nature immunology

Hypersensitive site 7 of the T_H2 locus control region is essential for expressing T_H2 cytokine genes and for long-range intrachromosomal interactions

Gap Ryol Lee[1], Charalampos G Spilianakis[1] & Richard A Flavell[1,2]

Several regulatory regions are important for the expression of genes encoding T helper type 2 (T_H2) cytokines, including T_H2-specific DNase I hypersensitivity sites in the T_H2 cytokine locus control region. Among these sites, *Rad50* hypersensitive site 7 (RHS7) shows rapid T_H2-specific demethylation after antigenic stimulation. To investigate the function of RHS7 in T_H2 cell differentiation, we have generated RHS7-deficient mice. $CD4^+$ T cells and mast cells showed a notable reduction in T_H2 cytokine expression *in vitro* and T_H2 responses *in vivo* were considerably impaired in RHS7-deficient mice. Deletion of RHS7 did not affect the expression of a linked *Rad50* gene, but it did reduce long-range intrachromosomal interactions between the locus control region and promoters of the T_H2 cytokine genes. Our findings show that RHS7 is essential for the proper regulation of T_H2 cytokine gene expression.

Naive $CD4^+$ T cells differentiate into distinct effector cell lineages, called T helper type 1 (T_H1) and T_H2, after antigenic stimulation. T_H1 cells produce interferon-γ and lymphotoxin-α and mediate immune responses against intracellular pathogens, whereas T_H2 cells produce interleukin 4 (IL-4), IL-5 and IL-13 and mediate immunity against extracellular parasites[1–4]. However, T_H1 cells can also cause autoimmunity and T_H2 cells can cause allergic diseases such as allergy and asthma. Differentiation into T_H1 and T_H2 cells is accompanied by chromatin changes in the genetic loci encoding the 'signature' cytokines interferon-γ and IL-4. The genes encoding IL-4, IL-5 and IL-13 are clustered in a 120-kilobase (kb) region in the mouse genome and a 160-kb region in the human genome. This locus also contains the gene encoding RAD50 (*Rad50*), which is constitutively expressed and involved in DNA repair.

The T_H2 cytokine locus has been studied intensively to investigate chromatin dynamics and gene regulation during cell fate determination and differentiation[5,6]. During T_H2 cell differentiation, the whole T_H2 cytokine locus undergoes chromatin modifications, manifested by the formation of DNase I hypersensitivity sites, DNA demethylation and histone acetylation[7–14]. Most of these chromatin changes occur at specific regulatory regions that are important for T_H2 cytokine gene regulation and lineage differentiation. These regulatory regions have been identified by analyses of DNase I hypersensitivity sites and by comparisons of DNA sequences in different species[15–23].

An evolutionarily conserved noncoding sequence (CNS) in the intergenic region between *Il4* and *Il13* (CNS-1; also called HSS) is important for the regulating of T_H2 cytokine genes[15,16]. Similarly, two hypersensitive sites located 3′ to *Il4*, HS5 and HS5a, are important for *Il4* regulation both in $CD4^+$ T cells and in mast cells[17,18]. In experiments using a transgenic reporter assay, the hypersensitive sites and conserved regions in the *Il4* locus were found to function as enhancers or repressors that can negatively regulate both *Il4* gene expression and regulators of differentiation specificity[19]. Experiments using transgenic mice generated from a bacterial artificial chromosome (BAC) containing essentially the whole T_H2 cytokine locus identified a locus control region (LCR) in the 3′ region of *Rad50* that coordinately regulates the expression of *Il4* and *Il13* (ref. 20). An LCR is an element that confers high-level tissue-specific expression to linked genes, presumably by overriding suppressive effects of flanking DNA sequences[21,22].

T_H2-specific DNase I hypersensitivity sites were subsequently identified in the LCR[23]. Several of these sites (*Rad50* hypersensitive site 5 (RHS5), RHS6 and RHS7) are highly conserved between species and are hyperacetylated during T_H2 cell differentiation. Combining these hypersensitive sites recapitulates, at least in part, the function of the T_H2 cytokine LCR, suggesting that these sites constitute the core of the LCR[23]. Experiments using the chromosome conformation capture technique[24], which provides information about the spatial organization of chromosomal regions *in vivo*[25], have shown that the T_H2 cytokine LCR is located near the *Il4*, *Il5* and *Il13* promoters in a T cell antigen receptor (TCR) activation–dependent way, suggesting the existence of long-range intrachromosomal interactions[24].

[1]Section of Immunobiology and [2]Howard Hughes Medical Institute, Yale School of Medicine, New Haven, Connecticut 06520, USA. Correspondence should be addressed to R.A.F. (richard.flavell@yale.edu).

Published online 19 December 2004; doi:10.1038/ni1148

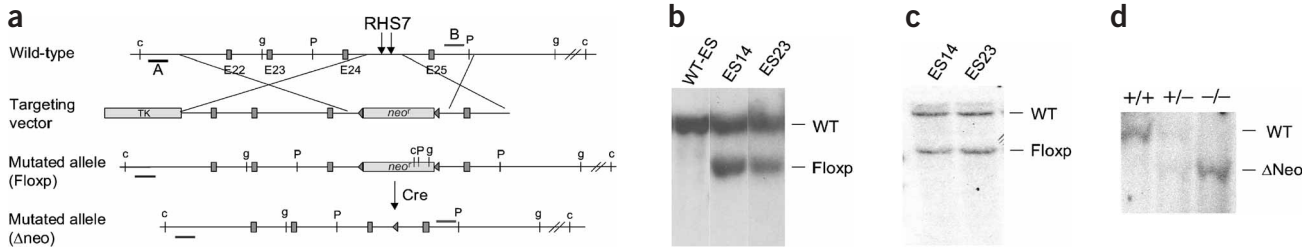

Figure 1 Targeted deletion of RHS7. (**a**) Targeted deletion strategy. RHS7 was replaced with *neo*^{r} flanked by two *loxP* sites. After selection of correctly targeted clones, *neo*^{r} was deleted by Cre-mediated recombination in embryonic stem cells. A and B, probes used for Southern blot; P, *Pst*I; c, *Bci*VI; g, *Bgl* I. Floxp, allele in which RSH7 is replaced with *neo*^{r}; ΔLCR, allele in which *neo*^{r} is deleted. (**b**,**c**) Confirmation of correct homologous recombination in embryonic stem cells. (**b**) DNA from embryonic stem cells was digested with *Bgl*I and probed with fragment B. Wild-type (WT) band, 9.1 kb; Floxp band, 4.8 kb. WT-ES is an embryonic stem cell clone containing two WT alleles; ES-14 and ES-23 are embryonic stem cell clones each containing one targeted allele (Floxp). (**c**) DNA from embryonic stem cells was digested with *Bci*VI and probed with fragment A. Wild-type band, 20.1 kb; Floxp band, 7.6 kb. (**d**) Genotyping of wild-type (+/+), heterozygous (+/–) and knockout (–/–) mice. *Pst*I-digested DNA from tail tissue was probed with fragment B. Wild-type band, 3.7 kb; ΔNeo band, 3.1 kb.

DNA methylation is generally considered to be a gene silencing mechanism and has been described in various systems in development, oncogenesis and cell differentiation[26–29]. The T_H2 cytokine locus is demethylated during T_H2 differentiation[8,10]; in addition, the promoter and 5′ regulatory region of *Il4* undergo progressive and presumably passive demethylation during T_H2 cell differentiation[10]. Notably, among the hypersensitive sites in the T_H2 cytokine LCR, RHS7 undergoes rapid, complete and T_H2-specific demethylation on TCR stimulation. The demethylation occurs almost completely within 2 d of stimulation[23]. Rapid site-specific demethylation also occurs in the promoter-enhancer region of *Il2* after TCR activation[30]. The functional importance of this rapid differentiation-specific demethylation in the endogenous locus, however, has not been studied in any system.

RHS7 is highly conserved between human and mouse[23]. RHS7 is also the RHS site that is hyperacetylated to the greatest extent during T_H2 differentiation[23]. RHS7 also shows the strongest enhancer function among individual RHSs in a transgenic reporter assay[23]. Together with the demethylation data, these results led us to speculate that this region may be important for LCR function and chromatin changes during T_H2 cell differentiation.

Here we have investigated the function of RHS7 in the endogenous locus by generating RHS7-deficient mice by homologous recombination. Using these mice, we have studied the function of RHS7 in T_H2 cell differentiation, T_H2 cytokine expression and chromatin changes on antigenic stimulation.

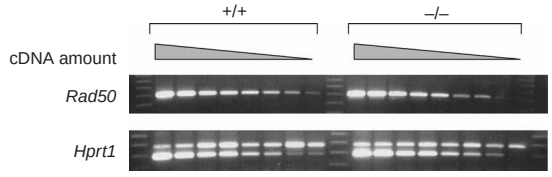

Figure 2 Expression of *Rad50* is unaffected in RHS7-deficient mice. Total RNA from *in vitro*–differentiated T_H2 cells from wild-type (+/+) and RHS7-deficient (–/–) mice was isolated and quantified by RT-PCR. The cDNA was serially diluted twofold (left to right; wedge). The same amount of cDNA was used in the *Rad50* and *Hprt1* PCR reactions. For accurate measurement of the amount of cDNA used in the PCR reaction, quantitative PCR was done in the *Hprt1* reaction with competitor cDNA (ref. 38). In the *Hprt1* image, the top band is added competitor and bottom band is endogenous *Hprt1*. Data are representative of one of three independent experiments with similar results.

RESULTS

Generation of RHS7-deficient mice

To investigate the function of RHS7 in its natural chromatin context, we deleted this region by homologous recombination in embryonic stem cells. We replaced RHS7, a conserved region of 637 base pairs (bp), with the gene encoding neomycin resistance (*neo*^{r}) flanked by *lox*P sites; *neo*^{r} was subsequently deleted by overexpression of Cre recombinase in the embryonic stem cells (**Fig. 1**). We implanted the RHS7-deleted embryonic stem cells in foster mothers to obtain chimeric mice. We bred mice showing germline transmission with C57BL/6 mice to obtain heterozygous mice, which we then crossed to generate RHS7-deficient mice.

We screened genotypes of the offspring of these crossings by PCR analysis of DNA obtained from tail tissue and confirmed them by Southern blot analysis (**Fig. 1**). Genotype data showed that the relative numbers of wild-type, heterozygous and homozygous-null mice followed a mendelian ratio, suggesting that the RHS7-deficient mice had no severe developmental defects. The RHS7-deficient mice showed no gross defects in appearance.

Rad50 expression

Because RHS7 is located in intron 24 of *Rad50*, it may act as an enhancer for *Rad50* expression as well as for T_H2 cytokine gene expression. We tested this possibility by examining the expression of *Rad50* by semiquantitative PCR with reverse transcription (RT-PCR) in $CD4^+$ T cells from RHS7-deficient mice and wild-type littermates. The expression of *Rad50* was similar in both types of mouse (**Fig. 2**), indicating that RHS7 has no effect on the expression of *Rad50*.

Expression of T_H2 cytokine genes *in vitro*

To investigate the function of RHS7 in the expression of T_H2 cytokines, we isolated naive $CD4^+$ T cells from RHS7-deficient and wild-type littermates, stimulated them with antibody to CD3 (anti-CD3) and antigen-presenting cells (APCs) in neutral or T_H2-polarizing conditions and measured IL-4, IL-5 and IL-13 in the culture supernatants by enzyme-linked immunosorbant assay (ELISA; **Fig. 3a**). In neutral stimulation conditions (T_H0), expression of IL-4, IL-13 and IL-5 was almost completely (IL-4 and IL-13) or substantially (IL-5) reduced in RHS7-deficient $CD4^+$ T cells compared with wild-type $CD4^+$ T cells (**Fig. 3a**, top). In T_H2 stimulation conditions, expression of IL-4 and IL-13 in RHS7-deficient mice was reduced to 40% of that in wild-type mice (**Fig. 3a**, bottom). In contrast, the

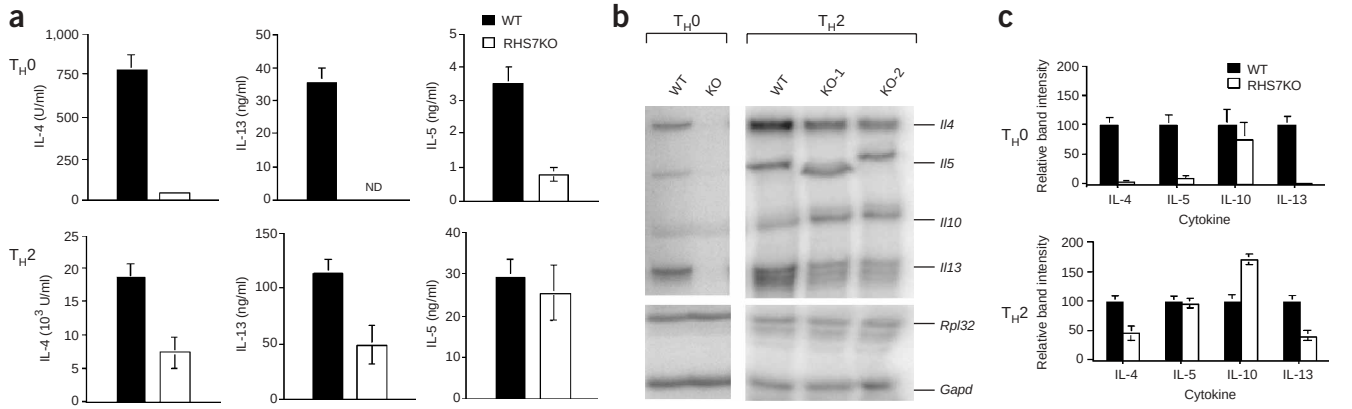

Figure 3 Expression of IL-4 and IL-13 is reduced in T_H0 and T_H2 cells from RHS7-deficient mice. (**a**) T_H2 cytokine protein expression. Naive $CD4^+$ T cells from wild-type (WT) and RHS7-deficient (knockout; KO) mice were differentiated for 5 d *in vitro* in either neutral (T_H0) or T_H2 conditions with soluble anti-CD3 and APCs. The cells were restimulated with plate-bound anti-CD3 for 24 h and cytokines in the supernatant were measured by ELISA. ND, not determined. Data represent the mean ± s.e.m. for three mice per group in one of three independent experiments with similar results. (**b**) RNase protection of T_H2 cytokine mRNAs. Total RNA was isolated from *in vitro*–differentiated T_H0 or T_H2 cells from wild-type and RHS7 knockout mice. The RNase protection assay used mCK-1b. Data are representative of one of three independent experiments with similar results. (**c**) Relative cytokine expression. The band intensity of each cytokine signal was measured with a phosphoimager and normalized to those of the *Rpl32* and *Gapd* signals. The band intensities of cytokines in cells from wild-type mice were arbitrarily set as 100%. Three independent experiments were combined for statistical analysis. Data represent the mean ± s.e.m. of three independent experiments.

expression of IL-5 in RHS7-deficient $CD4^+$ T cells was similar to that in wild-type $CD4^+$ T cells in T_H2 conditions (**Fig. 3a**, bottom).

We also measured the abundance of cytokine mRNA in T_H0 and T_H2 cells from wild-type and RHS7-deficient mice by RNase protection assay. Consistent with our ELISA results, the expression of *Il4*, *Il13* and *Il5* mRNA was almost completely eliminated in neutral stimulation conditions in $CD4^+$ T cells from RHS7-deficient mice (**Fig. 3b**). Quantification by densitometry confirmed a substantial reduction of band intensity for *Il4*, *Il5* and *Il13* mRNA in cells from RHS7-deficient mice compared with that in cells from wild-type mice (**Fig. 3c**). In T_H2 stimulation conditions, the expression of *Il4* and *Il13* mRNA was reduced in RHS7-deficient mice compared with that in wild-type mice, but the expression of *Il5* mRNA was similar in both types of mouse (**Fig. 3b**). Quantification by densitometry showed that the band intensity for *Il4* and *Il13* mRNA in RHS7-deficient mice was 50% less than that in wild-type mice, but there was no difference in the band intensity for *Il5* mRNA between RHS7-deficient and wild-type mice (**Fig. 3c**).

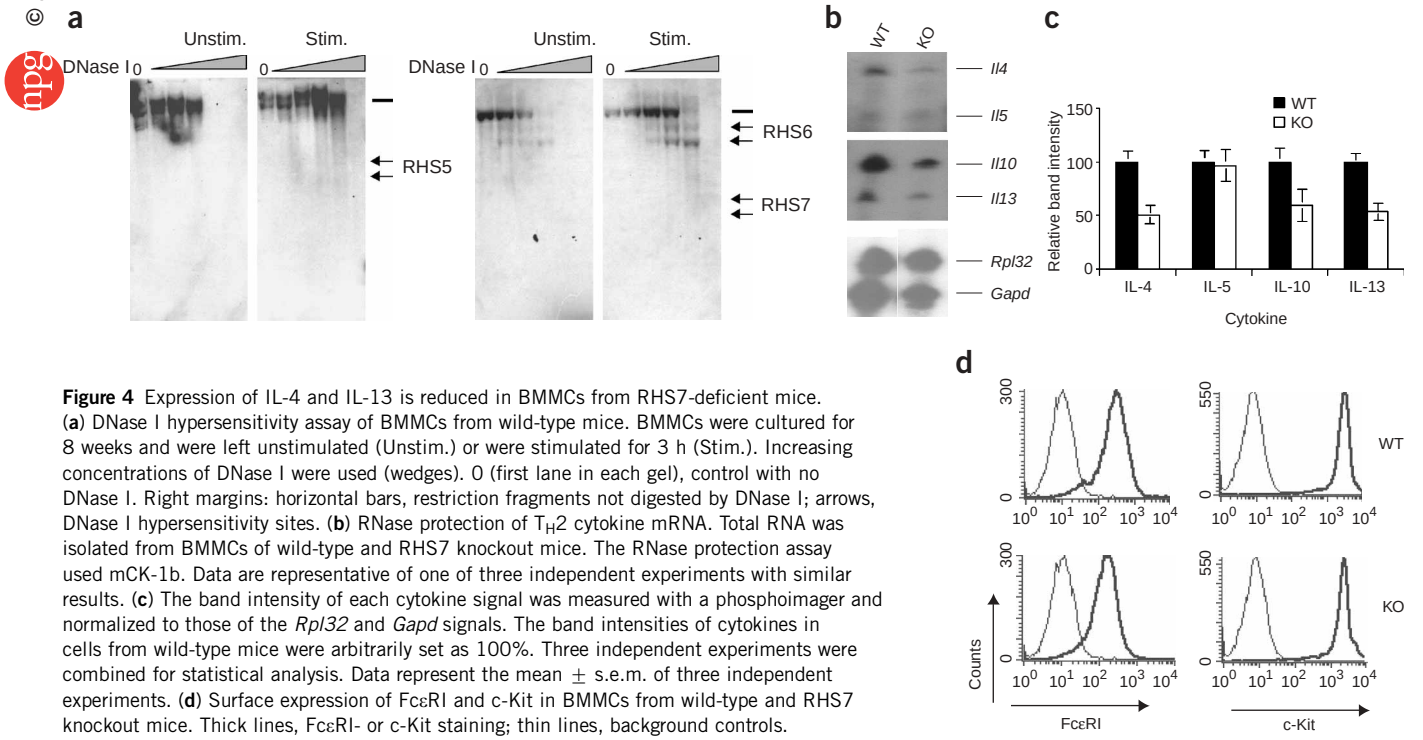

Figure 4 Expression of IL-4 and IL-13 is reduced in BMMCs from RHS7-deficient mice. (**a**) DNase I hypersensitivity assay of BMMCs from wild-type mice. BMMCs were cultured for 8 weeks and were left unstimulated (Unstim.) or were stimulated for 3 h (Stim.). Increasing concentrations of DNase I were used (wedges). 0 (first lane in each gel), control with no DNase I. Right margins: horizontal bars, restriction fragments not digested by DNase I; arrows, DNase I hypersensitivity sites. (**b**) RNase protection of T_H2 cytokine mRNA. Total RNA was isolated from BMMCs of wild-type and RHS7 knockout mice. The RNase protection assay used mCK-1b. Data are representative of one of three independent experiments with similar results. (**c**) The band intensity of each cytokine signal was measured with a phosphoimager and normalized to those of the *Rpl32* and *Gapd* signals. The band intensities of cytokines in cells from wild-type mice were arbitrarily set as 100%. Three independent experiments were combined for statistical analysis. Data represent the mean ± s.e.m. of three independent experiments. (**d**) Surface expression of FcεRI and c-Kit in BMMCs from wild-type and RHS7 knockout mice. Thick lines, FcεRI- or c-Kit staining; thin lines, background controls.

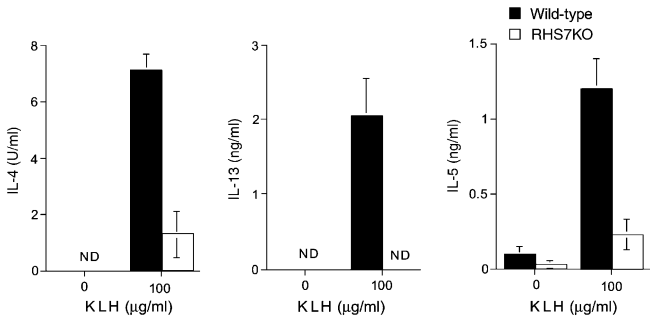

Figure 5 Reduced expression of T_H2 cytokines in RHS7-deficient mice on immunization with KLH. Alum-precipitated KLH (100 μg in 200 μl of PBS) was injected intraperitoneally into 8-week-old wild-type and RHS7 knockout mice. After 10 d, spleens from the immunized mice were removed and 1×10^7 spleen cells were cultured in the presence (100) or absence (0) of 100 μg/ml of KLH for 5 d. Concentrations of T_H2 cytokines in the culture supernatants were measured by ELISA. Data are expressed as the mean ± s.d. ($n = 4$) and are representative of one of three independent experiments with similar results. ND, not determined.

Mast cells constitute another source of T_H2 cytokines. Several regulatory regions in the T_H2 cytokine locus affect the expression of T_H2 cytokines similarly (HS5 and HS5a)[18] or differently (CNS-1)[16] in $CD4^+$ T cells and mast cells. We therefore examined whether mast cells form the same DNase I hypersensitivity sites that are present in the T_H2 LCR region in $CD4^+$ T cells. We stimulated bone marrow–derived mast cells (BMMCs) by crosslinking the FcεRI receptor and analyzed the formation of DNase I hypersensitivity sites by Southern blot. Mast cells formed the same DNase I hypersensitivity sites as did T_H2 cells in an activation-dependent way (**Fig. 4a**).

We also examined the expression of T_H2 cytokine mRNA in wild-type and RHS7-deficient mast cells by RNase protection assays. The expression of *Il4* and *Il13* mRNA in RHS7-deficient mast cells was 50% of that in wild-type mast cells (**Fig. 4b,c**), whereas that of *Il5* mRNA did not differ. This reduced expression of *Il4* and *Il13* was not due to defective mast cell differentiation, because surface expression of the mast cell markers FcεRI and c-Kit was similar between wild-type and RHS7-deficient mice (**Fig. 4d**). This result suggests that deletion of RHS7 affects T_H2 cytokine expression similarly in mast cells and in T_H2-polarized $CD4^+$ T cells.

Because *Il10* is expressed in T_H2 cells but is not located in the T_H2 cytokine locus, we examined whether its expression was affected by deletion of RHS7. The expression of *Il10* mRNA was slightly upregulated in T_H2 cells (**Fig. 3b,c**) and slightly downregulated in mast cells (**Fig. 4b,c**) from RHS7-deficient mice. This slight change in expression is probably a secondary outcome resulting from the effect of RHS7 on T_H2 cytokines involved in T_H2 differentiation. Together, these results show that RHS7 has a crucial function in regulating *Il4* and *Il13* expression coordinately as a component of the T_H2 cytokine LCR in T_H2 cells and mast cells.

T_H2 cytokine expression *in vivo*

We further investigated the function of RHS7 in the regulation of T_H2 cell differentiation by *in vivo* peptide immunization. We immunized wild-type and RHS7-deficient mice with alum-precipitated keyhole limpet hemocyanin (KLH). After 10 d, we collected splenic cells and stimulated them with KLH *in vitro* for 5 d. We measured expression of T_H2 cytokines in the culture supernatants by ELISA. Expression of the three cytokines IL-4, IL-13 and IL-5 was significantly lower in splenic cells from RHS7-deficient mice than in those from wild-type mice ($n = 4$; $P < 0.001$, Student's *t*-test; **Fig. 5**). This result suggests that RHS7 is essential in the *in vivo* differentiation of T_H2 cells in response to antigenic stimulus.

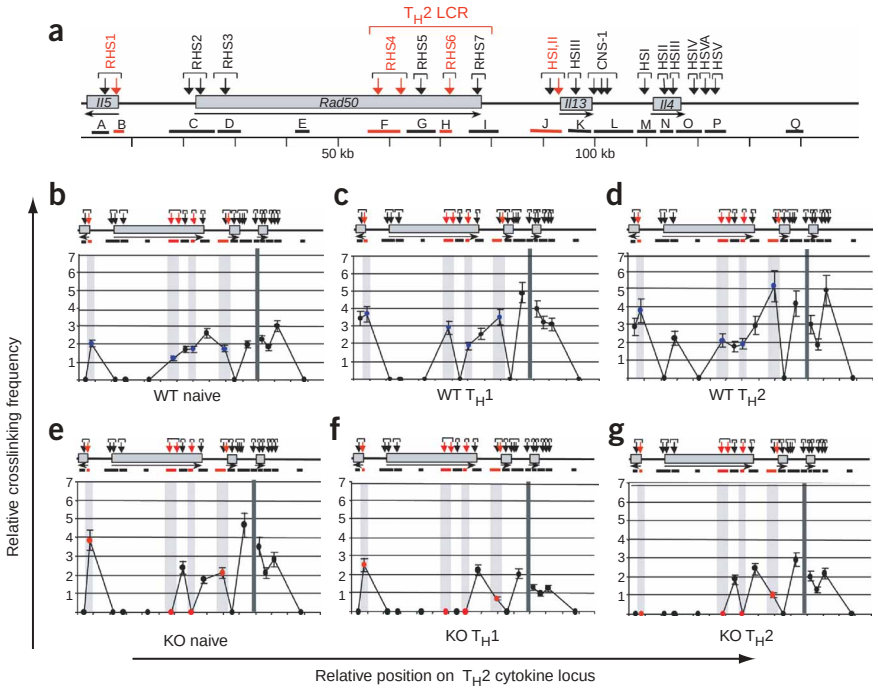

Figure 6 Impaired interaction of RHS4 and RHS6 with the *Il4* promoter in RHS7-deficient $CD4^+$ T cells. Chromosome conformation capture assay was done on the T_H2 cytokine locus in purified naive $CD4^+$ T cells or *in vitro*–stimulated T_H1 and T_H2 cells from wild-type and RHS7 knockout mice. (**a**) Relative positions of the cytokine genes in the T_H2 locus and DNase I hypersensitivity sites. Downward arrows, DNase I hypersensitivity sites; A–Q, digestion fragments produced by the *Bgl*II. (**b**–**g**) Relative positions of the *Bgl*II fragments in the locus plotted against their relative crosslinking frequency with *Bgl*II fragment M (the *Il4* promoter fragment)[24]. A relative crosslinking frequency of 1 was arbitrarily assigned to the crosslinking frequency between two neighboring fragments A and B in the *Gapd* locus, for which the *Bgl*II fragments analyzed are 559 bp apart[24]. Relative positions of RHS4, RHS6 and the promoters of *Il5* and *Il13* are in red above each graph; relative crosslinking frequencies of RHS4, RHS6 and the promoters of *Il5* and *Il13* are in blue for wild-type cells (**b**–**d**) and red for RHS7 knockout cells (**e**–**g**). Three independent experiments were combined for statistical analysis. Data represent the mean ± s.e.m. of three independent experiments.

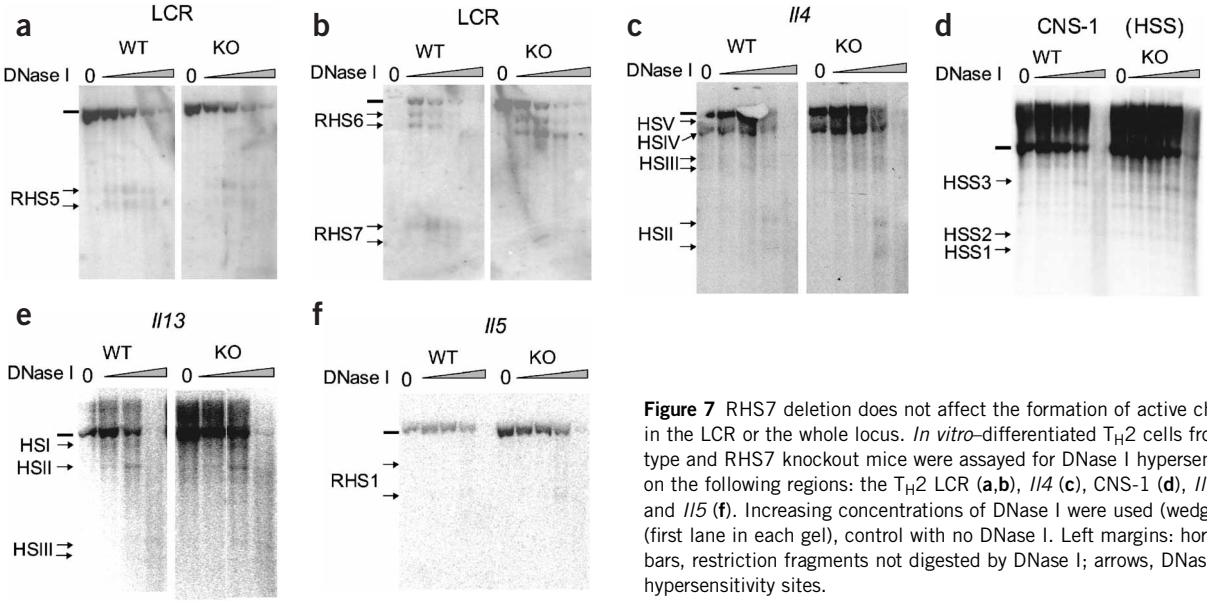

Figure 7 RHS7 deletion does not affect the formation of active chromatin in the LCR or the whole locus. *In vitro*–differentiated T_H2 cells from wild-type and RHS7 knockout mice were assayed for DNase I hypersensitivity on the following regions: the T_H2 LCR (**a**,**b**), *Il4* (**c**), CNS-1 (**d**), *Il13* (**e**) and *Il5* (**f**). Increasing concentrations of DNase I were used (wedges). 0 (first lane in each gel), control with no DNase I. Left margins: horizontal bars, restriction fragments not digested by DNase I; arrows, DNase I hypersensitivity sites.

Long-range intrachromosomal interactions

The T_H2 LCR and the promoters of *Il4*, *Il5* and *Il13* are closely juxtaposed in the nucleus[24], suggesting that there are long-range intrachromosomal interactions between these sequences. We therefore investigated whether RHS7 is functionally involved in this interaction. We stimulated naive $CD4^+$ T cells from wild-type and RHS7-deficient mice *in vitro* in T_H1 or T_H2 conditions and examined long-range interactions with the *Il4* promoter by chromosome conformation capture (**Fig. 6a**).

As shown before[24], the *Il4* promoter interacted with the LCR in naive, T_H1 and T_H2 cells from wild-type mice, with the exception in T_H1 cells of RHS5, which did not interact with the *Il4* promoter (**Fig. 6b–d**). Notably, deletion of RHS7 resulted in a complete loss of interaction between the *Il4* promoter and both RHS4 and RHS6 in naive, T_H1 and T_H2 cells (**Fig. 6e–g**). This result suggests that RHS7 is essential for maintaining the core chromatin configuration of the LCR in a 'poised' state. Notably, deletion of RHS7 also led to reduced interactions of the *Il4* promoter with the *Il13* and *Il5* promoters in T_H1 and T_H2 cells but not in naive cells, whereas the presence of RHS7 increased these interactions in T_H1 and T_H2 cells (**Fig. 6**). This result suggests that RHS7 is required for maintaining and stabilizing the 'active chromatin hub' on TCR stimulation. The idea of an active chromatin hub was first proposed to describe the clustering of all DNase I hypersensitivity sites, enhancers and promoters of active genes in the β-globin LCR[31,32].

These results suggest that RHS7 is essential in establishing, maintaining and stabilizing long-range intrachromosomal interactions between the T_H2 LCR and the promoters of *Il4*, *Il13* and *Il5* both in poised and activated states, thereby identifying RHS7 as a key component in the mechanism underlying the regulation of T_H2 cytokine genes.

Formation of overall active chromatin structure

We further tested whether RHS7 is important in establishing the overall active chromatin structure of the T_H2 cytokine locus. The RHS7 site is rapidly demethylated in T_H2 cells after TCR stimulation[23]; in addition, histone acetylation is strongly increased in RHS7 in T_H2 cells[23]. From these results, we speculated that RHS7 might be the site at which chromatin changes are initiated during T_H2 cell differentiation. We tested this hypothesis by examining whether the formation of DNase I hypersensitivity sites in the LCR was affected by deletion of RHS7.

Deletion of RHS7 did not affect the formation of RHS5 and RHS6, suggesting that RHS7 is not necessary for the formation of an overall active chromatin structure in the T_H2 LCR region (**Fig. 7a,b**). These data also suggested that other hypersensitive sites (RHS4, RHS5 and RHS6) may have a redundant function in the formation of open chromatin structure. We further examined the overall chromatin structure in the whole locus including *Il4*, *Il13*, *Il5* and CNS-1 regions by DNase I hypersensitivity assay; however, deletion of RHS7 did not affect the formation of DNase I hypersensitivity sites in the whole locus (**Fig. 7**).

DISCUSSION

Using targeted deletion of RHS7 in mice, we have investigated the function of RHS7 in the differentiation of T_H2 cells, in the expression of T_H2 cytokine genes and in the chromatin-remodeling events underlying these processes. We found that $CD4^+$ T cells and mast cells from RHS7-deficient mice show reduced T_H2 cytokine expression after antigenic stimulation both *in vivo* and *in vitro*. We also found that RHS7 is essential in the formation of a core chromatin configuration (the poised state) between the LCR and promoters of the T_H2 cytokine genes. Our study also suggests, however, that RHS7 is dispensable for the formation of an active chromatin structure in the T_H2 LCR region and in the whole T_H2 cytokine locus.

Deletion of RHS7 caused a reduction in the production of the three T_H2 cytokines IL-4, IL-5 and IL-13, showing that RHS7 is a crucial component of the T_H2 cytokine LCR that coordinately regulates the expression of the genes encoding these proteins. This result is consistent with studies showing strong enhancer function of RHS7 and the LCR in combination with RHS5 and RHS6 in a transgenic reporter assay[23]. The mechanism of this coordinate gene regulation needs further investigation. The T_H2 cytokine LCR colocalizes closely with the promoters of *Il4*, *Il5* and *Il13*, suggesting that there are long-range intrachromosomal interactions among these elements[24]. This close

proximity has been shown in naive, T_H1 and T_H2 cells, but not in non-expressing fibroblasts, suggesting that these elements adopt a poised configuration in $CD4^+$ lineage cells[24]. The T_H2 cytokine LCR does not, however, colocalize closely with the *Rad50* promoter, which suggests that *Rad50* is not present in the core chromatin configuration. This result supports a looping model in which cytokine promoters and the LCR associate by forming a chromatin complex for the coordinate regulation of T_H2 cytokine genes. Such a looping model has been proposed for the β-globin LCR[31–33].

Because RHS7 is the only distal regulatory site in the *Il4-Il5-Il13* locus that has been so far shown to be demethylated rapidly in a T_H2-specific way after antigenic stimulation, it will be useful to elucidate the functional relevance of this demethylation in gene transcription and chromatin-remodeling events during T_H2 cell differentiation. Previous work that examined the function of site-specific demethylation of the *Il2* promoter-enhancer region with a transient transfection assay using reporter constructs containing an *in vitro*–methylated or *in vitro*–demethylated enhancer-promoter region showed that this demethylation is essential for *Il2* transcription[30]. Our study here, which examined the functional involvement of site-specific demethylation in the context of endogenous chromatin, is consistent with the results found for *Il2*. Another feature of demethylation in the *Il2* enhancer-promoter region is that it occurs very rapidly, as early as 20 min after antigenic stimulation *in vivo*; thus, it has been proposed to be an active process[30]. Because demethylation in RHS7 is also rapid, occurring within 48 h of stimulation[23], it will be useful to determine whether an active process is involved in the demethylation in RHS7, as proposed for the *Il2* promoter-enhancer region[23].

The T_H2 LCR regulates the expression of the T_H2 cytokine genes[20]. Because the LCR is located in *Rad50*, it seemed possible that the LCR might affect the expression of *Rad50*. Our study shows, however, that the LCR regulates the T_H2 cytokine genes without affecting the expression of *Rad50*. Mechanistically, this result is further supported by our chromosome conformation capture experiment, which showed that *Rad50* is not located close to the promoters of the T_H2 cytokine genes and the LCR[24], suggesting that the interactions of the LCR are specific to cytokine promoters.

Differentiation-specific clustering of DNase I hypersensitivity sites and promoters at the core chromatin configuration has been shown in the β-globin LCR[32] and in the T_H2 cytokine LCR[24]. Our data further show that a component of the LCR is crucial in establishing, maintaining and stabilizing the long-range intrachromosomal interactions, providing a clue to the mechanisms underlying the function of LCRs. It will be useful to study the exact function of RHS7 in the formation of the active chromatin hub.

All RHSs (RHS4, RHS5, RHS6 and RHS7) interact with the promoters of T_H2 cytokine genes on TCR stimulation, suggesting that each hypersensitive site has an additive and presumably non-redundant function in the formation of a core chromatin configuration. Our results are consistent with the report that several interactions between hypersensitive sites in the β-globin LCR and the promoters of genes in the cluster are required to stabilize an active chromatin hub[34]. Our data have shown that the overall active chromatin structure of the locus is not affected by the deletion of RHS7, suggesting that the hypersensitive sites and their flanking sequences have a cooperative and redundant nature. This result is similar to previous findings from studies of the human β-globin LCR showing that the deletion of hypersensitive sites in the β-globin LCR in the endogenous locus does not affect the active chromatin structure, although it reduces the expression of globin genes[31,35–37]. It will be useful to see whether the combined deletion of RHS regions and/or the deletion of larger regions has even greater effects on the expression of the T_H2 cytokine genes and chromatin opening.

We found that defects in T_H2 cytokine gene expression caused by the deletion of RHS7 were greater after neutral stimulation conditions and *in vivo* immunization than after T_H2 differentiation conditions. One explanation might be differences in the amount of IL-4 available at the initial priming in neutral stimulation (including *in vivo*) versus T_H2 stimulation conditions. In neutral and *in vivo* conditions there is no exogenously added IL-4; thus, the initial production of IL-4 in neutral and *in vivo* conditions may be more dependent on the endogenous RHS7 locus. In contrast, large amounts of IL-4 added in T_H2 stimulation conditions may render the system relatively independent of the IL-4 output from the endogenous RHS7 locus.

The expression of *Il5* in cells from RHS7-deficient mice did not change after stimulation *in vitro* in T_H2 polarizing conditions, but was reduced greatly after both stimulation *in vitro* in neutral conditions and *in vivo* immunization. One explanation is the availability of IL-4 during initial stimulation as described above. Another explanation is that the expression of *Il5* might depend less on RHS7 than does the expression of *Il4* and *Il13*. Unlike the expression of *Il4* and *Il13*, the expression of *Il5* may not require RHS7 in conditions in which IL-4 is present. It will be useful to determine whether other regulatory regions are necessary for the proper regulation of *Il5*.

In summary, we have shown that RHS7 is essential for the proper and coordinate regulation of T_H2 cytokine gene expression and for the formation of a 'poised' chromatin configuration. Our results also shed light on how distal regulatory elements function during cell lineage commitment.

METHODS

Generation of RHS7-deficient mice. A BAC DNA containing *Rad50* (ref. 19) was used for construction of the targeting vector. A 5.6-kb *Bst*UI-*Sma*I fragment of *Rad50* was subcloned as the 5′ arm, and a 1.5-kb fragment containing exon 25 was subcloned as the 3′ arm into pEasyFlox (a gift from K. Rajewsky, Harvard University, Cambridge, Massachusetts). This vector was designed to replace the gene encoding RHS7 (637 bp, located 55,649–56,285 bp from the translation start site of *Rad50*) with the neo^r cassette flanked by two *lox*P sites, and a thymidine kinase gene was used to negatively select clones with random integration of the targeting vector. The vector was linearized by digestion with *Not*I and transferred into the TC1 embryonic stem cell line (a gift from P. Leder, Harvard University, Cambridge, Massachusetts) by electroporation. Clones resistant to G418 and gancyclovir were selected, and homologous recombination was confirmed by Southern blot analysis. We deleted neo^r by transfecting the Cre expression vector pGK-Cre (a gift from K. Rajewsky) into the embryonic stem cells. Clones with the correct deletion were screened by Southern blot analysis. Two independently targeted embryonic stem clones were injected into C57BL/6 blastocysts and were transferred into pseudopregnant foster mothers, and the resulting male chimeric mice were bred to C57BL/6 females to obtain heterozygous mice. Germline transmission was verified for both embryonic stem clones by Southern blot analysis of DNA obtained from tail tissue DNA from F_1 offspring. Heterozygous mice were interbred to generate homozygous RHS7-deficient mice. The phenotypes of the two lines were identical. Experiments with live mice were approved by the Yale University Institutional Animal Care and Use Committee.

RT-PCR. Total RNA was isolated from *in vitro*–differentiated T_H2 cells with TRIzol reagent (Invitrogen). Reverse transcription was done with Superscript II reverse transcriptase (Invitrogen). The PCR primers for *Rad50* were 5′-CGCAACTTCCAACTTCTGGTAATCACTCAC-3′ and 5′-TCAATGACTGT GGTTTCAGGGGAGACC-3′. For normalization of the amount of cDNA, quantitative PCR was done with hypoxanthine guanine phosphoribosyl transferase (*Hprt1*) primers and competitor cDNA as described[38].

***In vitro* CD4$^+$ cell differentiation.** We enriched CD4$^+$ T cells from the spleen cells of wild-type and RHS7-deficient mice by negative selection through depletion with monoclonal antibodies to major histocompatibility complex class II (M5/115), NK1.1 (HB191) and CD8 (T1B105), followed by depletion with a mixture of magnetic beads conjugated to anti-rat and anti-mouse immunoglobulin (Perseptive Biosystems). Naive CD4$^+$ T cells were sorted on the basis of the surface markers CD4^{hi}, CD62L^{hi} and CD44^{lo}. APCs were prepared by γ-irradiation and negative selection[19]. For neutral differentiation, 1×10^6 naive CD4$^+$ T cells were cultured with an equal number of APCs in 5 ml of Bruffs medium with 5% FCS (Life Technologies), penicillin and streptomycin in the presence of 2 μg/ml of soluble anti-CD3 (2C11) and 20 U/ml of IL-2. For T_H2 cell differentiation, 1,000 U/ml of IL-4 and anti–interferon-γ (XMG1.2) were added to the same culture. After 5 d, the cells were washed and restimulated for 24 h with plate-bound anti-CD3.

RNase protection assay. Total RNA was isolated from *in vitro*–differentiated T_H2 cells as described above. The RNase protection assay used a RiboQuant Multi-Probe RNase Protection Assay System according to the manufacturer's instructions (BD Bioscience). We used 10 μg of total RNA of each sample for hybridization with the [α-^{32}P]UTP-labeled mouse cytokine multiprobe template set mCK-1b (including templates for *Il4*, *Il5*, *Il10*, *Il13*, *Il15*, *Il9*, *Il2*, *Il3*, *Ifng*, ribosomal protein L32 (*Rpl32*) and glyceraldehyde phosphodehydrogenase (*Gapd*)). Protected fragments were separated by using a QuickPoint Gel System according to the manufacturer's instructions (Novex). Gels were dried for 1 h at 80 °C and were exposed to a STORM 820 phosphoimager (Amersham) for quantification of band intensities.

BMMC culture, stimulation and staining. Mast cells from wild-type and RHS7-deficient mice were generated from bone marrow and stimulated by crosslinking FcεRI as described[18]. We stained BMMCs for FcεRI and c-Kit as described[18].

KLH immunization. KLH in solution (1 mg/ml) was mixed with an equal volume of 4.5% alum and was precipitated by the dropwise addition of 1 M NaOH. We injected 100 μg of alum-precipitated KLH in 200 μl of PBS intraperitoneally into 8-week-old wild-type and RHS7-deficient mice. After 10 d, spleens from the immunized mice were obtained and 1×10^7 splenic cells were cultured for 5 d in the presence or absence of 100 μg/ml of KLH. T_H2 cytokines in the culture supernatants were measured by ELISA.

Chromosome conformation capture assay. Chromosome conformation capture assay was done as described[24] with purified naive CD4$^+$ T cells or *in vitro*–stimulated T_H1 and T_H2 cells from wild-type and RHS7-deficient mice.

DNase I hypersensitivity assay. DNase I hypersensitivity assays for T_H2 cells and BMMCs from wild-type and RHS7-deficient mice were done as described[7,9,23].

ACKNOWLEDGMENTS

We thank L. Evangelisti, C. Hughes and J. Stein for assisting with the generation of RHS7-deficient mice; P.E. Fields, S.T. Kim and E. Eynon for discussion; and F. Manzo for help with manuscript preparation. Supported by the National Institutes of Health (HL56389 to R.A.F.), Cancer Research Institute (C.S.) and Howard Hughes Medical Institute (R.A.F.). R.A.F. is an investigator of the Howard Hughes Medical Institute.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 25 August; accepted 17 November 2004
Published online at http://www.nature.com/natureimmunology/

1. Abbas, A.K., Murphy, K.M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383**, 787–793 (1996).
2. Dong, C. & Flavell, R.A. TH1 and TH2 cells. *Curr. Opin. Hematol.* **8**, 47–51 (2001).
3. Glimcher, L.H. & Murphy, K.M. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* **14**, 1693–1711 (2000).
4. O'Garra, A. & Arai, N. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell Biol.* **10**, 542–550 (2000).
5. Ansel, K.M., Lee, D.U. & Rao, A. An epigenetic view of helper T cell differentiation. *Nat. Immunol.* **4**, 616–623 (2003).
6. Smale, S.T. & Fisher, A.G. Chromatin structure and gene regulation in the immune system. *Annu. Rev. Immunol.* **20**, 427–462 (2002).
7. Agarwal, S. & Rao, A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* **9**, 765–775 (1998).
8. Bird, J.J. *et al.* Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9**, 229–237 (1998).
9. Takemoto, N. *et al.* TH2-specific DNase I-hypersensitive sites in the murine IL-13 and IL-4 intergenic region. *Int. Immunol.* **12**, 1981–1985 (1998).
10. Lee, D.U., Agarwal, S. & Rao, A. TH2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* **16**, 649–660 (2002).
11. Fields, P.E., Kim, S.T. & Flavell, R.A. Cutting edge: changes in histone acetylation at the IL-4 and IFN-γ loci accompany TH1/TH2 differentiation. *J. Immunol.* **169**, 647–650 (2002).
12. Avni, O. *et al.* T_H cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat. Immunol.* **3**, 643–651 (2002).
13. Yamashita, M. *et al.* Identification of a conserved GATA3 response element upstream proximal from the interleukin-13 gene locus. *J. Biol. Chem.* **277**, 42399–42408 (2002).
14. Makar, K.W. *et al.* Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. *Nat. Immunol.* **4**, 1183–1190 (2003).
15. Loots, G.G. *et al.* Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288**, 136–140 (2000).
16. Mohrs, M. *et al.* Deletion of a coordinate regulator of type 2 cytokine expression in mice. *Nat. Immunol.* **2**, 842–847 (2001).
17. Agarwal, S., Avni, O. & Rao, A. Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer *in vivo*. *Immunity* **12**, 643–652 (2000).
18. Solymar, D.C. *et al.* A 3′ enhancer in the IL-4 gene regulates cytokine production by TH2 cells and mast cells. *Immunity* **17**, 41–50 (2002).
19. Lee, G.R., Fields, P.E. & Flavell, R.A. Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity* **14**, 447–459 (2001).
20. Lee, G.R., Fields, P.E., Griffin, T.J., IV & Flavell, R.A. Regulation of the TH2 cytokine locus by a locus control region. *Immunity* **19**, 145–153 (2003).
21. Grosveld, F., van Assendelft, G.B., Greaves, D.R. & Kollias, G. Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell* **51**, 975–985 (1987).
22. Milot, E. *et al.* Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* **87**, 105–114 (1996).
23. Fields, P.E., Lee, G.R., Kim, S.T., Bartsevich, V. & Flavell, R.A. Chromatin domains and enhancer activity identify functional components of the TH2 cytokine locus control region. *Immunity* (in the press).
24. Spilianakis, C.G. & Flavell, R.A. Long range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nat. Immunol.* **5**, 1017–1027 (2004).
25. Dekker, J., Rippe, K., Dekker, M. & Kleckner, N. Capturing chromosome conformation. *Science* **295**, 1306–1311 (2002).
26. Bird, A.P. & Wolffe, A.P. Methylation-induced repression—belts, braces, and chromatin. *Cell* **99**, 451–454 (1999).
27. Attwood, J.T., Yung, R.L. & Richardson, B.C. DNA methylation and the regulation of gene transcription. *Cell. Mol. Life Sci.* **59**, 241–257 (2002).
28. Ehrlich, M. DNA methylation in cancer: too much, but also too little. *Oncogene* **21**, 5400–5413 (2002).
29. Fitzpatrick, D.R. & Wilson, C.B. Methylation and demethylation in the regulation of genes, cells, and responses in the immune system. *Clin. Immunol.* **109**, 37–45 (2003).
30. Bruniquel, D. & Schwartz, R.H. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat. Immunol.* **4**, 235–240 (2003).
31. Tolhuis, B. *et al.* Looping and interaction between hypersensitive sites in the active β-globin locus. *Mol. Cell* **10**, 1453–1465 (2002).
32. Palstra, R.J. *et al.* The β-globin nuclear compartment in development and erythroid differentiation. *Nat. Genet.* **35**, 190–194 (2003).
33. Carter, D. *et al.* Long-range chromatin regulatory interactions *in vivo*. *Nat. Genet.* **32**, 623–626 (2002).
34. Patrinos, G.P. *et al.* Multiple interactions between regulatory regions are required to stabilize an active chromatin hub. *Genes Dev.* **18**, 1495–1509 (2004).
35. Epner, E. *et al.* The β-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse β-globin locus. *Mol. Cell* **2**, 447–455 (1998).
36. Reik, A. *et al.* The locus control region is necessary for gene expression in the human β-globin locus but not the maintenance of an open chromatin structure in erythroid cells. *Mol. Cell. Biol.* **18**, 5992–6000 (1998).
37. Bender, M.A., Bulger, M., Close, J. & Groudine, M. β-Globin gene switching and DNase I sensitivity of the endogenous β-globin locus in mice do not require the locus control region. *Mol. Cell* **5**, 387–393 (2000).
38. Reiner, S.L., Zheng, S., Corry, D.B. & Locksley, R.M. Constructing polycompetitor cDNAs for quantitative PCR. *J. Immunol. Methods* **165**, 37–46 (1993).