nature immunology

Long-range intrachromosomal interactions in the T helper type 2 cytokine locus

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

The T helper type 2 (T_H2) locus control region is important in the regulation of the genes encoding the cytokines interleukins 4, 5 and 13. Using the chromosome conformation capture technique, we found that in T cells, natural killer cells, B cells and fibroblasts, the promoters for the genes encoding T_H2 cytokines are located in close spatial proximity, forming an initial chromatin core configuration. In $CD4^+$ T cells and natural killer cells, but not B cells and fibroblasts, the T_H2 locus control region participates in this configuration. The transcription factors GATA3 and STAT6 are essential for the establishment and/or maintenance of these interactions. Intrachromosomal interactions in the T_H2 cytokine locus may form the basis for the coordinated transcriptional regulation of cytokine-encoding genes by the T_H2 locus control region.

Il4, the gene encoding interleukin 4 (IL-4), is in the T helper type 2 (T_H2) cytokine locus, located on mouse chromosome 11. The genomic region of the T_H2 cytokine locus covers more than 120 kilobases (kb) and also includes two other T_H2 cytokine–encoding genes, *Il5* and *Il13*. These three cytokine-encoding genes are coordinately expressed in T_H2 cells, but little is known about the molecular mechanisms that underlie this coordinate transcriptional regulation. After transgenic methods were used to introduce different genomic fragments of the T_H2 cytokine locus, a region consisting of 25 kb at the 3′ end of *Rad50* (a constitutively expressed DNA repair gene) was identified that acts as a locus control region (LCR) for the expression of cytokine-encoding genes in this locus[1]. An LCR is a DNA element that supports high-level tissue-specific expression in linked genes regardless of any flanking suppressive sequences[2].

Before the identification of the T_H2 LCR, most studies of *Il4* transcriptional regulation concentrated on the identification and function of DNase I–hypersensitive sites in the *Il4* locus[3,4]. The identification of specific sites on DNA that are cleaved by very low concentrations of DNase I is usually indicative of the presence of regulatory elements such as promoters, enhancers, LCRs, silencers, insulators or matrix attachment regions[5]. In the IL-4 locus, two such hypersensitive sites, HSS3 (ref. 3) and HS-IV (ref. 4), are present in all T helper cell types. Additional DNase I–hypersensitive sites are present in differentiating and mature T_H2 cells. Conserved region I (CNS-I)[6], comprising the sites HSS2 and HSS1, is located between *Il4* and *Il13*; HS-I is located at the *Il4* promoter; HS-II and HS-III are located in the second *Il4* intron; and sites V_A and V are located 'downstream' of *Il4* (ref. 7). Extensive analysis throughout the T_H2 cytokine locus has demonstrated the existence of seven additional hypersensitive sites, four of which (RHS4–RHS7) are located in the T_H2 LCR at the 3′ of *Rad50* (data not shown).

The discovery of a LCR in the β-globin locus and the characterization of other LCRs in various systems, such as the T_H2 cytokine locus, suggest that tissue-specific developmentally regulated gene transcription relies not only on gene-proximal elements (promoters, enhancers and silencers) but also on long-range interactions of various *cis*-regulatory elements and complex secondary chromatin configurations[8]. Although chromatin rearrangement and its function in transcriptional gene regulation have been extensively studied in detail at the nucleosome level, less is known about higher orders of chromatin organization. Two techniques have been developed to identify long-range chromosomal interactions and to characterize the higher-order folding of chromatin in the regulation of genes encoding β-globin by an LCR[9,10]. The looping of large chromosomal regions that do not participate in active transcriptional networks can explain how gene-regulatory elements like LCRs, despite being at a great distance from their target, can nonetheless confer regulatory effects on the target genes[11]. The looping out of large intervening sequences between two regulatory elements has been suggested[12]. The looping model proposes that parts of an LCR act together as a 'holocomplex' to interact directly with individual genes. This type of interaction is influenced by the distance between the LCR and its target genes and the availability of specific transcription factors, which affect the interaction of the LCR with more distant genes[13].

The β-globin LCR activates only one gene at a time[14]. Using the chromosome conformation capture technique[10], we show here that the T_H2 cytokine gene loci form an initial core complex, after which the T_H2 LCR can confer its activatory effects. In the activation of these genes spanning a genomic distance of more than 120 kb, at least two levels of regulation occur. A cell-specific conformational change elicits interaction of the individual cytokine-encoding gene loci with the T_H2 LCR in cell types of the T cell lineage, regardless of expression of genes

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

Published online 19 September 2004; doi:10.1038/ni1115

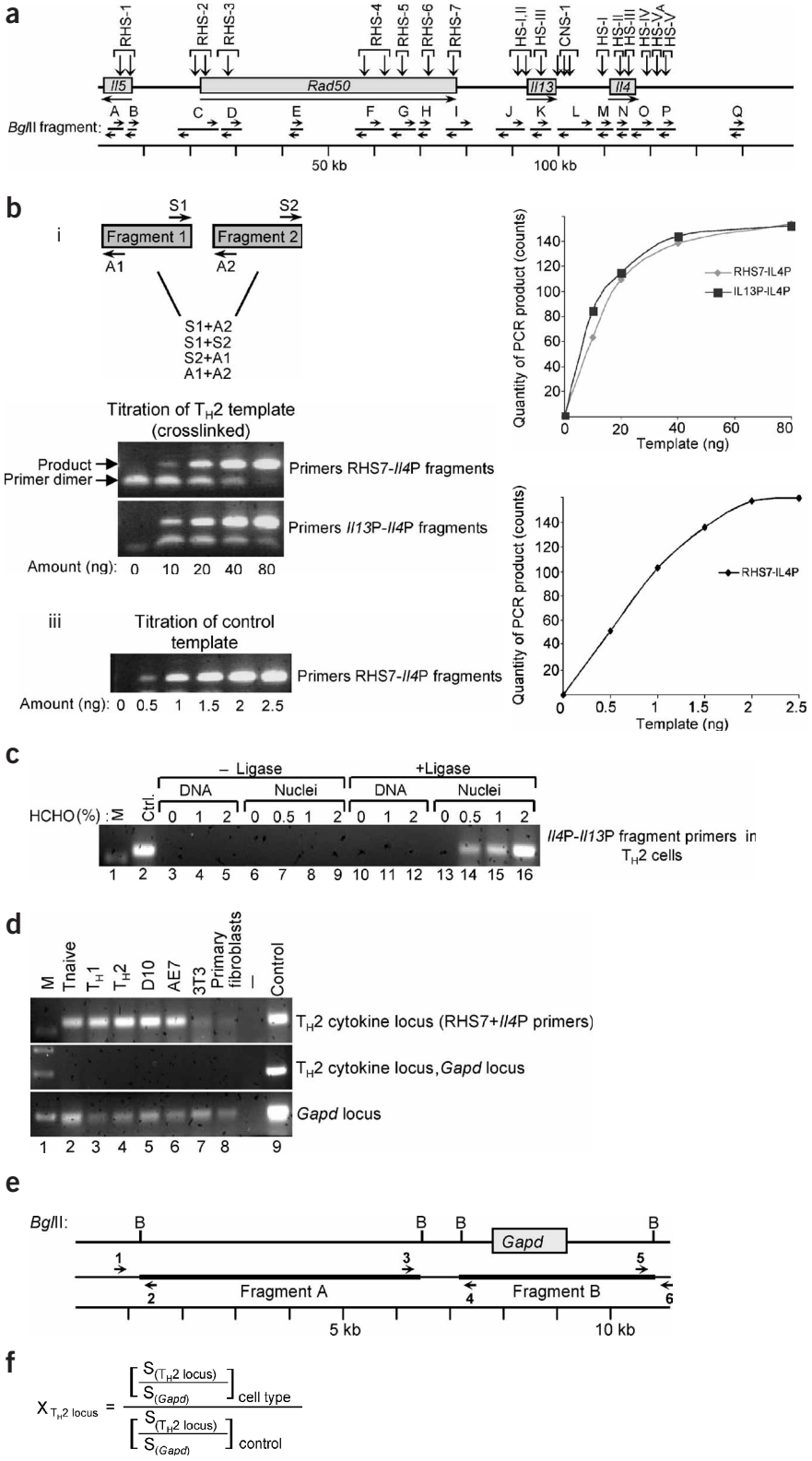

Figure 1 Chromosome conformation capture assay of the T_H2 cytokine locus. (**a**) T_H2 cytokine locus genes spanning a region of 120 kb. Counting on the T_H2 locus starts at −7,287 bp from the start site of *Il5*. Downward arrows above diagram indicate DNase I–hypersensitive sites characterized here (RHS) and elsewhere (HIS-III, HS-III, CNS-1, HS-I, HS-II, HS-III, HS-IV, HS-V_A and HS-V). Below, *Bgl*II restriction fragments (A–Q) used in the chromosome conformation capture analysis assay. The arrows above and below each fragment indicate primers used for each. (**b**) For each fragment of interest, a pair of primers was designed and used for PCR reactions in paired combinations (i). The signal of each reaction was measured to calculate the relative crosslinking frequency. ii, titration of the initial quantity of the template used in chromosome conformation capture assay derived from T_H2 cells and analyzed with two different sets of primers. Top, primers designed for fragments I and M (RHS7 site and *Il4* promoter (*Il4*P) fragments); bottom, primers designed for fragments J and M (*Il13* promoter and *Il4* promoter fragments). Similar titrations were done for every template used in the chromosome conformation capture to determine the initial quantity of the template to be used so the product would be in the exponential phase of amplification. iii, titrations for the randomly ligated control templates, similar to those for the crosslinked templates; in this example, with primers for fragments I and M (RHS7 site and *Il4* promoter fragments). (**c**) Control experiment with primer pairs for fragments M (*Il4* promoter) and J (*Il13* promoter) to detect ligation products on various templates. Lane 1, DNA marker (M); lane 2, control template (Ctrl). Lanes 3–9 (– Ligase), no ligation step; no PCR product. Lanes 10–12, purified mouse genomic DNA treated with formaldehyde (HCHO) and then digested with *Bgl*II, diluted in a DNA concentration of 2.8 ng/μl and ligated; no ligation product formation. Lanes 13–16, nuclei: no random intermolecular ligation products without formaldehyde crosslinking (lane 13); ligation product formation increases linearly with formaldehyde concentration (lanes 14–16). All subsequent chromosome conformation capture experiments used 2% formaldehyde. (**d**) PCR products obtained with primer pairs designed for fragments I (RHS7 site) and M (*Il4* promoter) of the T_H2 cytokine locus with different templates (top). Primer pair for fragment B of the *Gapd* locus combined with any other primer pair designed for every fragment of the T_H2 cytokine locus results in no product (middle). *Gapd* products obtained with primer pairs for fragments A and B of the *Gapd* locus, being 559 bp apart, are used for normalizing signals. Tnaive, naive T cells. (**e**) *Gapd* locus. B, *Bgl*II restriction enzyme sites. Fragments A and B, 559 bp apart, are analyzed by chromosome conformation capture assay. Numbered arrows indicate sites of the primers used for this locus. (**f**) Equation to calculate the relative crosslinking frequency between two fragments of the T_H2 locus ($X_{TH2\ locus}$). $S_{TH2\ locus}$ is the signal obtained with primer pairs for two different restriction enzyme fragments calculated for different cell types (T_H1, T_H2, 3T3 and so on) or for the control template (random ligated DNA). S_{Gapd} is the signal obtained with primer pairs for the *Gapd* locus fragments A and B, calculated for any cell type and the control template. This calculation gives a relative ligation crosslinking frequency for each cell type used, as it corrects for any differences in PCR amplification efficiencies, crosslinking and ligation efficiencies, the amounts of the template initially used, as well as the size of the PCR products.

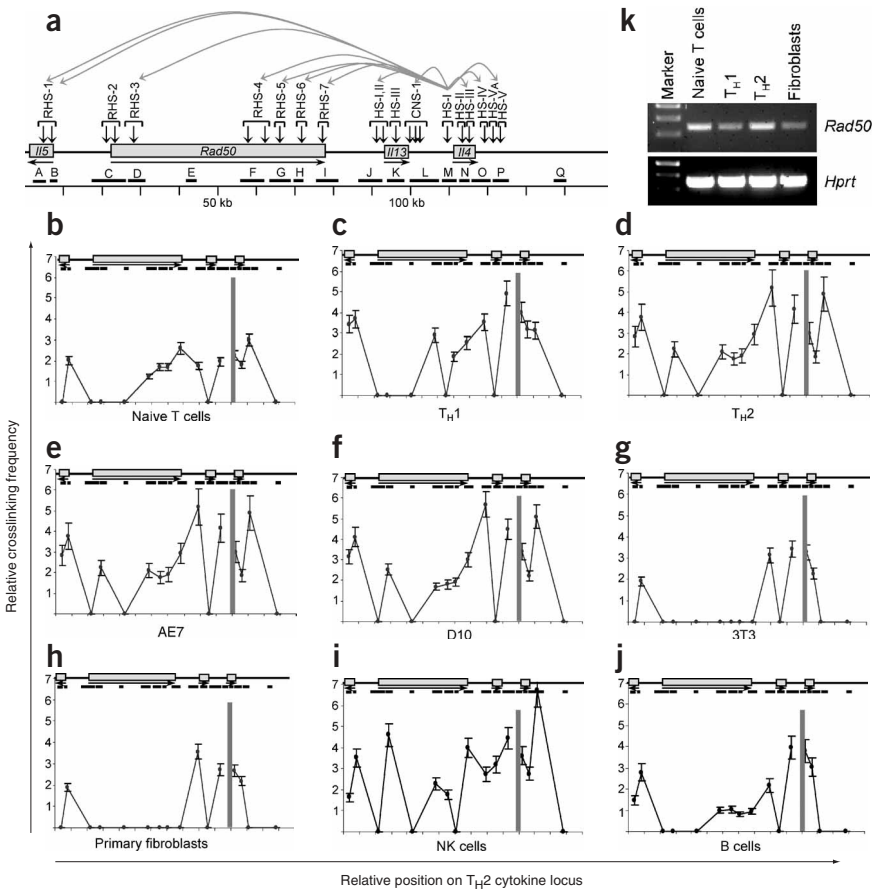

Figure 2 Poised chromatin conformation of the T_H2 cytokine gene loci and the T_H2 LCR in T cells and NK cells but not in fibroblasts. (**a**) Relative organization of the T_H2 cytokine locus genes with all characterized DNase I–hypersensitive sites. Gray arrows above indicate interactions of the *Il4* promoter fragment with other regions in the locus in T_H2 cells. (**b**–**j**) Relative crosslinking frequencies between stable fragment M (*Il4* promoter fragment) and the rest of the locus. (**b**–**d**) Different templates have been used for the analysis of naive T cells (**b**) and naive $CD4^+$ T mouse primary cells differentiated for 5 d with plate-bound anti-CD3 and anti-CD28 in T_H1 conditions (**c**) and T_H2 conditions (**d**). (**e**–**j**) Analysis of templates from the AE7 cell line (T_H1 phenotype; **e**), D10 cell line (T_H2 phenotype; **f**), 3T3 cell line (mouse fibroblasts; **g**), primary fibroblasts (**h**), NK cells (**i**) and B cells (**j**). Horizontal axes, position in the locus (1–150 kb); vertical axes, relative crosslinking frequency of fragment M (*Il4* promoter fragment) with all the other fragments of the locus. Gray bars, position of the *Il4* promoter (fragment M). For all similar graphs (**Figs. 2–6**): the relative crosslinking frequency with a value of 1 arbitrarily corresponds to the relative crosslinking frequency between the two neighboring fragments A and B of the *Gapd* locus, with fragments analyzed being 559 bp apart. The scaling on the vertical axes (1–7) allows direct comparison of all graphs. (**b**) RT-PCR showing the relative expression *Rad50* in T cells and fibroblasts. Cell cultures are the same used for the chromosome conformation capture analysis. *Hprt*, control.

encoding cytokines. In contrast, this change does not occur in B cells and fibroblasts. Further recruitment of cell type–specific transcription factors enables rapid activation of selective genes because these genes are present in a 'poised', preformed chromatin complex. Two T_H2-specific transcription factors, STAT6 and GATA3, are responsible for the formation and/or maintenance of the chromatin conformation changes in the T_H2 cytokine locus. Expression of GATA3 in fibroblasts, which lack the LCR–gene locus interaction, was sufficient to create this interaction. Furthermore, the *in vivo* binding of GATA3 occurs at discrete binding sites located in the RHS7 site of the T_H2 LCR.

Our data presented here provide a potential explanation in molecular terms for the coordinate expression of the TH2 cytokine–encoding genes *Il4*, *Il13* and *Il5*. The T_H2 LCR can thus coactivate the three cytokine-encoding genes participating in a preformed chromatin configuration without affecting the constitutively expressed *Rad50*, which is looped out of the active chromatin hub.

RESULTS

Chromosome conformation capture analysis

The chromosome conformation capture technique[15] enabled us to detect the frequency with which two different gene loci interact in the steady state in a given nucleus. To detect long-range interactions in the T_H2 cytokine locus, we used the same technique modified and adapted for mammalian systems[10]. In this technique, cells are collected and proteins are crosslinked to proteins and DNA with formaldehyde. The nuclei are isolated and, after removal of proteins not crosslinked to DNA, the chromatin is digested by restriction enzymes, followed by ligation at a very low DNA concentrations. In such conditions, ligation of intermolecular crosslinked restriction enzyme fragments is favored over ligation of random fragments[15]. After ligation, the protein-DNA crosslinks are reversed and all possible ligation products are detected by PCR with primer pairs designed at the 5′ and 3′ ends of each restriction enzyme site of interest. The crosslinking frequency of two restriction enzyme fragments, as measured by the quantitation of the PCR signals of a ligation product, is dependent on the relative proximity of the two fragments to each other captured at any time point. Thus, the chromosome conformation capture technique is a powerful tool for the identification of the spatial organization of a locus at a given time point *in vivo*.

The spatial organization of the T_H2 cytokine locus is presented in **Figure 1a**. A transgenic mouse reporter gene assay was used to identify a 25-kb region at the 3′ of *Rad50* with LCR activity[1]. Further characterization of this region identified seven new DNase I–hypersensitive sites, RHS1–RHS7, in the LCR (data not shown). We compiled all known DNase I–hypersensitive sites in the T_H2 cytokine locus into one diagram (**Fig. 1a**).

We used *Bgl*II to generate fragments for further analysis with the chromosome conformation capture assay. The *Bgl*II restriction fragments used for the analysis encompass either the promoter regions of the genes located in the T_H2 locus (fragments B, C, J and M; **Fig. 1a**) or (all of) the DNase I–hypersensitive sites in the locus (fragments D, F, G, I, K, L, N, O and P; **Fig. 1a**). We used regions with no functional activity as controls (fragments A, E and Q; **Fig. 1a**).

After preparing a template to be used in the chromosome conformation capture assay, we checked the efficiency of the restriction enzyme digestion by Southern blot and PCR to ensure that all

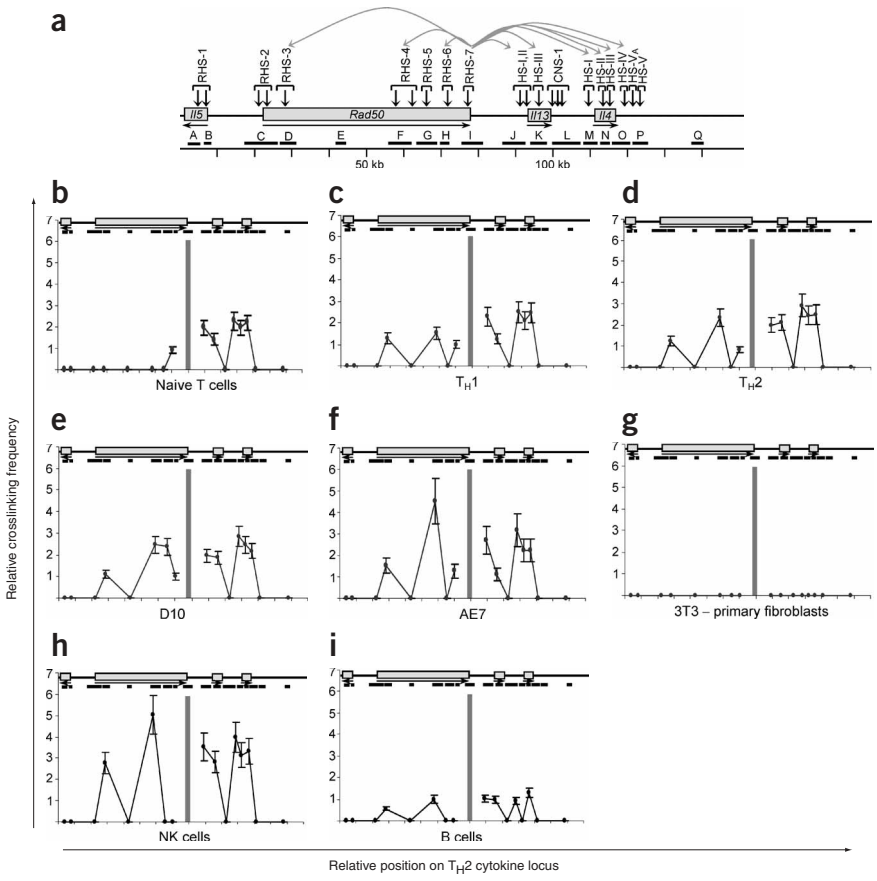

Figure 3 The RHS7 site of the T_H2 LCR interacts with other hypersensitive sites of the locus and the promoters of the cytokine-encoding genes in T cells but not in fibroblasts. (**a**) Relative organization of T_H2 cytokine locus; arrows above indicate interactions of the RHS7 site of the T_H2 LCR with other regions in the locus in T_H2 cells. (**b**–**i**) Relative crosslinking frequencies in templates from primary cultures of naive T cells (**b**), T_H1 (**c**), T_H2 (**d**), fibroblasts (**g**), NK cells (**h**) and B cells (**i**), as well as from cell lines D10 (**e**) and AE7 (**f**). Fragment I (RHS7 site) was used as a stable fragment and was combined with the fragments of the rest of the locus. Data represent standard errors of the mean values.

restriction enzyme sites were digested effectively to the same extent and without any 'preference' (data not shown). For quantitative as well as qualitative analysis, we 'titrated' all templates in PCR reactions using different primer pairs and quantified PCR products. We used a quantity of initial template such that the PCR product was in the exponential phase of amplification (**Fig. 1b**). We used the same quantitative titrations for PCR reactions for the control templates generated from digestion and random ligation of all possible fragments generated from a bacterial artificial chromosome clone containing the T_H2 cytokine locus (**Fig. 1b**) and then mixed these fragments with digested and ligated genomic DNA. In the control templates, all possible ligation products were present in equimolar amounts. To analyze the crosslinking efficiency of two specific restriction fragments, we used primers in paired combinations for PCR (**Fig. 1b**). For the formaldehyde crosslinking reactions, we used many controls to identify the optimal concentration of formaldehyde (**Fig. 1c**). We also used templates from different cell types and used various primer pairs in the PCR amplifications (**Fig. 1d**). As a control in this analysis, we used *Gapd*, a gene constitutively expressed in all cell types used without any known secondary chromatin conformation. In the *Gapd* locus, we used two *Bgl*II restriction fragments (A and B) that are near to each other (559 base pairs (bp) apart) in chromosome conformation capture analyses with primers 2, 3, 4 and 5. When we combined primer pairs for any fragment of the T_H2 cytokine locus (located in chromosome 11) with primers for the two *Bgl*II restriction fragments (A and B; **Fig. 1e**) of the *Gapd* locus (located in chromosome 2) no PCR product was detected. We measured the crosslinking and ligation efficiencies for each cell type used for the T_H2 cytokine locus and the *Gapd* locus. By normalizing each crosslinking frequency in the T_H2 cytokine locus to the crosslinking frequency of *Gapd* DNA, for a certain cell type, we corrected for differences in the quality and quantity of the template used. Also, by normalizing the crosslinking frequency between two different T_H2 cytokine locus fragments to that for the *Gapd* fragments, we corrected for differences in the amount of control templates between experiments. We calculated the relative crosslinking frequencies between two given fragments (equation, **Fig. 1f**). We arbitrarily set the relative crosslinking frequency with a value of 1 for the crosslinking frequency of the two *Gapd* fragments.

'Pre-poised' T_H2 chromatin conformations

We prepared crosslinked templates from sorted naive $CD4^+$ T cells and naive $CD4^+$ T cells differentiated for 5 d in T_H1 or T_H2 conditions, and from two T cell clones, D10.G4.1 (T_H2 phenotype) and AE7 (T_H1 phenotype). We also used non–T cell types to prepare crosslinked templates (natural killer (NK) cells, B cells, primary fibroblasts and a mouse fibroblast cell line (3T3)) for comparison with the T cells. We combined the primer pair for the *Il4* promoter *Bgl*II restriction fragment with primer pairs for every other restriction enzyme fragment in the T_H2 cytokine locus and measured relative crosslinking frequencies.

We tested primary fibroblasts, 3T3 fibroblasts and B cells, none of which expresses the T_H2 specific cytokine-encoding genes or is of a T cell lineage, for relative crosslinking frequencies of the *Il4* promoter fragment with all the other fragments of the locus and found that the *Il4* promoter comes in close proximity with the *Il5* and *Il13* promoters but not with the T_H2 LCR (**Fig. 2**). The crosslinking frequency of zero did not reflect impaired annealing of primers due to cell line polymorphisms (for example, fibroblasts), because when we used saturated amounts of template we were able to detect all the signals. But in these stringent quantitative experimental conditions, we could detect only the specific signals that were described.

In naive T cells, we found that the crosslinking frequency of the *Il4* promoter with the *Il5* and *Il13* promoters was twice that of nearby fragments in the control *Gapd* locus (**Fig. 2b**). Thus, even if the cytokine-encoding genes are not actively transcribed in naive T cells, the promoters are in a close proximity at the chromatin level. In contrast, the *Il4* promoter did not interact with the promoter of the constitutively expressed *Rad50*. We found the same positive interaction of the *Il4* promoter with the cytokine gene promoters

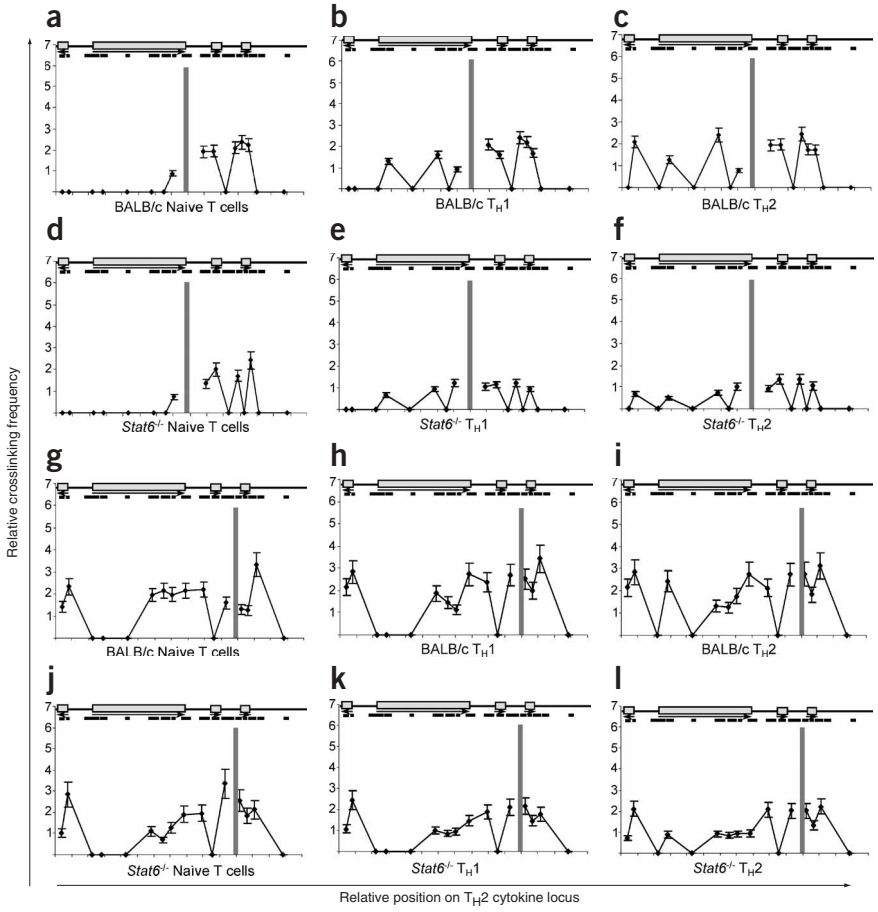

Figure 4 Absence of STAT6 leads to impairment of interactions between the T_H2 LCR and the rest of the T_H2 cytokine locus. (**a**–**f**) Relative crosslinking frequencies in naive T cells, T_H1 and T_H2 cells in BALB/c mice (**a**–**c**) and *Stat6*$^{-/-}$ mice (**d**–**f**) with fragment I (RHS7 site fragment) as a stable fragment versus the rest of the locus. (**g**–**l**) Relative crosslinking frequencies in the same cell types as in **a**–**f**, with fragment M (*Il4* promoter fragment) as a stable fragment versus the rest of the locus. Preparations of templates from BALB/c mice were used as a control for *Stat6*$^{-/-}$ mice, which are on the BALB/c genetic background. Data represent standard errors of the mean values.

plus the T_H2 LCR in T_H1 cells, NK cells and T_H2 cells (**Fig. 2**), although the relative crosslinking efficiencies were much higher in effector cells than in naive T cells (two- to three-fold increase in interactions of the *Il4* promoter with the *Il5* and *Il13* promoters). Thus, the promoters of the TH2 cytokine–encoding genes interact with each other in T cells, NK, B cells and fibroblasts.

Additionally, in naive T cells, the *Il4* promoter fragment, as judged from the relative crosslinking frequencies, seemed to be in close proximity to the T_H2 LCR and the *Il4* 3′ end DNase I–hypersensitive sites, known to have a regulatory function for the expression of *Il4* (**Fig. 2b**). Also, the *Il4* promoter seemed to interact with the DNase I–hypersensitive site RHS3 in T_H2 cells and NK cells but not in naive T cells and T_H1 cells. The same conclusions drawn for T_H1 and T_H2 cells can be extrapolated from our analysis of the T cell clones AE7 (**Fig. 2e**) and D10 (**Fig. 2f**). The constitutively expressed *Rad50* was expressed in all cell types examined, naive T cells, T_H1, T_H2 cells and fibroblasts, regardless of the interaction of the T_H2 LCR with the T_H2 cytokine promoters (**Fig. 2k**). The T_H2 LCR comes in close proximity to the T_H2 cytokine–encoding gene promoters both in T_H2 cytokine–expressing and T_H2 cytokine–nonexpressing T cell types and NK cells but not in fibroblasts and B cells.

The fact that almost the same qualitative interactions, albeit to a lesser magnitude, were detected in naive T cells as well as in effector cells might suggest that such interactions were due to the presence of contaminating effector cells in naive T cell cultures. We excluded this possibility by negatively selecting $CD4^+$ T cells using antibody-bound magnetic beads and subsequently sorting the $CD4^+$ T cell population and selecting for $CD4^+CD44^-CD62L^+$ cells, which are specific markers for $CD4^+$ naive T cells. We used this pure population of naive T cells in subsequent analyses to ensure that the interactions detected were characteristic of naive T cells and were not due to contamination of effector T_H1 and T_H2 cells (data not shown).

Thus, chromatin in the T_H2 cytokine locus has a 'pre-poised' conformation in all the cell types we examined, in which the promoters of the TH2 cytokine–encoding genes (*Il4*, *Il5* and *Il13*) come in close proximity forming an initial core chromatin structure. In addition, specific interaction between the T_H2 LCR with the T_H2 cytokine gene promoters is sustained in T cells and NK cells, whether or not they express the T_H2 cytokine–encoding genes, a configuration that we characterize here as 'poised'.

Cell-specific LCR-promoter interactions

The *Il4* promoter interacted with the T_H2 LCR in T cells and NK cells but not in B cells and fibroblasts. We sought to confirm these data by determining the interaction of the DNase I–hypersensitive site RHS7 of the LCR (which showed the strongest interaction with the *Il4* promoter) using all the other restriction enzyme fragments of the T_H2 locus. We prepared templates for the chromosome conformation capture assay from primary cultures and cell lines and measured the relative crosslinking frequencies of RHS7 with every other fragment in the locus. In naive $CD4^+$ T cells, T_H1 cells and the T_H1 clone AE7 cells as well as NK cells, the RHS7 site interacted with the promoters of *Il13* and *Il4* as well as with DNase I–hypersensitive sites located in *Il13* (HS-III) and the DNase I–hypersensitive sites HS-II, HS-III and HS-IV, near *Il4*. In differentiated T_H1 cells and the cloned T_H1 AE7 T cell line, the RHS7 site also interacted with the DNase I–hypersensitive sites RHS3 and RHS4 in introns of *Rad50* (**Fig. 3**). We obtained similar results by analysis of T_H2 primary cultures and the T_H2 clone D10.G4.1 (**Fig. 3**). Stable interactions were formed between the RHS7 site and different DNase I–hypersensitive sites of the LCR in addition to DNase I–hypersensitive sites located both within and 3′ of *Il4*. In contrast, no substantial interaction of RHS7 with any element tested was detected, indicating that the T_H2 LCR does not form any secondary chromatin configuration in fibroblasts and B cells (**Fig. 3g,i**).

Therefore, the initial long-range interactions between the cytokine gene promoters in the T_H2 locus occurred in $CD4^+$ cells and also in NK, B cells and fibroblasts, a phenomenon that was not cell lineage specific. These initial interactions were strengthened in $CD4^+$ T cells

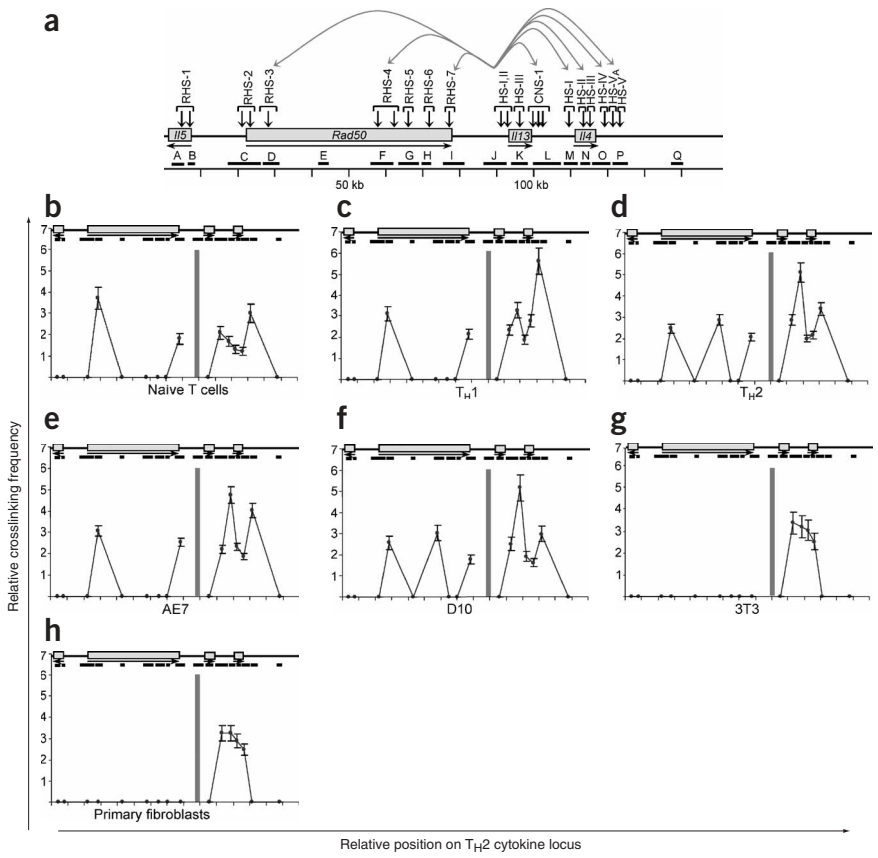

Figure 5 The *Il13* promoter interacts with the *Il4* promoter and several DNase I–hypersensitive sites on the T_H2 cytokine locus. (**a**) Relative organization of the T_H2 cytokine locus; arrows above indicate interactions of the *Il13* promoter fragment with other regions in the locus in T_H2 cells. (**b**–**h**) Relative crosslinking frequencies in templates from primary cultures of naive T cells (**b**), T_H1 (**c**), T_H2 (**d**) and fibroblasts (**h**) as well as from cell lines AE7 (**e**), D10 (**f**) and 3T3 (**g**). Fragment J (*Il13* promoter fragment) was used as a stable fragment and was combined with the fragments of the rest of the locus. Data represent standard errors of the mean values.

and NK cells by the interaction of the T_H2 LCR with this initially formed complex, a phenomenon that did not occur in fibroblasts and B cells.

Impaired interactions in the absence of STAT6

Because the T_H2 LCR participates in a secondary chromatin conformation with the promoters of the cytokine gene loci and other important DNase I–hypersensitive sites, we sought to determine the function of STAT6 in the establishment of this conformation. For this analysis, we prepared templates from *Stat6*$^{-/-}$ primary cultures of sorted naive $CD4^+$ T cells and differentiated T_H1 and T_H2 cells. All the previous templates presented so far were prepared from C57BL/6 mice. Because *Stat6*$^{-/-}$ mice are on a BALB/c background, we also prepared primary cultures from wild-type BALB/c mice. We measured the crosslinking frequencies of the RHS7 fragment with all the other fragments in the T_H2 cytokine locus. We found no substantial differences for the crosslinking frequencies in naive, T_H1 and T_H2 $CD4^+$ T cells derived from wild-type BALB/c mice (**Fig. 4**) and the crosslinking frequencies measured for the same cell types of C57BL/6 mice (**Fig. 2**).

For templates prepared from T_H1 and T_H2 cells from *Stat6*$^{-/-}$ mice (**Fig. 4e,f**, respectively), all the crosslinking frequencies measured for the RHS7 site and all the other fragments were substantially decreased (all values being close to or less than 1). A value of 1 is characteristic of very closely located restriction fragments of the genome and is considered background for the analysis. This phenomenon was most salient in effector T_H1 and T_H2 cells, whereas in the naive T cells the influence was minor.

We analyzed the same cell types to measure the crosslinking efficiencies of the *Il4* promoter fragment with the rest of the T_H2 locus (**Fig. 4g–l**). In the *Stat6*$^{-/-}$ cells, although the core conformation of the cytokine promoters was maintained, the interaction of the T_H2 LCR with the 'pre-poised' core configuration was impaired. Thus, the T_H2 cell–specific factor STAT6 is important for the interaction of the T_H2 LCR with the cytokine gene loci in both T_H1 and T_H2 cells.

Gene-specific interactions with the T_H2 LCR

To confirm the data obtained for the crosslinking frequencies of the *Il4* promoter or the RHS7 site with every other fragment in the T_H2 cytokine locus, we measured the crosslinking frequencies of the *Il13* promoter fragment with the fragments in the rest of the locus. The crosslinking frequencies of the *Il13* promoter with other fragments derived from cells of the T cell lineage indicated that the *Il13* promoter interacts with the *Il4* promoter, the T_H2 LCR and the *Il4*-proximal DNase I–hypersensitive sites (CNS-1, HS-II, HS-III, HS-IV, HS-V_A and HS-V). In templates prepared from B cells and fibroblasts, the *Il13* promoter interacted with the *Il4* promoter and the hypersensitive sites around the *Il4* locus but did not interact with the T_H2 LCR. Thus, the T_H2 LCR interacts only with the cytokine promoters in T cells, not in fibroblasts (**Fig. 5**).

We next measured the crosslinking frequencies of the *Il5* promoter fragment to the T_H2 cytokine locus. The crosslinking frequencies were similar in quality, with minor differences in the strength of interaction among all T cell types. In naive T cells, T_H1 cells, T_H2 cells, AE7 cells and D10.G4.1 cells (**Supplementary Fig. 1** online), the *Il5* promoter fragment interacted mainly with the *Il4* promoter, CNS-1, and HS-V_A and HS-V. In fibroblasts, the *Il5* promoter interacted with the *Il4* promoter but not with any other *cis* element (**Supplementary Fig. 1** online).

Rad50 is a constitutively expressed DNA repair gene with no known common transcriptional regulation with the cytokine-encoding genes in the T_H2 locus. We measured the crosslinking frequencies of the *Rad50* promoter with all the other restriction fragments in the locus (**Supplementary Fig. 2** online). In all the cell types for which we measured the crosslinking frequencies for *Rad50*, no interaction of the *Rad50* promoter with any other region of the T_H2 locus was detected. The crosslinking frequency measured (a value of 2–3) for fragment D (RHS3) simply reflects the fact that the two fragments (of the *Rad50* promoter and RHS3) analyzed were in a very close physical proximity in a linear configuration (**Supplementary Fig. 2** online). The conditions for all the PCR for this analysis were extremely

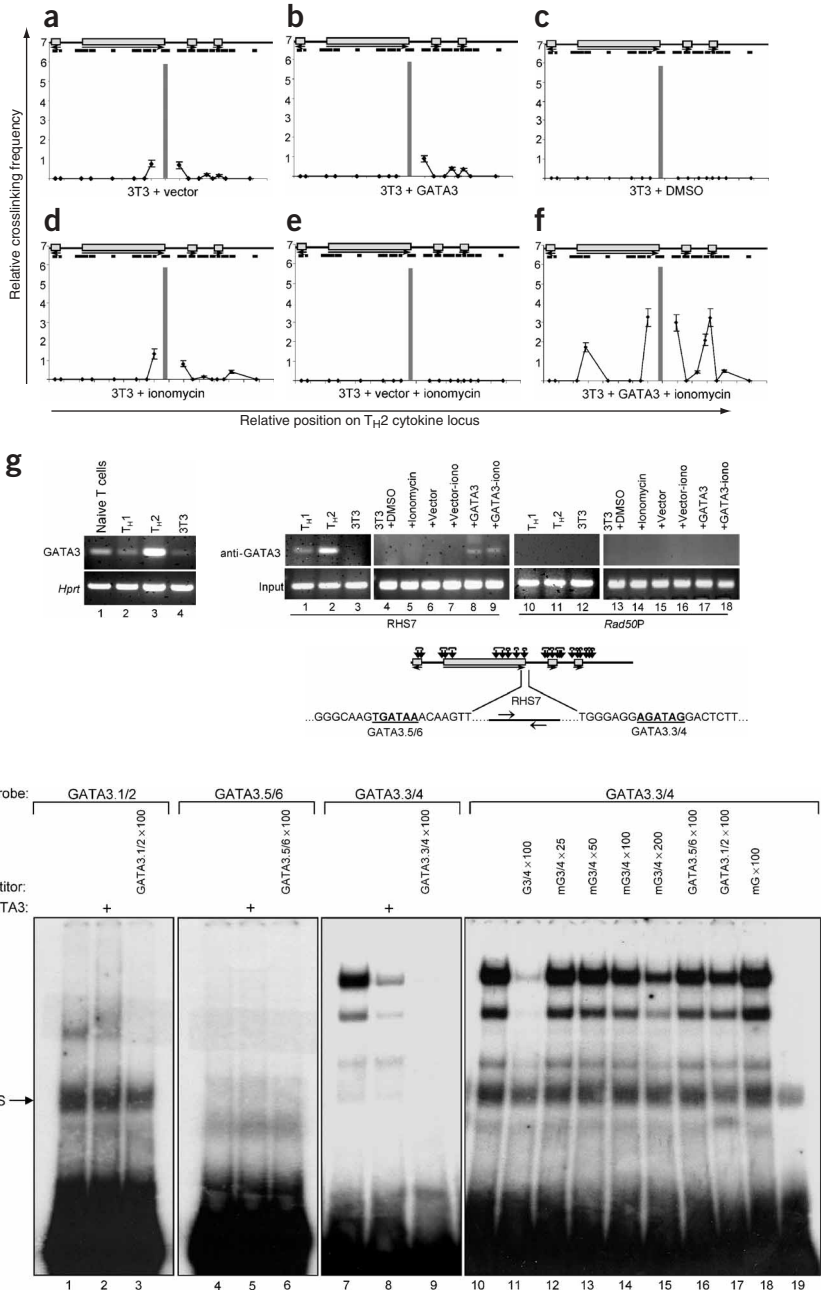

Figure 6 Calcium mobilization in 3T3 fibroblasts expressing GATA3 gain a secondary chromatin conformation in the T_H2 locus. (**a**–**f**) Relative crosslinking frequencies in templates from 3T3 cells transfected with a retrovirus vector (**a**) or a retrovirus vector expressing GATA3 (**b**) and treated for 3 h with 1 μM ionomycin (**e** and **f**). Controls, templates from 3T3 cells treated with dimethyl sulfoxide (DMSO; **c**) or ionomycin (**d**) only. Fragment I (RHS7 site) was used as a stable fragment and was combined with the fragments of the rest of the locus. Data represent standard errors of the mean values. (**g**) Left, RT-PCR showing the relative expression of mouse *Gata3* in primary naive T cells (lane 1) or naive $CD4^+$ T cells differentiated for 5 d in T_H1 conditions (lane 2) or T_H2 conditions (lane 3), or of mouse *Gata3* in 3T3 mouse fibroblasts (lane 4). Right, chromatin immunoprecipitation with an agarose-conjugated mouse monoclonal anti-GATA3. Soluble chromatin solution (20 μg) was immunoprecipitated from T_H1, T_H2 cells and 3T3 fibroblasts. Here, 10% of the material immunoprecipitated with anti-GATA3 was used for PCR and 2% of the total soluble chromatin solution was used for PCR as input. Primer pairs were designed for the amplification of a core region of the RHS7 DNase I–hypersensitive site and for the *Rad50* promoter. Vector-iono, vector plus ionomycin; GATA3-iono, GATA3 plus ionomycin. Bottom right, relative positions of the two consensus GATA3 binding sites in the RHS7 site and the positions of the primers used for the PCR reactions. The GATA3 binding sites are in bold; consensus sites are underlined. Above, T_H2 locus. In gray boxes are the genes present in the locus; arrows indicate positions of DNAseI–hypersensitive sites. (**h**) EMSAs with oligonucleotides containing two consensus GATA3 binding sites (GATA3.1/2), a GATA3 binding site at the 3′ end of the RHS7 site (GATA3.3/4) and a GATA3 binding site at the 5′ end of RHS7 site (GATA3.5/6); 5 μg of nuclear T_H2 cell extracts were used. Competition experiments used excess nonradiolabeled oligonucleotide (above lanes) as a competitor of either wild-type oligonucleotides or mutated oligonucleotides. NS, nonspecific band.

stringent to minimize the background PCR amplification and allowed us to detect only the specific interactions in the locus. Thus, these data support in molecular terms the hypothesis that *Rad50* is independently transcriptionally regulated in the T_H2 locus (also supported by **Fig. 2b**).

To augment further the data presented so far, we next examined the crosslinking frequencies of the DNase I–hypersensitive sites CNS-1, HS-VA and HS-V (**Supplementary Fig. 3** online). We confirmed that CNS-I, HS-VA and HS-V interact with the promoters of the TH2 cytokine–encoding genes in naive T cells and T_H2 cells as well as with various other DNase I–hypersensitive sites in the locus, but not with the T_H2 LCR. Thus, the T_H2 LCR specifically interacts with the promoters of the T_H2 cytokine–encoding genes, conferring its positive regulatory effects. It does not interact with the *Rad50* promoter, where another regulatory mechanism may be responsible for the constitutive expression of this gene.

GATA3 and calcium-induced chromatin reorganization

We hypothesized that expression of GATA3, a highly expressed T cell transcription factor with direct chromatin-remodeling activities, would support the interaction of the T_H2 LCR with the 'pre-poised' chromatin conformation in which the T_H2 cytokine–encoding genes participate. We used ionomycin to induce the calcineurin-regulated transcription factors, including nuclear factor of activated T cells (NFAT) proteins. NFATc1 cooperates with and binds to GATA3 at the DNase I–hypersensitive site $HS\text{-}V_A$ at the 3′ end of *Il4* (ref. 7).

We transfected 3T3 fibroblasts with a retrovirus vector consisting of GATA3, green fluorescent protein (GFP) and an internal ribosomal

entry site and sorted for GFP$^+$ cells. We calculated the crosslinking efficiencies for the RHS7 site fragment of the T_H2 LCR with all the other fragments of the locus. Interaction of the RHS7 site with other fragments was induced in 3T3 cells by treatment with *Gata3* plus ionomycin but not in any of the other conditions, including *Gata3* or ionomycin alone. In this template, the RHS7 fragment of the T_H2 LCR was induced to interact with RHS3, RHS6, the *Il13* promoter fragment, the *Il4* promoter and HS-II and HS-III of *Il4* (**Fig. 6a–f**). Thus, GATA3 is important for sustaining interactions of one DNase I–hypersensitive site (RHS7) of the T_H2 LCR with other elements in the T_H2 cytokine locus. Although GATA3 participates in sustaining such interactions, it did not support the expression of the T_H2 cytokine–encoding genes, as no T_H2 cytokines were detectable in the supernatants of the 3T3 cell cultures (**Supplementary Fig. 4** online).

We next assessed the relative abundance of *Gata3* mRNA in different cell types. Consistent with previous reports[16–19], *Gata3* was rarely expressed in naive T cells and even less so in differentiated T_H1 cells, but had much higher expression in T_H2 cells (**Fig. 6g**, left). The abundance of *Gata3* mRNA in fibroblasts was even lower than that in all other cell types examined (**Fig. 6g** left). As GATA3 is responsible for driving interactions of RHS7 with other regions in the T_H2 cytokine locus, we next examined the potential binding of GATA3 in the genomic region comprising the RHS7 site. The DNA sequence of the RHS7 DNase I–hypersensitive site is highly conserved between mouse and human, which contains two consensus GATA3 binding sites in the 5′ and 3′ segments of this region (**Fig. 6g**, right). We did chromatin immunoprecipitation assays using an agarose-conjugated mouse monoclonal antibody to GATA3 (anti-GATA3) on templates from differentiated T_H1 and T_H2 cells as well as 3T3 fibroblasts. The primers used for the amplification of the immunoprecipitated genomic fragments were located in the RHS7 site flanked by the two consensus GATA3 binding sites. GATA3 bound to the genomic region covered by the DNase I–hypersensitive site RHS7 of the T_H2 LCR in T_H2 cells, but much less so in T_H1 cells (**Fig. 6g**, right). There was no detectable binding in fibroblasts unless they were transduced with a retrovirus encoding GATA3 (**Fig. 6g**). We also analyzed the anti-GATA3 immunoprecipitates by PCR with primers in the *Rad50* promoter region but detected no signals, indicating that GATA3 did not bind to this chromatin region.

To further support the3 idea that GATA3 is responsible for conferring chromatin reorganization in the T_H2 locus, we examined its ability to bind in designated sites within the RHS7 site. Within the RHS7 site there were two potential GATA3 binding sites separated by 400 bp of DNA (**Fig. 6g**). To identify which site bound GATA3, we did electrophoretic mobility-shift assays (EMSAs) using nuclear extracts prepared from T_H2 cells and double-stranded oligonucleotides for the two GATA3 binding sites (**Fig. 6g**, lanes 4–6 and 7–9). We also used a commercially available oligonucleotide with two tandemly linked consensus GATA3 binding sites not in the context of the T_H2 locus (**Fig. 6h**, lanes 1–3). Using the oligonucleotide for the GATA3 site at the 3′ end of RHS7 (GATA3.3/4) and a mouse monoclonal antibody specific for GATA3, we did not detect a complex with altered mobility. The binding of the protein complex to the designated site was reduced by the addition of this antibody, indicating the presence of GATA3 in this complex (**Fig. 6h**, lane 7 versus lane 8). Also, when the GATA3 binding site in the oligonucleotide GATA3.G3/4 was mutated, the mutated oligonucleotide (mG3/4) could no longer compete for the binding of GATA3 in the wild-type GATA3.G3/4 oligonucleotide (**Fig. 6h**, lane 11 versus lanes 12–15). As indicated by the EMSA experiments, GATA3 bound the site at the 3′ end of RHS7. Thus, binding of GATA3 in RHS7 site is important for the participation of the T_H2 LCR in forming a secondary chromatin conformation in the T_H2 cytokine locus.

DISCUSSION

A key idea to emerge from analysis of the differentiation of naive CD4$^+$ T cells into T_H1 and T_H2 cells has been epigenetic changes in the chromatin of the loci of cytokine-encoding genes such as *Ifng*, *Il4*, *Il5* and *Il13*. In the nucleosomal structure of chromatin, post-translational modifications of histone tails have been well characterized, and hyperacetylation of histones in the vicinity of promoter regions as well as the binding of T cell–specific factors have been identified as key mechanisms regulating expression of the T_H2 cytokine–encoding genes[20–23]. Although description of epigenetic chromatin changes has enhanced the understanding of T cell differentiation, it does not explain how the coordinated regulation and expression of the T_H2 cytokine–encoding genes occurs, in terms of a common molecular mechanism.

Although LCRs are characterized mainly as DNA elements with strong enhancer function for transcription of the genes they regulate, the exact mechanism whereby LCRs act at a distance and in a chromatin environment is not clearly understood. At least two different groups using two different experimental approaches have presented data supporting the idea of a looping model for the action of the β-globin LCR[9,10,24]. On the basis of those findings, a looping model for the mode of action of the T_H2 LCR and the coordinated regulation of the TH2 cytokine–encoding genes has been proposed[25].

We aimed to elucidate how the T_H2 LCR can act over long distances and coordinately regulate the expression of the TH2 cytokine–encoding genes *Il4*, *Il13* and *Il5*. We have used the chromosome conformation capture technique[10,15] to identify long-range intrachromosomal interactions in the T_H2 cytokine locus. We found that the promoters of the T_H2 cytokine–encoding genes come into close spatial proximity and form a 'pre-poised' core chromatin configuration in cells of the T cell lineage that either express (T_H2, D10.4G.10) or do not express (naive T cells, T_H1, AE7) these cytokine-encoding genes. This configuration was present in NK cells, B cells and fibroblasts. The interactions were stronger in effector cells than in naive T cells, perhaps because of the expression of lineage-specific factors that stabilize these interactions. In this core complex in cells of the T cell and NK cell lineages, the LCR contributes further to this higher-order chromatin conformation to generate a 'poised' conformation. The expression of T_H2-specific factors may be responsible for directing specific expression of the T_H2 cytokine–encoding genes from this chromatin configuration as the intrachromosomal interactions were also detected in nonexpressing cells. The poised conformation of preassembled protein complexes on nontranscribing gene promoters has been described for several genes for which RNA polymerase II is bound to the promoter but elongation does not occur because of insufficient phosphorylation of the C-terminal domain of RNA polymerase II (refs. 26–31). Although a poised conformation of transcription complexes has been proposed for promoters, no evidence so far has supported such a hypothesis for the mode of action of an LCR. The β-globin LCR acquires such a conformation, interacting with other DNase I–hypersensitive sites in the locus, in erythroid progenitors that do not express the β-globin genes[24]. However the situation is much different in the T_H2 cytokine gene cluster because of the regulation of its coordinated expression.

The phenomenon in which a locus is heritably poised for gene expression even when it is transcriptionally inactive may be a unique feature of genes that require rapid transcriptional induction in response to a stimulus[32]. Indeed, the long-range chromatin conformation we detected even in naive T cells that did not express the T_H2

cytokines equips these cell types for rapid production of lineage-specific cytokines after stimulation. Polarized T_H1 or T_H2 cells, cultured in opposing conditions (T_H1 cells cultured in T_H2 conditions or T_H2 cells cultured in T_H1 conditions), still retain the potential to produce cytokines of their original phenotype[33,34], which is consistent with our data showing a preformed configuration in the T_H2 cytokine locus in T_H1 and T_H2 cells. In primary T_H1 cells or the T_H1 clone AE7, the RHS5 site of the T_H2 LCR does not interact with the *Il4* promoter fragment. The DNase I–hypersensitive site RHS5 is T_H2 specific, and thus the protein complexes that bind to this site may not be expressed in T_H1 cells. The same hypothesis could hold for the T_H2-specific interaction of the *Il13* promoter fragment with RHS4 site of the LCR. $CD4^+$ naive T cells, T_H1 and T_H2 cells initially have the potential to express T_H2 cytokines because of their 'poised' chromatin conformation, but additional cell-specific factors and post-translational histone modifications of chromatin determine whether a given event occurs.

$CD4^+$ naive T cells produce IL-4 in a STAT6-independent way[33], with peak mRNA production occurring 2 h after stimulation. Given our results here, we hypothesize that in naive T cells the chromatin configuration, as detected by the chromosome conformation capture assays, may support early expression of the T_H2 cytokines, but in a later developmental stage, such as in effector polarized cells, STAT6 is important for the maintenance of a higher-order chromatin structure. Additional factors may be needed to sustain memory epigenetic chromatin modifications that are not present in the *Stat6*$^{-/-}$ T cells. All interactions were shown by chromosome conformation capture analysis to be impaired in *Stat6*$^{-/-}$ effector cells, although the interactions between the cytokine promoters were sustained but not those with the T_H2 LCR. These data emphasize the importance of STAT6 for the formation of a secondary chromatin conformation and suggest that other factors are required to generate the 'pre-poised' conformation found in earlier developmental stages.

Another downstream effector of STAT6 is GATA3, a factor important for the expression of the T_H2 cytokines and for the formation and/or maintenance of interactions between the T_H2 LCR and the promoters of the genes encoding T_H2 cytokines. Increased expression of GATA3 in $CD4^+$ T cells causes T_H2 cytokine gene expression in developing T_H1 cells, in committed T_H1 cells and in the T_H1 clone HDK1 (refs. 17,34,35). Furthermore, ectopic expression of GATA3 in T_H1 cells or in a nonlymphoid, non-IL-5-producing cell line activates the *Il5* promoter[19]. We also analyzed the relative crosslinking frequencies of various fragments in the T_H2 cytokine locus in cell types that express GATA3, such as T helper cells and NK cells[36,37]. GATA3 might coordinately affect the expression of all three cytokine-encoding genes, *Il4*, *IL5* and *Il13*, in the T_H2 cytokine locus through effects imposed on an already preformed, 'pre-poised' chromatin configuration. Also, as GATA3 has direct chromatin-remodeling activities[38], it may assist the induction of the interaction of the T_H2 LCR with the promoters of the T_H2 cytokine–encoding genes.

Calcium mobilization leads to the rapid dephosphorylation of NFAT proteins and their translocation to the nucleus with increased affinity for DNA[39]. There are four members of the NFAT family of transcription factors, NFATc1–NFATc4, that are regulated by calcium and have important immune cell functions[39,40]. Protein complexes with transcriptional activity are composed by NFAT dimers or through cooperation of NFAT with transcription factors of the GATA family. NFATc1 and GATA3 bind to enhancer regions at the 3′ end of *Il4* in T cells[41]. In our studies, calcium mobilization in *Gata3*-transfected 3T3 fibroblasts induced activation of NFAT factors; this cooperation of NFAT with GATA3 may induce a higher-order chromatin conformation in the T_H2 cytokine locus. GATA3 bound to a GATA3 consensus binding site in the RHS7 site of the T_H2 LCR. Furthermore, chromatin immunoprecipitation experiments confirmed the binding of GATA3 protein to native chromatin. Also, direct binding of GATA3 to its target sites in a cell line (3T3) in which it is normally not expressed seemed to confer the regulatory effects of this factor in non–T cells. GATA3 binding sites are dispersed along the entire T_H2 cytokine locus (data not shown), providing the potential for GATA3 binding to exert its regulatory function. Although GATA3 confers spatial reorganization of the T_H2 cytokine locus, it does not have the ability, even in cooperation with calcium-induced factors, to drive T_H2 cytokine transcription in fibroblasts.

In summary, we have identified two different long-range intrachromosomal interactions in the T_H2 cytokine gene locus. In fibroblasts, B cells, NK cells and $CD4^+$ T cells, the promoters of all cytokine-encoding genes interact, forming a 'pre-poised', initial core chromatin configuration. In cells of the T cell lineage that express or do not express the T_H2 cytokines, the T_H2 LCR interacts with this initial core. A 'poised' chromatin conformation is created, and T_H2 cell–specific transcription factors can rapidly exert their activating effects through positive transcriptional regulation of the T_H2 cytokine–encoding genes in T_H2-polarized cells. STAT6 and GATA3 are two important factors that mediate the generation and/or maintenance of this chromatin configuration. Experiments in which specific regulatory genomic fragments are removed will demonstrate the importance of each regulatory domain in the formation of chromatin structures and the positive regulation of expression. Characterization of the protein complexes bound to the T_H2 LCR and identification of protein complexes with specific remodeling activity acting at a long distance in chromatin rather than at promoter and enhancer elements are also needed.

METHODS

Mice and cell cultures. C57BL/6, BALB/c and *Stat6*$^{-/-}$ mice were all purchased from Jackson Laboratories. The D10.G4.1 conalbumin-specific T_H2 line derived from the AKR/J mouse was obtained from American Type Culture Collection. The AE7 cell line (T_H1 phenotype) was maintained in Bruff's media with 5% FBS, 2 mM L-glutamate, 30 μg/ml of IL-2 (R&D Systems), 50 U/ml of penicillin and 50 μg/ml of streptomycin. Every 10 d, 1×10^5 cells were stimulated with 5×10^5 irradiated antigen-presenting cells and antigen (0.5 μg/ml of cyto-1 peptide)[42]. The 3T3 cells were transfected using the Lipofectamine-2000 system (Invitrogen) with an empty GFP–internal ribosomal entry site retrovirus vector or the same vector expressing mouse *Gata3* cDNA (gifts from K. Murphy, Washington University School of Medicine, St. Louis, Missouri). Cells were collected after 48 h and sorted for GFP expression. GFP^+ cells were used for the chromosome conformation capture assay.

Isolation and differentiation of naive $CD4^+$ T cells. Usually, 10–20 spleens from 4- to 6-week-old BALB/c or C57BL/6 mice were used to make single-cell suspensions. Isolation and differentiation of naive $CD4^+$ T cells was done as described[20]. Naive $CD4^+$ T cells (1×10^6 cells/ml) were stimulated with plate-bound mouse anti-CD3 (145-2C11; American Type Culture Collection) and anti-CD28 (Pharmingen) and were collected 5 d later. The chromosome conformation capture assays used 1×10^7 cells of each type; 0.2×10^6 cells were restimulated for 24 h with plate-bound anti-CD3 (2C11, American Type Culture Collection) and cytokine in supernatants was measured by enzyme-linked immunosorbent assay. NK cells were negatively selected with anti-CD8 (TIB105; American Type Culture Collection), anti-CD4 (GK1.5; American Type Culture Collection) and anti–major histocompatibility complex class II (m5114; American Type Culture Collection) and then were sorted for the population of NK cells after staining with anti–CD49b/Pan-NKCells (DX5; Pharmingen) and anti-TCRα3 (H57-597; Pharmingen). B cells were positively selected with a biotin-conjugated anti–mouse CD45R/B220 (01122D; Pharmingen) and MACS columns (Miltenyl Biotech) and were sorted

after being stained with anti-CD19 (1D3; Pharmingen) and anti-B220 (RA3-6B2; Pharmingen).

Chromosome conformation capture assay. We used a chromosome conformation capture assay initially developed for yeast cells[15], which was modified and adapted for mammalian cells[10]. *Bgl*II (NEB) was used to generate the restriction fragments in the T_H2 locus. For all control templates, PCR products were digested with the appropriate restriction enzyme to check for the existence of the restriction enzyme site on the fragment. The size of all *Bgl*II restriction fragments on the T_H2 cytokine locus was, on average, 5 kb. The control template for the chromosome conformation capture assay, for the T_H2 locus, was prepared with a bacterial artificial chromosome clone spanning the entire T_H2 cytokine locus (clone B182; Genome Systems). The bacterial artificial chromosome clone (30 μg) was digested with 300 U *Bgl*II overnight at 37 °C. DNA was extracted with phenol and chloroform and was precipitated with ethanol. DNA fragments were ligated with T4 DNA ligase (NEB) at a DNA concentration of 300 ng/μl. A bacterial artificial chromosome clone was used to prepare the control template, and all possible restriction fragments after *Bgl*II digestion were present in equimolar amounts and after ligation at a high DNA concentration, all possible ligation products were present in the sample. For preparation of the control template for the *Gapd* locus, PCR products spanning the *Bgl*II restriction sites of interest were gel-purified and the DNA concentration was determined. Equimolar amounts of the different PCR products were mixed and digested with *Bgl*II. DNA was precipitated after phenol-chloroform extraction and was ligated at a high DNA concentration using T4 DNA ligase. For preparation of the mixed control template for the *Gapd* and T_H2 loci, the same procedure was used, with digestion and ligation of equimolar amounts of all *Bgl*II restriction fragments from the bacterial artificial chromosome–T_H2 clone and the *Gapd* PCR fragments.

PCR analysis of the ligation products. The linear range of PCR amplification was determined for the control and crosslinked DNA templates of the *Gapd* locus, the T_H2 locus and the combined *Gapd* with T_H2 loci. By comparing the PCR signals of the crosslinked templates versus the PCR signals of the control templates, we corrected for differences in amplification efficiency between primer sets and also for differences in signal intensities due to the size of PCR products (all of the primers were designed to give PCR products of 80–180 bp). All the primers were designed to have an annealing temperature of 56–58 °C, and they all yielded a product when used with the control templates. PCR cycles were as follows: an initial denaturing step for 3 min at 94 °C; 36 cycles of 30 s at 94 °C, 20 s at 56 °C and 15 s at 72 °C; followed by a final step of 2 min at 72 °C. For a 20-μl PCR reaction, 20–60 ng of each crosslinked template was used with 1× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 pmol of each primer, 1.5 U Tsg DNA polymerase (Centaur) and 2.5% dimethyl sulfoxide (Sigma). PCR products were separated by 2% agarose gel electrophoresis and specific products were quantified with the AlphaInnotech Fluorchem 8800 image analyzer and ScionImage analysis software. The templates were prepared two to three times in independent experiments and the whole set of PCR reactions for the analysis of interaction of one restriction fragment with all other fragments was repeated at least three times, for a maximum of seven times. Data presented are the average of results for all PCR reactions, with no substantial differences between experiments. Crosslinking frequencies were calculated with the equation in **Figure 1f**. All PCR products with signals that were considered positive were isolated, gel-extracted (Qiaquick; Qiagen), cloned in a TA vector (TA cloning Kit; Invitrogen) and sequenced to confirm the presence of the sequences of two different restriction enzyme fragments ligated in a *Bgl*II site. Primers used are in **Supplementary Figure 5** online.

Chromatin immunoprecipitation. T_H1 and T_H2 primary cells as well as 3T3 cells (1.2×10^7 cells) were used to prepare soluble chromatin solutions as described[26]. Chromatin immunoprecipitation used 20 μg soluble chromatin solution and 20 μl agarose-conjugated mouse monoclonal anti-GATA3 (HG3-31; sc-268AC; Santa Cruz Biotechnology). The sequences of the primers used for the PCR analysis of the immunoprecipitated chromatin were as follows: RHS7.S, 5′-TCACTCATAGCCGATACCTCA-3′; RHS7.A, 5′-AGTGAGGGGAATAACACTACC-3′; *Rad50*P.S, 5′-CAGAGCTAGACCCGATCTCA-3′; and *Rad50*P.A, 5′-CGAGCCAGCAACCGTAAG-3′.

EMSA. These experiments used 5 μg of T_H2 cell nuclear extracts bound on 2 ng double-stranded DNA oligonucleotide. The binding reactions proceeded for 20 min in a buffer containing 50 μg/ml of poly(dI:dC), 200 μg/ml of BSA, 10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 50 mM NaCl, 10% glycerol and protease inhibitors. The samples were loaded on a native 5% polyacrylamide gel (0.5× Tris–boric acid–EDTA buffer and 2% glycerol) and were separated by electrophoresis for 2 h at 200 V. Gels were dried and exposed to X-ray film. For competition assays, a 100-fold excess of a cold competitor was included or otherwise as indicated (**Fig. 6**, lanes 12,13,15). In supershift experiments, 2 μg of mouse monoclonal anti-GATA3 (HG3-31; sc-268X; Santa Cruz Biotechnology) was preincubated for 30 min on ice with the extracts before the addition of the probe.

The sequences of the two strands of the oligonucleotides used were as follows (consensus GATA3 binding site sequences in bold.): GATA3.1, 5′-GATCCACT**TGATAA**CAGAAAG**TGATAA**CTCT-3′; GATA3.2, 5′-GATCAGAGTTATCACTTTCTGTTATCAAGTG-3′; GATA3.3, 5′-GATCTGTGGGAGG**AGATAG**GACTCTTAC-3′; GATA3.4, 5′-GATCGTAAGAGTCCTATCTCCTCCCACA-3′; GATA3.5, 5′-GATCCCGGGCAAG**TGATAA**ACAAGTTTG-3′; GATA3.6, 5′-GATCCAAACTTGTTTATCACTTGCCCGG-3′. The sequences of the two strands of the oligonucleotides with the mutated consensus GATA3 binding sites were as follows (mutated nucleotides in bold): mG1, 5′-CACTT**CT**TAACAGAAAGT**CT**TAACTCT-3′, and mG2, 5′-AGAGTTAAGACTTTCTGTTAAGAAGTG-3′. The sequences of the two strands for the oligonucleotide GATA3. 3/4 with the mutated GATA3 binding site were as follows (point mutations in bold): mG3/4.sense, 5′-GATCTGTGGGAGGA**CT**TAGGACTCTTAC-3′, and mG3/4.antisense, 5′-GATCGTAAGAGTCCTAAGTCCTCCCACA-3′.

RNA preparation and reverse transcription. RNA was prepared with TRIzol reagent (GibcoBRL) following the manufacturer's instructions. Reverse transcription used 2 μg of RNA, oligo(dT) primer and SuperScript II RNase H^- Reverse transcriptase (Invitrogen). PCR reactions used 5% of the cDNA produced. The sequences of the primers used were as follows: *Hprt*.S, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; *Hprt*.A, 5′-GAGGGTAGGCTGGCCTATAGGCT-3′; *Rad50*.S, 5′-CCGGATCGAAAAGATGAGCATT-3′; *Rad50*.A, 5′-GGGCCCGCACGTCTGTTTC-3′; and *Gata3*.S, 5′-CAAGGCAACCACGTCCCGTCC-3′; and *Gata3*.A, 5′-AGCAGAGGCGGGCGGCATAC-3′.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank F. Manzo for assistance with manuscript preparation; C. Hughes for technical assistance; P.E. Fields, G.R. Lee, S.T. Kim and L. Jones for discussions; F. Grosveld and W. de Laat for protocols on chromosome conformation capture technique and for suggestions on improving the manuscript; and Wyeth Laboratories for the donation of IL-12. Experiments in this study were approved by the Yale University Institutional Animal Care and Use Committee. Supported with a Cancer Research Institute fellowship (C.S.) and Howard Hughes Medical Institute (R.A.F.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 10 May; accepted 18 August 2004
Published online at http://www.nature.com/natureimmunology/

1. Lee, G.R., Fields, P.E., Griffin, T.J. IV, & Flavell, R.A. Regulation of the T_H2 cytokine locus by a locus control region. *Immunity* **19**, 145–153 (2003).
2. 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).
3. Takemoto, N. *et al.* T_H2-specific DNase I-hypersensitive sites in the murine IL-13 and IL-4 intergenic region. *Int. Immunol.* **10**, 1981–1985 (1998).
4. Agarwal, S. & Rao, A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* **9**, 765–775 (1998).
5. Carey, M. & Smale, S. *Transcriptional regulation in eukaryotes: concepts, strategies, and techniques.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2000).
6. 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).

7. 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).
8. Li, Q., Peterson, K.R., Fang, X. & Stamatoyannopoulos, G. Locus control regions. *Blood* **100**, 3077–3086 (2002).
9. Carter, D., Chakalova, L., Osborne, C., Dai, Y.-F. & Fraser, P. Long-range chromatin regulatory interactions *in vivo*. *Nat. Genet.* **32**, 1–4 (2002).
10. Tolhuis, B., Palstra, R.-J., Splinter, E., Grosveld, F. & de Laat, W. Looping and interaction between hypersensitive sites in the active β-globin locus. *Mol. Cell* **10**, 1453–1465 (2002).
11. Dekker, J. A closer look at long-range chromosomal interactions. *Trends Biochem. Sci.* **28**, 277–280 (2003).
12. Choi, O. & Engel, J.D. Developmental regulation of β-globin gene switching. *Cell* **55**, 17–26 (1988).
13. Dillon, N., Trimborn, T., Strouboulis, J., Fraser, P. & Grosveld, F. The effect of distance on long-range chromatin interactions. *Mol. Cell* **1**, 131–139 (1997).
14. Wijgerde, M., Grosveld, F. & Fraser, P. Transcription complex stability and chromatin dynamics *in vivo*. *Nature* **377**, 209–213 (1995).
15. Dekker, J., Rippe, K., Dekker, M. & Kleckner, N. Capturing chromosome conformation. *Science* **295**, 1306–1311 (2002).
16. Zhu, J., Guo, L., Watson, C.J., Hu-Li, J. & Paul, W.E. STAT6 is necessary and sufficient for IL-4's role in T_H2 differentiation and cell expansion. *J. Immunol.* **166**, 7276–7281 (2001).
17. Zheng, W.P. & Flavell, R.A. The transcription factor GATA-3 is necessary and sufficient for T_H2 cytokine gene expression in CD4 T cells. *Cell* **89**, 587–596 (1997).
18. Zhang, D.-H., Cohn, L., Ray, P., Bottomly, K. & Ray, A. Transcription factor GATA-3 is differentially expressed in murine T_H1 and T_H2 cells and controls T_H2-specific expression of the interleukin-5 gene. *J. Biol. Chem.* **272**, 21597–21603 (1997).
19. Gorelik, L., Fields, P.E. & Flavell, R.A. Cutting edge: TGF-β inhibits T_H type 2 development through inhibition of GATA-3 expression. *J. Immunol.* **165**, 4773–4777 (2000).
20. Fields, P.E., Kim, S.T. & Flavell, R.A. Cutting edge: changes in histone acetylation at the IL-4 and IFN-γ loci accompany T_H1/T_H2 differentiation. *J. Immunol.* **169**, 647–650 (2002).
21. 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).
22. Messi, M. *et al.* Memory and flexibility of cytokine gene expression as separable properties of human T_H1 and T_H2 lymphocytes. *Nat. Immunol.* **4**, 78–86 (2002).
23. Guo, L. *et al.* In T_H2 cells the IL-4 gene has a series of accessibility states associated with distinctive probabilities of IL-4 production. *Proc. Natl. Acad. Sci. USA* **99**, 10623–10628 (2002).
24. Palstra, R.-J. *et al.* The β-globin nuclear compartment in development and erythroid differentiation. *Nat. Genet.* **35**, 190–194 (2003).
25. Ansel, K.M., Lee, D.U. & Rao, A. An epigenetic view of helper T cell differentiation. *Nat. Immunol.* **4**, 616–623 (2003).
26. Spilianakis, C. *et al.* CIITA regulates transcription onset via Ser5-phosphorylation of RNA Pol II. *EMBO J.* **22**, 5125–5136 (2003).
27. Boehm, A.K., Saunders, A., Werner, J. & Lis, J.T. Transcription factor and polymerase recruitment, modification, and movement on dHSP70 *in vivo* in the minutes following heat shock. *Mol. Cell. Biol.* **23**, 7628–7637 (2003).
28. Gilmour, D.S. & Lis, J.T. RNA polymerase II interacts with the promoter region of the noninduced HSP70 gene in *Drosophila melanogaster* cells. *Mol. Cell. Biol.* **6**, 3984–3989 (1986).
29. Graunke, D.M., Fornace A.J. Jr, & Pieper, R.O. Presetting of chromatin structure and transcription factor binding poise the human GADD45 gene for rapid transcriptional up-regulation. *Nucleic Acids Res.* **27**, 3881–3890 (1999).
30. Wang, X.Y., Kolb, A., Cannon, W. & Buck, M. Nucleoprotein complex formation by the enhancer binding protein NifA. *Nucleic Acids Res.* **25**, 3478–3485 (1997).
31. Ljungman, M. & Hanawalt, P.C. Presence of negative torsional tension in the promoter region of the transcriptionally poised dihydrofolate reductase gene *in vivo*. *Nucleic Acids Res.* **23**, 1782–1789 (1995).
32. Smale, S.T. & Fisher, A.G. Chromatin structure and gene regulation in the immune system. *Annu. Rev. Immunol.* **20**, 427–462 (2002).
33. Grogan, J.L. *et al.* Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* **14**, 205–215 (2001).
34. Lee, B.H. *et al.* GATA-3 induces T helper cell type 2 (T_H2) cytokine expression and chromatin remodeling in committed T_H1 cells. *J. Exp. Med.* **192**, 105–115 (2000).
35. Ouyang, W. *et al.* Inhibition of T_H1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* **9**, 745–755 (1998).
36. Rosmaraki, E.E. *et al.* Identification of committed NK cell progenitors in adult murine bone marrow. *Eur. J. Immunol.* **31**, 1900–1909 (2001).
37. Samson, S.I. *et al.* GATA-3 promotes maturation, IFN-γ production, and liver-specific homing of NK cells. *Immunity* **19**, 701–711 (2003).
38. Takemoto, N., Arai, K. & Miyatake, S. Cutting edge: the differential involvement of the N-finger of GATA-3 in chromatin remodeling and transactivation during T_H2 development. *J. Immunol.* **169**, 4103–4107 (2002).
39. Rao, A., Luo, C. & Hogan, P.G. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707–747 (1997).
40. Feske, S., Giltrane, J., Dolmetsch, R., Staudt, L. & Rao, A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat. Immunol.* **2**, 316–324 (2001).
41. Okamura, H. & Rao, A. Transcriptional regulation in lymphocytes. *Curr. Opin. Cell Biol.* **13**, 239–243 (2001).
42. Kovac, Z. & Schwartz, R. The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. *J. Immunol.* **134**, 3233–3240 (1985).