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Cell-Type-Restricted Binding of the Transcription Factor NFAT to a Distal IL-4 Enhancer In Vivo

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Summary

By DNase I hypersensitivity analysis, we have identified an inducible, cyclosporin A–sensitive enhancer located 3′ of the interleukin-4 (IL-4) gene. The enhancer binds the Th2-specific transcription factor GATA3 in vivo but is not perceptibly influenced by the absence of a second Th2-specific factor, cMaf. The antigen-inducible transcription factor NFAT1 binds the IL-4 enhancer and the IL-4 promoter only in stimulated Th2 cells; conversely, NFAT1 binds to the interferon (IFN)-γ promoter only in stimulated Th1 cells. Our results support a model whereby transcription factors such as NFAT1, which are nonselectively induced in antigen-stimulated T cells, gain access to cytokine regulatory regions only in the appropriate subset of differentiated T cells in vivo. This restricted access enables antigen-dependent and subset-specific transcription of cytokine genes.

Introduction

The immunomodulatory cytokine interleukin-4 (IL-4) plays a central role in the development of effector B and T cells and is critical for host defense during parasitic infections. Dysregulation of IL-4 expression results in uncontrolled allergic inflammation and aberrant immune responses to pathogens (reviewed in Abbas et al., 1996; O'Garra, 1998). IL-4 expression is, in general, mutually exclusive with the expression of interferon (IFN)-γ, a cytokine with a major influence on the inflammatory response. T helper (Th) cells that produce the inflammatory cytokines IFN-γ and TNF but not IL-4 are classified in the Th1 subset, while Th cells that produce IL-4 but not IFN-γ are classified in the Th2 subset (reviewed in Abbas et al., 1996; Mosmann and Sad, 1996; O'Garra, 1998). In addition, Th2 cells show coordinate expression of the closely linked IL-5 and IL-13 genes.

Transcription of the IL-4 gene by T cells is regulated in two distinct steps (reviewed in Rincon and Flavell, 1997a; Szabo et al., 1997b; Agarwal and Rao, 1998; Murphy, 1998; O'Garra, 1998; Agarwal et al., 1999). The first step involves the differentiation of naive T cells into mature effector Th2 cells, while the second step is the inducible transcription of the IL-4 gene by the differentiated Th2 cells. Th2 differentiation is triggered when naive T cells initially encounter antigen in the periphery and is strongly potentiated by IL-4 and the IL-4-induced transcription factor STAT6 (reviewed in Abbas et al., 1996; O'Garra, 1998). In contrast, the second step of acute IL-4 gene transcription is much less dependent on STAT6 and IL-4 (Lederer et al., 1996; Huang et al., 1997). The early stages of Th2 differentiation are accompanied by long-range changes in the DNase I hypersensitivity pattern and DNA methylation status of the IL-4/IL-5/ IL-13 gene cluster (Agarwal and Rao, 1998; Bird et al., 1998; Takemoto et al., 1998). They are also marked by selective up- or downregulation of transcription factors and cell surface receptors characteristic of the Th2 lineage (Ho et al., 1996; Szabo et al., 1997a; Zheng and Flavell, 1997; Sallusto et al., 1998).

The antigen-induced transcription factor NFAT and its cooperating transcription factor AP-1 (Fos/Jun) are implicated in the second phase of induced IL-4 gene transcription by differentiated Th2 cells (reviewed in Rao et al., 1997). NFAT is a family of proteins that includes the four calcium-regulated transcription factors NFAT1 (NFATp, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), and NFAT4 (NFATc3) (reviewed in Rao et al., 1997; Crabtree, 1999; Kiani et al., 2000); a fifth NFAT protein, NFAT5/ TonEBP, is regulated by osmotic shock (Lopez-Rodriguez et al., 1999; Miyakawa et al., 1999). Activation of the four calcium-regulated NFAT proteins (hereafter abbreviated NFAT) requires the calcium/calmodulin-dependent phosphatase calcineurin and is inhibited by the immunosuppressive agents cyclosporin A (CsA) and FK506. In transgenic mice, reporter activity driven by multiple copies of a composite NFAT-AP-1 site was higher in Th2 than in Th1 cells (Rincon and Flavell, 1997b; Wenner et al., 1997), possibly because Th2 cells express several-fold higher levels of the AP-1 family member JunB (Li et al., 1999). It is generally assumed, however, that because NFAT itself is nonselectively induced in both Th1 and Th2 cells (reviewed in Szabo et al., 1997b; Kuo and Leiden, 1999), it cannot explain the subset specificity of cytokine gene expression. Rather, transcription factors that are selectively expressed in Th1 or Th2 lineage cells are likely to be involved.

The transcription factors cMaf (Maf) and GATA3 show a striking level of Th2 restriction. Both Maf and GATA3 are expressed at low levels in naive T cells and become upregulated during Th2 differentiation; both are constitutively expressed in differentiated Th2 cells but not in differentiated Th1 cells; and both are targets of the IL-4-dependent transcription factor STAT6 (Ho et al., 1996; Zhang et al., 1997; Zheng and Flavell, 1997; Kurata et al., 1999). Analysis of Maf-deficient and Maf-transgenic mice indicates that Maf exerts a selective effect on IL-4 gene expression without directly affecting the expression of other Th2 cytokines including IL-5, IL-6, IL-10, and IL-13 (Ho et al., 1998; Kim et al., 1999). In contrast, GATA3 appears to have a global effect on Th2 differentiation. Differentiated Th1 cells from GATA3-transgenic mice showed increased expression of mRNAs encoding IL-4, IL-6, and IL-10 (Zheng and Flavell, 1997); GATA3-overexpressing B and primary Th1 cell lines showed

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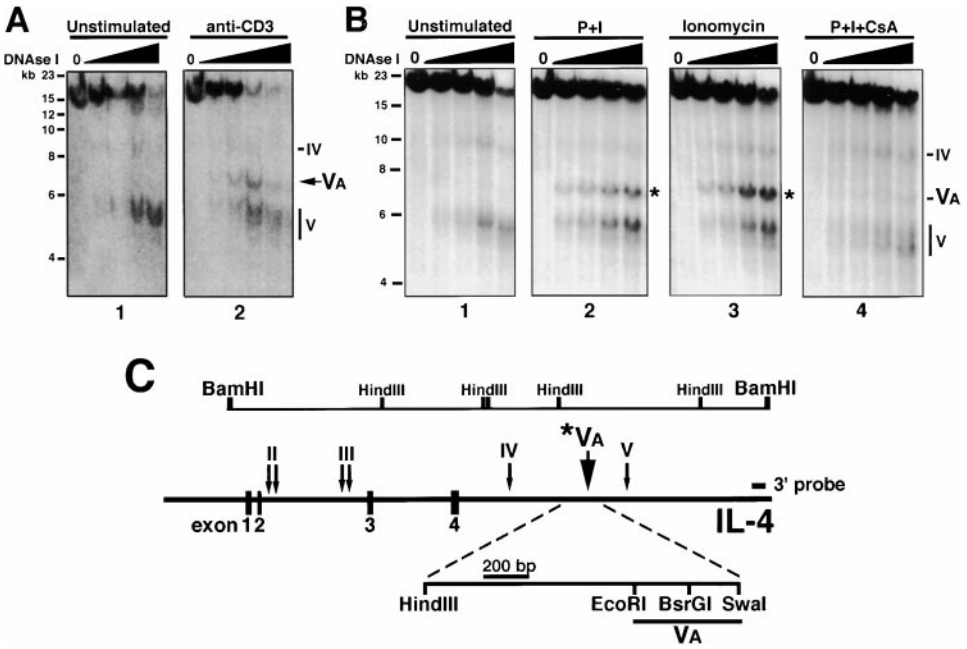

Figure 1. TCR or Ionomycin Stimulation of Primary Th2 Cells Induces a Novel DNase I HS Site Located 3′ of the IL-4 Gene

(A) Stimulation with anti-CD3. Primary Th2 cells differentiated for 2 weeks were either left unstimulated (panel 1) or stimulated for 6 hr using plate-bound anti-CD3ε (panel 2). Cells were subjected to DNase I HS analysis and membranes were hybridized using a probe from the 3′ end of the 19 kb BamHI fragment spanning the IL-4 gene (Agarwal and Rao, 1998). The constitutive HS sites IV and V (Agarwal and Rao, 1998) and the inducible site VA are indicated. The use of a 5′ promoter probe did not reveal any other inducible sites.

(B) Requirement for calcium and calcineurin. DNase I HS analysis of primary Th2 cells differentiated for 1 week and then either left unstimulated (panel 1) or stimulated for 6 hr with PMA and ionomycin (P + I, panel 2), ionomycin alone (panel 3), or PMA, ionomycin, and CsA (P + I + CsA, panel 4). Membranes were hybridized using the 3′ probe described in (A). The inducible site VA is indicated by an asterisk in panels 2 and 3.

(C) Map of the murine IL-4 locus showing the approximate location of the constitutive DNase I HS sites and the location of the inducible HS site VA. The 3′ probe used in (A) and (B) is indicated. The expanded view shows the 1.2 kb HindIII–SwaI fragment containing the inducible site VA, with the restriction sites used in Figure 3A indicated.

striking induction of IL-5 (Ranganath et al., 1998; Zhang et al., 1998); expression of antisense GATA3 in a differentiated Th2 clone led to decreased expression of IL-4, IL-5, IL-6, and IL-10 (Zheng and Flavell, 1997); expression of dominant-negative GATA3 in transgenic mice led to reduced expression of IL-4, IL-5, and IL-13 (Zhang et al., 1999); and expression of GATA3, even in STAT6-deficient T cells, resulted in production of Th2 cytokines and appearance of the differentiated Th2 pattern of DNase I hypersensitivity on the IL-4 locus (Ouyang et al., 2000).

The regulation of IL-4 gene transcription has been studied by many laboratories. With a few exceptions (Henkel et al., 1992; Kubo et al., 1997; Agarwal and Rao, 1998; Bird et al., 1998; Takemoto et al., 1998), essentially all the focus has been on the proximal IL-4 promoter (reviewed in Brown and Hural, 1997; Rincon and Flavell, 1997a; Szabo et al., 1997b; Murphy, 1998). Transgenic experiments have documented, however, that the IL-4 promoter does not suffice for optimal IL-4 gene transcription in vivo (Wenner et al., 1997). The levels of inducible transcription from an IL-4 promoter-reporter transgene were several orders of magnitude lower than those observed for the endogenous IL-4 gene. Furthermore, although the integrated promoter-reporter construct achieved a considerable degree of Th2 specificity (~40-fold), the level of differential expression was much less impressive than that of the endogenous IL-4 gene. These results point to the existence of distal enhancer elements that cooperate with the IL-4 promoter to modulate IL-4 gene expression in vivo.

Here we have used DNase I hypersensitivity mapping and transient transfection assays to identify a distal IL-4 enhancer located 3′ of the IL-4 gene. The enhancer was discovered as an inducible DNase I hypersensitive (HS) site appearing in stimulated Th2 cells but not in either resting or stimulated Th1 cells. T cells lacking Maf continued to show appearance of both the constitutive and the inducible pattern of DNase I HS sites, suggesting that Maf is not critical for chromatin remodeling of the IL-4 gene by naive T cells or for the activity of the IL-4 enhancer. In contrast, chromatin immunoprecipitation assays showed that the enhancer binds GATA3 in differentiated Th2 cells in vivo, consistent with the possibility that GATA3 is required for enhancer function. Both the IL-4 enhancer and the IL-4 promoter bind the NFAT family member NFAT1 only in stimulated Th2 cells; conversely, the IFN-γ promoter binds NFAT1 only in stimulated Th1 cells. These results define an antigen-responsive, NFAT-dependent, GATA3 binding distal regulatory element in the IL-4 gene, and suggest a mechanism by which nonselectively induced transcription factors such as NFAT may regulate subset-specific transcription of cytokine genes.

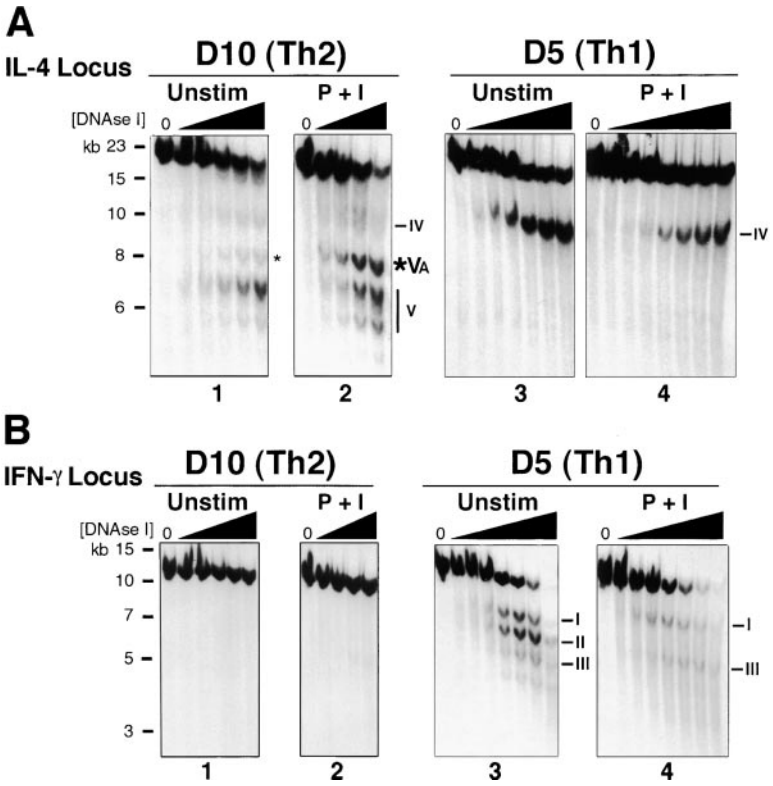

Figure 2. Only Remodeled Cytokine Loci Respond to Acute Stimulation with Further Changes in Chromatin Structure

(A) DNase I HS analysis of the IL-4 locus in D10 (Th2) and D5 (Th1) cells either left unstimulated (panels 1 and 3) or stimulated for 6 hr with PMA and ionomycin (P + I, panels 2 and 4). Membranes were hybridized using the 3′ probe indicated in Figure 1. Constitutive HS sites are indicated, and the inducible HS site V_A is marked with an asterisk.

(B) DNase I HS analysis of the IFN-γ locus in unstimulated and stimulated (P + I) D10 and D5 cells. Blots from (A) were stripped and rehybridized using an IFN-γ exon 4 probe. Th1-specific constitutive HS sites (Agarwal and Rao, 1998) are indicated in panel 3.

Results

Identification of Inducible Hypersensitive Site V_A in the IL-4 Gene

To identify promoter-distal regulatory elements associated with stimulated transcription of the IL-4 gene, we compared DNase I hypersensitivity patterns on the IL-4 locus of unstimulated and stimulated Th2 cells (Figure 1). CD4 T cells, differentiated under polarizing Th2 conditions for 1 week, showed the expected constitutive pattern of DNase I hypersensitivity (Agarwal and Rao, 1998) (Figure 1A, left panel). These resting cells did not produce detectable levels of IL-4 transcripts. In contrast, cells stimulated with plate-bound anti-CD3 produced abundant levels of IL-4 mRNA and exhibited a novel, inducible HS site (Figure 1A, right panel). We termed this inducible HS site "V_A" ("five A") based on its proximity to the Th2-specific constitutive HS site V ("five") and its dependence on activation. Site V_A was also observed in primary Th2 cells and Th2 clones stimulated either with phorbol ester (PMA) plus calcium ionophore (ionomycin) or with ionomycin alone (Figure 1B, panels 2 and 3), and its appearance was abrogated by the immunosuppressive agent cyclosporin A (CsA) (Figure 1B, panel 4). Site V_A mapped ~12 kb 3′ of the IL-4 promoter and 5 kb 3′ of the end of the IL-4 coding region (Figure 1C).

Appearance of Inducible HS Site V_A Is Th2 Restricted but Does Not Require the Transcription Factor cMaf

The formation of site V_A was strictly Th2 restricted in vivo (Figure 2). Stimulation of the D10 Th2 clone with PMA plus ionomycin resulted in strong induction of site V_A (Figure 2A, panels 1 and 2). In contrast, stimulation of the D5 (Ar-5) Th1 clone did not result in induction of site V_A, nor did it cause any other change in DNase I HS patterns throughout the IL-4 locus (Figure 2A, panels 3 and 4). However, D5 cells displayed clear changes in the chromatin structure of the IFN-γ locus following PMA plus ionomycin stimulation, with the most prominent effect being disappearance of HS site II (Figure 2B, panels 3 and 4). No marked changes were observed in the DNase I HS pattern of the IFN-γ locus in stimulated D10 cells (Figure 2B, panels 1 and 2). Therefore, despite identical stimulation of the D5 Th1 and D10 Th2 cell clones, the changes in chromatin structure that correlated with active transcription were restricted to those cytokine loci that had undergone stable chromatin remodeling during T cell differentiation.

The transcription factor Maf, which is expressed at elevated levels in Th2 cells relative to Th1 cells, has been implicated in IL-4 gene transcription (Ho et al., 1996, 1998; Kim et al., 1999). To determine whether Maf contributes to the changes in chromatin structure on the IL-4 locus, either during Th2 differentiation or during acute stimulation of differentiated Th2 cells, we incubated CD4 T cells from normal and Maf-deficient mice with IL-4 and anti-IL-12 (Th2 differentiation conditions). Following this period of differentiation, we compared DNase I HS patterns in resting and PMA plus ionomycin stimulated cells (Figure 3). DNase I hypersensitivity mapping with a 5′ probe encompassing the IL-4 promoter revealed no differences in the resting DNase I HS pattern of normal versus Maf-deficient T cells (Figure 3A), indicating that Maf is not required for the long-range changes in chromatin structure that occur in the IL-4 genetic locus during Th2 differentiation. Likewise, the

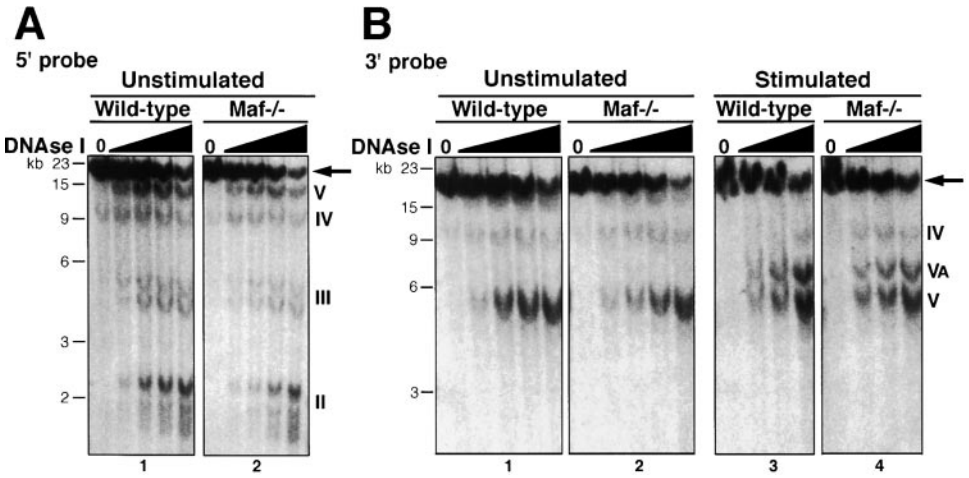

Figure 3. The Th2-Restricted Transcription Factor Maf Is Not Required for Formation of Constitutive or Inducible DNase I HS Sites in the IL-4 Locus

CD4 T cells were purified from the lymph nodes and spleens of wild-type or Maf-deficient mice and differentiated under strongly polarizing Th2 conditions for one week.

(A) DNase I HS analysis was performed on resting cultures of wild-type and Maf$^{-/-}$ Th2 cells. Genomic DNA was digested to completion with BamHI and subjected to Southern analysis using a probe corresponding to the 5′ end of the IL-4 locus.

(B) Wild-type and Maf$^{-/-}$ Th2 cells were either left unstimulated (panels 1 and 2) or stimulated for 6 hr with 20 nM PMA and 2 μM ionomycin (panels 3 and 4). Cells were harvested and subjected to DNase I HS analysis. Membranes were hybridized using a probe corresponding to the 3′ end of the IL-4 locus (as in Figure 1).

use of a probe from the 3′ end of the IL-4 locus revealed no differences in DNase I HS patterns in either resting or stimulated cells (Figure 3B), indicating that Maf also does not play a role in the stimulation-dependent appearance of the inducible HS site VA. These results are consistent with the finding that Maf regulates expression of only the IL-4 gene in the IL-4/IL-5/IL-13 gene cluster by virtue of its ability to bind a site in the IL-4 promoter (Ho et al., 1996, 1998; Kim et al., 1999).

Hypersensitive Site VA Exhibits Inducible, CsA-Sensitive Enhancer Activity in Th2 Cells

To determine whether the inducible HS site VA plays a functional role in IL-4 gene transcription, we localized it to a short restriction fragment and tested its function in transient transfection assays (Figure 4). First, we used HS analysis to map site VA to a 438 bp EcoRI–SwaI fragment, hereafter termed the core IL-4 enhancer (Figure 4A). The restriction map of this region is shown in Figure 1C and its sequence in Figure 4B. Restriction enzyme accessibility experiments confirmed that the SwaI site was localized to the vicinity of the inducible hypersensitive site (Figure 4C). When SwaI was diffused into intact nuclei, its ability to cleave the IL-4 locus increased after stimulation of D10 but not D5 cells (Figure 4C, compare lanes 2 and 4 with 6 and 8), indicating that the "accessibility" of this genomic region to both DNase I and SwaI increased in a Th2-specific manner upon stimulation.

DNA fragments containing HS site VA behaved as inducible, CsA-sensitive enhancer elements in transient transfection assays in D10 cells (Figure 5). A single copy of a 438 bp EcoRI–SwaI fragment spanning the core enhancer conferred both PMA/ionomycin responsiveness and CsA sensitivity on a luciferase reporter plasmid driven by a minimal SV40 promoter (Figure 5A; pGL3-VA 438). A shorter (300 bp) PstI–SwaI fragment retained essentially all the enhancer activity of the 438 bp fragment (Figure 5A; pGL3-VA 300). When tested with the IL-4 promoter, the fragment conferred 60-fold enhancement of induced transcription in stimulated D10 cells compared to 30-fold enhancement with the IL-4 promoter alone (Figure 5B). The lower effect of the enhancer in combination with the IL-4 promoter reflects the fact that the IL-4 promoter itself is highly active in stimulated cells. The enhancer conferred no increase in basal transcription from either the SV40 promoter or the IL-4 promoter in unstimulated D10 T cells, indicating that stimulation was absolutely required for enhancer activity (Figures 5A and 5B).

Subset-Restricted Binding of NFAT to Cytokine Regulatory Regions In Vivo

The requirements for appearance of site VA (Figure 1) and the properties of the site VA enhancer (Figure 5) paralleled the requirements for nuclear translocation and activation of NFAT (Rao et al., 1997). Inspection of the nucleotide sequence of the enhancer fragment (Figure 4B) revealed four potential NFAT binding sites (T/AGGAA; Rao et al., 1997), which bound NFAT when tested in electrophoretic mobility shift assays (EMSA) in vitro. Binding was nonselective, since it was observed with nuclear extracts from stimulated D5 as well as D10 T cells (data not shown). These results confirm previous work demonstrating equivalent induction of NFAT in nuclear extracts from stimulated Th1 and Th2 cells (Rooney et al., 1994; Li-Weber et al., 1997; Rincon and Flavell, 1997b).

To reconcile the nonselective binding of NFAT to site VA in vitro with the Th2-specific appearance of site VA in vivo (Figure 2), we used chromatin immunoprecipitation

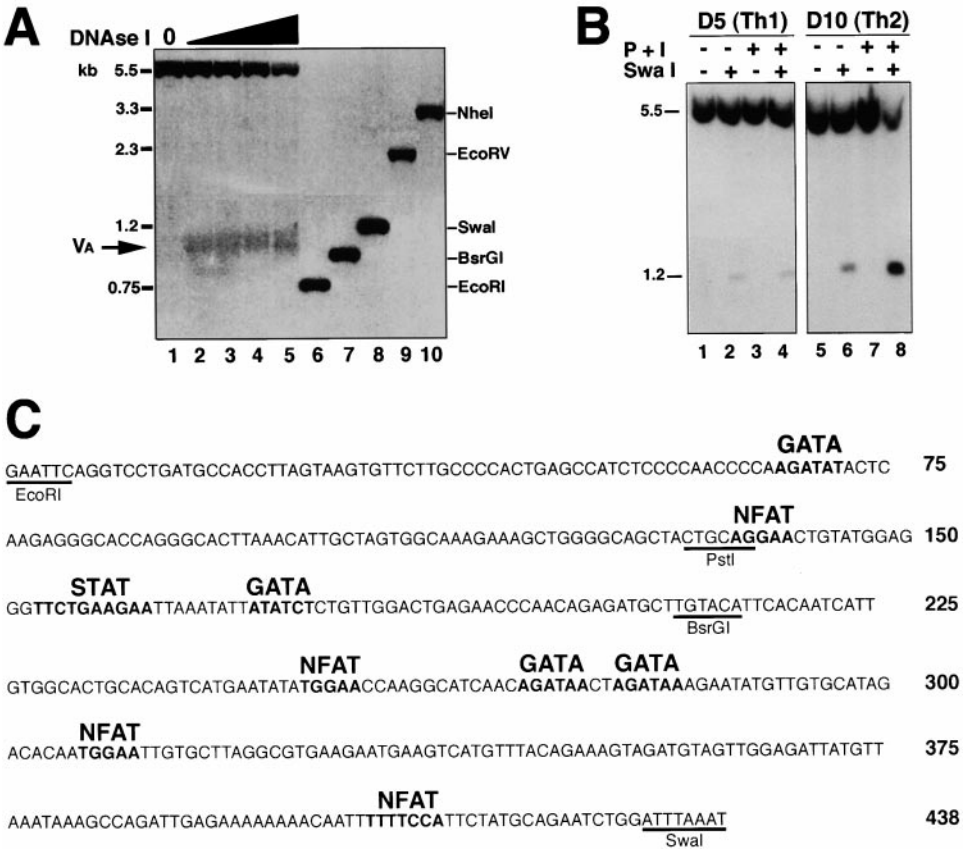

Figure 4. Localization and Sequence of Inducible HS Site VA

(A) Mapping of DNase I HS site VA to a 438 bp fragment EcoRI–SwaI fragment 3′ of the IL-4 gene. Lanes 1–5, D10 nuclei were subjected to DNase I titration, and the genomic DNA was digested to completion with HindIII and analyzed on a Southern blot. Lanes 6–10, markers were provided by D10 genomic DNA digested to completion with Hind III and the indicated restriction enzymes. Membranes were hybridized with a probe derived from the 5′ end of a 5.5 kb HindIII fragment containing both sites V and VA (see Figure 1C). The top half of this autoradiogram and the lanes containing the markers were exposed for a shorter time than the bottom half, necessitated by the fact that the intensity of the parent band and of the markers was much greater than that of the hypersensitive band.

(B) Restriction enzyme accessibility analysis of the SwaI site in resting and stimulated D5 and D10 cells. Cells were left unstimulated (−) or were stimulated (+) for 6 hr with PMA and ionomycin (P + I). Nuclei were prepared and left untreated (−) or treated (+) for 1 hr with SwaI, following which DNA was purified and digested to completion with HindIII. Southern blots were hybridized as described in (A). The relative intensities of the 1.2 kb band in lanes 2, 4, 6, and 8 (expressed as a percentage of the total intensity in the lane) were 2.5%, 3.0%, 7.7%, and 40.6%, respectively.

(C) Nucleotide sequence of the 438 bp EcoRI–SwaI fragment encompassing the IL-4 enhancer. Relevant restriction enzyme sites are underlined and labeled. Sequences that match consensus binding motifs (on either DNA strand) for NFAT (A/TGGAA) (Rao et al., 1997), GATA (A/TGATAA/G) (Ko and Engel, 1993), and STAT [TTC(X)$_{3-4}$GAA] (Schindler et al., 1995) transcription factors are shown in bold.

(ChIP) assays (Figure 6). The ChIP technique can establish whether a known transcription factor truly binds in the vicinity of a known regulatory element in living cells (Parekh and Maniatis, 1999). Three of the four calcium-regulated NFAT proteins, NFAT1, NFAT2 and NFAT4, are expressed in T cells (Timmerman et al., 1996; Lyakh et al., 1997); of these, we chose to examine NFAT1 since it is the most abundant NFAT protein in T cells, constituting 80%–90% of total NFAT (Xanthoudakis et al., 1996). Moreover, NFAT1 can potently transactivate the IL-4 promoter, especially in combination with Maf (Ho et al., 1996; Hodge et al., 1996a). ChIP assays using anti-NFAT1 antibodies showed that NFAT1 bound to both the IL-4 promoter and IL-4 enhancer regions in an inducible and Th2-specific manner in vivo: that is, PCR products were generated with the IL-4 promoter and enhancer primers only when the DNA–protein complexes ("chromatin") were immunoprecipitated from stimulated D10 (Th2) but not D5 (Th1) cells (Figure 6A, compare lanes 3 and 4). Conversely, NFAT1 bound to the endogenous IFN-γ promoter, which contains a putative NFAT site (Rao et al., 1997), only in stimulated D5 cells (Figure 6B, compare lanes 3 and 4). Control experiments showed that the IL-4 promoter and enhancer sequences were present at equivalent concentrations in template DNA from D5 and D10 cells (Figure 6C). ChIP analysis using primary differentiated Th1 and Th2 cells confirmed that NFAT1 binding to the IL-4 enhancer was dependent on stimulation, as expected from the fact that NFAT1 is nuclear only in stimulated cells (Rao et al., 1997) (Figure 6D). We conclude that the IL-4 promoter and enhancer are selectively accessible to NFAT1 binding in Th2 cells, while the IFN-γ promoter is selectively accessible to NFAT1 binding in Th1 cells.

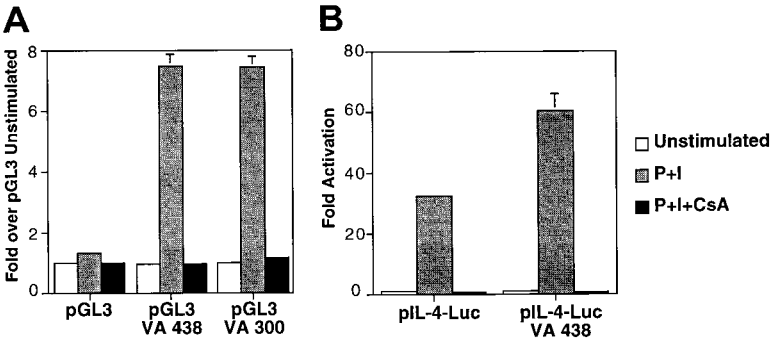

Figure 5. The Inducible HS Site VA Contains an Inducible, CsA-Sensitive Transcriptional Enhancer

(A) SV40 minimal promoter: D10 cells were transfected with an SV40 promoter-luciferase plasmid (pGL3) or the same plasmid containing either the 438 bp EcoRI–SwaI fragment (pGL3-VA 438) or the 300 bp PstI–SwaI fragment (pGL3-VA 300). Twenty-four hours after transfection, cells were left unstimulated or stimulated for 8 hr with PMA and ionomycin (P + I) or PMA, ionomycin, and CsA (P + I + CsA). Results are represented as fold activation relative to the luciferase activity of pGL3 in unstimulated cells (set at 1; range 30,000–100,000 relative luciferase units). Bars indicate standard deviations of three independent experiments, which in several cases are too small to be visible.

(B) IL-4 promoter: D10 cells were transfected with an IL-4 promoter-luciferase construct (pIL4-Luc) or the same construct containing the 438 bp EcoRI–SwaI fragment containing HS site VA (pIL4-Luc VA). Twenty-four hours after transfection, cells were left unstimulated or stimulated for 8 hr with PMA and ionomycin (P + I) or PMA, ionomycin, and CsA (P + I + CsA). Results are represented as fold activation relative to the luciferase activity of pIL-4-Luc in unstimulated cells (set at 1; range 150,000–200,000 relative luciferase units). Bars indicate standard deviations of three independent experiments, which in several cases are too small to be visible.

The Th2-Restricted Transcription Factor GATA3 Binds to the IL-4 Enhancer In Vivo

The core IL-4 enhancer also contained several putative binding sites for the Th2-restricted transcription factor GATA3, identified by visual inspection as related to the consensus sequence A/TGATAA/G (Ko and Engel, 1993) (Figure 4B). To determine whether GATA3 indeed bound to the IL-4 enhancer region in vivo, we again used ChIP assays. The results showed that GATA3 binds in vivo to the IL-4 enhancer region in D10 Th2 cells but not in D5 Th1 cells that do not express GATA3 (Figure 7A, compare lanes 3 and 4). GATA3 was not observed to bind to the IL-4 promoter in Th2 cells (Figure 7B, lane 4), despite the presence of several reasonably good GATA binding sites (Ranganath et al., 1998; Zhang et al., 1998). In control experiments, the IL-4 promoter region could be detected with the same primers after immunoprecipitation with anti-NFAT1 (Figure 7C, lane 4). Presumably, GATA3 binds with higher affinity to the putative sites in the IL-4 enhancer, relative to the sites present in the IL-4 promoter (Ko and Engel, 1993). However, because of the intrinsic limitations of the ChIP technique (the immunoprecipitated chromatin contains DNA fragments of average size ~1 kb), we cannot definitively identify the specific GATA sites to which GATA3 is bound, although they are clearly located in the vicinity of the IL-4 enhancer region and are likely to include those identified as residing within the core IL-4 enhancer in Figure 4C.

Discussion

In this work, we have defined an inducible DNase I HS site, site VA, located 3′ of the IL-4 gene. Several arguments support the hypothesis that site VA functions as a distal IL-4 enhancer in vivo, cooperating with the IL-4 promoter to enable optimal, antigen-dependent and Th2-restricted expression of IL-4. First, the IL-4 promoter is clearly insufficient to support high-level and fully Th2-specific transcription of a linked reporter gene in vivo (Wenner et al., 1997), and thus distal regulatory regions are implicated. Second, the inducible, CsA-sensitive appearance of site VA in primary Th2 cells mirrors the features of endogenous IL-4 transcription, as well as the properties of the site VA enhancer in transient transfection assays. Third, the site VA enhancer is the only inducible HS site in the immediate vicinity of the IL-4 gene. The locus is bounded at its 5′ and 3′ ends by the IL-13 gene and the CNS-specific gene KIF3 (Frazer et al., 1997). The DNase I HS pattern across this 31 kb interval has been mapped, and HS site VA is the only HS site that is completely restricted to activated Th2 cells (Takemoto et al., 1998; this report; S. A., unpublished data).

Our results are consistent with a large body of other evidence, suggesting that the Th2-specific transcription factor Maf is involved in regulating the inducible phase of IL-4 gene transcription rather than in broadly implementing a Th2 genetic program (Ho et al., 1996, 1998; Kim et al., 1999). Maf-deficient T cells show a relative defect in IL-4 but not IL-5 or IL-13 production (Kim et al., 1999); conversely, Th2 cells from Maf-transgenic mice show a tendency to overexpress IL-4 but not the other Th2 cytokines (Ho et al., 1998). Most importantly, Maf overexpression in transgenic mice does not result in IL-4 production by differentiated Th1 cells, indicating that Maf does not force a Th2 program when ectopically expressed (Ho et al., 1998). In complete accordance with these findings, we have shown that Maf-deficient T cells are as competent as wild-type T cells in acquiring the differentiated Th2 pattern of DNase I hypersensitivity during the first stimulation of primary T cells with antigen. Thus, despite the fact that Maf is a direct or indirect target of STAT6 and GATA3 (Kurata et al., 1999; Ouyang et al., 2000), it is unlikely to play a major role in regulating the acquisition of transcriptional competence on the IL-4 locus during Th2 differentiation. Differentiated Maf-deficient Th2 cells also showed no impairment, relative to wild-type Th2 cells, in the appearance of the inducible DNase I HS site VA upon stimulation. This result indicates that Maf does not play a critical role in the function of the IL-4 enhancer. Rather, the effect of Maf may be localized to its known binding site in the proximal promoter (Ho et al., 1996; Cron et al., 1999).

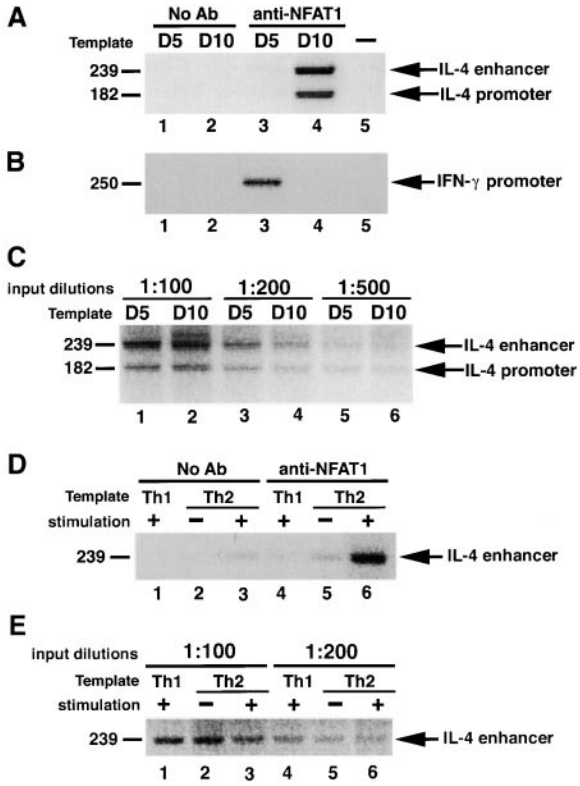

Figure 6. Specific In Vivo Binding of NFAT to the IL-4 Promoter and Enhancer in Th2 Cells, and the IFN-γ Promoter in Th1 Cells

(A) In vivo binding of NFAT1 to the IL-4 promoter and the IL-4 enhancer in stimulated D5 (Th1) and D10 (Th2) cells was assessed by chromatin immunoprecipitation (ChIP). DNA–protein complexes were immunoprecipitated without antibodies ("No Ab") or with a cocktail of antibodies specific for NFAT1. PCR primers specific for the IL-4 enhancer ("site VA") and the IL-4 promoter amplify products of 239 bp and 182 bp, respectively.

(B) The same DNA purified in (A) was amplified using primers specific for the IFN-γ promoter, which amplify a product of 250 bp.

(C) As a control for parts (A) and (B), to show that DNA–protein complexes from D5 Th1 and D10 Th2 cells contained equivalent levels of IL-4 promoter/enhancer target sequences, PCR was performed directly on dilutions of DNA from complexes not subjected to immunoprecipitation.

(D) ChIP analysis of CD4 T cells differentiated under Th1 or Th2 conditions for 1 week and either left unstimulated (−) or stimulated (+) for 6 hr with PMA plus ionomycin. DNA was amplified with primers corresponding to the IL-4 enhancer.

(E) As a control for part (D), to show that DNA–protein complexes from stimulated Th1 and unstimulated or stimulated Th2 cells contained equivalent levels of IL-4 enhancer target sequences, PCR was performed directly on dilutions of DNA from complexes not subjected to immunoprecipitation.

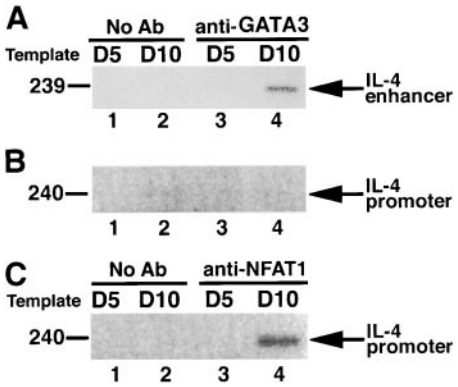

Figure 7. GATA3 Binds In Vivo to the IL-4 Enhancer but Not the Promoter

In vivo binding of GATA3 to the IL-4 promoter and the IL-4 enhancer in stimulated D5 (Th1) and D10 (Th2) cells was assessed by ChIP.

(A) DNA–protein complexes were immunoprecipitated without antibodies ("No Ab") or with anti-GATA3 antibodies. The PCR primers were specific for the IL-4 enhancer (239 bp).

(B) The same DNA purified in (A) was amplified using primers specific for the IL-4 promoter.

(C) As a control, the IL-4 promoter region could be detected with the same primers as in (B) after immunoprecipitation of DNA–protein complexes with anti-NFAT1 antibodies.

In contrast to Maf, GATA3 appears to play a major role in specifying the Th2 phenotype (Zheng and Flavell, 1997; Zhang et al., 1999; Ouyang et al., 2000). GATA3 is essential for T cell development (Hattori et al., 1996; Ting et al., 1996); thus, analyses of GATA3-deficient T cells await the development of a conditional GATA3 knockout mouse. Ectopic overexpression of GATA3 in cell lines and in mice results in increased expression of a spectrum of Th2 cytokines; conversely, overexpression of antisense and dominant-negative GATA3 in cell lines and in mice results in impaired Th2 cytokine production (Zheng and Flavell, 1997; Ranganath et al., 1998; Zhang et al., 1998, 1999; Ouyang et al., 2000). While it is clear that GATA3 acts directly on the IL-5 promoter (Zhang et al., 1997; Lee et al., 1998), the mechanism through which it promotes IL-4 gene expression has been controversial (Zheng and Flavell, 1997; Ranganath et al., 1998; Zhang et al., 1998, 1999). Our results showing that GATA3 binds to the site VA enhancer provide a site of action for this key transcription factor in regulating IL-4 expression. In addition, GATA3 may act through other GATA binding elements dispersed throughout the IL-4/IL-13 locus, which have been shown to possess enhancer function in transient reporter assays (Ranganath et al., 1998).

We have shown that the distal site VA enhancer contains binding sites for the antigen-induced transcription factor NFAT and binds the family member NFAT1 in vivo. The ChIP assay only detects binding and cannot by itself establish whether a given protein acts as a transcriptional activator or repressor when bound to a specific genomic region in vivo. Nevertheless, careful analysis of the phenotype of NFAT1-deficient mice (Kiani et al., 1997) suggests that NFAT1 activates transcription when bound to the IL-4 promoter and enhancer sites. T cells from NFAT1-deficient mice show a mild degree of Th2 skewing and hyperproduce Th2 cytokines (Hodge et al., 1996b; Xanthoudakis et al., 1996), a phenotype that is greatly exaggerated in mice lacking both NFAT1 and NFAT4 (Ranger et al., 1998a); in contrast, T cells lacking NFAT2 show a relative impairment of IL-4 production (Ranger et al., 1998b; Yoshida et al., 1998). These observations have led to the hypothesis that NFAT2 is a positive regulator of IL-4 gene expression, while NFAT1 and NFAT4 act solely as negative regulators. This interpretation cannot be strictly accurate: NFAT1-deficient T cells

show no difference in peak levels of IL-4 transcripts relative to wild-type T cells but rather a prolongation of the late phase of IL-4 transcription (Kiani et al., 1997). If NFAT1 acted solely as a transcriptional repressor, a 5- to 10-fold increase in peak levels would be expected, since NFAT1 accounts for 80%–90% of total NFAT and is present in wild-type cells at 5- to 10-fold higher levels than in NFAT2 (Xanthoudakis et al., 1996). Thus, it is likely that NFAT1, NFAT2, and NFAT4 all activate the early phase of IL-4 gene transcription, but only NFAT1 and NFAT4 are able to induce negative feedback mechanisms that blunt the late phase of IL-4 gene transcription (Kiani et al., 1997; Miaw et al., 2000) (reviewed in Agarwal et al., 1999; Kiani et al., 2000).

The most surprising aspect of our studies was that NFAT1 binding to cytokine regulatory regions was highly subset-specific in vivo. NFAT proteins are known to be expressed at equivalent levels in Th1 and Th2 cells and to bind equivalently in vitro to naked DNA containing the IL-4 enhancer and the IL-4 promoter (Rooney et al., 1994; Li-Weber et al., 1997; Rincon and Flavell, 1997b; O. A., unpublished data). Nevertheless, NFAT1 bound to the IL-4 promoter/enhancer regions in vivo only in Th2 cells and to the IFN-γ promoter region only in differentiated Th1 cells. These results predict that mechanisms operating in the chromatin context restrict the access of NFAT to cytokine regulatory regions in vivo. One hypothesis is that although NFAT1 can bind independently to the regulatory regions in vitro, it is only capable of binding them in the context of chromatin if subset-specific transcription factors such as GATA3 are also present in the nucleus. Another, not mutually exclusive, hypothesis is that the chromatin structure of the IL-4 enhancer and promoter is selectively altered during Th2 differentiation so that these regions become accessible to NFAT1 only in differentiated (and stimulated) Th2 cells. For instance, transcription factors such as STAT6 and NFAT, which are acutely induced during the first antigen plus cytokine stimulation of naive T cells, might bind transiently to regulatory regions of the IL-4 gene; this binding might then promote stable alterations in chromatin structure, histone acetylation, or DNA methylation status of the adjacent regions that persist in the differentiated Th2 cells after the initial stimulus has died away. In this context, it is noteworthy that both the IL-4 promoter (Lederer et al., 1996) and the site V_A enhancer (Figure 4B) contain binding sites for STAT and NFAT. The functions of NFAT and STAT6 would diverge in the secondary stimulation of differentiated T cells, since acute transcription of the IL-4 gene requires antigen stimulation and NFAT but is known not to be as dependent on IL-4 stimulation and STAT6 (Huang et al., 1997). A precedent for such a model is provided by studies of the HO endonuclease gene in yeast (Cosma et al., 1999). In this system, transient binding of the SWI5 transcription factor results in recruitment of the SWI/SNF chromatin remodeling complex and the SAGA histone acetyltransferase complex to the HO gene; these enzyme complexes induce chromatin structural changes that promote the stable binding of a cell-type-specific transcription factor, SBF. We are currently investigating whether analogous mechanisms are involved in chromatin remodeling and acquisition of transcriptional competence by the IL-4 genetic locus.

A prediction from our data is that distal enhancer elements may be involved in regulating the expression of many if not all NFAT-dependent genes. This is especially likely in the case of the IFN-γ gene and other genes that might possess similarly weak and poorly cell-type-restricted proximal promoter regions (Penix et al., 1993, 1996; Campbell et al., 1996; Young, 1996; Aune et al., 1997; Sweetser et al., 1998). The array of NFAT sites in the IL-4 enhancer is reminiscent of the multiple NFAT sites present in the IL-4 and IL-2 promoters and the IL-3 and GM-CSF enhancers (reviewed in Rao et al., 1997). The apparent redundancy of NFAT binding to these cytokine promoter and enhancer regions resembles the situation at the β-globin locus, where related transcriptional activators bind at different stages of erythroid development to recurring sequence motifs in the locus control region and in the globin gene promoters (Orkin, 1995). The use of widely dispersed regulatory regions, containing redundant binding sites for the same family of transcription factors, may be a general strategy to limit the inappropriate expression of cell-type-specific genes.

Experimental Procedures

Mice

C57BL/6J and CAF1/J mice (Jackson Laboratories, Bar Harbor, Maine) were maintained in pathogen-free conditions in barrier facilities at the Center for Animal Resources and Comparative Medicine, Harvard Medical School. c-Maf$^{-/-}$ mice (Kim et al., 1999) were kindly provided by L. H. Glimcher (Harvard School of Public Health).

DNase I Hypersensitivity Analysis

The murine T cell clones D5 (Ar-5; Rao et al., 1984) and D10 (D10.G4.1; Kaye et al., 1983) were maintained as previously described (Agarwal and Rao, 1998). CD4 T cells were purified from C57BL/6J mice and differentiated in the Th1 or Th2 direction as previously described (Agarwal and Rao, 1998). Isolation and DNase I digestion of nuclei and purification of genomic DNA were performed as previously described (Agarwal and Rao, 1998; Cockerill, 2000) using primary Th2 cells differentiated for 2 weeks, which were either left unstimulated or stimulated for 6 hr with immobilized anti-CD3ε or with 20 nM PMA plus 2 μM ionomycin in the presence or absence of 2 μM CsA.

Probe locations are depicted in locus diagrams. The probe from the 5′ end of a 19 kb BamHI fragment spanning the IL-4 locus has been described (Agarwal and Rao, 1998). A similar probe was generated from the 3′ end of the same fragment (Figures 1–3; location indicated in Figure 1C). For fine mapping of HS site V_A, a probe from the 5′ end of a 5.5 kb HindIII fragment spanning HS sites V and V_A was used. The IL-4 probes (5′ BamHI 19 kb, 3′ BamHI 19 kb, and 5′ HS V/V_A) were created by digestion of plasmids containing these probes and gel purification of the fragments. The exon 4 probe from the IFN-γ gene (Agarwal and Rao, 1998) was generated by PCR and gel purified. The template for PCR was a murine IFN-γ genomic clone (kindly provided by H. A. Young).

Restriction Enzyme Accessibility

Accessibility of the IL-4 locus to the restriction enzyme SwaI was assessed as described (Boyes and Felsenfeld, 1996). Briefly, SwaI (150 U) was incubated for 1 hr at room temperature with intact nuclei from unstimulated and stimulated T cells (20 nM PMA plus 2 μM ionomycin for 6 hr). DNA was purified and digested to completion with HindIII. The probe is the same as that used for the fine mapping of site V_A (5′ end of a 5.5 kb HindIII fragment encompassing both sites V and V_A). Relative hybridization intensities were assessed using the program NIH *Image* 1.61.

Transient Transfection Assays

A single copy of the 438 bp EcoRI–SwaI fragment or the 300 bp PstI–SwaI fragment spanning HS site V_A was cloned 5′ of the minimal SV40 promoter in the luciferase reporter plasmid pGL3 (Promega). D10 cells were electroporated (960 μF/270 V) and rested overnight. Transfected cells were divided and either left unstimulated or stimulated for 8 hr with PMA (20 nM) and ionomycin (2 μM) or PMA, ionomycin, and CsA (2 μM). CsA was added 20 min before the stimuli. Cell lysates were harvested and luciferase activity was assessed using an automated luminometer (Berthold).

Chromatin Immunoprecipitation (ChIP) Assays

ChIP analysis was carried out essentially as described (Parekh and Maniatis, 1999). Unstimulated or stimulated (6 hr, 20 nM PMA plus 2 μM ionomycin) D5 Th1 cells and D10 Th2 cells (5 to 15 × 10^7) or CD4 T cells differentiated under Th1 or Th2 conditions for 1 week (Agarwal and Rao, 1998) were cross-linked using formaldehyde. Nuclei were isolated and sonicated, and DNA–protein complexes were purified by CsCl gradient centrifugation. The chromatin was immunoprecipitated using a cocktail (5 μg) of anti-67.1 and anti-NFAT1-C antibodies specific for the family member NFAT1 (Wang et al., 1995) or with 5 μg of anti-GATA3 antibodies (Santa Cruz). Following deproteination and reversal of cross-links, the presence of selected DNA sequences was assessed by PCR. The primers used were as follows: site V_A, 5′-AGGGCACTTAAACATTGC-3′ and 5′-ACGCCTAAGCACAATTCC-3′ (239 bp product); IL-4 promoter, 5′-TTGGTCTGATTTCACAGG-3′ and either 5′-AACAATGCAATGCT GGC-3′ (182 bp product) or 5′-ATCAATAGCTCTGTGCCG-3′ (240 bp product); IFN-γ promoter, 5′-GCTCTGTGGATGAGAAAT-3′ and 5′-AAGATGGTGACAGATAGG-3′ (250 bp product).

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