Chromatin-based regulation of cytokine transcription in Th2 cells and mast cells

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Abstract

Th2 cells and mast cells are major sources of IL4, IL5 and IL13, cytokines that mediate immunity against parasites and are also central players in the pathophysiology of asthma, allergy and atopic disease. We asked whether Th2 cells and mast cells, which belong to the lymphoid and myeloid lineages, respectively, use different cis-acting regulatory regions to transcribe the cytokine genes. Comparison of DNase I hypersensitivity patterns at the RAD50/IL4/IL13 locus revealed that most hypersensitive sites (HSs) are common to Th2 and mast cells, but two regions [conserved non-coding sequence (CNS) 1 and mast cell HSs] show cell type-specific differences. CNS-1, one of the most highly conserved CNS regions in the RAD50/IL13/IL4 locus, displays two strong DNase I HSs in Th2 cells but is not DNase I hypersensitive in mast cells, explaining a previous finding that deletion of CNS-1 impairs cytokine expression in Th2 cells but not in mast cells. Conversely, two constitutive HSs (mast cell HSs) in the first intron of the IL13 gene are present in mast cells but not in Th2 cells; these sites develop early during mast cell differentiation and may have a role in maintaining accessibility of the IL13 locus to high-level transcription in stimulated cells.

Introduction

The cytokines IL4, IL5 and IL13 mediate immunity against parasites and extracellular pathogens, and are also central players in the pathophysiology of asthma, allergy and atopic disease (1–3). These cytokines are produced by Th2 cells, NKT cells and mast cells, which are all important sources of the cytokines during in vivo immune responses. The major factor controlling expression of these cytokines is IL4 itself, which acts in an autocrine fashion to promote differentiation of naive T cells into Th2 cells that strongly up-regulate transcription of all three cytokine genes. Mast cells are best known for producing mediators of allergic responses and as critical effector cells in IgE-dependent immediate-type hypersensitivity reactions; however, activated mast cells are also well established as an important source of several cytokines including IL4 and IL13. Mast cells are widely distributed throughout the body, providing the high local concentration of cytokines that is critical in many immune responses (4, 5).

A central question in gene regulation is whether cell types of different lineages use different trans-acting factors and cis-regulatory elements to transcribe the same genes. Analysis of cytokine gene expression in Th2 and mast cells has provided important insights into this question. Although Th2 cells and mast cells are both capable of expressing the IL4 and IL13 genes, they utilize different subsets of transcriptional regulators to activate cytokine gene transcription. For instance, IL4 and IL13 gene transcription are NFAT dependent in both mast cells and T cells, but the two cell types use distinct NFAT proteins to activate gene expression (6–8). GATA3 and Maf, transcription factors that are essential for maximal IL4 gene expression by Th2 cells (9, 10), are not expressed in mast cells (11, 12); conversely, an intact STAT6-signalling pathway is required for Th2 differentiation (13–15), but IL4 production by mast cells does not require STAT6 (16).

The IL4, IL5 and IL13 cytokine genes are closely linked in an evolutionarily conserved gene cluster located within a 220-kb genomic region on mouse chromosome 11 and human chromosome 5 (17–19). Multi-species sequence comparisons of a 1-megabase region surrounding this locus revealed 90 conserved non-coding sequences (CNSs) with presumed regulatory function (18). The region surrounding the IL4 and
IL13 genes has been especially well studied; it contains many CNSs located not only in the immediate vicinity of the two genes (18) but also at the 3' end of the neighbouring RAD50 gene (20–23). The longest highly conserved region is termed CNS-1; it is located 3' of the IL13 gene in the intergenic region between IL13 and IL4. The RAD50 gene is located 5' of the IL13 gene, and encodes an essential and ubiquitously expressed DNA repair enzyme whose function is unrelated to T\(_\text{h}\)2 cytokine expression; surprisingly, its 3' end contains several CNS regions and enhancer elements which together function as a locus control region (LCR) for the IL4 and IL13 genes (20).

While bioinformatic approaches are useful for tentative identification of CNS regions as conserved regulatory regions, they cannot establish whether a given CNS is actually utilized as a regulatory region by any particular cell type. A relatively simple method of assigning potential regulatory function is by examining the accessibility of a CNS region to restriction enzymes and/or DNase I; such regions of increased accessibility often correspond to cis-regulatory regions where binding of trans-acting factors leads to local disruption of a regular nucleosomal array (24–26). The approach of using DNase I hypersensitivity to identify functional cis-regulatory elements has been well validated in the RAD50/IL13/IL4 locus, where a remarkably large fraction of CNS regions correspond to sites that are DNase I hypersensitive in T\(_\text{h}\)2 cells (26). The biological importance of several such regulatory elements has been established by deleting them from the mouse genome (see Discussion).

In this study we have used DNase I hypersensitivity mapping to examine the utilization of CNS regions in the RAD50/IL13/IL4 locus by T\(_\text{h}\)2 cells and mast cells, which derive from two distinct cellular lineages (lymphoid and myeloid, respectively). Our results indicate that these cell types show considerable overlap, but also cell type-specific differences, in their pattern of DNase I hypersensitivity in the RAD50/IL13/IL4 locus. Surprisingly, mast cells show no DNase I hypersensitivity at CNS-1, which displays two strong DNase I hypersensitive sites (HSs) in T\(_\text{h}\)2 cells and is one of the most highly conserved CNS regions in the 1-megabase region surrounding the RAD50/IL13/IL4 locus. This observation is likely to explain the fact that CNS-1 functions as an enhancer in T\(_\text{h}\)2 cells but not in mast cells, as judged by the fact that murine T\(_\text{h}\)2 cells with genomic deletion of CNS-1 show decreased expression of both IL4 and IL13, whereas mast cells from the same mice show no impairment in the production of either cytokine. In addition, we have identified two new constitutive, MCHSs in the first intron of the IL13 gene, which develop early during mast cell differentiation and so may have an important developmental role. These sites could have a role in maintaining accessibility of the IL13 locus to high-level transcription in stimulated cells.

**Methods**

**Mice**

Four- to 6-week old BALB/c or C57BL/6 mice were obtained from Jackson Laboratories and maintained in pathogen-free conditions in a barrier facility.

**Bone marrow-derived mast cell and embryonic stem cell-derived mast cell differentiation and stimulation**

By culturing whole bone marrow with IL3, or by in vitro differentiation of embryonic stem (ES) cells, it is possible to obtain cells that resemble early mast cells before they enter the tissues (27, 28). Bone marrow-derived mast cells (BMMCs) were generated from bone marrow cells isolated from femurs and tibias of 8- to 12-week old C57BL/6 mice as described (29). Cells were maintained for 4–12 weeks in media containing 50% WEHI-3 (American Type Tissue Collection, Manassas, VA, USA) conditioned supernatant as a source of IL3. For generation of embryonic stem cell-derived mast cells (ESMCs), wild-type ES cells were differentiated to embryoid bodies as described (6, 27, 30). ES cells were maintained in DMEM on fibroblast-feeder cells in the presence of 1000 U ml\(^{-1}\) LIF (Invitrogen, Carlsbad, CA, USA). Prior to differentiation, ES cells were passaged two times on gelatinized plates, and media was changed to IMDM. ES cells were then trypsinized and resuspended at a concentration of 7.5–12.5 × 10\(^6\) cells ml\(^{-1}\) in IMDM differentiation media (containing 15% FCS, 2 mM l-glutamine (Invitrogen), 300 μg ml\(^{-1}\) transferrin (Boehringer Ingelheim, Ridgefield, CT, USA), 55 μM β-mercaptoethanol, 50 μg ml\(^{-1}\) ascorbic acid (Sigma, St. Louis, MO, USA), 5% protein-free ham's medium (Invitrogen)) in Petri-grade dishes for ~11 days. Embryoid bodies were then disrupted in trypsin and single-cell suspensions were cultured in mast cell media [RPMI with 50% WEHI-3 conditioned supernatant, and c-Kit ligand (20 ng ml\(^{-1}\))]. For both BMMCs and ESMCs, differentiation was assessed by metachromatic staining of cytoplasmic basophilic granules with toluidine blue, and by surface staining of membrane FcεRI and c-Kit (29). In experiments requiring stimulation, BMMCs and ESMCs were treated with 20 nM phorbol myristate acetate (PMA) and 2 μM ionomycin (Calbiochem, San Diego, CA, USA) for the indicated times. Alternatively, cells were sensitized in 3 μg ml\(^{-1}\) anti-dinitrophenol rat IgE (Zymed) for 1 h at 37°C. After removal of the cells from IgE-containing medium, FcεRI-bound IgE was then cross-linked for 4 h with mouse anti-rat IgG F(ab\(_{1}\))\(_{2}\) fragments (Jackson Immunoresearch Laboratories) at 25 μg ml\(^{-1}\).

**T cell differentiation and stimulation**

T cell differentiation was performed as described (31). Primary CD4+ T cells were isolated from spleen and lymph nodes of 6-week-old C57BL/6 mice, stimulated for 2 days with plate-bound anti-CD3 and anti-CD28 and cultured 1 week in T\(_\text{h}\)2-polarizing conditions (IL4, anti-IL12 and anti-IFN-γ antibody were added). For stimulation, cells were treated with 20 nM PMA and 2 μM ionomycin for the indicated times. Plate-bound stimulation of T\(_\text{h}\)2 cells with anti-CD3/anti-CD28 was performed exactly as described (29).

**RNase protection assay**

Total RNA was purified using Ultraspec reagents (Biotecx Laboratories, Houston, TX, USA). Transcript levels were analysed using the RiboQuant multiprobe RNase protection kit (Pharmingen, San Diego, CA, USA) with the mck-1 probe, and quantified by PhosphorImager. Cytokine mRNA levels
were normalized to the corresponding L32 mRNA standard levels in each lane, and relative intensity values were plotted.

**DNase I hypersensitivity and restriction enzyme accessibility analysis**

Nuclei were isolated from T\(_{h2}\) and mast cells as described (31). Varying amounts of DNase I ( Worthington Biochemical, Lakewood, NJ, USA) were added to aliquots of nuclei and incubated at room temperature for 3 min. Purification of genomic DNA, Southern blotting and hybridization were performed as described (31). Restriction enzyme accessibility was performed as described (32–34). Briefly, nuclei were prepared as for DNase I HS analysis, but resuspended in buffer F [100 mM NaCl, 50 mM Tris–HCl (pH 8), 5 mM MgCl\(_2\), 0.1 mM ethyleneglycol-bis(aminohetero)-tetraacetic acid (EGTA) and 1 mM 2-mercaptoethanol] and split into aliquots.

After addition of 50–200 U of restriction enzyme, samples were incubated at room temperature for 30 min. Purified DNA was digested to completion with BamHI and analysed by Southern blot. All probes used were designed to hybridize to one end of the restriction fragment to be analysed and were generated by PCR from genomic DNA.

**Micrococcal nuclease analysis**

Nuclei were isolated from 10\(^6\) cells in NP-40 lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 3 mM MgCl\(_2\), 0.5% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine) and resuspended in MNase digestion buffer (10 mM Tris–HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) containing 1 mM CaCl\(_2\). Varying amounts of MNase were added and nuclei were incubated at room temperature for 5 min. The reaction was stopped by adding 20 μl of MNase stop buffer (100 mM EDTA, 10 mM EGTA, pH 7.5). After overnight incubation with proteinase K, phenol–chloroform extraction and ethanol precipitation, the purified DNA was digested to completion with PstI and run on a 1% agarose gel. After over night incubation with proteinase K, phenol–chloroform extraction and ethanol precipitation, the purified DNA was digested to completion with PstI and run on a 1% agarose gel. Southern blot was performed overnight in 10 \(×\) standard saline citrate. The probe used for hybridization corresponded to the 5’ end of a PstI fragment of the IL13 locus containing also the 3’ end of the first IL13 exon. For these experiments, the IL3-dependent mast cell line CFTL15 was used (35).

**Visualization Tool for Alignment analysis**

VISTA (Visualization Tool for Alignment) analysis was extensively described in (26). Comparison of human and mouse sequences was performed using web-based software available at the Lawrence Berkeley Laboratory Genome website (http://www-gsd.lbl.gov/vista). Human and mouse sequences were obtained from the Ensembl Genome database (http://www.ensembl.org).

**Results**

**Relative levels of cytokine expression by T\(_{h2}\) cells and mast cells**

We first compared the ability of mast cells and T\(_{h2}\) cells to produce a panel of ‘Th2’ cytokine genes (Fig. 1). ESMCs and primary T\(_{h2}\) cells from C57BL/6 mice were stimulated with PMA and ionomycin for different times ranging from 5 min to 24 h, and transcript levels of several cytokines were assessed by RNase protection assay (RPA). Mast cells showed much lower levels of IL4 transcripts relative to T\(_{h2}\) cells, but expressed comparable levels of IL5 mRNA and higher levels of IL13 mRNA; T\(_{h2}\) cells selectively transcribed the IL10 gene, whereas mast cells selectively transcribed IL6 (Fig. 1A and B).

In separate experiments, very similar patterns of cytokine production—low IL4 expression but higher IL13 expression relative to T\(_{h2}\) cells—were observed for primary BMMCs differentiated with IL3 for 8 weeks in culture and stimulated for 4 h with PMA and ionomycin (Fig. 1C).

We compared cytokine production by ESMC and BMMC after stimulation for 4 h with either PMA and ionomycin or FcγRI cross-linking (Fig. 2). PMA and ionomycin consistently gave a stronger stimulation of the cells when compared with FcγRI cross-linking (Fig. 2A), but the ratio of IL13/IL4 mRNA levels was identical for both kinds of stimuli, even though reproducibly higher in ESMC than in BMMCs (Fig. 2A and B; please note the difference in time of exposure to the autoradiographic film). This difference most likely reflects the different culture conditions that we used to generate these two kinds of mast cells: ESMCs were differentiated in the presence of both IL3 and c-KIT ligand, while BMMCs were differentiated in the presence of IL3 alone (see Methods). In fact, BMMCs grown in a combination of IL3 and c-KIT ligand display increased capacity to secrete cytokines relative to BMMCs grown in IL3 alone (36), which represent a population of relatively immature c-KIT-expressing mast cells with the potential to differentiate further along either the mucosal or the connective tissue-type mast cell lineage both in vitro and in vivo (36).

Figure 2B (lower panel) also shows the IL13/IL4 ratio obtained performing RPA on primary T\(_{h2}\) cells cultured in vitro for 3 weeks and re-stimulated for 4 h with either PMA and ionomycin or anti-CD3 and anti-CD28 antibodies to cross-link the TCR. Strikingly, while the IL13/IL4 ratio in mast cells ranged from 50 (BMMC) to 200 (ESMC), T\(_{h2}\) cells showed consistently very similar levels of IL4 and IL13 expression, with a ratio between these two cytokine expression levels always close to one [please, note the difference in scale between the upper (mast cells) and lower (T\(_{h2}\) cells) panels]. These data verify that the cytokine responses of mast cells and T\(_{h2}\) cells treated with PMA and ionomycin recapitulate their physiological responses to ligation of their surface FcγRI and TCR.

**DNase I hypersensitivity patterns at the IL4/IL13 locus in T\(_{h2}\) cells and mast cells**

We next compared DNase I hypersensitivity patterns in the extended IL4/IL13/RAD50 locus in T\(_{h2}\) cells and mast cells (Fig. 3). As observed previously, the two cell types showed very similar DHS patterns in the vicinity of the IL4 gene (29). However analysis of more distal regions revealed several striking differences (Fig. 3; also see summary Fig. 4). (i) As previously reported, (31) T\(_{h2}\) cells displayed three DHS sites I, II and III, in the IL13 gene, located ~3 kb 5’ of the transcription start site, in the proximal promoter and in intron 2, respectively. Mast cells showed all three of these sites as well as two additional strong constitutive DHS sites located in the first
Fig. 1. Cytokine mRNA expression by Th2 cells and mast cells. Primary CD4+ T cells were isolated from 6-week-old C57Bl/6 mouse spleen and lymph nodes, stimulated for 2 days with plate-bound anti-CD3 and anti-CD28 and cultured for 1 week in Th2-skewing conditions. Mast cells were derived from wild-type ESMC or from bone marrow of 6- to 8-week old C57Bl/6 mice. (A) Primary 1 week-differentiated Th2 cells and ESMCs were stimulated for the indicated times with PMA and ionomycin, total RNA was extracted, and cytokine mRNA levels were evaluated by RPA. (B) Quantification of the experiment shown in (A). (C) In a separate experiment from that shown in Fig. 1(A) and quantified in Fig. 1(B), primary Th2 cells differentiated for 1 week and primary BMMCs differentiated for 8 weeks were stimulated for 4 h with PMA and ionomycin, total RNA was extracted and RPA was performed. PhosphorImager quantification of the RPA data is shown.
intron of the IL13 gene (Fig. 3B). These sites [termed MCHS (mast cell hypersensitive sites)] were not observed in either resting or stimulated Th2 cells, and their intensity in mast cells was not altered by treatment with the calcineurin inhibitor CsA (data not shown). Also, the MCHS sites were observed in mast cells derived from either C57Bl6 or Balb/c background (data not shown) and they were present in mast cells of both bone marrow and stem cell origin (compare Figs 3B and 6B). (ii) The intergenic region located between the IL4 and IL13 genes contains HSS3, a DHS site present in naive T cells, Th1 cells and Th2 cells (31, 37). This site was also apparent in mast cells (Fig. 3C). However, mast cells showed no evidence of two nearby intergenic DHS sites, HSS1 and HSS2, which are located within the CNS-1 (Fig. 3D). (iii) The 3’ end of the RAD50 gene, which contains a LCR for the IL4 and IL13 genes, contains several DHS sites that correspond to CNS regions (20–23). Three of these, RAD50-A, RAD50-B and RAD50-C, are located within introns 21, 22 and 24 of the RAD50 gene, respectively, and are hypersensitive in Th2 cells; these sites are also present in stimulated BMMCs (Fig. 3E). As in Th2 cells (21), the RAD50-A and RAD50-B sites are constitutive, while the RAD50-C site is induced following acute stimulation (Fig. 3E).

The data are summarized in Fig. 4(A). Of a total of 19 HSs apparent in ~70 kb of the analysed IL4/IL13/RAD50 locus, a region encompassing the IL13 and IL4 genes as well as part of the LCR in the 3’ end of the RAD50 gene (21, 23), 15 sites are present in both Th2 cells and mast cells, two (the MCHS sites) are mast cell-specific and two (the HSS1 and HSS2 sites located in CNS-1) are Th2-specific. We mapped the locations of the DHS sites in relation to CNS regions, using the VISTA alignment/display program to generate an inter-species (mouse–human) comparison of the genomic DNA sequence of the cytokine locus (Fig. 4B). A remarkable fraction of the DHS sites, among them the RAD50-A, RAD50-B and RAD50-C HSs (21, 23), the IL13 DHS site I (31) [also known as conserved GATA3 response element; 38], the IL13 DHS site II which corresponds to the IL13 promoter (31), HSS1 and 2 (CNS-1) (37) and IL4 DHS I, II, IV and V (31), coincide with CNS regions that are strongly conserved between human and mouse (Fig. 4B) (reviewed in 24, 26).

Restriction enzyme accessibility and nucleosome positioning at the MCHS sites in Th2 cells and mast cells

Even though the MCHS sites are only moderately well conserved (60–70% sequence identity) between human and mouse (Fig. 4B), they are very strongly DNase I hypersensitive in mast cells (Fig. 3B) and they are the only mast cell-specific DHS sites in the entire region mapped in Figs 3 and 4(A). We therefore investigated them in more detail. Using genomic DNA digested with restriction enzymes as size markers (as described in 34, 39), we mapped the location of the MCHS within a 1690-bp region bounded by HaeII and EcoRV sites, somewhat closer to the HaeII site (Fig. 5B). To confirm that the
MCHS region was selectively accessible in mast cells, we used a restriction enzyme accessibility assay with NcoI (Fig. 5C). When NcoI was diffused into intact nuclei, its ability to cleave at the IL13 locus was significantly higher in mast cells compared with T\(_h\)2 cells (Fig. 5C; compare lanes 2–4 with lanes 9–11), indicating that the 'accessibility' of this genomic region to both DNase I and NcoI is specifically increased in mast cells. As control, we used the restriction enzyme EcoRV, which cuts outside the MCHS region (see Fig. 5B and the schematic diagram in Fig. 5A).

Increased accessibility of a genomic region to DNase I and restriction enzymes often indicates that the region is nucleosome free or is located in a region with an altered nucleosome configuration. To test this hypothesis, we assessed nucleosome positioning by MNase digestion (Fig. 5D). Ethidium bromide staining yielded the expected nucleosomal ladder for total genomic DNA (left panel), but Southern blotting with a probe for IL13 exon 1 showed a regular pattern of nucleosomes positioned between exons 1 and 2 of the IL13 gene, except in the region of the MCHS sites. The absence of nucleosomes in the MCHS region is likely to explain the DNase I hypersensitivity and increased accessibility of this region to restriction enzymes in mast cells.

**Fig. 3.** DNase hypersensitivity analysis of the IL4/IL13 locus. Primary T cells were obtained from mouse spleen and lymph nodes, and mast cells were derived from wild-type ES cells or from mouse bone marrow as described for Fig. 1. DNase I hypersensitivity analyses were performed on resting cells except in panel E, where cells were left untreated or were stimulated for 2 h with PMA and ionomycin. (A) Schematic diagram of the IL5/RAD50/IL13/IL4/Klf3a cytokine locus, showing the IL13 and IL4 genes and flanking genes. (B) DHS sites in the IL13 locus. Note the two strong constitutive DHS sites (MCHS) observed in mast cells but not in T\(_h\)2 cells. Also indicated are DHS sites I [conserved GATA3 response element (CGRE)], II and III of the IL13 gene. (C) DHS sites in the BamHI intergenic fragment between IL13 and IL4. Both mast cells and T\(_h\)2 cells display the DHS site HSS3. (D) DHS sites in the ScaI intergenic fragment between IL13 and IL4. ScaI was used instead of BamHI for complete digestion of the genomic DNA; this allows detection of CNS-1, which is otherwise too close to the probe used in Fig. 3(C). (E) DHS sites at the 3’ end of the RAD50 gene; note that KpnI was used in this experiment because it allows the visualization of all three DHS sites in this region (RAD50-A and RAD50-B which are constitutive and RAD50-C which is inducible).

**Early appearance of the MCHS sites during mast cell development**

We next asked how early the MCHS sites appeared during mast cell differentiation. Bone marrow precursors were incubated for 1 week with IL3; at this time, differentiated mast cells are just beginning to emerge from the cultures, as assessed by metachromatic staining for cytoplasmic granules (data not shown) and surface staining for the mast cell markers c-KIT and FcεRI (Fig. 6A, top panels; 40). At this point the
The population contains both c-KIT-positive and c-KIT negative cells, suggesting a heterogenous mixture of cell types (Fig. 6A, top right panel). By 4 weeks of differentiation the cultures are fully differentiated and homogenous with respect to c-KIT and FcεRI expression (Fig. 6A, bottom panels). The 1-week cultures are still not completely differentiated, as judged not only by the heterogeneity of c-KIT expression but also by the low expression level of FcεRI relative to more fully differentiated 4-week BMMCs (Fig. 6A, compare top and bottom left panels).

Parallel analysis of DNase I hypersensitivity showed that all DHS sites in the IL13 gene appear rapidly, within 1 week after the onset of mast cell differentiation (Fig. 6B). The fact that BMMCs differentiated for only 1 week already display the DNase I hypersensitivity pattern characteristic of fully differentiated mast cells suggests that the most critical changes in chromatin configuration characteristic of this cellular lineage have already occurred at this early stage. Although it is formally possible that the hypersensitivity pattern is due to differentiated mast cells present in the bone marrow which have been expanded under our culture conditions, this explanation seems unlikely since almost no cells in the population displayed the high-level FcεRI levels expected of...
mature mast cells (Fig. 6A, top panel), and the sensitivity of the DHS assay is such that contaminating mature mast cells would have to constitute a very significant fraction (>15%) of all cells in the culture to be detected in the assay. Because of the difficulty in identifying mast cell precursors in the bone marrow and isolating sufficient numbers for biochemical analysis, it is not yet possible to examine the DNase hypersensitivity pattern of the \( \text{IL}4/\text{IL}13 \) locus in primary \( \text{T}_{2} \) cells and ESMCs. Nuclei were prepared and left untreated (–) or treated with increasing amounts of \( \text{NcoI} \) (which cuts within the MCHS) or \( \text{EcoRV} \) (which cuts outside the MCHS) for 30 min. Purified DNA was digested to completion with \( \text{BamHI} \) and Southern blots were hybridized as described in (A). (i) Parental (\( \text{NcoI} \) or \( \text{EcoRV} \) undigested) band, (ii) band due to incomplete digestion, (iii) complete digestion at the MCHS \( \text{NcoI} \) site and (iv) complete digestion at the \( \text{EcoRV} \) site. (C) Micrococcal nuclease analysis of the MCHS region. Nuclei of CFTL15 cells, amurine IL3-dependent mast cell line derived from foetal liver (35), were incubated with increasing amounts of MNase and the purified DNA was digested to completion with \( \text{PstI} \) prior to staining with ethidium bromide (EtBr, left panel) or Southern blotting with a probe corresponding to the 5’ end of a \( \text{PstI} \) fragment containing exon 1 of the \( \text{IL}13 \) gene (right panel).

![Fig. 5](image_url)

**Fig. 5.** MCHS is hypersensitive to restriction enzyme and micrococcal nuclease digestion. (A) Schematic diagram of the \( \text{IL}13 \) cytokine locus, showing the restriction sites used in DNase I hypersensitivity assays, as well as for restriction enzyme accessibility and micrococcal nuclease digestion. All probes were designed to hybridize to one end of the selected restriction fragment; the position of the probes used in panels B–D is indicated. (B) Mapping of DNase I MCHS sites to a 1690-bp \( \text{HaeIII}–\text{EcoRV} \) fragment between exons 1 and 2 of the \( \text{IL}13 \) gene. Left panel, ESMC nuclei were subjected to DNase I titration, and the genomic DNA was digested to completion with \( \text{BamHI} \) prior to Southern blot analysis. Right panel, markers were provided by genomic DNA digested to completion with \( \text{BamHI} \) and the indicated restriction enzyme. Membranes were hybridized with a probe derived from exon 4 of the \( \text{IL}13 \) gene. (B) Restriction enzyme accessibility analysis of the \( \text{NcoI} \) and \( \text{EcoRV} \) restriction sites in primary \( \text{T}_{2} \) cells and ESMCs. Nuclei were prepared and left untreated (–) or treated with increasing amounts of \( \text{NcoI} \) (which cuts within the MCHS) or \( \text{EcoRV} \) (which cuts outside the MCHS) for 30 min. Purified DNA was digested to completion with \( \text{BamHI} \) and Southern blots were hybridized as described in (A). (i) Parental (\( \text{NcoI} \) or \( \text{EcoRV} \) undigested) band, (ii) band due to incomplete digestion, (iii) complete digestion at the MCHS \( \text{NcoI} \) site and (iv) complete digestion at the \( \text{EcoRV} \) site. (C) Micrococcal nuclease analysis of the MCHS region. Nuclei of CFTL15 cells, a murine IL3-dependent mast cell line derived from foetal liver (35), were incubated with increasing amounts of MNase and the purified DNA was digested to completion with \( \text{PstI} \) prior to staining with ethidium bromide (EtBr, left panel) or Southern blotting with a probe corresponding to the 5’ end of a \( \text{PstI} \) fragment containing exon 1 of the \( \text{IL}13 \) gene (right panel).
Discussion

Multi-species genomic sequence comparisons have revealed that in addition to the expected evolutionary conservation of protein-coding sequences, a large number of highly conserved non-coding sequences (CNSs) are scattered throughout the genome (18, 44). Where tested, a remarkable fraction of all CNSs corresponds to functional cis-regulatory elements which can be identified in relevant cell types by their increased accessibility to restriction enzymes and/or DNase I (26). A central question in gene regulation is whether cell types of different lineages utilize different subsets of cis-regulatory elements to transcribe the same gene; alternatively, a given set of cis-regulatory elements might always be essential to regulate gene expression even in unrelated cell types that express a particular gene. Here we have addressed this question by comparing IL4 and IL13 cytokine gene regulation in T\(_{h2}\) and mast cells, representatives of the lymphoid and myeloid lineages, respectively. We find evidence for both hypotheses presented above: the pattern of DNase I hypersensitivity is clearly similar in both cell types, but some CNS/cis-regulatory elements are utilized in a cell type-specific way (Figs 2A and 3). Specifically, the majority of cis-regulatory elements located within the RAD50(IL4)/IL13 locus (15 of 19) is utilized in common by T\(_{h2}\) cells and mast cells, as judged by...
overlapping patterns of DNase I hypersensitivity (see Figs 2A and 3). Only two regions of difference were identified: the highly conserved intergenic sequence CNS-1 displayed two DNase I HSs, HSS1 and HSS2, selectively in T<sub>h</sub>2 cells, and conversely, a moderately conserved sequence in the first intron of the *IL13* gene displayed DNase I hypersensitivity selectively in mast cells (Fig. 2A). Thus despite representing very different cellular lineages, T<sub>h</sub>2 cells and mast cells overlap substantially in their use of conserved regulatory regions in the RAD50/IL4/IL13 locus, although cell type-specific differences are observed as documented in other systems as well (45).

The biological importance of several CNS regions/DNase I HSs in the RAD50/IL4/IL13 locus has been established by examining the consequences of deleting them from the mouse genome (22, 29, 39, 46). Our results show clearly that the cis-regulatory function of a particular CNS region in a given cell type can be predicted from whether or not it displays DNase I hypersensitivity in that cell type. (i) Specifically, two CNSs, HSS3 and DHS IV, are located 3' of the *IL13* and *IL4* genes, respectively, and correspond to DNase I hypersensitive (DHS) sites in naive T cells and mast cells as well as in differentiated T<sub>h</sub>1 and T<sub>h</sub>2 cells. Deletion of DHS IV resulted in variegated increases in IL4 expression in T<sub>h</sub>1 cells which normally silence the IL4 gene, as well as in mast cells which normally express IL4 (39). These results suggest strongly that the DHS IV region possesses silencer function in both T<sub>h</sub>1 cells and mast cells. (ii) Similarly, CNS-2 located 3' of the IL4 gene corresponds to a DHS site (DHS V) in T<sub>h</sub>2 cells as well as mast cells: an adjacent less well-conserved region, DHS V<sub>a</sub>, is inducibly DNase I hypersensitive in both cell types. Deletion of the DHS V/V<sub>a</sub> region resulted in decreased IL4 expression by T<sub>h</sub>2 cells as well as mast cells, identifying this region as an IL4 enhancer in both types of cells (29). (iii) Likewise, a well-demarcated CNS region in the RAD503' LCR (termed RAD50-C or 7) is not DNase I hypersensitive in naive T cells or in T<sub>h</sub>1 cells, but is inducibly hypersensitive in differentiated T<sub>h</sub>2 cells (21, 22). Deletion of this site also results in decreased IL4 and IL13 expression by T<sub>h</sub>2 cells and mast cells, identifying this region as an enhancer for both genes in both lineages of cells (22). Thus, all these regions are bona fide cis-regulatory regions utilized by T cells as well as mast cells to regulate expression of the *IL4* and *IL13* cytokine genes. (iv) In contrast, the highly conserved sequence CNS-1, which corresponds precisely to two DNase I hypersensitive sites present in T<sub>h</sub>2 cells (HSS1 and HSS2), displays DNase I hypersensitivity in T<sub>h</sub>2 cells but not in mast cells. Consistent with this finding, deletion of CNS-1 resulted in a significant decrease in IL4, IL5 and IL13 production by T cells, but had no effect on cytokine expression by primary mast cells (18, 46). Together these data suggest strongly that bioinformatic searches for conserved regulatory regions, followed by DNase I hypersensitivity mapping, constitute a generally feasible method for identifying functional cis-regulatory regions of genes (24, 26).

The selective DNase I hypersensitivity and restriction enzyme accessibility of the MCHS sites in mast cells suggests a specific regulatory function for this intronic region in mast cells. However, the exact nature of this function remains to be elucidated. Consistent with constitutive expression of the MCHS sites in resting mast cells, the MCHS region did not behave as a conventional inducible enhancer element in transient reporter assays in mast cells (data not shown). Nor did the MCHS region behave as a constitutive mast cell-specific enhancer when tested in conjunction with a minimal promoter (data not shown). Potentially, the MCHS sites could have a developmental role, conferring (or being associated with the appearance of) a ‘permissive’ chromatin status in the *IL13* locus during the differentiation of mast cell precursors into mature mast cells, and the presence of constitutively bound trans-acting factors at these sites might be important for high-level transcription of the *IL13* gene relative to the *IL4* gene in mast cells (Fig. 1). Acquisition of a cytokine-producing phenotype occurs in two steps: (i) developmental signals confer locus opening and (ii) activation signals in differentiated cells promote transcriptional gene activation. Consistent with the hypothesis that it has a role in mast cell differentiation, the MCHS region is not DNase I hypersensitive in the *Pu.1−/−* ‘precursor’ cell line which does not express the IL4 or IL13 cytokine genes. Deletion of the MCHS region in the mouse genome will be required to demonstrate whether this intron region and its associated trans-acting factors contribute in a mast cell-specific manner to *IL13* gene expression by directing locus remodelling during mast cell differentiation and/or by functioning as a mast cell-specific regulatory element in the chromatin context.

In keeping with our finding of a mast cell-specific intronic HSs in the *IL13* gene, Henkel et al. (47) and Henkel and Brown (48) reported a mast cell-specific enhancer in the second intron of the *IL4* gene. This enhancer is DNase I hypersensitive and augments *IL4* gene transcription in transient transfection assays in mast cell lines. Although the authors showed that this enhancer does not function in the transformed T cell line EL-4 (which expresses IL4 upon activation), our group (31) subsequently showed that this region is DNase I hypersensitive in the T<sub>h</sub>2 cell line D10 and T<sub>h</sub>2 cells differentiating from naive CD4 T cells. Taken together, the data indicate that the intronic enhancer in the *IL4* gene is utilized both by mast cells and by untransformed T<sub>h</sub>2 cells, whereas the putative intronic regulatory elements in the MCHS region of the *IL13* gene may be selectively active in mast cells. However, because mast cells and T<sub>h</sub>2 cells utilize different sets of NFAT, GATA and other transcription factors potentially capable of binding the intronic enhancer (6, 48, 49), this cis-regulatory region may nevertheless have a cell type-specific function.

Is there a correlation between DNase I hypersensitivity in the vicinity of a cytokine gene and the expression level of that cytokine gene in T<sub>h</sub>2 cells versus mast cells? The picture is again complicated by the fact that these two cell types express different sets of trans-activating elements. Although the *IL4* gene is expressed at significantly lower levels in mast cells relative to T<sub>h</sub>2 cells (Fig. 1), the DNase I hypersensitivity patterns around the *IL4* gene are very similar in the two cell types (Fig. 3A; 29), with the minor difference that the *IL4* 3' enhancer (site V<sub>a</sub>) is basally hypersensitive in mast cells but becomes hypersensitive in T<sub>h</sub>2 cells only after stimulation (29, 34). The fact that mast cells transcribe relatively low levels of *IL4* compared with T<sub>h</sub>2 cells may therefore reflect the fact that they do not express factors like c-Maf, which is essential for high *IL4* (but not *IL13*) expression in T<sub>h</sub>2 cells (11, 50). In contrast, the *IL13* gene shows significant differences in
intrinsic hypersensitivity in mast cells and Th2 cells, with the MCHS sites only apparent in mast cells, and these differences may account for the significantly higher level of IL13 transcripts observed in mast cells relative to Th2 cells. In this regard it is also striking that CNS-1, a 401-bp region which is the most highly conserved CNS in a 1-megabase region containing the IL5/IL4/IL13 cytokine gene cluster (18), is DNase I hypersensitive and functions as an enhancer element only in Th2 cells (Figs 3 and 4; 46). Overall our data indicate that while there is a good correspondence between CNS regions, DHS sites and functional cis-regulatory elements in genes, the actual level of gene expression observed in a given cell type under given conditions of stimulation is likely to be regulated by complex developmental interactions involving cell type-specific trans-acting factors which bind the cis-regulatory elements.

In summary, we have described and compared the chromatin structure of the RAD50/IL4/IL13 locus in Th2 and in mast cells. By showing that CNS-1 is DNase I hypersensitive in Th2 cells but not in mast cells, we have solved the paradox of why deletion of this region impaired IL4 and IL13 expression in Th2 cells but was without effect in mast cells. We have also identified two constitutive, MCHSs in the first intron of the IL13 gene; these sites develop early during mast cell differentiation and may be important for maintaining an accessible configuration of the IL13 locus so as to achieve immediate high-level transcription of the IL13 gene in response to stimulation.

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