hypersensitivity and non-placental mammalian conservation appears to separate necessary distal regulatory elements from distal regulatory elements that are not necessary for *IFNG* expression.

Consistent with our results, other reports have demonstrated functional roles for CNS-4 in responding to Stat5a, Stat5b, NFAT and T-bet transcription factors (11-13, 18, 19). IL-2 signaling through Jak3 induces Stat5 binding at CNS-4 and is important for remodeling of the *Ifng* locus after 72 hours of culture (12), consistent with a role for CNS-4 in effector cultures. CNS+20 has little enhancer activity on its own, but acts synergistically with CNS-4 in reporter assays (17) and loops into CNS-4 upon Th1 differentiation (25). In the *IFNG*-BAC transgenic system, CNS+20 is required for IFN-γ expression from *in vivo* generated memory cells. One model to explain these observations would be that *IFNG* distal regulation has successive requirements during T cell maturation. As Th1 cells develop further away from an initial polarization signal more distal regulatory elements would become necessary. In this model CNS-30 is required for human IFN-γ expression in primary cultures and later stages of differentiation, CNS-4 is required in effector Th1 cells, and CNS+20 is required in memory Th1 cells. An alternative hypothesis would be that there are yet-undefined *in vivo* factors which regulate IFN-γ expression and distal regulatory element utilization. In this model, an *in vivo* factor allows CNS-4 to be dispensable in primary cultures and makes CNS+20 absolutely required during *in vivo* differentiation. Future work will differentiate between these hypothesizes.

Counter to our initial hypothesis, removing CNS-16 leads to relatively high human IFN-γ expression by both Th1 and Th2 cells and is necessary for IFN-γ expression only in NK cells. These results are surprising because in a similar BAC transgenic system, the murine CNS-16 ortholog is absolutely necessary for transcription of an *Ifng*-BAC transgene Thy1.1 reporter by both T cells and NK cells (9). However, CNS-16 is not conserved beyond non-placental mammals (Fig. 1), is not required for IFN-γ expression in our human BAC system, and is therefore not universally required for IFN-γ expression by T cells. Numerous reports have demonstrated species-specific roles of distal regulatory elements (5, 26, 27). Thus, it is

likely that the CNS-16 ortholog, mCNS-22, is required for murine IFN-γ expression in T cells while CNS-16 plays an opposing role in human *IFNG* regulation repressing IFN-γ expression. Indeed, the mouse IFN-γ locus has undergone substantial rearrangements relative to non-rodent species (28). Future comparison of the mouse and human interferon gamma loci will provide additional insights into the species-specific functions of distal regulatory elements.

Although regulation of human IFN-γ expression from the 190 kb *IFNG*-BAC transgene correctly depends upon the transcription factors T-bet and STAT4 (14) and is Th1/Th2 selective, it is likely that regulation of *IFNG*-BAC transgenes does not perfectly mirror regulation in humans. Analysis is based upon the assumption that regulation of transgenic human IFN-γ in a mouse fully recapitulates regulation in the endogenous human genome, which may not be true. As such, these findings need to be confirmed in humans.

Transgenic T cells with a 190 kb or 210 kb IFNG-BAC transgene express human IFN-γ at equivalent levels. The two transgenes differ mainly by the inclusion of CNS+120 in the 210 kb *IFNG*-BAC. CNS+120 is thought to facilitate three-dimensional organization of the *IFNG* locus via CCCTC-binding factor (CTCF) binding at CNS+120, CNS-63 and the *IFNG* first intron (29, 30). CTCF has been described as a transcriptional activator (31), insulator (32) and repressor (33). However, most of these functional studies come from reporter assay systems which do not take genomic context into account and a relevant question is the exact function of CTCF binding sites in the genome. Arguing against an insulating role of the +120 CTCF site in the *IFNG* locus are previous experiments reporting copy-number dependence for 190 kb *IFNG*-BAC transgenes (14), and equivalent expression between 190 kb and 210 kb *IFNG* BAC transgenes. Another hypothesis would be that the +120 kb and −63 kb CTCF sites serve to bring the *IFNG* locus into close physical location to other genes in the surrounding regions. *IFNG* is adjacent to *IL22* and *IL26*, both of which are expressed in Th17 cell subsets upon T cell receptor signaling. *IFNG* locus CTCF sites may play a role in co-regulation of *IFNG, IL22* and *IL26*. Alternatively, the CTCF sites may govern intrachromosomal looping interactions, such as those observed between the *Ifng* locus and the *Il4* locus (25).

Transgene deletion effects are dissimilar between NK cells and T cells. In NK cell assays, each distal regulatory element is required for a fraction of human IFN-γ expression. These results support models proposing that distal regulation serves to allow expression under sub-optimal signaling conditions (7) and functions in evolution to modify the level of gene expression in individual species (27). A second model is that distal regulatory elements are both cell-type and stimulus selective and function in evolution by allowing changes in the timing and location of gene expression (5, 27). This model is consistent with distal regulatory element usage in T cells. In T cell assays, CNS-30 is needed in primary cultures, CNS-4 in effector cultures and CNS+20 in memory experiments. Thus, our results support both models of distal regulation. More importantly, choice of cell type determines which model is supported. These results illustrate the importance of analyzing multiple cell types and phenotypes when analyzing the key contributions distal regulatory elements make to achieve proper gene regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

BAC   Bacterial artificial chromosome

CNS   Conserved noncoding sequence

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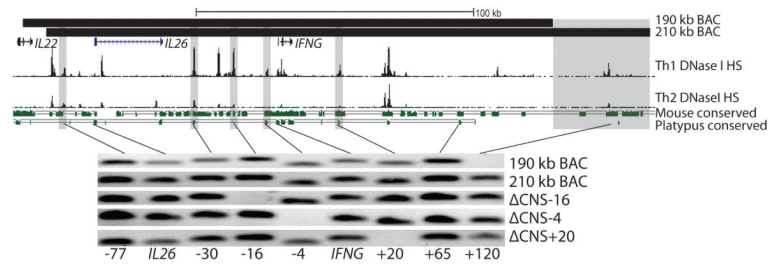
FIGURE 1.
BAC transgenes used in this study. Top: Locations of 190 kb and 210 kb BAC transgenes, Th1 and Th2 DNase I hypersensitivity and conserved sequences between humans and mice or between humans and platypus. Grey bars represent deletion locations. For conservation analysis, green bars are conserved sequences and solid lines represent a fully aligned genome. Bottom: Integration check of transgenes. Shown are human-specific PCR integration checks for BAC transgenic mice.
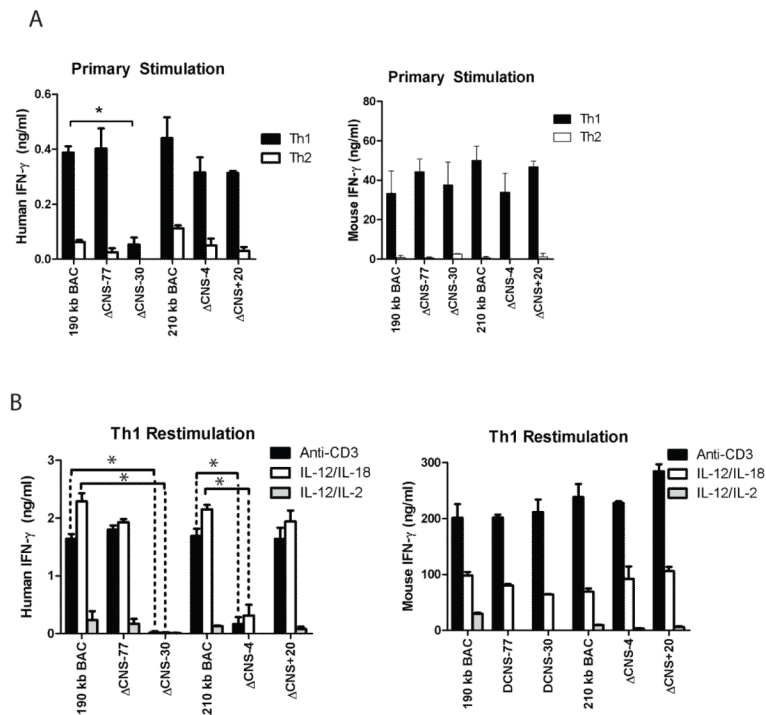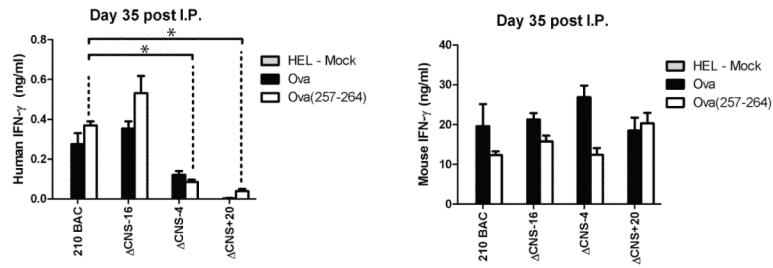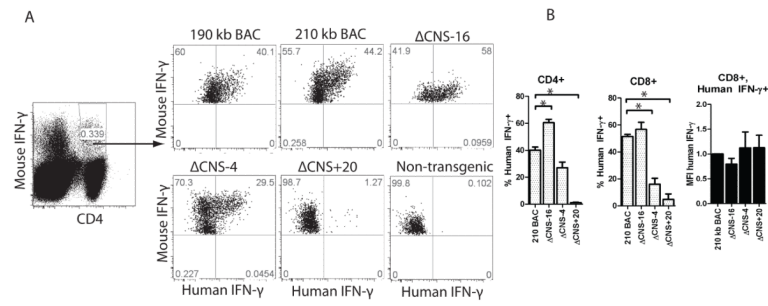
FIGURE 2.
CNS-4 is required for human IFN-γ production. *A*, Transgenic CD4+ cells were cultured under Th1 and Th2 polarizing conditions for three days. Human IFN-γ (left) or mouse IFN-γ (right) concentrations were determined from cultures. *B*, Day five CD4+ Th1 cultures were restimulated with anti-CD3, or IL-12 and IL-18, or IL-12 and IL-2. Human and mouse IFN-γ concentrations were determined by ELISA. Results are representative of independent replicates. Error bars are standard deviations. * P < 0.05.
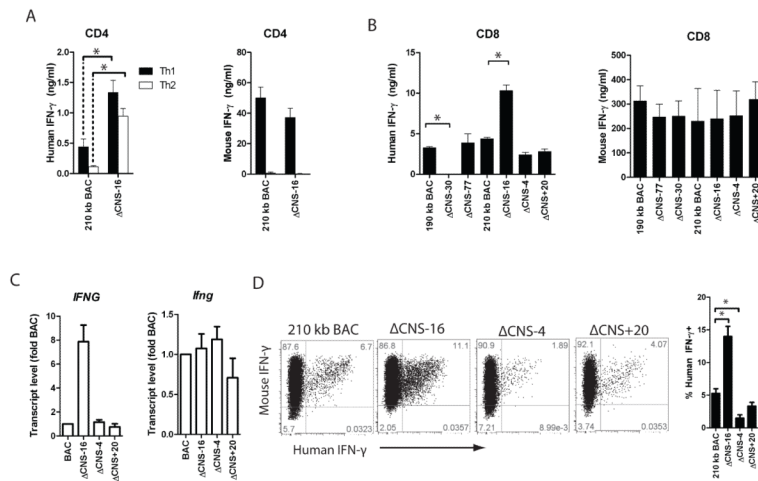
FIGURE 3.

CNS-16 represses human IFN-γ production. *A*, Transgenic CD4+ cells were cultured under Th1 and Th2 polarizing culture conditions for three days. Human IFN-γ (left) or mouse IFN-γ (right) concentrations were determined in cultures. *B*, Transgenic CD8+ cells were cultured under Th1 polarizing conditions for three days. IFN-γ was measured in culture by ELSIA. Results are representative of independent replicates. *C*, Transgenic CD4+ cells were cultured under Th1 conditions for three days and mRNA levels were determined by quantitative PCR. Error bars are standard deviation. *D*, Transgenic CD8+ T cells were cultured under Th1 polarizing conditions for seven days and restimulated with PMA/ Ionomycin. Interferon gamma was determined by intracellular cytokine staining. Quantification of the percentages of human IFN-γ+ cells from three independent replicates is shown. * P < 0.05.
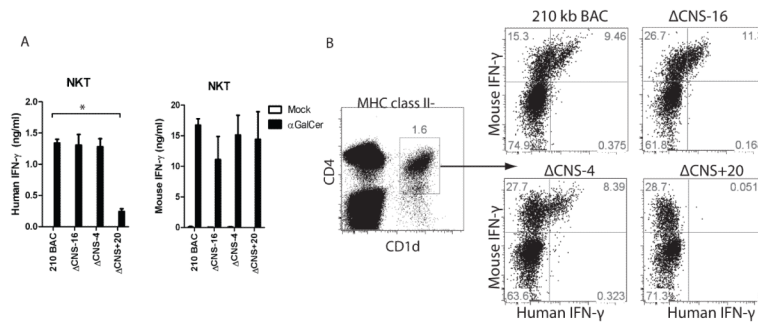
FIGURE 4.
CNS+20 is required for antigen specific recall responses. Transgenic mice were immunized with OVA in CFA, and boosted at day 10 with OVA in IFA. At day 35 mice splenocytes were restimulated with HEL protein, OVA, or OVA 257-264 peptide. IFN-γ concentrations were determined by ELISA. Results are means of three independent replicates. Error bars are standard error of the mean. * $P < 0.05$.
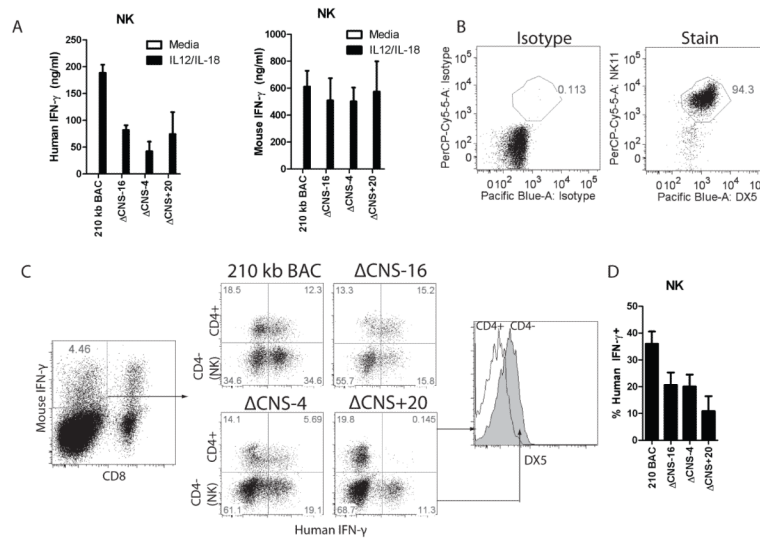
FIGURE 5.
CNS+20 is required for *in vivo* generated Th1 cells. *A*, Splenocytes from day 35 immunized transgenic mice were restimulated with IL-12 and IL-18 and IFN-γ was assayed by intracellular cytokine staining. *B*, Quantification of the percentages of human IFN-γ positive cells in mouse IFN-γ+, CD4+ or mouse IFN-γ+, CD8+ populations or MFI of human IFN-γ positive cells. Results are means of three independent replicates and error bars are standard error of the mean. * $P < 0.05$.

FIGURE 6.
CNS+20 is required for IFN-γ production by antigen-stimulated NKT cells. *A*, Splenocytes were isolated from transgenic mice and stimulated with a mock treatment or with alpha GalCer. Human and mouse IFN-γ concentrations in culture supernatants were determined by ELISA. Results are representative of three independent experiments. Error bars are standard error of the mean. *B*, Splenocytes from transgenic mice were stimulated with IL-12 and IL-18 and IFN-γ was assayed by intracellular cytokine staining. NKT cells were gated on MHC class II−, CD1d+ populations. * $P < 0.05$.

FIGURE 7.
CNS-16, CNS-4 and CNS+20 are required for IFN-γ expression by NK cells. *A*, NK cells were purified from transgenic splenocytes and stimulated with IL-12 and IL-18, or given a mock stimulus. IFN-γ concentrations were determined by ELISA. Results are representative of four independent experiments and error bars represent standard deviation. *B*, Representative cell purity of purified NK cells. *C*, Splenocytes from day 35 immunized transgenic mice were restimulated with IL-12 and IL-18 and IFN-γ was determined by intracellular cytokine staining. NK cells were determined as CD8−, CD4−, DX5+. *D*, Quantification of the percentages of human IFN-γ positive cells in mouse IFN-γ+ CD4−, CD8-populations. Results are means of three independent replicates and error bars represent standard error of the mean.