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Modulation of Chromatin Structure Regulates Cytokine Gene Expression during T Cell Differentiation

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Summary

Differentiating cells undergo programmed alterations in their patterns of gene expression, which are often regulated by structural changes in chromatin. Here we demonstrate that T cell differentiation results in long-range changes in the chromatin structure of effector cytokine genes, which persist in resting Th1 and Th2 cells in the absence of further stimulation. Differentiation of naive T helper cells into mature Th2 cells is associated with chromatin remodeling of the IL-4 and IL-13 genes, whereas differentiation into Th1 cells evokes remodeling of the IFNγ but not IL-4 or IL-13 genes. IL-4 locus remodeling is accompanied by demethylation and requires both antigen stimulation and STAT6 activation. We propose that chromatin remodeling of cytokine gene loci is functionally associated with productive T cell differentiation and may explain the coordinate regulation of Th2 cytokine genes.

Introduction

Helper T (Th) lymphocytes undergo two spatially and temporally distinct phases of differentiation. Following the first developmental phase, which occurs in the thymus, a second phase triggered by initial encounter with antigen in the periphery leads to the development of effector T helper cell subsets displaying mutually exclusive patterns of cytokine gene expression (Mosmann and Coffman, 1989; Paul and Seder, 1994; Murphy, 1998). T helper type-1 (Th1) cells characteristically transcribe the IFNγ, interleukin-2 (IL-2), and lymphotoxin genes, whereas Th2 cells express the IL-4, IL-5, IL-6, IL-10, and IL-13 genes. Both Th1 and Th2 cells appear to derive from a common naive precursor cell whose differentiation pathway is determined by cytokine and costimulatory signals during primary antigenic stimulation (Bluestone, 1995; Abbas et al., 1996; O'Garra, 1998). Specifically, Th1 differentiation is driven by IL-12 and requires the IL-12-responsive transcription factor STAT4 (Kaplan et al., 1996a; Thierfelder et al., 1996), while Th2 differentiation is elicited by IL-4 and requires the IL-4-responsive transcription factor STAT6 (Kaplan et al., 1996b; Shimoda et al., 1996; Takeda et al., 1996). In vivo, progressive polarization of the cytokine response occurs in response to chronic antigenic stimulation as a result of the self-amplification and negative cross-regulation inherent in T cell differentiation (Abbas et al., 1996). Clinically, Th1 patterns of cytokine production are associated with inflammation and autoimmune disease, while Th2 patterns are characteristic of allergic responses and asthma (Abbas et al., 1996; Romagnani, 1997; O'Garra, 1998).

Differential cytokine production by activated Th1 and Th2 cells is controlled at the level of gene transcription. In particular, transcriptional regulation of the IL-4 gene has been intensely investigated due to the potent and broad immunomodulatory effects of IL-4 in states of both health and disease. IL-4 gene expression is tightly regulated, displaying pronounced tissue specificity and a strict requirement for antigen activation (Rincon and Flavell, 1997; Szabo et al., 1997). The basis for activation-induced expression of IL-4 has been elucidated by detailed molecular analysis of the IL-4 promoter (reviewed in Casolaro et al., 1996; Brown and Hural, 1997). Transcription factors induced and synthesized in response to antigen-receptor stimulation, such as NFAT and AP-1 family members, bind to specific regions of the IL-4 proximal promoter to activate transcription in IL-4 producing cells (Chuvpilo et al., 1993; Szabo et al., 1993; Rooney et al., 1995). Moreover, the Th2-specific transcription factors c-Maf (Maf) and GATA-3 have been shown to *trans*-activate IL-4 promoter–reporter constructs and elicit transcription of the endogenous IL-4 gene when ectopically expressed in IL-4 nonproducing cells (Ho et al., 1996; Zheng and Flavell, 1997).

While analysis of proximal promoter regions has yielded considerable insight into control of IL-4 gene expression, transcriptional regulation of endogenous gene loci occurs at multiple levels in eukaryotes (Ernst and Smale, 1995). Several considerations prompted us to examine the chromatin structure and locus accessibility of cytokine genetic loci during T cell differentiation. First, differentiated effector T cells show much higher levels and more rapid kinetics of cytokine gene transcription than naive T helper cells. For instance, IL-4 transcripts peak at high levels in effector Th2 cells after only 4 hr of stimulation, while IL-4 transcripts are expressed at ~100-fold lower levels in naive cells and their detection requires 16–24 hr of stimulation (Le Gros et al., 1990; Croft and Swain, 1995; Lederer et al., 1996). Conceivably, changes in chromatin structure could explain both the increase in transcriptional competence of cytokine gene loci during Th differentiation and the differential expression of cytokine genes in differentiated Th1 and Th2 cells. Second, cytokine expression by a mature T cell is effectively a "memory" response, since the context of initial antigenic stimulation is reflected in the pattern of inducible gene expression at much later times; mechanistically, this memory could be imprinted through chromatin changes that occur at the time of initial differentiation and are maintained through cell division (Wolffe, 1994; Hagstrom and Schedl, 1997).

We show here that long-range differences in chromatin structure reflect the transcriptional competence of the IL-4, IL-13, and IFNγ genes in differentiated T helper cells and may underlie the ability of these T cells to support rapid, high-level transcription of cytokine genes. By detailed study of the IL-4 locus, we have shown that

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differentiating Th2 cells develop a polarized chromatin structure on the IL-4 locus that is stably maintained in the absence of transcription through the locus. Development of this accessible chromatin structure occurs rapidly and specifically in differentiating Th2 cells, and requires both antigenic stimulation and IL-4-mediated STAT6 activation. We conclude that chromatin remodeling of cytokine gene loci is functionally associated with the acquisition of T helper cell phenotype and may explain the coordinate regulation of Th2 cytokine genes.

Results

Accessibility of the IL-4 And IFNγ Genetic Loci Reflects the Ability to Transcribe These Cytokine Genes

We analyzed the chromatin structure and accessibility of the IL-4 gene locus in terminally differentiated T cell clones using DNase I hypersensitivity analysis. D5 (Ar-5 [Rao et al., 1984]) cells are of the Th1 phenotype, expressing IFNγ and IL-2 transcripts after stimulation with immobilized anti-CD3, whereas D10 (D10.G4.1 [Kaye et al., 1983]) cells are of the Th2 phenotype and express IL-4, IL-5, IL-6, IL-10, and IL-13 transcripts upon stimulation (Figure 1A). DNase I hypersensitivity analysis was conducted using unstimulated D5 and D10 cells, which express undetectable or very low levels of cytokine mRNA transcripts, so that chromatin structure and locus accessibility could be assessed under conditions of minimal gene transcription (Figure 1A, lanes 1 and 3).

Our experiments indicated that resting D5 and D10 cells display marked differences in chromatin configuration on the IL-4 locus (Figure 1B). The Th2 clone D10 displayed an accessible chromatin configuration with a complex pattern of DNase I hypersensitivity comprised of 5 clusters of hypersensitive (HS) sites within a 19 kb BamHI fragment spanning the IL-4 gene (Figure 1B, panel 2). HS site I maps to the IL-4 promoter, HS sites II and III to the second intron, and HS sites IV and V to locations 3′ of the IL-4 gene (Figure 1C). By comparison, the Th1 clone D5 exhibited a relatively "closed" chromatin configuration with only one HS site (site IV) over 19 kb of the IL-4 locus (Figure 1B, panel 1).

We also examined DNase I hypersensitivity of the IL-4 locus in other IL-4 producing and nonproducing cells. Resting P815 mast cells and primary bone marrow–derived mast cells, which (like D10 cells) produce IL-4 upon stimulation, showed a dispersed pattern of DNase I hypersensitivity that was similar to that of D10 cells (Figure 1B, panel 4; data not shown). Fibroblast NIH 3T3 and macrophage/monocyte P388D1 cells, which do not express IL-4 upon stimulation, showed none of the DNase I HS sites observed on the IL-4 locus in lymphoid cells (Figure 1B, panel 3; data not shown). To summarize, both Th1 and Th2 cells possessed HS site IV, while the presence of HS sites II, III, and V correlated completely with the ability of resting Th2 cells and mast cells to produce IL-4 upon stimulation.

The patterns of DNase I hypersensitivity observed on the IFNγ locus in D5 and D10 cells were the converse of those on the IL-4 locus, consistent with mutually exclusive production of these cytokines by differentiated Th1 and Th2 cells. Thus, the resting Th1 clone D5, which transcribes the IFNγ gene upon stimulation, displayed an open chromatin structure on the IFNγ locus characterized by three prominent DNase I hypersensitive sites (Figure 1D, panel 1). In contrast, the resting Th2 clone D10, which is incapable of producing IFNγ upon stimulation, displayed an inaccessible IFNγ locus with none of the hypersensitive sites found in the Th1 clone (Figure 1D, panel 2). NIH 3T3 cells, which do not produce IFNγ mRNA, also lacked the open chromatin structure observed in the Th1 cells, and displayed only a weak HS site II (Figure 1D, panel 3). A similar correlation between DNase I hypersensitivity and ability to transcribe the IFNγ gene has been reported in human cells (Hardy et al., 1985, 1987). Therefore, the differential expression of IL-4 and IFNγ in T helper clones correlates completely with differential chromatin accessibility of the IL-4 and IFNγ genetic loci.

Chromatin Remodeling of the IL-4 Locus in Differentiating Th2 Cells

D5 and D10 cells represent stable, terminally differentiated T helper clones. The differences in locus accessibility observed in these clones could reflect differences in their strain background or could be due to the establishment of atypical chromatin structures during long-term culture. We asked whether differentiated Th1 and Th2 T cells, recently derived from a common precursor pool of naive T cells, acquired polarized chromatin configurations over the IL-4 locus. $CD4^+$ T cells were purified from lymph nodes and spleen of C57BL/6 mice and sorted to isolate naive ($Mel14^{hi}$) T cells. Purified cells were divided into two aliquots and differentiated for 2 weeks with anti-CD3 and cytokines under strongly polarizing Th1 or Th2 conditions. As expected, Th1 cells obtained by differentiation in the presence of IL-12 and anti-IL-4 expressed high levels of IFNγ transcript but no IL-4 mRNA upon stimulation (Figure 2A, lane 2), whereas Th2 cells obtained by differentiation in the presence of IL-4 and anti-IL-12 showed high-level expression of IL-4 but no IFNγ mRNA (Figure 2A, lane 4). Again, DNase I hypersensitivity analysis was performed using unstimulated Th1 and Th2 cells in which transcripts of IL-4 or IFNγ mRNA were completely undetectable (Figure 2A, lanes 1 and 3).

Figure 2B demonstrates that the differentiated Th2 population displayed an accessible chromatin structure with five clusters of HS sites over the IL-4 locus, identical to that observed for the D10 Th2 clone (compare Figure 1B, panel 2, with Figure 2B, panel 2). In contrast, the primary Th1 population possessed only the strong HS site IV seen in the D5 Th1 clone (Figures 1B, panel 1, and 2B, panel 1). Thus, primary Th1 and Th2 populations derived from a common pool of naive precursors acquire polarized patterns of IL-4 locus accessibility identical to those observed in the fully differentiated Th1 and Th2 clones.

Differences in IL-4 locus accessibility observed in primary Th1 versus Th2 cells could result either from selective repression of the IL-4 locus in differentiating Th1 cells or from selective activation of the IL-4 locus in Th2 cells. To distinguish between these possibilities, we

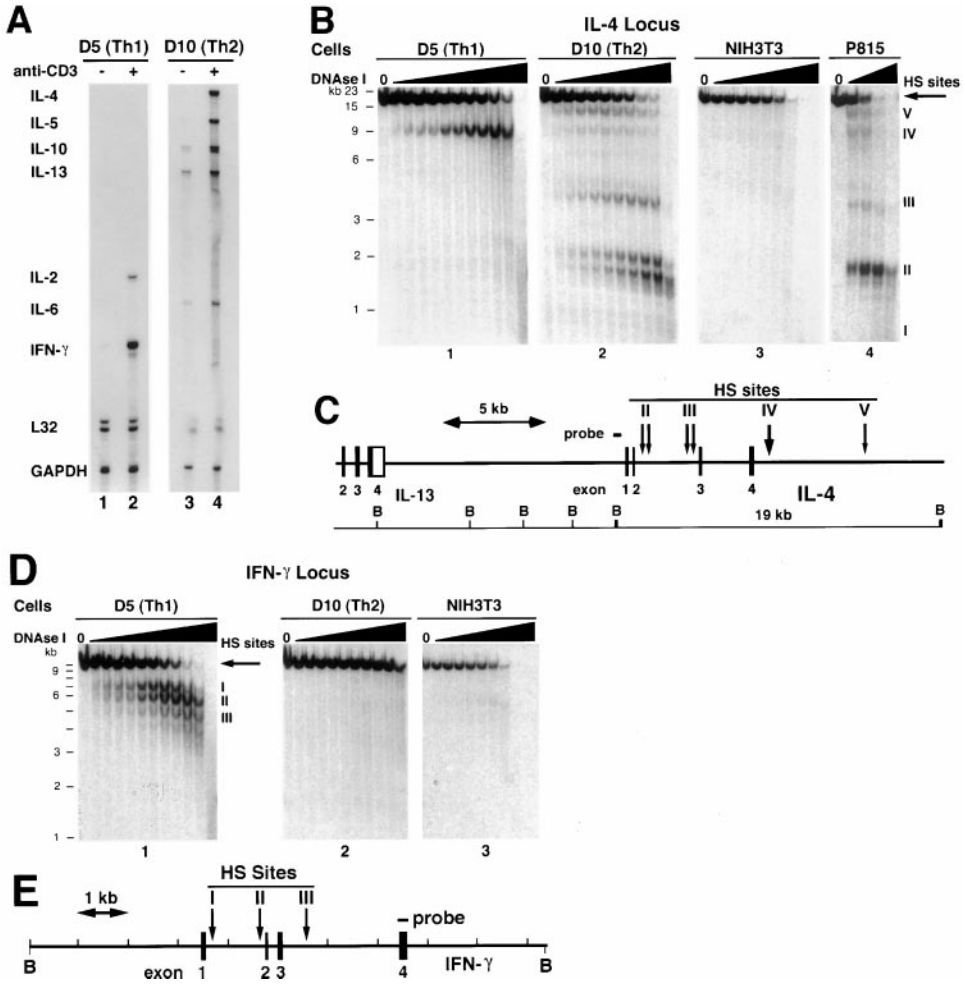

Figure 1. Accessibility of the IL-4 and IFNγ Genetic Loci Reflects the Ability to Transcribe These Cytokine Genes

(A) Multiprobe RNase protection assay of cytokine transcripts from D5 (Th1) and D10 (Th2) cells unstimulated (lanes 1 and 3) or stimulated with immobilized anti-CD3ε for 6 hr (lanes 2 and 4). Cytoplasmic RNA (5 μg) was hybridized for each lane.

(B) DNase I HS pattern on the IL-4 locus in unstimulated D5, D10, NIH 3T3 (fibroblast), and P815 (mastocytoma) cells. Titrations of DNase I were 0–50 U/ml for D5, D10, and P815 cells and 0–75 U/ml for NIH 3T3 cells. The intact genomic fragment in the Southern is indicated by the arrow.

(C) Map of the murine IL-4 locus showing the approximate locations of the DNase I HS sites. The location of the probe used in (B) and in subsequent analyses of the IL-4 locus is indicated. Mapping of sites IV and V was refined using a probe from the 3′ end of the BamHI fragment (data not shown). B, BamHI site.

(D) DNase I HS pattern of the IFNγ locus in unstimulated D5, D10, and NIH 3T3 cells. Southern blots from (B) were stripped and rehybridized with a probe from the murine IFNγ locus.

(E) Map of the murine IFNγ locus showing the approximate locations of the DNase I HS sites. The exon 4 probe used in (D) and in subsequent analyses of the IFNγ locus is indicated. A probe from the 3′ end of the BamHI fragment was used to confirm the approximate locations of the three indicated HS sites (data not shown).

assessed the accessibility of the IL-4 locus in naive precursor T cells. $CD4^+$ T cells were purified from spleen and lymph nodes of BALB/c DO11.10 transgenic mice (Murphy et al., 1990) on the *scid* background. All $CD4^+$ T cells on this background are naive, since they express a single T cell receptor specific for an ovalbumin peptide that is not encountered in the mouse, and endogenous receptor rearrangements are unproductive. Figure 3A (panel 1) demonstrates that naive T cells displayed a chromatin configuration corresponding to a transcriptionally incompetent locus, exhibiting only HS site IV on the IL-4 locus. Differentiation of these naive cells for 7 days under Th2 conditions led to chromatin remodeling and establishment of a transcriptionally competent IL-4 locus, assessed both by measurement of cytokine transcripts (data not shown) and by DNase I hypersensitivity analysis (Figure 3A, panel 2). The IL-4 locus was not remodeled when identical precursors were cultured under Th1 conditions (as in Figure 2B; data not shown).

Further kinetic analysis revealed the appearance of DNase I HS sites characteristic of an accessible IL-4 locus as early as 48 hr after primary stimulation with antigen plus IL-4 (Figure 3A, panel 4). The Th2-specific HS sites were not as clear-cut as those in the fully differentiated D10 clone or in 2 week Th2 cultures (Figures 1B and 2B). This may reflect heterogeneity of the T cell

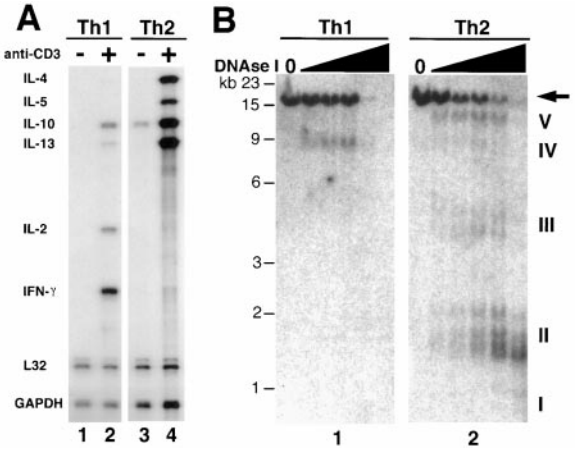

Figure 2. Primary Th1 and Th2 Cells Display Polarized Chromatin Structures on the IL-4 Locus

(A) Naive (Mel14^{hi}) CD4$^+$ T cells from C57BL/6 mice were differentiated in vitro for 2 weeks under Th1 or Th2 conditions and analyzed for cytokine expression and DNase I HS pattern. Multiprobe RNase protection assay of cytokine transcripts from unstimulated (lanes 1 and 3) and stimulated (immobilized anti-CD3ε, 6 hr; lanes 2 and 4) primary Th1 and Th2 cells.

(B) DNase I HS analysis of the IL-4 locus in unstimulated primary Th1 and Th2 cells corresponding to cells used in lanes 1 and 3 of (A). Titrations of DNase I were 0–30 U/ml. The intact genomic fragment is indicated by the arrow.

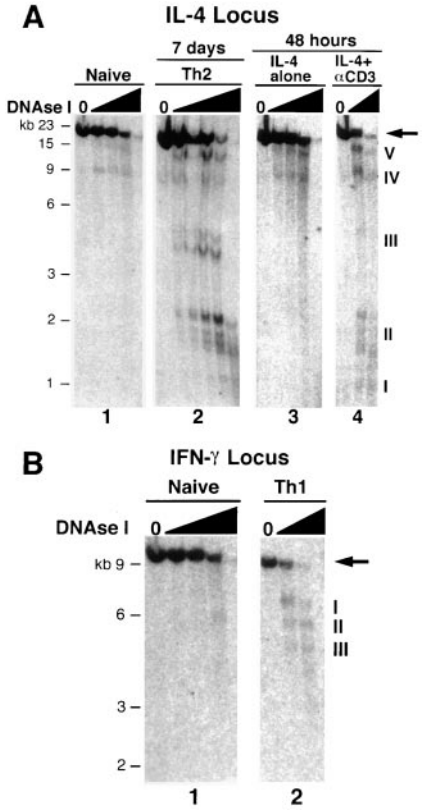

Figure 3. Th2 Cells Rapidly Acquire a Transcriptionally Competent Chromatin Structure on the IL-4 Locus in Response to Antigen Plus Cytokine Stimulation

(A) Panels (1) and (2): CD4$^+$ T cells purified from BALB/c DO11.10 TCR transgenic *scid* mice were directly analyzed (naive) or differentiated for 7 days under Th2 conditions. DNase I HS analysis of the IL-4 locus in resting naive cells (panel 1) and in vitro differentiated Th2 cells (panel 2) is shown. Although a band at the apparent position of HS site V was detected in naive cells using the 5′ probe (panel 1), rehybridization with a probe from the 3′ end of the BamHI fragment indicated that it was not a true HS site. Titrations of DNase I were 0–30 U/ml. The intact genomic fragment is indicated by the arrow. Panels (3) and (4): DNase I HS analysis of the IL-4 locus in CD4$^+$ T cells purified from lymph nodes of C57BL/6 mice cultured for 48 hr with either 1000 U/ml of IL-4 alone (panel 3) or 1000 U/ml IL-4 plus immobilized anti-CD3ε (1 μg/ml) (panel 4). We note that genetic background (BALB/c versus C57BL/6) does not appear to influence the pattern of HS on the IL-4 locus in naive or Th2 differentiated cells (compare panels 1 and 2 with panels 3 and 4, respectively).

(B) DNase I HS analysis of the IFNγ locus in unstimulated naive (panel 1) and Th1 differentiated (7 days; panel 2) CD4$^+$ T cells purified from BALB/c DO11.10 TCR transgenic *scid* mice. Titrations of DNase I used were 0–30 U/ml. The intact genomic fragment is indicated by the arrow. The probe used is the one shown in Figure 1E.

population at this time point, since complete polarization typically requires multiple rounds of stimulation (Murphy et al., 1996). In the presence of IL-4 alone, chromatin remodeling did not occur, and cells retained the closed chromatin configuration on the IL-4 locus (Figure 3A, panel 3). Taken together, these experiments indicate that the open, transcriptionally competent chromatin configuration of the IL-4 locus is not apparent in naive T cells but is rapidly acquired by differentiating Th2 populations as a result of stimulation with both antigen and IL-4.

Although Th1 cells did not remodel the IL-4 locus, they acquired an accessible pattern of DNase I hypersensitivity on the IFNγ locus (Figure 3B, panel 2), consistent with their ability to produce high levels of IFNγ transcripts upon stimulation; naive cells displayed only a weak HS site II, similar to NIH 3T3 fibroblasts (Figure 3B, panel 1).

Chromatin Remodeling of the IL-4 Locus Occurs by a Mechanism Involving STAT6

The IL-4-responsive transcription factor STAT6 plays an essential role in Th2 differentiation, since T helper cells from *STAT6*$^{-/-}$ mice are incapable of producing Th2 cytokines such as IL-4 (Kaplan et al., 1996b; Shimoda et al., 1996; Takeda et al., 1996). The lack of IL-4 gene transcription in *STAT6*$^{-/-}$ cells might be due to an inability to establish an accessible chromatin structure on the IL-4 locus; alternatively, STAT6 could be necessary for acute activation of IL-4 transcription after a transcriptionally competent IL-4 locus had been formed through a STAT6-independent mechanism. CD4$^+$ T cells isolated from *STAT4*$^{-/-}$ and *STAT6*$^{-/-}$ mice on the BALB/c background were cultured for two weeks under strongly polarizing Th2 conditions. *STAT4*$^{-/-}$ Th2 cells produced abundant IL-4 transcripts when stimulated with anti-CD3, whereas IL-4 transcription by *STAT6*$^{-/-}$ cells differentiated under Th2 conditions was severely diminished (Figure 4A, compare lanes 2 and 4). DNase I hypersensitivity analysis demonstrated that resting *STAT4*$^{-/-}$ Th2 cells had remodeled the IL-4 locus as efficiently as wild-type Th2 cells, consistent with their ability to produce

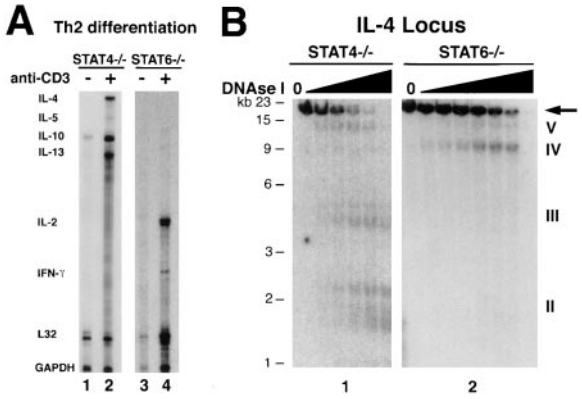

Figure 4. STAT6 Is Required for Chromatin Remodeling of the IL-4 Locus during Th2 Differentiation

(A and B) $CD4^+$ T cells were purified from spleen and lymph nodes of BALB/c *STAT4*$^{-/-}$ and *STAT6*$^{-/-}$ mice, and differentiated for 2 weeks under Th2 conditions. (A) RNase protection assay of cytoplasmic RNA (5 μg) from unstimulated (lanes 1 and 3) and stimulated (immobilized anti-CD3ε, 6 hr; lanes 2 and 4) cells. (B) DNase I hypersensitivity analysis of the IL-4 locus of unstimulated *STAT4*$^{-/-}$ (panel 1) and *STAT6*$^{-/-}$ (panel 2) cells obtained by differentiation under Th2 conditions. Titrations of DNase I were 0–30 U/ml. The intact genomic fragment is indicated by the arrow.

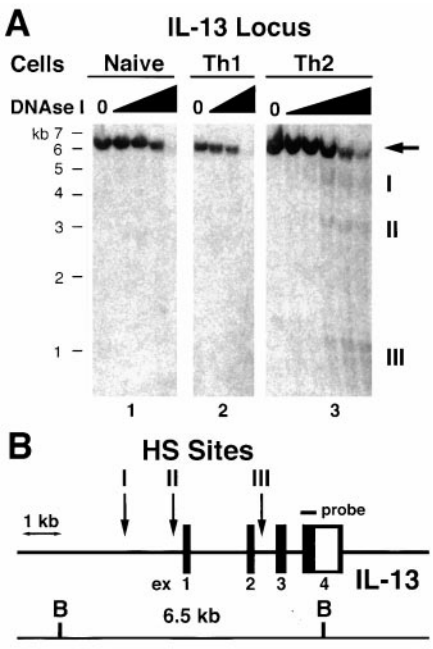

Figure 5. Coordinate Remodeling of the IL-4 and IL-13 Genetic Loci during Th2 Differentiation

(A) DNase I HS pattern of the IL-13 locus in primary BALB/c DO11.10 TCR transgenic *scid* $CD4^+$ T cells, analyzed either directly (naive) or after differentiation under Th1 or Th2 conditions. Southern blots from Figure 3A (panels 1 and 2) and Figure 3B (panel 2) were stripped and rehybridized with a probe from the murine IL-13 locus. The intact genomic fragment is indicated by the arrow.

(B) Map of the murine IL-13 locus showing the approximate locations of the DNase I HS sites. The exon 4 probe used in (A) is indicated. B, BamHI site.

high levels of IL-4 transcripts upon stimulation (Figure 4B, panel 1). In contrast, resting *STAT6*$^{-/-}$ cells displayed a clear defect in the establishment of a transcriptionally competent IL-4 locus, retaining only the strong HS site IV characteristic of naive and Th1 cells (Figure 4B, panel 2). These results suggest that STAT6 is required for long-range remodeling of the IL-4 locus during Th2 differentiation. However, we cannot rule out an alternative possibility raised by the "selective" model of T helper cell differentiation (Bix and Locksley, 1998), which is that STAT6 selectively promotes the expansion of cells that have remodeled the IL-4 locus. We note also that our results apply only to Th2 cells, as populations of IL-4 producing cells (perhaps NK T cells) can be recovered from *IL-4Rα*$^{-/-}$ mice, which are arguably deficient in STAT6 activation (Noben-Trauth et al., 1997).

Activation of the IL-4 Locus Is Accompanied by CpG Demethylation

CpG methylation strongly correlates with transcriptional silencing of genes in differentiated cells, possibly because it induces the formation of repressive higher order chromatin structures that restrict locus accessibility (Kass et al., 1997). We therefore examined the methylation status of the IL-4 locus in differentiated Th1 and Th2 cells. Digestion of genomic DNA with methylation-sensitive restriction enzymes revealed that the Th1 clone D5 possessed a hypermethylated IL-4 locus, whereas the IL-4-producing D10 clone displayed extensive hypomethylation of the IL-4 locus (data not shown). Confirming a recent observation (Bird et al., 1998), Th2 differentiation was strikingly correlated with demethylation of the IL-4 locus. Consistent with a closed chromatin structure, the IL-4 locus in naive T helper cells was completely methylated at two SmaI sites in the 19 kb BamHI fragment encompassing the locus (data not shown). Differentiation of these cells under strongly polarizing conditions for 2 weeks resulted in Th2-specific demethylation of the SmaI sites (data not shown). These results indicate that chromatin remodeling of the IL-4 locus is accompanied by CpG demethylation during Th2 differentiation, whereas neither of these changes occurs during Th1 differentiation.

Coordinate Remodeling of the IL-4 and IL-13 Genetic Loci during Th2 Differentiation

The IL-13 gene is located only 12 kb from the IL-4 gene (Figure 1C), and both the IL-13 and IL-4 genes are transcribed by activated Th2 cells and mast cells. Therefore, we asked whether Th2 differentiation was associated with coordinate activation of both the IL-4 and IL-13 genetic loci at the level of chromatin accessibility. DNase I hypersensitivity analysis of primary differentiating T cells revealed that, like the IL-4 gene, the IL-13 gene in naive T helper cells was found in a repressed, transcriptionally incompetent chromatin structure, which was unchanged upon Th1 differentiation (Figure 5A, panels 1 and 2). In contrast, differentiating Th2 cells rapidly acquired an accessible chromatin configuration on the IL-13 locus, as judged by the appearance of three clusters of HS sites within a 6.5 kb BamHI fragment spanning the IL-13 gene (Figures 5A, panel 3, and 5B). This pattern

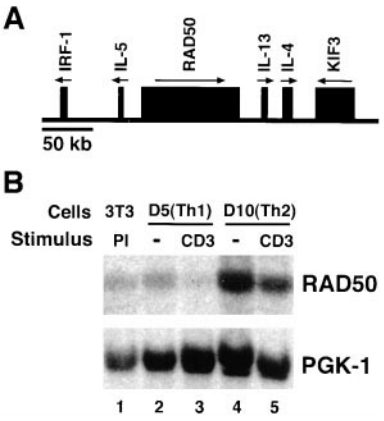

Figure 6. Increased Basal Transcription of the *RAD50* Gene in Th2 Cells

(A) Organization of the human IL-4/IL-5/IL-13 cytokine gene cluster based on data derived from sequencing of the human 5q23–31 chromosomal region (Frazer et al., 1997). A syntenic region on mouse chromosome 11q displays the same relative organization of genetic loci. The *RAD50* gene occupies the 100 kb interval between the IL-13 and IL-5 gene loci. Transcriptional orientation is indicated by the direction of the arrow.

(B) Northern analysis of 10 μg of total cellular RNA from stimulated (2 hr; PI) NIH 3T3 fibroblasts (lane 1) and unstimulated (lanes 2 and 4) or stimulated (immobilized anti-CD3ε [CD3]; 6 hr; lanes 3 and 5) D5 and D10 cells. Blots were probed with full-length murine *RAD50* cDNA, then stripped and reprobed with a full-length murine *PGK-1* (phosphoglycerate kinase-1) probe. PI, 20 nM PMA plus 2 μM ionomycin.

of DNase I hypersensitivity on the IL-13 locus was also apparent in D10 Th2 cells, but not in D5 Th1 cells or NIH 3T3 fibroblasts (data not shown), further confirming that the presence of the HS sites correlated with an accessible, transcriptionally competent genetic locus in cells capable of producing IL-13. Moreover, analysis of CpG methylation in the IL-13 locus revealed that two SmaI sites in the IL-13 promoter were completely methylated in the D5 Th1 clone and completely unmethylated in the D10 Th2 clone (data not shown). Thus, by the criteria of both long-range accessibility and DNA methylation, the IL-13 locus and the IL-4 locus share a common repressed chromatin environment in Th1 cells and a common activated chromatin environment in Th2 cells.

The precise organization of the IL-4, IL-5, and IL-13 genes has been revealed by sequencing of the human 5q23–31 chromosomal region (Frazer et al., 1997), which is syntenic with the corresponding region of murine chromosome 11. The murine *RAD50* gene, which is involved in chromosomal double-stranded break repair and is expressed in diverse tissues (Kim et al., 1996), occupies the 100 kb interval between the genes for IL-13 and IL-5 (Figure 6A). We reasoned that if the accessibility of the entire IL-4/IL-5/IL-13 chromosomal region was increased in Th2 cells as a result of Th2 differentiation, the basal transcription of the *RAD50* gene in Th2 clones might be elevated relative to Th1 clones merely as a consequence of being located in a region of extended chromatin accessibility. Northern analysis revealed that the basal expression of the murine *RAD50* gene is indeed higher in the D10 Th2 clone than in the D5 Th1 clone (Figure 6B, lanes 2–5). Transcription of the *RAD50* gene is not activated by anti-CD3 stimulation (Figure 6B, lanes 3 and 5), further suggesting that differential accessibility of the locus underlies the observed difference in transcript levels. Interestingly, the level of *RAD50* transcription in D5 cells is similar to that in NIH 3T3 cells (Figure 6B, lanes 1–3); both cell lines display closed chromatin structures over the IL-4 and IL-13 loci (Figure 1A; data not shown). Taken together, these data suggest that the IL-4/IL-5/IL-13 locus is coordinately activated in Th2 cells at the level of chromatin accessibility.

Discussion

Regulation of Cytokine Gene Loci at the Level of Chromatin Remodeling: A Two-Step Model for Cytokine Gene Expression

Our results suggest strongly that productive T cell differentiation is associated with chromatin remodeling of genes that confer the effector phenotype of the differentiated cells (Figure 7A). Thus differentiation of naive T cells into mature Th2 effector cells is accompanied by long-range changes in the chromatin structure of the IL-4 and IL-13 genes; conversely, Th1 differentiation is accompanied by long-range changes in the structure of the IFNγ locus. On the IL-4 locus, chromatin remodeling occurs rapidly (within 48 hr) following initial stimulation at a time when IL-4 transcripts are barely detectable in the differentiating naive cell (Le Gros et al., 1990; Croft and Swain, 1995; Lederer et al., 1996), suggesting that it parallels or precedes initial transcription through the locus and thus constitutes a very early differentiative event. Importantly, all experiments were performed on resting cells that were not actively transcribing the cytokine genes; thus, our results indicate differences in the transcriptional competence of the genetic loci rather than overt transcription of the genes.

Confirming a recent report (Bird et al., 1998), we have also demonstrated that naive T cells possess a hypermethylated IL-4 locus, which undergoes demethylation specifically during Th2 differentiation. Activation of genetic loci by demethylation is observed in several genes in the immune system (Bergman and Mostoslavsky, 1998) and coincides with chromatin remodeling. Recent studies have provided a mechanistic link between methylation of DNA and the establishment of repressive chromatin structures (Jones et al., 1998; Nan et al., 1998). Thus, Th2 differentiation is accompanied by regulated demethylation and chromatin remodeling of the IL-4 locus, which together may result in the establishment of an accessible chromatin structure on the IL-4 gene.

In general, activation of tissue-specific genes during differentiation occurs first at the level of chromatin accessibility and results in the formation of transcriptionally competent genetic loci characterized by increased sensitivity to digestion with DNase I (Groudine et al., 1983; Tuan et al., 1985; Forrester et al., 1986). Loci in the open conformation can subsequently respond to acutely activating signals, often conveyed by non-tissue-specific transcription factors that can gain access to the open locus and recruit or activate the basal transcriptional machinery. Our data suggest a similar two-step model for activation of cytokine genes during T

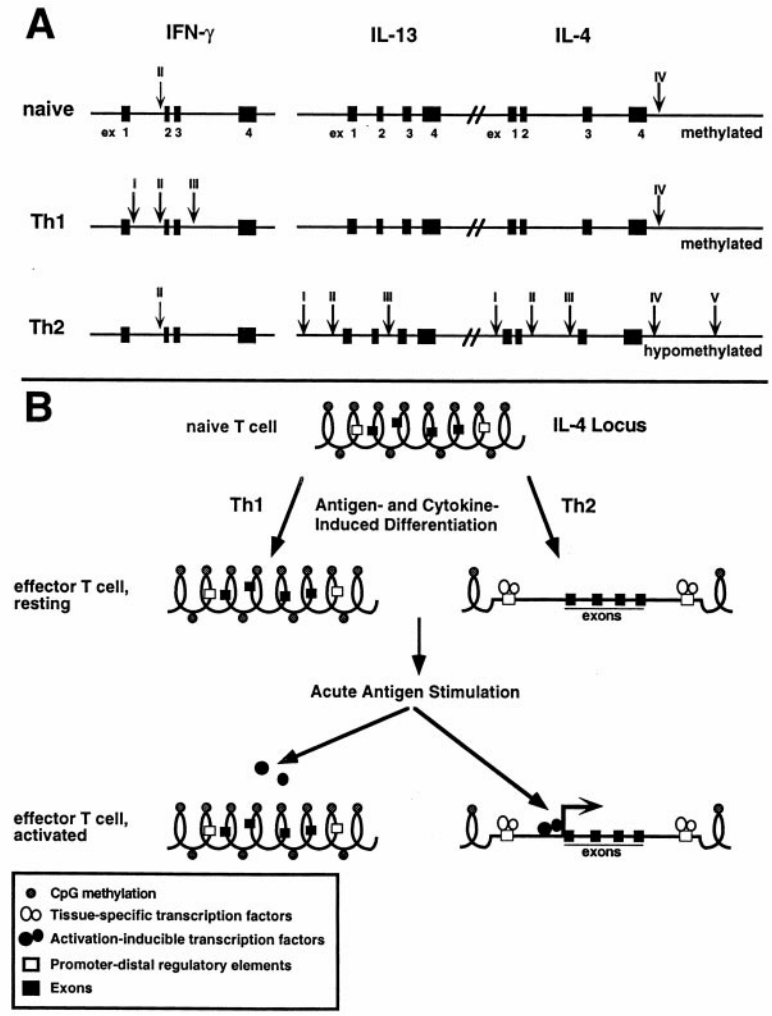

Figure 7. Regulation of Cytokine Gene Expression at the Level of Chromatin

(A) Summary of changes in the DNase I hypersensitive pattern and methylation status of the IFNγ, IL-13, and IL-4 gene loci during T cell differentiation. Arrows denote approximate locations of hypersensitive sites.

(B) A two-step model for regulation of the endogenous IL-4 gene locus in T cells. Step 1: the IL-4 gene (exons shown as black squares) resides in a poorly accessible chromatin structure (depicted as looped DNA structure with dense methylation) in naive T cells. Th2 differentiation results in the induction of tissue-specific factors (open ovals) such as GATA-3 and Maf, which bind to dispersed regulatory elements (open squares) to effect and maintain an accessible IL-4 locus structure (unlooped DNA) in the absence of active transcription. Chromatin remodeling in Th2 cells is accompanied by demethylation of the IL-4 locus. Th1 differentiation does not induce these tissue-specific factors and thus the IL-4 locus remains inaccessible. Step 2: subsequent antigen stimulation results in activation of inducible transcription factors (closed ovals), some of which may be non-tissue-specific such as NFAT and AP-1. These factors can access the IL-4 locus only in Th2 cells, where they bind DNA (perhaps cooperatively with the tissue-specific transcription factors) and stimulate rapid, high-level IL-4 transcription. This model may be generalizable to other cytokine genes.

cell differentiation (Figure 7B) (Agarwal and Rao, 1998). Naive T cells display a repressed, transcriptionally incompetent chromatin structure that is likely to account for their slow kinetics and low levels of cytokine gene transcription, facts especially well documented for the IL-4 gene (Le Gros et al., 1990; Croft and Swain, 1995; Lederer et al., 1996). Differentiative signals induced upon primary contact with antigen and polarizing cytokine elicit Th1-specific remodeling of the IFNγ locus and Th2-specific remodeling of the IL-4 and IL-13 genes, presumably via nuclear factors expressed selectively in the relevant T helper subset. The resulting accessible chromatin configurations persist in the differentiated cells in the absence of active transcription. Subsequent antigen stimulation results in the activation and synthesis of inducible transcription factors, many of which (e.g., NFAT) may be non-subset-specific (Rooney et al., 1994); these factors gain access to loci that are in the open configuration and promote rapid, high-level transcription of the cytokine genes.

This model is consistent with several features of cytokine expression by T cells. First, it may explain the observation that the cytokine expression patterns of effector T cells continue to reflect the context of primary antigenic stimulation long after the antigen has been withdrawn. This memory may be a consequence of the persistence and heritable propagation of accessible chromatin structures on specific cytokine loci. Second, it may account for the monoallelic expression of IL-2, IL-4, and perhaps other cytokine genes, especially in the early stages of differentiation (Bix and Locksley, 1998; Hollander et al., 1998; Riviere et al., 1998). The stochastic nature of this process and its dependence on signal strength in the initial antigen stimulation suggest that it is governed by the availability of tissue-specific nuclear factors that regulate chromatin remodeling and locus demethylation but are present in limiting amounts in the naive cell. Third, the model may explain the fact that naive T cells exhibit a much greater dependence on costimulation for cytokine production relative to differentiated T cells (Bluestone, 1995): again, nuclear factors upregulated by costimulation may be needed for chromatin remodeling rather than just for acute transcription. Fourth, the ability of naive T cells to express IL-2 but not IL-4 may be explained by postulating that remodeling of the IL-2 locus occurs during thymic differentiation, while remodeling of the IL-4 locus occurs during peripheral differentiation as shown here. This explanation is consistent with the observation that immature thymocytes lacking the TCR can produce IL-2 when stimulated pharmacologically with phorbol esters and calcium ionophores (Rothenberg and Ward, 1996). Finally, the model

explains the observation that treatment of naive T cells with azacytidine and trichostatin (inhibitors of the maintenance methylase and of histone deacetylases, respectively) results in more rapid kinetics of IL-4 gene expression (Bird et al., 1998): the global demethylation and histone acetylation associated with use of these drugs may facilitate the initial locus remodeling steps.

Factors Involved in Chromatin Remodeling

The DNase I–hypersensitive sites detected on the IL-4 locus in T helper cells may correspond to novel regulatory elements of the IL-4 gene. Transgenic experiments strongly indicate that promoter-distal regulatory elements are necessary for high-level and position-independent expression of the IL-4 gene (Wenner et al., 1997). Moreover, HS site IV of the IL-4 gene, the only HS site observed in Th1 cells, overlaps partially with a silencer element characterized in transient transfection experiments that functions only in Th1 cells (Kubo et al., 1997). Further characterization of this element may reveal a mechanism for repression of the IL-4 locus in naive and Th1 cells. Likewise, the location of HS site II corresponds to an intronic HS site that was previously characterized as an enhancer element in mast cells (Henkel et al., 1992); this region may serve a similar function in Th2 cells. The intronic HS sites of the IL-4, IL-13, and IFNγ loci are also consistent with the presence of a paused RNA polymerase II complex, as described for HS sites in the TATA box of the IL-2 gene (Brunvand et al., 1993), and in the first introns of the c-*myc* and c-*myb* genes (Bentley and Groudine, 1986; Bender et al., 1987). Polymerase occupancy of resting genetic loci would permit rapid gene transcription in response to stimulation.

Which nuclear factors might play a role in locus remodeling of cytokine genes? We have shown that STAT6 is required for the establishment of an accessible chromatin structure on the IL-4 locus during Th2 differentiation. We have also shown that remodeling of the IL-4 locus cannot be achieved through IL-4 stimulation alone, indicating that stimulation through the antigen receptor is essential. Following the instructive model of T helper cell differentiation (O'Garra, 1998) in which cytokines drive the differentiation of naive cells, one possibility is that STAT6 and antigen-induced transcription factors such as NFAT and NFκB are directly involved in remodeling the IL-4 locus. While these factors could potentially initiate locus remodeling, they are unlikely to be involved in *maintaining* the accessible chromatin structure on the IL-4 locus, since their nuclear localization is rapidly terminated in the absence of continued stimulation (Finco and Baldwin, 1995; Darnell, 1997; Rao et al., 1997), whereas the accessible IL-4 locus structure and the differentiated Th2 phenotype are maintained. We propose instead that STAT6 and antigen-responsive transcription factors induced during primary stimulation activate a differentiation-specific genetic program that results in the stable induction of Th2-specific nuclear factors. These factors then bind to dispersed regulatory elements of specific cytokine loci and recruit chromatin remodeling enzymes (see Figure 7B). Changes in chromatin configuration are inherited by subsequent generations of differentiated cells (Wolffe, 1994; Kass et al., 1997), thus becoming independent of continued IL-4 stimulation or STAT6 activation. A parallel program involving STAT4 may be involved in IFNγ locus opening.

Our model suggests that Th2-specific nuclear factors are directly involved in chromatin remodeling and formation of hypersensitive sites on the IL-4 locus in resting Th2 cells. Based on overexpression, gene disruption, and transgenic experiments, the transcription factors Maf, GATA-3, and BCL6 have been proposed as regulators of Th2 differentiation (Ho et al., 1996; Dent et al., 1997; Szabo et al., 1997; Zhang et al., 1997; Zheng and Flavell, 1997). BCL6 (whose deletion potentiates Th2 differentiation) has been suggested to function by competing with STAT6 (Dent et al., 1997), while Maf and GATA-3, which are expressed at high levels in Th2 but not Th1 cells, have been postulated to act by binding to promoter elements of the IL-4 and IL-5 genes (Ho et al., 1996; Zhang et al., 1997; Zheng and Flavell, 1997). Members of the GATA and Maf families are constitutively expressed and localized to the nucleus in resting cells (Yang et al., 1994; Blank and Andrews, 1997) and are known to regulate erythroid cell differentiation via formation of HS sites and long-range chromatin remodeling on the β-globin locus (Orkin, 1995; Stamatoyannopoulos et al., 1995; Armstrong and Emerson, 1996; Motohashi et al., 1997). By analogy, the Th2-restricted transcription factors GATA-3 and Maf may mediate chromatin remodeling in the IL-4/IL-5/IL-13 locus. Overexpression of Maf in M12 B cells, which do not produce IL-4, results in activation of the endogenous IL-4 gene (Ho et al., 1996), again consistent with a role for Maf in regulating the accessibility of the IL-4 genetic locus.

The IL-4, IL-5, and IL-13 Genes: A Coordinately Regulated Chromosomal Locus?

Our studies also support the intriguing possibility that the IL-4, IL-5, and IL-13 genes, which are clustered within 150 kb on both human and mouse chromosomes and are coordinately expressed by Th2 cells, constitute a single chromosomal locus regulated by long-range alterations in chromatin structure. Further, the linkage of the IL-13 and IL-4 loci within 12 kb and their coordinate activation in Th2 cells and mast cells suggests that these loci share common regulatory elements and nuclear factors involved in locus opening. By DNase I hypersensitivity analysis, the development and maintenance of IL-13 locus accessibility directly paralleled derepression of the IL-4 locus; these data provide evidence of coordinated regulation of the IL-4 and IL-13 gene loci at the level of chromatin accessibility. Furthermore, Th2 clones showed upregulated basal transcription, relative to Th1 cells, of the *RAD50* gene located within the IL-4/IL-5/IL-13 gene cluster, possibly due to increased accessibility of this entire locus in Th2 cells. One or more of the identified hypersensitive sites in the IL-4/IL-13 locus may constitute a long-range *cis*-acting regulatory element, analogous to the locus control region of the β-globin gene cluster (Kioussis and Festenstein, 1997), that governs accessibility of this entire chromosomal region. Thus, our data provide the molecular framework for identification and functional characterization of promoter-distal regulatory elements potentially involved in long-range regulation of the IL-4, IL-5, and IL-13 genes.

Experimental Procedures

Mice, Cell Cultures, and In Vitro T Cell Differentiation

All mice were maintained in pathogen-free conditions in barrier facilities at the Center for Animal Resources and Comparative Medicine, Harvard Medical School.

All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, penicillin-streptomycin, nonessential amino acids, sodium pyruvate, vitamins, HEPES, and 2-mercaptoethanol. Cultures of the murine T cell clones D5 (Ar-5 [Rao et al., 1984]) and D10 (D10.G4.1 [Kaye et al., 1983]) were supplemented with 10 U/ml purified rat IL-2 (Collaborative Biomedical Products) and, for D10 cells, 25 U/ml recombinant IL-4 (added as supernatant from the I3L6 cell line, which was transfected with a constitutively expressed murine IL-4 cDNA [Tepper et al., 1989]). T cell clones were restimulated with antigen and irradiated antigen-presenting cells every 4 weeks; cells were expanded and used for analysis only after 2 weeks of rest following restimulation.

For primary cell analyses, spleen and lymph nodes were isolated from C57BL/6 mice (typically 3–6 weeks old). $CD4^+$ T cells were purified using magnetic beads (Dynal), and naive ($Mel14^{hi}$) cells were isolated by FACS after incubation with FITC-Mel14 antibody (Pharmingen). For Th1 differentiation, cells were stimulated (5×10^5 cells/0.5 ml) with 1 μg/ml plate-bound anti-CD3ε (145.2C11; Pharmingen) in the presence of 10 ng/ml recombinant mouse IL-12 (courtesy of Hoffman-La Roche) and 10 μg/ml purified anti-IL-4 antibody (11B11; a gift from the Biological Response Modifiers Program, National Cancer Institute). For Th2 differentiation, cells were stimulated in the presence of 1000 U/ml mouse IL-4, 10 μg/ml anti-IFNγ (R4–6A2; Pharmingen), and 6 μg/ml anti-IL-12 (courtesy of Hoffman-La Roche). After 24 hr, both cultures were supplemented with 20 U/ml IL-2. For cultures longer than 4 days, cells were expanded (4-fold) on the fourth day after stimulation in the absence of anti-CD3ε antibody but in the continued presence of cytokines and antibodies. After a total of 7 days of culture, cells were restimulated as above but omitting anti-IL-12 in the Th2 culture; after 48 hr of restimulation, cultures were expanded as necessary in the presence of only IL-2 for Th1 cells or IL-2 plus IL-4 for Th2 cells. At the appropriate time point (48 hr or 7 or 14 days; indicated in each figure), Th1 and Th2 cultures were extensively washed and 2×10^7 cells of each population were harvested for multiprobe RNase protection assay (RPA; Pharmingen), DNase I HS analysis, and/or methylation analysis.

Analysis of chromatin structure in naive cell populations required cell numbers technically difficult to obtain by FACS sorting of $Mel14^{hi}$ cells. Instead, $CD4^+$ T cells were purified using magnetic beads from the spleen and lymph nodes of BALB/c DO11.10 TCR transgenic mice (Murphy et al., 1990) bred onto the *scid* background (kindly provided by A. K. Abbas, Harvard Medical School), in which $CD4^+$ T cells are naive due to the presence of a single TCR, specific for a foreign antigen, on all peripheral T cells. Following purification, 2×10^7 cells were directly processed for DNase I HS analysis, while 4×10^6 cells were differentiated under either Th1 or Th2 conditions as described above. Cells were harvested 7 days after primary stimulation, extensively washed, and analyzed by RPA and DNase I HS analysis.

DNase I Hypersensitivity and Methylation Analyses

Isolation and DNase I digestion of nuclei and purification of genomic DNA were performed as described by Cockerill et al. (1993). Briefly, nuclei were isolated from $2–10 \times 10^7$ cells by lysis in 0.1% NP-40 and resuspended to approximately 0.4 mg/ml nucleic acid as determined by measurement of absorbance at 260 nm. Aliquots of nuclei were incubated for 3 min at 25°C with 0–75 U/ml DNase I (Worthington). DNA was purified by phenol/chloroform extraction and digested to completion with BamHI. Samples were resolved on a 0.6% agarose gel, transferred to Nytran membranes (Schleicher and Schuell), and hybridized with probes labeled by random priming. Probe locations are depicted in locus diagrams and correspond to an 800 bp promoter fragment from the IL-4 locus, a 450 bp exon 4 probe from the IFNγ locus, and a 450 bp exon 4 probe from the IL-13 locus. Hybridized membranes were exposed to Biomax MS film (Kodak) for 2–7 days.

Genomic DNA for methylation analyses was digested to completion with BamHI, purified by phenol/chloroform extraction, ethanol precipitated, and resuspended in 10 mM Tris (pH 8.0) and 1mM EDTA buffer. Concentrations were determined by measurement of absorbance at 260 nm, and 10 μg of BamHI-digested DNA was further digested by SmaI (80 U), XmaI (50 U), HpaII (100 U), MspI (50 U), or McrBC (50 U) for 16 hr. All enzymes were obtained from New England Biolabs. Digested samples were further processed for Southern analysis identically to samples for DNase I hypersensitivity analyses.

RNase Protection Assay

Cells (5×10^6) were left unstimulated or stimulated in 60 mm dishes for 6 hr using 1 μg/ml plate-bound anti-CD3ε antibody in the absence of any cytokines or cytokine antibodies. Cytoplasmic RNA was purified and 5 μg of RNA was analyzed using the RiboQuant multiprobe RNase protection kit (Pharmingen) according to manufacturer's instructions. In brief, sample RNA was hybridized to ^{32}P-labeled probes overnight and subjected to RNase digestion; protected RNAs were purified and resolved on a 6% denaturing polyacrylamide gel.

Northern Blot Analysis

Total cellular RNA (10 μg) was separated on a 1% agarose/formaldehyde gel and transferred to Nytran membrane (Schleicher and Schuell). A full-length (5.3 kb) cDNA probe to the murine *RAD50* gene was used for detection of RAD50 transcripts. The blots were stripped in formamide and reprobed with a cDNA (1.6 kb) for the housekeeping gene phosphoglycerate kinase-1 (*PGK-1*).

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